Glucose-induced Cellular Dysfunction:

The Role of Protein Kinase C

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by

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I would like to express my gratitude to Professor John Walls for introducing me to the importance of basic science, for providing me with my first opportunities in laboratory science and for encouraging me to channel my enthusiasm into this field of research.
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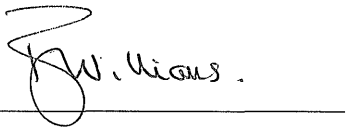
I am also grateful to Professor John D. Swales for the ever open door, the generous gift of his time and wisdom and for his encouragement and support, all of which have been instrumental in allowing me to continue the research work contained in this thesis.
Finally, I thank my wife Sue and children Tom and Amy for their selfless encouragement and understanding of my commitment to this work.

Statement of Originality

All of the work contained in this thesis is completely original. The hypothesis tested in this thesis was formulated by myself. The experimental design was devised by myself. All of the experimental work was performed by me, except some of the later work on vascular permeability factor when I had some technical assistance from two technicians (Anne Quinn-Baker and Barbara Gallacher), under my direct supervision and supported by research grants acquired by me. The work was begun at the University of Leicester, continued at the University of Colorado, USA, and has continued in the department of Medicine and Therapeutics, University of Leicester. The manuscripts of all of the published works contained in this thesis report the results of my own work and were researched and written by myself.

The work contained in this thesis has not been submitted, and is not currently being submitted for any other degree, at this, or any other University.



Bryan Williams, December 1995.

"It is often what we think we know already
that prevents us from learning..."

Claude Bernard 1878

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The structure of this thesis.

This thesis is divided into four sections. The first section contains the introductory overview in which I review the previous hypotheses that have implicated high extracellular glucose concentrations in the pathogenesis of cellular dysfunction and injury. These previous studies set the stage for the presentation of my work in which I have explored the novel hypothesis that glucose-induced protein kinase C activation may provide a final common path for many aspects of cellular dysfunction in diabetes mellitus.

The second section contains my published works which reported the first detailed characterisation of glucose-induced protein kinase C activity and subsequently explored the potential biological significance of this ubiquitous intracellular signalling pathway with regard to a variety of pathophysiological changes in cell function.

The third section of this thesis also contains my published works which illustrate how my studies have subsequently developed with specific reference to our description of the potentially important role of a recently discovered vascular permeability factor in the development of vascular dysfunction in diabetes mellitus. This section culminates in the presentation of my latest study which has just been submitted for publication to the Journal of Clinical Investigation. This study demonstrates that high extracellular glucose concentrations directly stimulate vascular permeability gene expression and peptide production by human vascular smooth muscles via a protein kinase C-dependent mechanism. In so doing, this latest study illustrates the consistency of the theme that began and continues throughout this thesis; notably, the role of protein kinase C in mediating many diverse aspects of glucose-induced cellular dysfunction.

Finally, in section four, I conclude the thesis by reviewing the main features of the published works contained within it and I describe how future work could extend these observations and provide the basis for possible new therapeutic interventions designed to attenuate glucose-induced cellular injury.

Chapter 1.

Review of the previously proposed mechanisms for glucose-induced cellular dysfunction in diabetes mellitus.

Introduction.

Insulin dependent diabetes mellitus and non-insulin dependent diabetes mellitus are both characterised by the development of chronic hyperglcaemia. Both forms of diabetes are also associated with premature cardiovascular morbidity and mortality [1-8]. In this regard, the diabetic state is characterised by the accelerated development of macrovascular disease ie. atheroma disease of the major blood vessels leading to stroke, coronary heart disease and peripheral vascular disease. Diabetes is also associated with the development of a very specific form of microangiopathy which plays a fundamental role in the pathogenesis of disease complications such as nephropathy, retinopathy and also neuropathy.

It was inevitable that hyperglycaemia *per se,* would be implicated in the pathogenesis of diabetic macro- and microangiopathy. Nevertheless, until recently, definitive evidence that strict glycaemic control could delay or prevent diabetic complications was lacking. Although the studies of animals models of diabetes and epidemiological data supported that notion that hyperglycaemia was an important contributor to the development of

diabetic complications [9-25], it was not until the recent publication of two prospective clinical trials that this notion was substantiated [26,27].

The first of these important studies was reported by Reichard, et. al. in 1993. Their study examined the effect of long-term intensified insulin treatment on the development of the microvascular complications of diabetes mellitus in 102 insulin dependent diabetics were randomly assigned to an intensive insulin therapy regimen or remained on standard insulin therapy [26]. The development of nephropathy, retinopathy and neuropathy was then assessed in the two groups over a seven and a half year follow-up. The intensively treated group had better long-term glycaemic control than the group treated with standard insulin therapy and this was associated with a significant reduction in the development of all microvascular disease complications in the intensively treated group.

This report was followed only two months later by the results of the much larger Diabetes Control and Complications Trial (DCCT) which also prospectively examined the effect of intensive insulin therapy on the development of the microvascular complications of insulin dependent diabetes mellitus [27]. In this study, a total of 1441 insulin dependent diabetics were randomly assigned to intensive or standard insulin therapy and followed for a mean of six and a half years. At the end of this follow-up period, improved glycaemic control as a consequence of intensive insulin therapy was associated with a significant delay in the onset and significant retardation of the progression of retinopathy, nephropathy and neuropathy.

Together these studies provide consistent and substantial evidence that the microvascular complications of insulin dependent diabetic subjects can be ameliorated by strict glycaemic control. By inference, they also imply that elevated glucose concentrations *per se,* play an important role in the pathogenesis of these complications. Nevertheless, important questions remain unanswered: Although elevated blood glucose levels have been identified in epidemiological studies as an important independent risk factor for the development of macrovascular disease [28], the crucial question as to whether strict glycaemic control can limit the development of macrovascular disease has not been addressed by prospective clinical studies. Moreover, almost all studies have focused on insulin dependent diabetes whereas a majority (by a ratio approximating 10:1) of diabetic subjects are non-insulin dependent. The development of macro- and microvascular disease in non-insulin dependent diabetics is no less severe than it is in insulin dependent diabetes. It cannot necessarily be assumed that the impressive effect of strict glycaemic control in retarding the development of microvascular disease in insulin dependent diabetics will be reproduced in non-insulin dependent diabetes. Moreover, whether or not strict glycaemic control is efficacious at reducing the development of macrovascular complications in non-insulin dependent diabetes, also remains unanswered. These latter two important questions are currently being addressed by the United Kingdom Prospective Diabetes Study (UKPDS) which will report and hopefully clarify some of these important clinical issues within the next few years.

Epidemiological studies, retrospective clinical studies and the latest prospective clinical trial data thus lend considerable support to the hypothesis that elevated glucose concentrations are directly implicated in the pathogenesis of diabetic complications. Nevertheless, the case against glucose would be much stronger if plausible mechanisms to account for glucose-induced tissue injury could be demonstrated. Such experimental evidence is essential for a variety of reasons: First, the demonstration of cellular mechanisms directly implicating glucose in the pathogenesis of vascular injury would enhance our clinical resolve to modify this risk factor. Secondly, increasing our understanding of the biological basis of glucose-mediated cell toxicity may allow the development of novel therapeutic strategies designed to minimise this deleterious action of glucose. Such an approach is attractive because achieving the strict levels of glycaemic control required to retard tissue injury is not feasible in all patients. Moreover, even when strict glycaemic control is achieved, it delays but does not necessarily prevent the development of diabetic complications [26,27]. For these reasons, I believe that the development of adjunctive therapies, designed to selectively inhibit glucose-driven toxic pathways within vulnerable cells, may provide the ultimate therapeutic approach to minimise the diabetic complications.

In this thesis I have explored a novel hypothesis in which I propose a specific biological pathway whereby elevated glucose concentrations may directly influence various aspects of cell function. This pathway involves the direct, glucose-driven activation of protein kinase C (PKC), an important, ubiquitous and multifunctional kinase system that plays a pivotal role in regulating a diverse array of cell functions (see chapter 7). Before I

embarked on these studies, I reviewed the previous and existing hypotheses that have implicated elevated glucose concentrations in the pathogenesis of the various manifestations of tissue injury that have been described in diabetes mellitus. This was a very instructive and important exercise. The first thing that captured my attention was the fact that blood glucose levels in man are so tightly regulated and maintained almost on the verge of hypoglycaemia. This is remarkable when one considers that glucose is an essential metabolite for the brain and that hypoglycaemia is potentially lethal over a short period of time whereas moderate hyperglycaemia is not. This perhaps reflects the fact that evolutionary forces have recognised that moderate hyperglycaemia is not benign and that preventing sustained hyperglycaemia is essential for normal cell function and survival. It is also intriguing that the cells of the vascular endothelium and the vascular smooth muscle coat within vessels appear to have no intrinsic mechanism to protect themselves from hyperglycaemia. These cells are in constant and intimate contact with circulating glucose and the uptake of glucose by these cells is largely insulin independent and predominantly dependent on the extracellular-to-intracellular glucose gradient. Thus, when the extracellular glucose concentration is chronically elevated, increased quantities of glucose flux into these cells. Perhaps it is no co-incidence that dysfunction of such cells and the resulting vascular disease, is a prominent clinical manifestation of chronic diabetes mellitus.

Accepting that glucose appears to have free access to vascular and perhaps other cell types, by what mechanisms or biological pathways does it induce cell injury and dysfunction? In this thesis I explore protein kinase C dependent pathways, but before

describing my studies, it is important and appropriate to consider the other biological mechanisms that have been proposed for glucose-induced cellular dysfunction. Not least of all because it is unlikely that disturbances in the regulation of a single biochemical pathway will provide all of the answers.

1. The non-enzymatic glycation hypothesis.

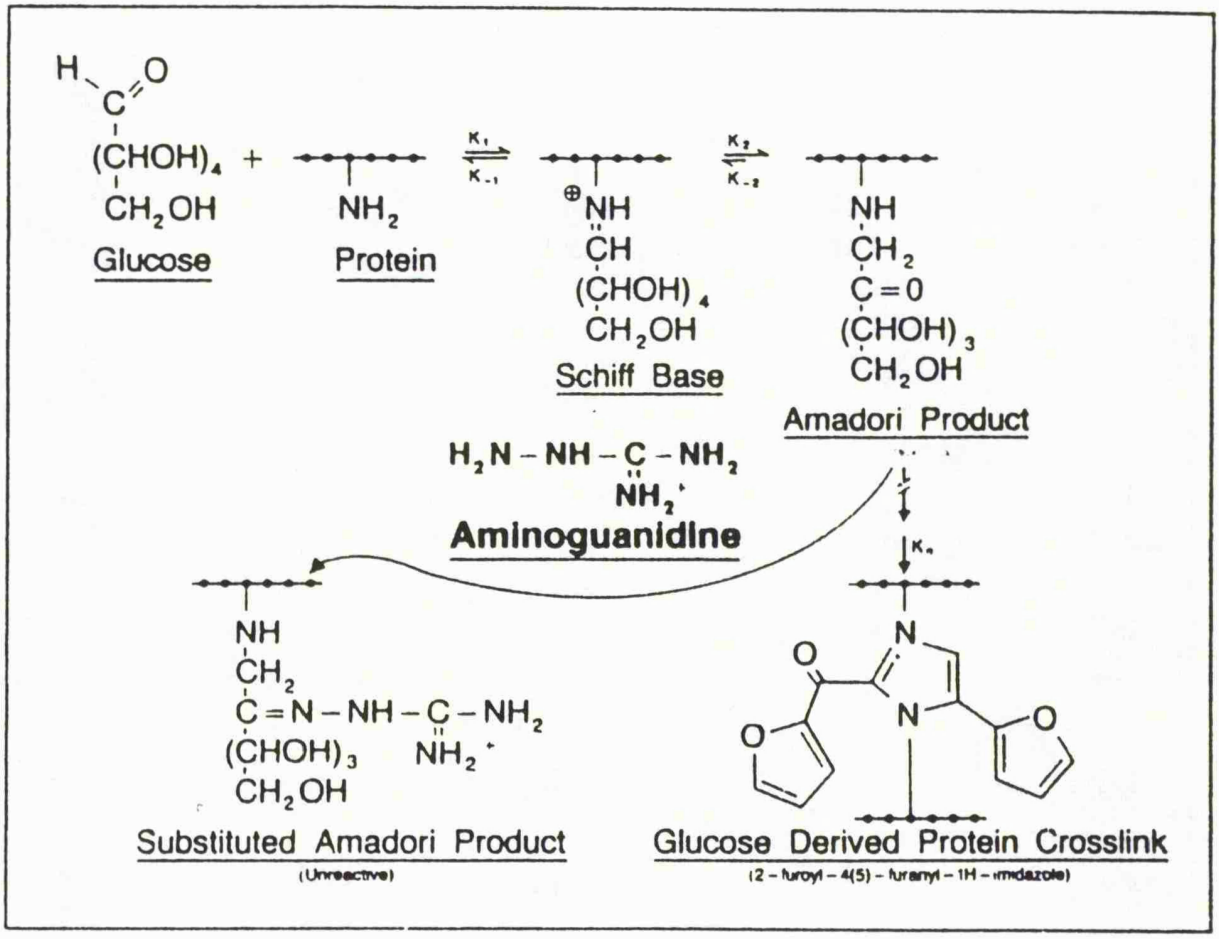
Introduction: In pursuit of a mechanism whereby prolonged hyperglycaemia may contribute to the development of diabetic complications, one major area of research has focused on the glycosylation of proteins. Sugars and proteins can spontaneously interact leading to the non-enzymatic glycation of proteins [29,30]. This is a ubiquitous process because conceivably any primary amino group within any protein has the potential to react with glucose and other sugars to form Schiff bases and subsequently the more stable Amadori product. This spontaneous biochemical sequence represents the *early* phase of the glycation process. At this stage the glycation reaction is reversible and probably has little direct role to play in the pathogenesis of diabetic complications. However, some of the early glycation products develop further. By slowly undergoing a complex series of chemical rearrangements, the early reversible glycation product is converted to a series of intermediate and then advanced glycosylation end products (AGEs) which progressively accumulate in a variety of tissues with time . Unlike the early products of glycation, the AGEs are certainly not benign and their accumulation may play a key role in the development of structural and functional disturbances in tissues exposed to chronic hyperglycaemia in diabetic patients.

"Early" Non-Enzymatic Glycation; Schiff bases and Amadori products: It has now been established that the aldehyde or keto groups of reducing sugars are capable of reacting with amino groups of amino acids or nucleic acids to form Schiff bases. The Schiff bases can then undergo spontaneous rearrangement to form more stable glycosylation products; the Amadori products, named for the chemist who first described this type of chemical rearrangement (figure 1).

This non-enzymatic glycation process is glucose-concentration dependent, reversible and ultimately reaches a steady state plateau, occasionally in hours and invariably within a few weeks [31,32]. The amount of glycosylation on proteins will thus increase when blood glucose levels are high and quickly returns to normal after restoration of the blood glucose level to normal. The absolute level of Amadori product formation, *in vivo*, is thus constantly changing and is reflective of short term glycaemic control. Moreover, as the level of Amadori product formation rapidly reaches a maximum, these early glycosylation products do not continue to accumulate on stable tissue proteins [33,34]. Thus, the level of Amadori products forming in diabetic subjects can only be 2-fold to 3-fold higher than those in the tissues of non-diabetic subjects, even after many years of hyperglycaemia.

Potential Pathological Significance of Amadori Products: Given the broad distribution of the early glycoslyation products, their reversible nature and the fact that Amadori products do not increase with time, it is thought to be unlikely that the Amadori

Figure 1



The generation of Schiff Bases and Amadori product formation. This illustration also indicates how Aminoguanidine can inhibit the formation of advanced glycosylation end-products by binding preferentially to reactive precursors of advanced glycosylation ie. the Amadori products and forms instead a series of unreactive substituted products that can no longer form cross links.

products contribute significantly to diabetic complications. Nevertheless, a number attempts have been made to link these early glucose-mediated post-translational modification of proteins with the development of diabetic complications. For example; the glycosylation of albumin has been shown to markedly influence its transport through the endothelial barrier [35-37]. The glycosylation of low-density lipoprotein (LDL) impairs its removal by tissue fibroblasts and macrophages [38,39], perhaps contributing to the atherogenic process of diabetes. The glycosylation of high-density lipoprotein (HDL) impairs its capability to transport cholesterol through impaired recognition of the HDL receptor [40], further increasing the cholesterol burden on the diabetic patient. Other studies have focused on the impact of glycosylation on the function of proteins within the clotting cascade. Glycated fibrin is less susceptible to plasmin digestion *in vitro* [41] and the activity of antithrombin III is also impaired by *in vitro* glycation [42]. Both of these mechanisms could contribute to a prothrombotic state in diabetes but their significance *in vivo* is unknown. Further studies have demonstrated that the glycation of haemoglobin A [43], erythrocyte and platelet membrane proteins [44], immunoglobulins and complement components [45], can lead to subtle changes in their functional characteristics, the clinical significance of which remains unproven and unclear.

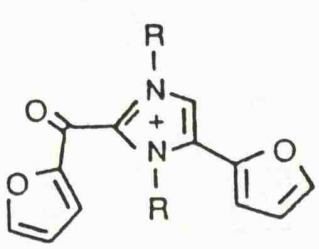
Structural proteins such as lens crystallins, vascular wall collagen and peripheral nerve myelin also undergo glucose modification *in vivo* at a rate which is increased in diabetic patients [46-48]. The amount of glycosylation in these proteins is particularly high due to the accessibility of free amino groups within these proteins and the prolonged half life of the proteins. The study of these particular glycated proteins has been intense because

disturbances to their structure and function is prominent in diabetic patients. Heretofore, however, there is little evidence to support the hypothesis that the "early" products of non-enzymatic glycation are sufficient to produce clinically significant pathological effects. Nevertheless, the Amadori products are not pathologically innocent because they may slowly transform into a variety of advances glycosylation end products which are thought to represent a very important glucose-driven mechanism for tissue injury in diabetes mellitus.

Advanced Glycosylation End Products (AGEs): As indicated above, the Amadori product may undergo a complex series of chemical rearrangements leading to the formation of AGEs [26,31,32]. Details of the structure of AGEs has been somewhat elusive due to their marked heterogeneity and their instability during their isolation from tissues. To date, only a few of what are likely to be many AGEs have been described (figure 2).

Various properties of AGEs have now been defined. These products can form on proteins (extracellular and intracellular), lipids and nucleic acids and they can bind to AGE-specific receptors on a variety of tissues, including; macrophages, vascular smooth muscle and endothelial cells, fibroblasts and lymphocytes [32]. Unlike early glycosylation, the AGE-modification of proteins is irreversible and does not return to normal when glucose levels are normalised. Instead, AGEs continue to accumulate in tissues over the lifetime of the constituent proteins [49-51]. AGE-modified proteins are resistant to proteolytic digestion and they are highly reactive substances which have the capacity to form covalent bonds with amino groups from other proteins leading to their cross-linking

Figure 2



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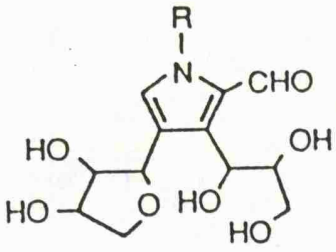
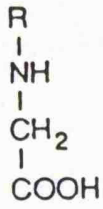


AFGP



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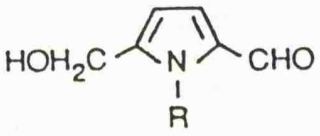
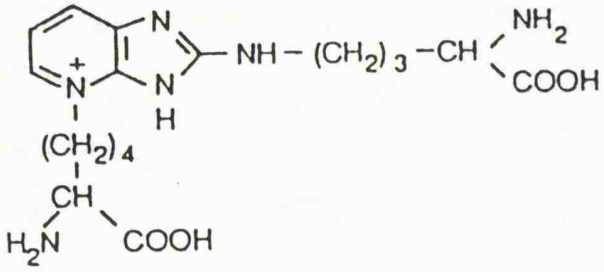


Pyrraline



Pentosidine

The chemical structures of the advanced glycosylation end-products that have been identified to date, they are: 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole, 1-alkyl-2-formyl-3, 4-diglucosyl pyrrole, or N'-carboxymethyl-lysine, lyrraline and pentosidine.

via nucleophilic addition reactions [49-51]. A classic example of such cross-linking has been demonstrated for collagen [52]. Such a long-lived protein is particularly susceptible to the chronic accumulation of AGEs which can promote collagen-collagen cross links leading to increased tissue rigidity. The rate of accumulation of AGEs on blood vessel wall proteins is proportional to the time integrated blood glucose levels over long periods of time. A related property is their capacity to continue the process of cross link formation even in the absence of free glucose [31,32]. The irreversibility and progressive accumulation of AGEs on cellular proteins, lipids and nucleic acids at an accelerated rate in diabetic subjects suggests that this mechanism is much more likely to be relevant to the pathogenesis of diabetic complications than the aforementioned early glycation products.

Potential Pathological Significance of AGEs: Many properties of AGEs have been described *in vitro* and *in vivo* that imply they could play a fundamental role in the pathogenesis of a variety of diabetic complications. I will focus on the evidence pertinent to vascular disease as this is the main focus of this thesis. Increased vascular permeability to circulating macromolecules, endothelial dysfunction and increased vascular matrix deposition and the accelerated development of atherosclerosis are characteristic features of vascular disease in diabetic subjects. Evidence is fast accumulating to support the hypothesis that AGEs may contribute to the development of these abnormalities.

AGEs and vascular matrix: Covalent bonds readily form between reactive AGEs and long-lived vascular matrix proteins and other soluble matrix proteins. Collagen was the first matrix protein to be studied in this context. Collagen forms covalent heat-stable

intermolecular bonds with AGEs and the amount of cross-linked collagen peptides formed increases as a function of time and glucose concentration [31,32,52,53]. Normal collagen cross-links form at two discrete sites at the C-terminal and N-terminal but in the presence of AGEs, multiple cross links form [54]. In one study of diabetic rats, aortic collagen was three times more cross linked than aortic collagen from non-diabetic animals [53]. These changes are sufficient to disturb the structural organisation of collagen and its association with basement membrane components thereby inhibiting the formation of ordered polymeric matrix complexes. Moreover, the AGE-mediated cross links decreases the solubility of proteins and renders them less susceptible to enzymatic digestion [49-51]. These properties are particularly relevant to connective tissue and extracellular matrix collagen as they would be associated with increased thickness and rigidity of the vascular matrix compartment leading to reduced vascular compliance; a characteristic development in diabetic subjects.

AGEs and increased vascular endothelial permeability: Recently, two AGE-binding proteins were identified on endothelial cells. The first was a 35 kDa protein, a member of the immunoglobulin superfamily of receptors and an 80 kDa protein with complete sequence homology to lactoferrin [55,56]. The function of these endothelial cell surface binding proteins is unclear. They do not appear to function as classic scavenger receptors for AGE-modified proteins. Instead, the binding of AGE-modified proteins appears to regulate some important aspects of endothelial function. For example; AGE binding to the endothelium can enhance the procoagulant activity of endothelial cells and directly increase endothelial permeability to circulating macromolecules [57].

The aforementioned AGE-induced changes in the extracellular matrix structure and function may also contribute to endothelial permeability changes. Anionic proteoglycans are markedly reduced in the basement membranes of diabetic subjects [58,59]. The quantity of anionic proteoglycans within the basement membrane is inversely proportional to protein permeability because the electrostatic forces generated by the proteoglycans act to repel the egress of proteins from the circulation. The binding of the anionic proteoglycans to matrix proteins is dramatically reduced when the matrix proteins are AGE-modified [60,61]. This suggests that the integral cross-linking of basement membrane components by AGEs distorts important basement membrane recognition sites for proteoglycans [60,61].

In addition to the aforementioned mechanisms for AGE-induced endothelial dysfunction in diabetic subjects, recent studies also suggest that AGEs may inhibit the actions of nitric oxide (NO). NO is continuously liberated by the endothelium and acts as a direct relaxant of the underlying vascular smooth muscle cells, leading to vasodilatation [62]. There is considerable evidence to suggest that NO release by the endothelium is impaired in diabetic subjects [63,64]. It has been shown that AGE- proteins "quench" the vasodilator actions of NO *in vitro* [65]. Moreover, in studies of experimental diabetes, the administration of the AGE-specific inhibitor aminoguanidine (see below) reversed the diabetes-induced defect in vasodilatation [65]. Further confirmation of the capacity of AGEs to inhibit endothelial modulation of vascular tone was provided by more recent studies examining the effects of the administration of AGE-modified proteins to non-diabetic animals [66]. Exogenous AGE-modified proteins induced marked abnormalities in vascular

relaxation in otherwise healthy normoglycaemic rats and rabbits, an effect that was reversed by the co-administration of aminoguanidine [66].

AGE-induced cross linking of extravasated macromolecules: When endothelial permeability is increased in diabetic subjects, macromolecules such a s albumin, lipoproteins and fibrinogen can escape from the circulation at an increased rate. The extracellular accumulation of such macromolecules is of major pathophysiological significance in the initiation and propagation of atherosclerosis [67]. The development of AGEs could enhance this process via at least two mechanisms. First, by promoting a generalised increase in vascular endothelial permeability as described above. Secondly, by promoting the increased entrapment of macromolecules by vascular matrix proteins in the subendothelial space. Extravasated albumin, lipids and other macromolecules can be entrapped by covalent cross linking to matrix proteins by reactive AGEs. *In vitro,* LDL cross linking to collagen has been shown to increase linearly with the amount of AGE-modified collagen [68]. Moreover, *in vivo*, the amount of lipoprotein cross-linked to aortic collagen in diabetic rats has been reported to be almost three times greater than that observed in non-diabetic animals, despite similar circulating lipoprotein levels [69]. It is now believed by many that the microvasculature of patients with diabetes progressively accumulates lipoproteins and a variety of other plasma proteins via a process identical to that described above. For example, the addition of IgG or albumin to collagen bound AGEs *in vitro*, leads to the irreversible covalent binding of these proteins to collagen[70]. Similar results have been observed using AGE-modified basement membrane *in vitro* [71] inasmuch that the amount of IgG covalently cross-linked to AGE-modified glomerular

basement membranes has been shown to be five times higher in diabetic rats when compared to non-diabetic rats *in vivo*[72].

These observations suggest a mechanism whereby macromolecules implicated in the pathogenesis of atherosclerosis can readily gain access to the subendothelial space and once there, progressively accumulate due to their covalent entrapment by AGE-modified proteins. Furthermore, these covalently bound macromolecules can themselves be modified by AGEs thus providing an expanded focus for further macromolecular entrapment.

Macrophage activation and AGEs: Macrophages express a membrane associated receptor that specifically recognises AGE-modified proteins. Interestingly, proteins modified by the *early* glycosylation (Amadori) process are not recognised by these receptors. The AGE-specific macrophage has been extensively characterised [73-75]. Monocyte-macrophages have long been implicated in tissue maintenance and remodelling as well as in the process of atherosclerosis [67]. It has been suggested that the AGE-specific receptor acts to recognise AGE-modified proteins leading to their internalisation, degradation and thus removal by macrophages. Such a mechanism would allow macrophages to preferentially remove senescent AGE-modified macromolecules [31]. However, when AGE-modified proteins are ingested by macrophages, low levels of cytokines such as tumour necrosis factors α (TNFα), interleukin 1α (IL1α), platelet derived growth factor (PDGF) and insulin-like growth factor-1A (IGF-1A) are liberated by the macrophage [76-78]. These cytokines have all been implicated in the pathogenesis of

the atherosclerotic proliferative lesion and increased vascular matrix deposition. It is conceivable that in diabetes, the gross excess of AGE-modified structural and entrapped proteins induces the over-activation of macrophages and the overproduction of the aforementioned cytokine cocktail. In addition, AGE-modified proteins such as albumin and lipoproteins have been shown to be selectively chemotactic for monocytes thereby promoting the recruitment of macrophages to the vascular wall[76]. Studies *in vitro* have indicated that human monocytes exhibit marked increase in transendothelial migration when the endothelial monolayers are growth on AGE-containing substrates [76]. This observation suggest that enhanced AGE-modification of blood vessel wall proteins could induce the migration of monocytes to the subendothelial space and simultaneously promote their activation thereby generating a substrate for atherogenesis.

Potential Clinical Significance of AGE Accumulation by Diabetic Vascular Tissues:

The aforementioned scientific evidence suggests that the excess accumulation of AGEs in vascular and other tissues of diabetic subjects could contribute to the premature development of vascular disease and associated complications of this condition. AGEs could impact at multiple sites along the chain of events that contribute to; disturbed endothelial function and permeability, abnormal and excessive vascular matrix accumulation, macromolecular entrapment, enhanced monocyte-macrophage recruitment and activation, and finally, the enhanced production of a cytokine cocktail that could further amplify this sequence and promote matrix synthesis, vascular smooth cell and fibroblast proliferation [31,32].

Supportive evidence for such a causal relationship between AGEs and diabetic vascular disease comes from experimental studies in animals using the specific AGE-inhibitor aminoguanidine. Aminoguanidine-HCl is a small hydrazine-like compound [79]. This and related compounds do not inhibit the early glycosylation process ie. Amadori product formation. However, aminoguanidine does inhibit the rearrangements of Amadori products into intermediate and advanced glycosylation end products. Aminoguanidine has a high affinity for Amadori products and promotes the formation of stable complexes with these products which do not react further and do not induce protein cross-linking [80]. This appeared to represent an attractive mechanism to inhibit AGEs formation and subsequent studies in various experimental models of diabetes lend support to this assumption. In studies of diabetic rats, their treatment for four months with aminoguanidine prevented abnormal collagen-to-collagen cross-linking and decreased lipoprotein trapping within the vascular wall [81]. Other studies in diabetic animals have shown similar promising results with regard to the prevention of AGE-mediated basement membrane thickening, collagen structural changes, the prevention of lens protein cross-linking, reversal of renal glomerular lesions and proteinuria, prevention of structural and functional abnormalities in peripheral nerves and retinal vessels [82-88]. Presently, the efficacy of aminoguanidine and like compounds in diabetic humans awaits evaluation.

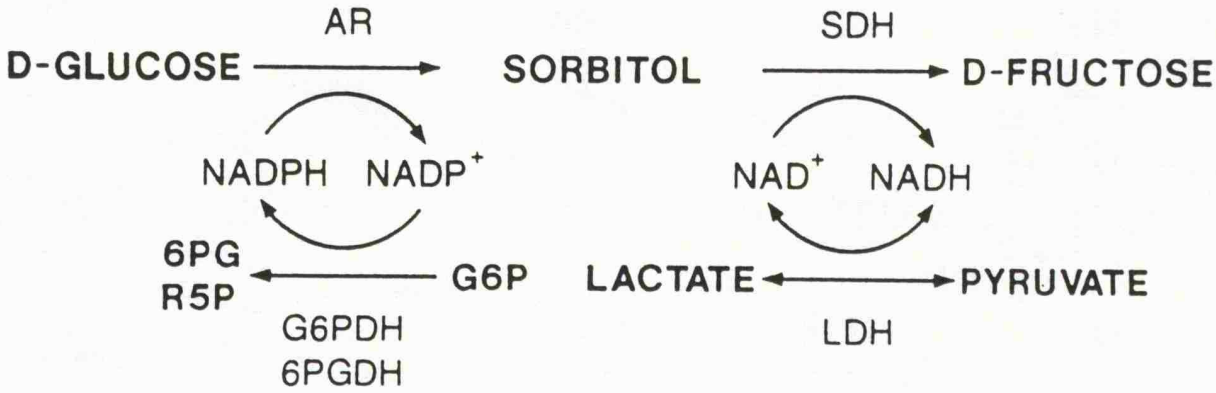
2. The polyol pathway and sorbitol hypothesis.

The polyol pathway and sorbitol hypothesis: After its uptake by various cells, glucose undergoes a variety of metabolic fates. In the presence of hyperglycaemia, cellular glucose uptake is markedly increased and its flux through numerous metabolic pathways is enhanced. For example, under normal physiological conditions, intracellular glucose is predominantly oxidised by glycolysis and the flux of glucose through alternative metabolic pathways is less significant. In contrast, in the presence of hyperglycaemia, the extracellular-intracellular flux of glucose is increased and the excess free intracellular glucose is reduced to sorbitol by the action of aldose reductase [89]. In most tissues, the accumulated sorbitol is then reduced by sorbitol dehydrogenase to fructose (figure 3). This pathway is usually referred to as the "polyol pathway".

The polyol pathway is ubiquitous in mammalian cells but its physiological function is poorly understood. For example, although the polyol pathway is known to be important for the production of seminal fluid fructose within the male reproduction system [90]. In addition, The renal papillary endothelium normally maintains extraordinarily high sorbitol levels as part of its adaptation to its hypertonic environment and its aldose reductase content has been shown to be regulated by the tonicity of the extracellular fluid [91]. The function of the polyol pathway elsewhere has not been established.

The sorbitol hypothesis: Proposed mechanisms of tissue damage: In the last twenty years there has been considerable controversy as to whether the polyol pathway plays a role in the pathogenesis of diabetic complications, particularly the initial enzymatic step

Figure 3.



The polyol pathway. This diagram indicates that glucose is reduced by aldose reductase (AR) to sorbitol which is then oxidised sorbitol dehydrogenase (SDH) to fructose. The reduction of glucose to sorbitol is coupled to the oxidation of NADPH to $NADP^+$. $NADP^+$ is then reduced back to NADPH by the hexose monophosphate pathway. The oxidation of sorbitol to fructose is coupled to the reduction of NAD^+ to NADH. Note that cytosolic ratio of NADH/NAD^+ is in equilibrium with lactate and pyruvate.

in the polyol pathway, notably the reduction of glucose to sorbitol by aldose reductase. It was more than thirty years ago that a British biochemist, Ruth Van Heyningen, first demonstrated an excess of sorbitol in the lenses of diabetic rats [92]. Since then, a substantial accumulation of evidence has been cited to support the hypothesis that increased activity of the polyol pathway, leading to sorbitol accumulation in various tissues, may contribute to the development of diabetic complications in humans and experimental animals [93]. The polyol pathway has been most commonly implicated in the development cataract and neuropathy in diabetic subjects and experimental animals. Two principle mechanisms have been proposed:

1. Intracellular accumulation of sorbitol leading to cell swelling, dysfunction and subsequently irreversible injury.
2. Depletion of cellular myoinositol leading to disturbances in phosphoinositide metabolism, abnormal intracellular signalling and perturbed regulation of key ion transporters such as sodium-potassium-ATPase.

Osmotic effects of intracellular sorbitol accumulation: The first diabetic complication attributed to activation of the polyol pathway and sorbitol accumulation was the development of cataract. It has been proposed that the excess accumulation of sorbitol within the lens of diabetics leads to the influx of water into the lens, driven by the osmotic forces generated by sorbitol accumulation. This could lead to swelling of the lens, dissociation of the lens fibres and the initiation of cataract formation [94-97]. Support for this hypothesis comes from the observation that the development of cataracts in diabetic rats can be delayed, if not prevented by the administration of aldose reductase

inhibitors (ARIs) which prevented sorbitol accumulation within the lens of these animals [93]. However, in humans, the role of the polyol pathway and sorbitol accumulation in cataract formation is less clear cut. Although sorbitol levels are increased in human ocular lenses with cataracts [98], the sorbitol levels are much lower than those observed in animals with experimental diabetes and thus perhaps insufficient to induce osmotic damage [99,100].

The second diabetic complication attributed to the polyol pathway is the development of neuropathy. Sorbitol has been shown to accumulate in the peripheral nerves of diabetic animals [101]. This is associated with a substantial reduction in motor nerve conduction velocity in diabetic animals and a similar abnormality in nerve conduction velocity in diabetic humans. It has been proposed that nerve swelling due to a sorbitol-driven osmotic gradient may contribute to the development of diabetic neuropathy, presently however, there is no substantial experimental evidence to support this hypothetical mechanism. Moreover, extrapolation of the "osmotic hypothesis" to peripheral nerves is problematical, since concentrations of sorbitol in nervous tissue is much lower than that in the ocular lens [102], thus any osmotic effects of the sorbitol are likely to be much less substantial in nervous tissues.

The polyol pathway and cellular myoinositol depletion: Myoinositol is a ubiquitous cyclic hexahydoxyhexanol that is present and concentrated in most animal cells [103]. It has been shown that when some tissues are exposed to hyperglycaemia, the accumulation of sorbitol is accompanied by depletion of intracellular myoinositol levels [104-111]. Furthermore, it was an unexpected finding that ARIs that prevent sorbitol accumulation

in the tissues of diabetic animals also prevent myoinositol depletion [107-109,112]. The mechanism whereby aldose reductase activity influences myoinositol metabolism in diabetes is unknown but the aforementioned evidence suggested that activation of the polyol pathway by hyperglycaemia is essential for myoinositol depletion to occur.

The myoinositol depletion hypothesis has been most extensively explored in peripheral nerves of diabetic rats. The hypothesis proposed that depletion of myoinositol would lead to decreased basal and stimulated phosphoinositide turnover in a variety of tissues due to the fact that myoinositol is required for the synthesis of phosphatidylinositol. Phosphoinositide (PI) turnover is an important intracellular mechanism regulating the activity of sodium-potassium-ATPase (ATPase). Thus, the proposed sequence of events was; 1) hyperglycaemia leading to increased polyol pathway activity, 2) an associated decrease in intracellular myoinositol levels, 3) decreased PI turnover due to the decrease in myoinositol levels (required for PI synthesis), and 4) decreased membrane associated ATPase activity due to a reduction in PI turnover. A decrease in ATPase activity would lead to an increase in intracellular sodium concentration and a decrease in the membrane potential. In nervous tissue, it was proposed that abnormalities in the membrane potential would disturb nerve conduction and repolarisation and thus provide a basis for diabetic neuropathy [113]. Support for the hypothesis came from observations demonstrating that; 1) in some cells myoinositol does play a role in regulating ATPase activity, 2) ATPase activity and myoinositol are decreased in nerves from diabetic rats, 3) the function of normal nerves is impaired when myoinositol is depleted *in vitro*, 4) decreased ATPase activity in the nerves of diabetic rats can be prevented by dietary supplementation of

myoinositol, 5) the use of ARIs in diabetic rats corrects the myoinositol levels in nervous tissue and restores the ATPase activity to normal [113].

Subsequently the hypothesis was extended and it was proposed that the reason why decreased PI metabolism (in response to depleted myoinositol levels) resulted in decreased ATPase activity was that decreased PI turnover would lead to decreased diacylglycerol generation which would in turn reduce the activity level of protein kinase C as the latter in known to phosphorylate and regulate ATPase activity. Support for this hypothesis comes from the observation that direct activation of PKC with the tumour promoter phorbol ester; phorbol myristate acetate (PMA), increased the depressed ATPase activity of nervous tissues from diabetic rats but did not influence ATPase activity in non-diabetic animals [114].

Critique of the polyol pathway and the myoinositol depletion hypothesis: If sorbitol accumulation in various tissues is sufficient to induce tissue dysfunction and subsequently tissue destruction then inhibition of aldose reductase might be expected to prevent the development of a wide variety of diabetic complications. A variety of aldose reductase inhibitors have been produced and tested in diabetic subjects and experimental animals with diabetes. The results of these studies have been conflicting and generally disappointing [115-122]. There is still some optimism that aldose reductase inhibitors may have a role to play in the prevention or amelioration of diabetic neuropathy in man [123]. However, the recent Sorbinil-Retinopathy trial (sorbinil is an orally active aldose reductase inhibitor) showed that aldose reductase inhibition did not prevent the

development of diabetic retinopathy [124,125]. This result may not necessarily mean that sorbitol plays no role in the development of diabetic retinopathy because it is unclear from the study whether the doses of sorbinil used were sufficient to completely inhibit aldose reductase and thus prevent sorbitol accumulation. It is clear that the study of aldose reductase inhibitors in clinical practice has been bedevilled by the fact that few studies have established that the drug used was effective at inhibiting aldose reductase and prevented sorbitol accumulation in target tissues. Moreover, no study has attempted to assess their impact on cardiovascular disease, the major cause of premature mortality in diabetic patients. The aldose reductase controversy lingers on and the stigma of failed, albeit inadequate clinical trials means that it is unlikely to be clarified by further clinical trials. In effect the jury is still out but may never return.

The second and often quoted component of the polyol pathway and sorbitol hypothesis proposes intracellular myoinositol depletion as a precursor of glucose-induced cellular dysfunction and injury. Central to this hypothesis is the concept that increased glucose flux through the polyol pathway leads to depleted tissue stores of myoinositol. Most of the original studies *in vitro* and *in vivo* concentrated on the acute effects of hyperglycaemia on tissue myoinositol levels whereas diabetic complications develop over many years. It was only recently that the more chronic effects of hyperglycaemia on tissue myoinositol levels were evaluated in a model of experimental diabetes in the rabbit [126]. These studies examined glucose, sorbitol and myoinositol levels in the eye, aorta, heart and kidneys of non-diabetic and severely diabetic rabbits after two months. Paradoxically, despite the predictable increases in glucose and sorbitol levels in most of

these tissues, there was no evidence of myoinositol depletion. In contrast, total tissue myoinositol levels were actually higher in the cornea, aqueous humour, cardiac conducting tissues and aorta of the diabetic animals when compared to non-diabetic control animals [126]. Moreover, in patients with chronic diabetes, there appears to be no change in myoinositol levels in nerve biopsy specimens [127]. These results prompt reconsideration of the polyol-myoinositol depletion hypothesis. These studies fail to support the hypothesis that the late complications of diabetes are mediated through depleted tissue myoinositol levels secondary to chronic hyperglycaemia. One possible caveat to salvage this hypothesis has been suggested by its original proponents, notably that there may be discrete, glucose-sensitive, subcellular pools of myoinositol that are depleted by hyperglycaemia, which in turn disturb discrete cellular functions [113]. If this were true, there would have to be a remarkable dissociation between total tissue concentrations of myoinositol and its concentration in the proposed subcellular pools. This remains an unlikely possibility and the prevailing evidence suggests that the generalised myoinositol depletion hypothesis is an inadequate explanation for the development of most of the diverse manifestations of diabetic complications.

The polyol pathway; conclusions: The polyol pathway represents the first detailed attempt to define a biochemical mechanism whereby chronic hyperglycaemia could contribute to the development of diabetic complications. Research in this area has been intense but has rarely focused on vascular biology or disease. Nevertheless, it remains an enduring area of controversy and no overview of the mechanisms of glucose-mediated cellular dysfunction and/or injury would be complete without reference to the polyol

pathway even though its immediate relevance to vascular disease has never been established.

3. The free radical and lipid peroxidation hypothesis.

Introduction: There has recently been considerable interest in the possibility that hyperglycaemia may give rise to increased free radical generation in diabetic patients and that these free radicals may contribute to the accelerated development of macrovascular disease via a variety of mechanisms, including; lipid peroxidation, macrophage dysfunction, endothelial injury and disordered haemostasis.

The generation and biological function of free radicals: Free radical is a term given to an ion atom with a single unpaired electron in the outer shell of the electron orbit. This single unpaired electron confers two properties on free radical ions or atoms; reactivity and paramagnetism. Free radicals are usually short-lived as the presence of a lone electron makes them inherently unstable as the free radicals actively scavenge for free electrons to restore their paired electron status. This is usually achieved by removing an ^{electron} from an adjacent atom. This is the process of oxidation, thus all free radicals have the potential to function as highly reactive oxidants.

Free radicals are readily produced as a normal biological response by neutrophils via a burst of oxidative metabolism which consumes glucose and generates NADPH [128]. This

reduced co-enzyme is a substrate for membrane bound NADPH oxidase which uses NADPH and water to generate a superoxide anion, a free radical species of oxygen. The superoxide anion is a relatively weak oxidant but is capable of undergoing a further series of reactions which generate peroxide and hydroxyl radicals which are highly reactive compounds capable of destroying invading bacteria [129].

It is now well established that free radical production is not confined to phagocytes. Many tissues produce and utilise free radicals in oxidative reactions [130]. Inevitably, complex mechanisms also exist to control free radical generation, thereby preventing oxidative damage to proteins, lipids and other cellular substrates within surrounding tissues. These mechanisms include; strict intracellular compartmentalisation of such reactions ie. within mitochondria, the presence of detoxifying enzyme systems ie. catalase and superoxide dismutase and the presence of non-specific free radical scavengers (antioxidants) such as vitamins C and E, magnesium and glutathione. It is clear that the generation and detoxification of such a complex and reactive biological system is normally finely controlled and that disturbances of this regulatory process at a variety of sites could lead to increased free radical production. To date a number of reports have implicated increased free radical production in the pathogenesis of vascular disease [130,132]. There is also accumulating evidence to suggest that free radical production may be increased in diabetic subjects, perhaps as a direct consequence of hyperglycaemia, thereby suggesting another biological mechanism whereby hyperglycaemia may contribute to vascular dysfunction and disease [132-138].

Free radicals, diabetes and hyperglycaemia: Free radical activity appears to be increased in diabetic patients with or without complications [132-138]. A definitive explanation for this increased free radical activity is currently unavailable but it could reflect increased free radical production, reduced free radical clearance or a combination of both mechanisms.

It has been proposed that free radical production may be increased as a direct consequence of hyperglycaemia: 1. Glucose has the capacity to act as an oxidising agent in the presence of trace amounts of transition metals, generating free radicals, hydrogen peroxide and reactive ketoaldehydes [138,139]. Moreover, the *in vitro* exposure of albumin to high glucose concentrations results in oxidative damage to the protein [139]. Exposing albumin to high glucose concentrations (25mM) in the presence of trace amounts of transition metals resulted in free radical production and a steady increase in protein fragmentation [138]. However, the oxidative ability of glucose is not restricted to proteins because high glucose concentrations can also induce lipid peroxidation [138]. With regard to oxidation of lipid fractions, in diabetic subjects, it has been reported that low density lipoproteins and high density lipoproteins are all vehicles for lipid peroxidation and the low density fraction is particularly susceptible [140]. In human plasma it is clear that the low density lipoprotein fraction is the most susceptible to peroxidation [141] and that this process results in many alterations to their biological properties and greatly enhances their potential to initiate and propagate atherosclerosis [142] (see below). 2. It has also been proposed that the process of non-enzymatic glycosylation (see above) can drive free

radical production because the Amadori product can auto-oxidise [143]. Oxidation of lipoprotein has been demonstrated during *in vitro* glycosylation [138]. It thus seems likely that both free glucose and early glycosylation products are capable of contributing to the oxidative damage of proteins and lipids observed in diabetic patients.

In addition to increased free radical production, there is also evidence that antioxidant capacity is also reduced in diabetic subjects [132-134]. Although this could in part represent increased consumption of antioxidants by the chronic increase in free radical activity, a number of mechanisms have been proposed which could directly decrease antioxidant activity. The first of these also directly implicates hyperglycaemia and links decreased antioxidant activity to the aforementioned polyol pathway activation. The polyol pathway is activated by the increased metabolism of glucose by aldose reductase (see above). The coenzyme for this pathway is nicotinamide-adenine dinucleotide phosphate (NADPH) which would thus be consumed by the increased activity of aldose reductase. As the reduced form of NADPH in essential in cells to regenerate the reduced forms of key antioxidants such as glutathione, increased polyol pathway activity is associated with glutathione depletion. It has been proposed that such a scenario could potentially expose any tissue with evidence increased glucose-driven sorbitol metabolism to increased free radical mediated injury. In this regard, glutathione levels have been shown to be reduced in the plasma, platelets and neutrophils of diabetic subjects [146]. In addition, there is clinical evidence that the levels of other antioxidants such as vitamins C and E and magnesium are also reduced in diabetic subjects [144-148]. It is thus possible to construct a

hypothetical mechanism for increased free radical activity and decreased antioxidant activity in diabetes, at least some of which may be directly dependent on hyperglycaemia.

Mechanisms of free radical-induced vascular injury: It is accepted that the oxidation of lipids to form lipid peroxides has an important role in the pathogenesis of atherocsclerosis. Oxidised LDL but not native LDL is a powerful chemotactic stimuli for macrophages [149] and is readily taken up by these cells via scavenger receptors (as opposed to the normal apoprotein receptor [150,151]. Unlike apoprotein receptor-mediated LDL uptake by monocytes and macrophages, LDL uptake by scavenger receptors is not regulated [152,155] thereby culminating in the progressive accumulation of oxidised LDL by monocytes or macrophages, promoting their transformation into foam cells, a characteristics pathological feature of the early atherosclerotic lesion [155]. Lipid peroxides are also directly cytotoxic to the vascular endothelium [153-156]. This promotes increased endothelial permeability and the release of a powerful cocktail of cytokines such as endothelin, platelet derived growth factor and tumour necrosis factor [142,155]. Lipid peroxides are also capable of inhibiting antithrombin III activity [157], producing procoagulant activity [158] and enhancing platelet aggregation all of which could contribute to the development of intravascular thrombosis and vascular occlusion. Furthermore, lipid peroxides directly inhibit endothelial production of vasodilatory prostaglandins ie. prostacyclin or PGI_2, while promoting the increased synthesis of the vasoconstrictor prostanoid, thromboxane A_2. This imbalance could contribute to local vasoconstriction and thrombosis [142].

Clinical support for the involvement free radical mediated lipid peroxidation in atherosclerosis comes from the observation that lipid peroxides are high in patients with atherosclerosis and in diabetic patients [131]. In addition, fluorescent proteins, products of oxidative damage have been shown to be increased in patients with microangiopathic complications [159]. Moreover, it is likely that hyperglycaemia is playing a fundamental role in free radical production because the structural and functional modification of LDL as a consequence of lipid peroxidation in diabetic patients *in vivo*, can be reproduced by exposing LDL to high glucose concentrations *in vitro* [138,141]. These observation are important because they imply that even if the LDL levels are not elevated in diabetic subjects, their increased peroxidation as a consequence of hyperglycaemia would transform LDL into a much more potent atherogenic moiety [142].

Hyperglycaemia, free radicals and diabetic complications; conclusions: The aforementioned studies suggest that free radical production is increased in diabetic subjects as a consequence of a direct increase in their generation and decreased elimination of free radicals due to depressed antioxidant activity. There is also data to support the concept that the net increase in production of free radicals is, at least in part, dependent on glucose-mediated mechanisms such as; direct glucose-mediated auto-oxidation of proteins and lipids, free radical production as a by-product of non-enzymatic glycosylation, and decreased anti-oxidant activity (reduced glutathione synthesis) resulting from enhanced NADPH consumption secondary to an increase in polyol pathway activity. There is no doubt that the oxidation of proteins and lipids modifies

their biological structure and function in such a way that they could play a key role in the accelerated pathogenesis of vascular disease and other complications of diabetes mellitus.

4. The hyperglycaemic pseudohypoxia hypothesis.

Introduction: One of the latest hypotheses put forward to explain the development of glucose-induced cellular dysfunction focuses on the possibility that elevated glucose concentrations may generate a state of pseudohypoxia [160]. This hypothesis is founded on the observation that many of the functional vascular changes observed in diabetic subjects, particularly in the earlier stages of their metabolic disturbance, resemble those seen in hypoxia [161-164]. Cellular hypoxia is associated with an increase in the ratio of NADH to NAD^+ because of the impaired oxygenation of NADH to NAD^+. A similar imbalance in the cytosolic NAD to NAD^+ ratio has also been documented in tissues exposed to elevated glucose levels *in vitro* and *in vivo*, in the presence of normal oxygen availability [165-171]. It has thus been proposed that this glucose-derived "pseudohypoxia" may account for many aspects of glucose-induced cellular dysfunction [160].

Mechanisms of Glucose-induced Pseudohypoxia: Two mechanisms have been proposed to account for the development of cellular pseudohypoxia in the presence of high glucose concentrations. The first implicates the polyol pathway (see above), the second suggest that increased glycolysis may be sufficient to generate a state of pseudohypoxia.

Increased metabolism of glucose via the polyol pathway may be the most important mechanism to account for an increase in cytosolic NADH/NAD^+ (see figure 3). The increased generation of sorbitol via this pathway leads to the increased oxidation of sorbitol to fructose by sorbitol dehydrogenase, a reaction which is coupled to the reduction of NAD^+ to NADH. Support for this hypothesis comes from the observation that aldose reductase inhibitors, which reduce the glucose-derived increase in sorbitol production, also prevent the glucose-induced redox imbalance in a variety of tissues [165,167,169,171]. Moreover, exposing human erythrocytes to elevated sorbitol levels (in the presence of normal glucose concentrations) *in vitro*, has been shown to markedly increase cytosolic NADH/NAD^+ [172]. Furthermore, more recent studies have shown that a recently developed sorbitol dehydrogenase inhibitor was able to reduce the diabetes-induced increase in the NADH/NAD^+ ratio in the retinal tissues of diabetic rats [173]. Together, these observations suggest that the glucose-induced redox imbalances are at least in part, due to increased metabolism of glucose via the polyol pathway and in particular, the increased rate of oxidation of sorbitol to fructose.

The second mechanism whereby hyperglycaemia could induce a redox imbalance mimicking that generated by hypoxia, involves increased glycolysis. An increased rate of glycolysis can alter the cellular NADH/NAD^+ ratio. The mechanism is thought to result from a dysequilibrium between the rate of oxidation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate and the rate of reduction of pyruvate to lactate [174,175] but the full details of the underlying mechanism are incompletely understood.

Consequences of an increased cytosolic NADH/NAD$^+$ ratio: A glucose-driven increase in NADH/NAD$^+$ could disturb the activity of a variety of cytosolic and mitochondrial enzymes that use NADH and NAD$^+$ as cofactors and/or are inhibited by changes in the redox state. Various metabolic consequences of an increase in cytosolic NADH/NAD$^+$ have been demonstrated:

Increased de novo synthesis of diacylglycerol: An increased cytosolic NADH/NAD$^+$ ratio favours the increased de-novo synthesis of diacylglycerol (DAG) by two mechanisms [176]. Both of these mechanisms depend on the fact that the increased NADH/NAD$^+$ ratio shifts the equilibrium of reactions in the DAG synthetic pathway in favour of increased DAG synthesis (figure 8). DAG is an endogenous activator of protein kinase C. Protein kinase C activation by glucose is the main focus of this thesis and the subsequent chapters of this thesis will reveal how the activation of this kinase system has been demonstrated in a number of tissues and cell types in diabetes and may be an important final common path for glucose-induced cellular dysfunction.

Effect of increased cellular NADH/NAD$^+$ on free radical production: The potential importance of changes in free radical production or their decreased clearance in the development of diabetic complications has been discussed above. Superoxide ions are the normal by-product of oxidation of NADH to NAD$^+$ by the electron transport chain in mitochondria [177,178]. NADH has been reported to increase superoxide ion production by cultured fibroblasts exposed to interleukin-1 [179] via mechanisms which are presently

unclear. Nevertheless this represents another mechanism whereby the cellular changes mimicking pseudohypoxia and induced by elevated glucose could contribute to cellular injury in diabetic subjects.

Potential clinical consequences of increased cytosolic NADH/NAD$^+$ ratio in diabetic subjects: The aforementioned hypothesis proposes that hyperglycaemia has the capacity to induce a state of pseudohypoxia. One attractive feature of this hypothesis is that is provides a simple mechanism whereby elevated glucose concentrations could induce profound disturbances in cell function. Moreover, it suggests a cellular basis for the observation that the tissues of diabetic subjects are particularly susceptible to injury in the presence of hypoxia or ischaemia. It has been proposed that cells with an established redox imbalance due to hyperglycaemic pseudohypoxia would require less severe hypoxia or ischaemia to further increase the cellular NADH/NAD$^+$ ratio to the same injurious level caused by hypoxia or ischaemia alone [160]. Finally, the pseudohypoxia hypothesis also illustrates how the various mechanisms that have been proposed to account for glucose-induced cellular dysfunction are not necessarily mutually exclusive. On the contrary, the pseudohypoxia hypothesis demonstrates how a simple disturbance to cellular metabolism could link two of the aforementioned alternative hypotheses for glucose-induced cellular injury, notably; involvement of the polyol pathway and increased free radical production. Moreover, the pseudohypoxia hypothesis also suggests a mechanism to account for increased DAG synthesis and protein kinase C activation in the presence of hyperglycaemia. This latter suggestion has considerable relevance to this thesis in which I will characterise the activation of protein kinase C in vascular smooth

muscle cells and glomerular mesangial cells *in vitro* and demonstrate how this may contribute to cellular dysfunction in diabetes mellitus.

This review has illustrated the various hypotheses that have implicated hyperglycaemia in the pathogenesis of cell injury. Many of these mechanisms interact and overlap with each other, supporting the view that no single mechanism is dominant. Many of the previous studies have focused on microvascular injury, and in particular neuropathy and retinopathy. Few studies have directly assessed the effects of elevated glucose concentrations on vascular cells. This is a significant omission, bearing in mind that various manifestations of vascular disease is the major cause of premature morbidity and mortality in diabetic patients.

In 1989, I began studying intracellular signalling mechanisms in vascular smooth muscle cells, *in vitro*, and became aware of the pivotal role played by protein kinase C in co-ordinating so many different aspects of cell function. Simultaneously, I had noted a couple of abstracts from scientific congresses suggesting that protein kinase C activity was elevated in various tissues from diabetic rats. I thus decided to examine whether an elevated glucose concentration *per se,* was capable of activating protein kinase C. I elected to focus these studies on the vascular smooth muscle cell because of the importance of protein kinase C in regulating the function of this cell and the importance of vascular cell dysfunction in the pathogenesis of diabetic complications. My work in this area forms the major part of the published works presented in this thesis and the

study of glucose-induced protein kinase C activation in a variety of cell types has become an area of intense scientific interest.

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Chapter 2.

Characterisation of Glucose-induced In-Situ Protein Kinase C Activity in Cultured Human Vascular Smooth Muscle Cells.

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Characterization of Glucose-Induced In Situ Protein Kinase C Activity in Cultured Vascular Smooth Muscle Cells

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The VSMC is an important target for the injurious effects of hyperglycemia in vivo. PKC plays a key role in the regulation of VSMC contraction and growth. This study examines whether elevated extracellular glucose concentrations (10–30 mM [180–540 mg/dl]) activate PKC in cultured rat VSMCs in vitro. A new, rapid, and highly specific assay was used to determine in situ PKC activity in digitonin-permeabilized VSMCs. PKC activity in VSMCs responded rapidly to variations in extracellular glucose concentrations. PKC was activated significantly within 10 min of exposure to D-glucose (20 mM) versus glucose (5 mM). Moreover, with continued exposure to D-glucose (20 mM), PKC activation was sustained for up to 48 h. Reducing D-glucose concentrations to 5 mM restored PKC activity to control values within 1 h. PKC activation was also glucose-concentration dependent. A threshold of only 15 mM (270 mg/dl) was required to significantly and maximally activate PKC in VSMC. PKC was not activated in the presence of osmotic control media that contained either elevated mannitol or L-glucose concentrations. In marked contrast to the sustained PKC activation induced by D-glucose in VSMCs, the normal physiological PKC response to the pressor hormones, AII and AVP, was short-lived and returned to base line within minutes. Sustained PKC activation in the presence of elevated D-glucose concentrations in vitro could disturb the normal physiological regulation of VSMC function and growth and thereby may contribute to the apparent vasotoxicity of hyperglycemia in vivo. *Diabetes* 41:1464–72, 1992

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VSMC, vascular smooth muscle cell; PKC, protein kinase C; AII, angiotensin II; AVP, arginine vasopressin; DAG, diacylglycerol; MEM, minimum essential medium; FCS, fetal calf serum; cpm, counts/min; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; PMA, phorbol 12-myristate 13-acetate; diC_8, *sn*-1,2-dioctanoylglycerol; ANOVA, analysis of variance; H-7, 1-[5-isoquinolinesulfonyl]-2-methylpiperazine, di-HCl.

Long-standing diabetes mellitus is characterized by the development of widespread micro- and macroangiopathy (1–3), and hyperglycemia has been implicated as a risk factor for this vascular injury (3–7). The cellular mechanisms by which hyperglycemia may predispose to vascular injury, however, remain to be defined.

PKC is a serine-threonine protein kinase that is relatively abundant in vascular tissue and plays an important role in the regulation of VSMC growth and contraction (8–13). In view of the importance of PKC in regulating vascular function, and the clinical and experimental evidence of vascular dysfunction in diabetes mellitus, it is intriguing that recent studies suggest that PKC is activated in a variety of tissues from the diabetic rat (14–16). In vitro studies indicate that PKC also is activated in cultured retinal endothelial cells (14,17) and isolated glomeruli in vitro (15) after exposure to elevated extracellular glucose concentrations, thus suggesting an effect of hyperglycemia as an important determinant of PKC activation. This concept is supported by in vitro studies that demonstrate that elevated extracellular glucose concentrations increase the de novo synthesis of DAG, which in turn directly promotes the activation of PKC by increasing the affinity of the kinase for Ca^{2+} (14,15).

It is important to note, however, that in the aforementioned studies, estimates of cellular PKC activity have been based exclusively on the observation that the inactive, loosely membrane-bound form of PKC fractionates with the cytosol, whereas the activated form of PKC remains tightly associated with the membrane (18,19). Thus, although PKC translocation from cytosol to membrane has been demonstrated in some tissues in the presence of elevated extracellular glucose concentrations, whether this corresponds to in situ activation of PKC has never been determined. Moreover, assays

based on the measurement of apparent PKC translocation can be susceptible to homogenization artifacts. In particular, the affinity of the plasma membrane for PKC may differ markedly, depending on whether the enzyme is activated by hormones, phorbol esters, membrane-permeable diglycerides, or endogenous DAG (20). This consideration may be particularly relevant to the diabetic state, which is known to induce changes in membrane biochemistry (21) that could contribute further to cell-fractionation artifacts. For these reasons, the assumption that the magnitude and temporal characteristics of PKC translocation always correspond directly to in situ PKC activity may be unwarranted. Another concern is that the H-1 histone substrate routinely used to assay the subcellular redistribution of PKC is not specific for PKC and can be phosphorylated by many other kinase systems that may be activated simultaneously (22). Furthermore, basic proteins such as H-1 histone have been shown to influence directly the kinetic properties of PKC (23).

In an effort to circumvent some of these limitations for determining PKC activity, Heasely and Johnson (24,25) recently used a highly specific peptide substrate for PKC in digitonin-permeabilized, cultured PC12 pheochromocytoma cells to monitor in situ PKC activation in response to growth factors. This novel experimental approach has several advantages over the translocation protocols. These advantages include the ease and specificity of the assay and the ability to perform rapid and accurate time courses. Most importantly, it provides a direct, in situ, and highly specific measurement of PKC activity, independent of the need for cell homogenization and fractionation.

In view of the potential pathophysiological significance of glucose-induced PKC activity in vascular tissue, it is important that the existing data demonstrating glucose-induced subcellular PKC distribution are complemented by studies confirming that this phenomenon actually is associated with an increase in glucose-induced in situ PKC activity. To achieve this aim, we adapted the method of Heasely and Johnson (24,25) to provide the first direct measurements of the effects of elevated extracellular glucose concentrations on in situ PKC activity in cultured VSMCs. Moreover, to gauge the potential pathophysiological importance of glucose-induced PKC activation, the same method has been used to examine the temporal characteristics, glucose concentration dependency, and potency of glucose-induced PKC activation compared with the in situ PKC response to physiological and pharmacological activators of PKC in VSMCs.

RESEARCH DESIGN AND METHODS

Preparation of cultured rat VSMC. Aortic VSMCs were isolated from the Sprague Dawley rats and cultured as described previously (11,12,26). VSMCs were grown in MEM (Gibco, Grand Island, NY), supplemented with 2 mM L-glutamine, 2 g/L $NaHCO_3$, 60 mg/L penicillin, 135 mg/L streptomycin, and 10% FCS in an incubator at 37°C in 95% humidified O_2 and 5% CO_2. Every 5–10 days, the cells were passaged after trypsin EDTA harvesting. For all experiments, 2nd through 4th passaged VSMCs were used.

Assay of glucose-induced PKC activity in permeabilized, cultured VSMC. For the in situ measurement of PKC activity in VSMC, the method described by Heasley and Johnson (24,25) in cultured PC12 cells was adapted for use in VSMCs. VSMCs were seeded into 96-well flat-bottomed microtiter plates at a density of 20,000 cells/well. VSMCs were maintained in regular growth medium for 1–3 days until confluent. The growth medium then was aspirated, and the VSMCs were rinsed with 200 μl of Hank's balanced salt solution before being incubated for varying time periods with one of four test media: *1*) Control medium—Regular MEM containing 0.5% FCS and a normal glucose concentration of 5 mM (90 mg/dl); *2*) High-glucose medium—Similar to the control medium, except that it was supplemented with D-glucose to increase the glucose concentration from 10 to 30 mM (180–540 mg/dl); *3*) Mannitol osmotic control medium—Similar to control medium but supplemented with the relatively impermeable hexose 20 mM (360 mg/dl) mannitol, an osmotic control for the high-glucose medium; *4*) L-glucose control medium—Similar to control medium but supplemented with the permeable but poorly metabolized hexose 20 mM (360 mg/dl) L-glucose.

To permeabilize the VSMCs and initiate the PKC assay, the test medium was aspirated and replaced with 40 μl of a buffered salt solution containing 137 mM NaCL, 5.4 mM KCl, 10 mM magnesium chloride, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 25 mM β-glycerophosphate, 5.5 mM D-glucose, 5 mM EGTA, 1 mM $CaCl_2$ (this combination yields ~100 nM Ca^{2+} and thus is designed to provide a fixed and stable Ca^{2+} concentration, approximating the normal basal-free intracellular Ca^{2+} concentration), 100 μM [γ-^{32}P]ATP (~5000 cpm/pmol), 50 μg/ml digitonin, and 20 mM HEPES (pH 7.2, 30°C). In addition, a 100 μM PKC-specific peptide substrate (VRKRTLRRL) was added to the buffer. This short synthetic peptide is based on the sequence surrounding a major PKC phosphorylation site within the epidermal growth factor receptor (27). This peptide substrate is not phosphorylated by cyclic nucleotide-dependent or Ca^{2+}/calmodulin-dependent protein kinases or by S6 kinase and has been characterized extensively to be a highly specific substrate for PKC in cultured PC12 cells (24,25). By permeabilizing VSMCs with digitonin, the VRKRTLRRL peptide enters VSMCs with [γ-^{32}P]ATP to allow a selective and rapid analysis of in situ PKC activity. The kinase reaction proceeds for 10 min at 30°C before termination by the addition of 10 μl of 25% (wt/vol) TCA (final TCA concentration, 5%). Aliquots (45 μl) of the acidified reaction mixture are spotted onto 2-cm phosphocellulose circles (Whatman P-81) and washed batches; 3 washes with 75 mM phosphoric acid and 1 wash with 75 mM sodium phosphate (pH 7.5; 500 ml/wash for 2 min). Because of the basicity of the VRKRTLRRL substrate, it is retained by the phosphocellulose filter at neutral pH, whereas contaminating free [γ-^{32}P]ATP is removed, reducing the assay blanks. The PKC-dependent phosphorylation of the peptide substrate bound to the filter was quantified by scintillation counting with a Packard β-counter (Downers Grove, IL). VSMC protein per well was solubilized in 0.1% SDS, 0.1N NaOH and

quantified by using a modification of the method of Lowry et al. (28). The background phosphorylation was assessed in the following two ways. First, to assess background phosphorylation of the VRKRTLRRL peptide substrate, immediately before adding the reaction buffer, 40 μl of 10% ice-cold TCA was added to the cell monolayer for 10 min to precipitate cell protein and eliminate the possibility of kinase activity. TCA was then aspirated, the reaction buffer added, and the assay of kinase activity allowed to proceed in the usual manner to determine kinase-independent phosphorylation of the VRKRTLRRL peptide. Second, to determine whether there was a significant kinase-dependent background phosphorylation of cell proteins other than the VRKRTL RRL substrate, the assay was performed as usual except that the VRKRTLRRL substrate was eliminated from the reaction buffer. These two controls for background phosphorylation were always <0.05% of added cpm and were not different after exposing VSMC to various glucose concentrations, 10^{-6} M PMA, H-7, or after PKC down-regulation after prolonged exposure to PMA. Taken together, these data suggest that the low level of background phosphorylation detected by this assay is constant, not PKC dependent, truly nonspecific, and thus does not influence the interpretation of the final result. Results are expressed as PKC-dependent peptide phosphorylation, pmol · min^{-1} · mg^{-1} VSMC protein.

Assay of effector-induced PKC activity in permeabilized, cultured VSMC. To examine the effects of AVP, AII, PMA, and diC_8 on PKC activity, the control medium was aspirated and the VSMCs were exposed to fresh control medium, supplemented with the effectors. At the end of the desired exposure time, the effector was aspirated and the VSMCs were permeabilized with the reaction buffer, thereby providing an instantaneous assay of effector-stimulated in situ PKC activity.

Statistical analysis. Results are expressed as mean ± SE. An unpaired Student's *t* test or ANOVA with Bonferroni correction was used for statistical analysis. Each assay was performed in quadruplicate, and the mean of these four results represents a single experiment or an *n* value of 1. Results are expressed as PKC-dependent peptide phosphorylation, pmol · min^{-1} · mg^{-1} VSMC protein. A *P* value of <0.05 was considered significant.

AVP, AII, PMA, and DiC_8 were purchased from Sigma (St. Louis, MO), H-7 was purchased from Calbiochem (La Jolla, CA), and [γ-^{32}P]ATP was purchased from Amersham (Arlington Heights, IL). VRKRTLRRL, the PKC-specific synthetic peptide substrate, was a generous gift from Lynn E. Heasley and Gary L. Johnson, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO.

RESULTS

Determining the optimal conditions for the assay of in situ PKC activity in permeabilized VSMC cells. To assay the PKC-dependent phosphorylation of the synthetic peptide substrate VRKRTLRRL, VSMCs were permeabilized with digitonin. Preliminary investigations were performed to define the optimal digitonin concentration required to permeabilize adequately VSMCs without producing excessive membrane damage that would depress PKC activity. VSMCs were stimulated with PMA (10^{-7} M) for 10 min and then permeabilized with different digitonin concentrations (Fig. 1). In the absence of digitonin, no detectable phosphorylation of the VRKRTLRRL peptide above background was present, demonstrating that cell permeabilization was necessary to allow the peptide substrate access to PKC. In the presence of digitonin (50 μg/ml), a highly significant increase in peptide phosphorylation occurred in response to PMA, indicative of in situ PKC activity. Digitonin (50 μg/ml) did not modify VSMC morphology or promote cell detachment. Moreover, with this digitonin concentration, PMA-induced PKC activity was retained by the cell monolayer postpermeabilization (PKC activity in the supernatant postpermeabilization was <10% total PKC activity). This latter finding is consistent with the concept that the PKC activity being measured by this assay is tightly associated with the cell membrane, as has been proposed for the active form of PKC (8,9,18,19). Similar results were obtained with a digitonin concentration of 100 μg/ml. However, when the digitonin concentration was increased to 200 μg/ml, peptide phosphorylation diminished. This presumably represents excessive digitonin-induced membrane disruption, resulting in a loss of membrane-associated PKC activity. Based on these observations, digitonin (50 μg/ml) was used to permeabilize VSMCs for subsequent experiments.

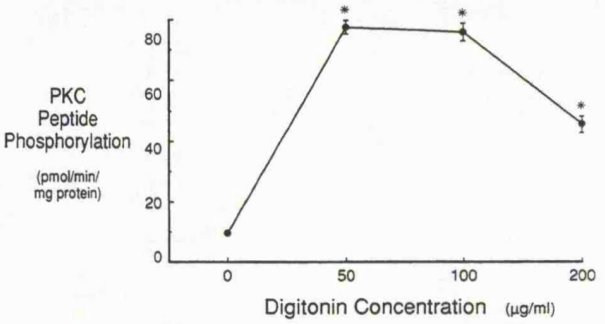

FIG. 1. Dose-dependent digitonin permeabilization of VSMCs. VRKRTLRRL peptide gained access to PMA-stimulated (10^{-7} M) PKC activity after permeabilizing VSMCs with different digitonin concentrations. VSMCs were exposed to the VRKRTLRRL substrate for 10 min. *$P < 0.01$ vs. background VRKRTLRRL phosphorylation in the absence of VSMC permeabilization, $n = 3$.

Next, the optimal time for the kinase reaction was defined. VSMCs grown in culture medium containing 5 mM glucose were exposed to PMA (10^{-7} M) for 10 min and then permeabilized (digitonin, 50 μg/ml). Figure 2 shows that the kinase reaction was linear with time for up to 20 min at 30°C. A similar linear curve (data not shown) was obtained in further experiments by using VSMCs that had been preexposed for 48 h to medium containing 20 mM glucose. This suggests that changing the glucose concentration per se did not influence the specific activity of [γ-^{32}P]ATP, and that it was appropriate to use a kinase reaction time of 10 min for subsequent experiments, irrespective of the glucose concentration.

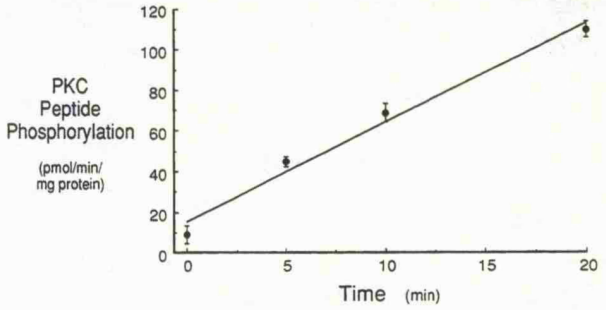

FIG. 2. Time course of VRKRTLRRL phosphorylation in permeabilized VSMCs after exposure to PMA (10^{-7} M). PKC-dependent phosphorylation of VRKRTLRRL substrate in VSMCs was linear with time for up to 20 min after previous exposure to PMA and permeabilization of VSMCs with digitonin (50 μg/ml), $r = 0.975$, $P < 0.002$, $n = 2$.

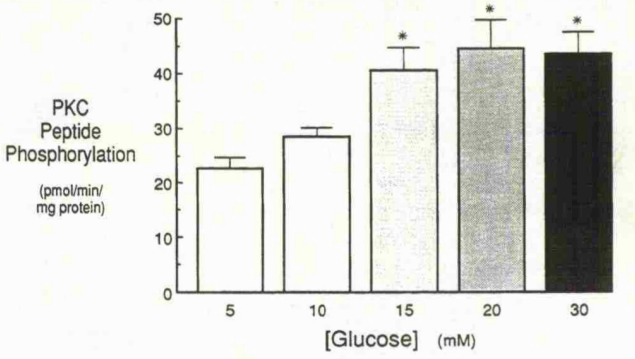

FIG. 4. Glucose concentration dependency of PKC activation in VSMCs. VSMCs were exposed to culture medium containing different D-glucose concentrations (5–30 mM) for 48 h before measurement of in situ PKC activity. *$P < 0.05$ vs. glucose (5 mM), $n = 3$.

Time dependency of glucose-induced PKC activation in VSMC. Having defined the optimal conditions for the direct assay of in situ PKC activity in cultured VSMCs, we used this strategy to assay glucose-induced PKC activity. VSMCs were exposed to either control medium (D-glucose, 5 mM) or a high-glucose medium (D-glucose, 20 mM) for various time periods before determining in situ PKC activity (Fig. 3). PKC activity in the presence of control medium remained constant throughout. A significantly greater activation of PKC was observed within 10 min of exposure to the high-glucose medium. Importantly, the increase in PKC activity was sustained and did not downregulate for up to 48 h of continued exposure to elevated D-glucose concentrations. The PKC assay was performed with 96-well microtiter plates. After 48 h in culture, confluent VSMCs occupying the small surface area of these plates tended to overgrow and detach. For this reason, the time-course studies were not extended beyond 48 h.

Glucose concentration dependency of PKC activation in VSMC. The next study defined the concentration of D-glucose required both to initiate PKC activation and to maximally activate PKC in VSMCs. VSMCs were exposed to culture medium containing various D-glucose concentrations for 48 h before we measured PKC activity. PKC was activated significantly in the presence of an extracellular glucose concentration of only 15 mM and maximally activated when the D-glucose concentration reached 20 mM compared with 5 mM (Fig. 4). In additional experiments, exposing VSMCs to PMA (10^{-6} M) for 10 min after a 48-h preexposure to a high D-glucose medium (20 mM) did not influence the maximal PKC response seen with PMA (10^{-6} M) alone (PMA-stimulated PKC activity after D-glucose exposure: 118.0 ± 11.2 pmol · min^{-1} · mg^{-1} protein vs. without D-glucose preexposure: 109.0 ± 9.6 pmol · min^{-1} · mg^{-1} protein, NS, $n = 3$). This suggests that glucose activates a subspecies of PKC that is also activated by PMA.

Recovery of glucose-induced PKC activation in VSMCs. The next study examined the rate of PKC inactivation once extracellular glucose concentrations were restored to normal. VSMCs were exposed to a high D-glucose (20 mM) culture medium for 48 h to activate PKC. The culture medium then was changed to control medium (glucose, 5 mM) to determine the time required

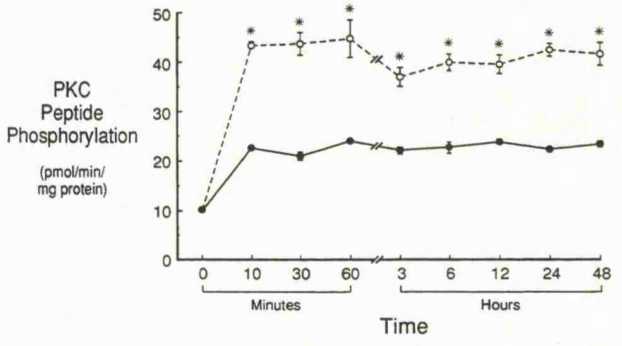

FIG. 3. Time course of glucose-induced PKC activity in VSMCs. VSMCs were exposed to control medium (glucose, 5 mM; *closed circles*) or a high-glucose medium (20 mM; *open circles*) for as long as 48 h before measurement of in situ PKC activity. PKC activity in VSMCs increased in presence of high-glucose medium within 10 min and remained activated with continued exposure to high-glucose medium. *$P < 0.01$ vs. glucose (5 mM), $n = 3$.

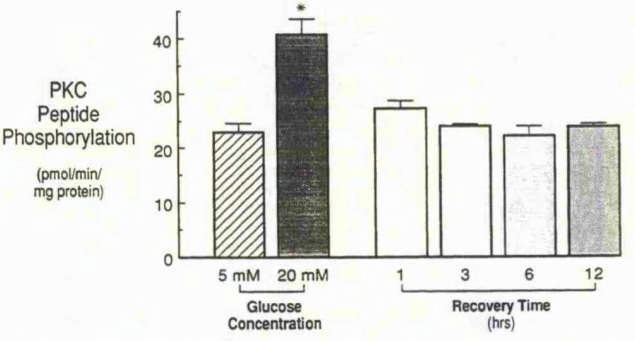

FIG. 5. Recovery of PKC activity in VSMC after previous exposure to high-glucose medium. VSMCs were exposed to either 5 or 20 mM glucose for 48 h. PKC was activated significantly in presence of 20 versus 5 mM glucose, $P < 0.05$. High-glucose medium then was replaced with medium containing 5 mM glucose to measure the rate at which PKC activity returned to values equivalent to those measured in VSMCs exposed to 5 mM glucose. Recovery of PKC activity to control values occurred within 1 h, $n = 3$.

TABLE 1
Confluent VSMCs were exposed to culture media containing either 0.5 or 10% FCS for 48 h before measurement of in situ PKC activity

Glucose concentration (mM)	PKC activity (pmol · min^{-1} · mg^{-1} VSMC protein)	
	0.5% FCS	10% FCS
5	22.8 ± 2.2	33.3 ± 4.3
10	29.7 ± 2.9	39.5 ± 3.1
15	38.8 ± 3.8*	45.7 ± 3.5
10	43.1 ± 3.8*	53.2 ± 4.0*
30	41.9 ± 4.5*	55.7 ± 4.7*

In the presence of either FCS concentration, elevating the extracellular D-glucose concentration for 48 h resulted in sustained activation of in situ PKC activity in VSMCs (mean ± SE). For each glucose concentration, differences were not in significant PKC activity between 0.5% and 10% FCS (unpaired Student's *t* test).
*$P < 0.05$ vs. 5 mM glucose at similar FCS concentration (ANOVA with Bonferroni correction, $n = 4$).

to restore PKC activity to control values. Figure 5 shows that PKC activity was restored to control values within 1 h of normalizing the extracellular glucose concentration. The rapid reversibility of the glucose-induced effect on PKC activity excludes the possibility that elevated extracellular glucose concentrations activated PKC as a consequence of VSMC damage. In support of this conclusion, additional morphological and biochemical studies confirm that 48-h exposure to culture medium containing up to 30 mM D-glucose does not impair VSMC viability (data not shown).

Effects of 10% FCS on glucose-induced PKC activity in VSMC. After demonstrating that elevated extracellular glucose concentrations induce a significant activation of in situ PKC activity in VSMC in the presence of 0.5% FCS, it was important to determine whether similar results could be obtained with VSMCs that had been exposed continuously to 10% FCS. Ten percent FCS is the concentration routinely used to encourage active growth of VSMCs in culture. Table 1 shows that when the D-glucose concentration of the 10% FCS culture medium was increased, a significant increase in situ PKC activity in VSMCs, occurred reproducing the effect seen with 0.5% FCS. Interestingly, at each level of D-glucose concentration, in situ PKC activity was increased numerically but not significantly in the presence of 10% FCS versus 0.5% FCS. This presumably represents activation of PKC by the various growth factors in FCS. Importantly, however, irrespective of the FCS concentration, the same concentration threshold of ~20 mM D-glucose was required to maximally activate PKC. Further studies confirmed that the temporal characteristics and recovery from glucose-induced PKC activation also were unchanged (data not shown) when the FCS concentration of the culture medium was increased from 0.5 to 10%.

Determining the specificity of the D-glucose effect on PKC activity in VSMC. To investigate the possibility that D-glucose activated PKC by a mechanism depending on changes in extracellular osmolality and therefore unrelated to the metabolism of D-glucose, the PKC activity

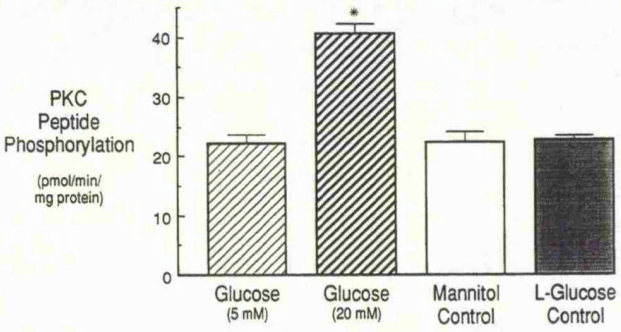

FIG. 6. Effects of elevated mannitol or L-glucose on PKC activity in VSMCs. VSMCs were exposed to control medium (glucose, 5 mM) or control medium supplemented with D-glucose (20 mM), mannitol (20 mM), or L-glucose (20 mM) for 48 h before measuring in situ PKC activity. *$P < 0.01$ vs. all other media tested, $n = 3$.

levels of VSMCs exposed to two control media were examined. The first contained mannitol (20 mM), a relatively impermeant hexose, and the second contained L-glucose (20 mM), a cell-permeable, poorly metabolized isomer of D-glucose. Unlike elevated concentrations of D-glucose, neither of these media activated PKC in VSMCs (Fig. 6). These results demonstrate that PKC activation by D-glucose was unrelated to changes in extracellular osmolality and depended on the capacity of VSMCs to metabolize D-glucose.

The next series of studies were designed to compare the characteristics and potency of glucose-induced PKC activation with the PKC response to experimental exogenous activators of PKC, i.e., PMA or DAG, or physiological activators of PKC, i.e., AVP and AII.

The effects of PMA on in situ PKC activity in VSMC. Phorbol esters such as PMA have been shown to be potent activators of PKC in many cell types (8,9,29). However, the effects of PMA on in situ PKC activity in VSMC have never been reported. Exposing VSMCs to PMA (10^{-6} M) activated PKC and induced a maximal response within 10 min (Fig. 7). This response to PMA was ~3 times greater than the maximal response to elevated D-glucose concentrations. However, unlike the

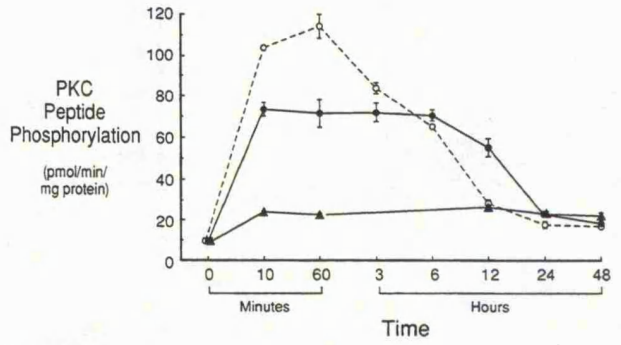

FIG. 7. Effects of PMA on in situ PKC activity in VSMCs. PMA (10^{-6} M, ○; 10^{-7} M, ●) increased PKC activity in VSMCs vs. PKC activity in cells exposed to control medium (▲). With continued exposure to PMA, PKC activity down-regulated with time, with rate of down-regulation being most rapid in VSMCs exposed to PMA (10^{-6} M), $n = 3$.

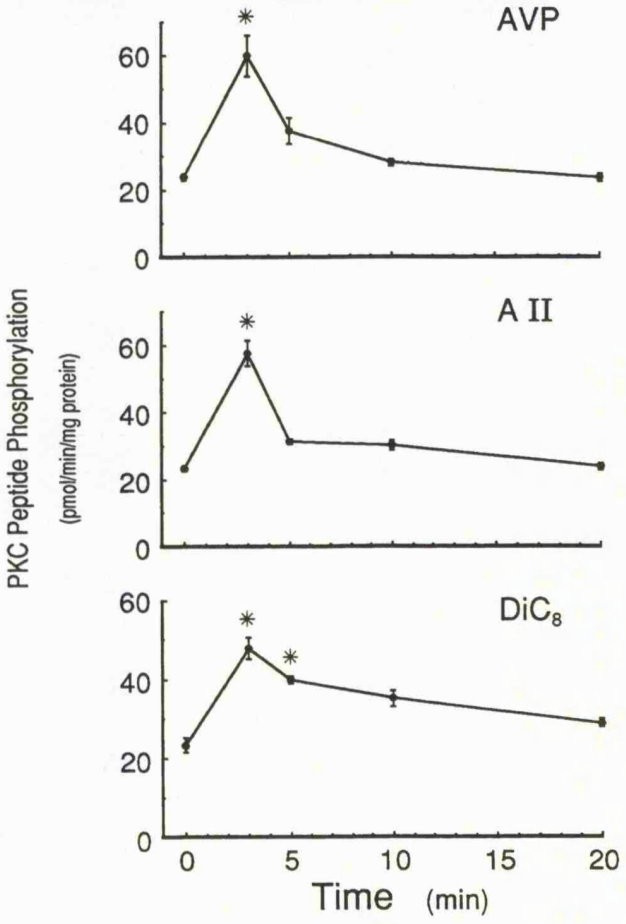

FIG. 8. Effect of DiC_8, AVP, and AII on PKC activity in VSMCs. DiC_8 (10^{-6} M) induced a transient increase in PKC activity in VSMCs. AVP (10^{-7} M) or AII (10^{-7} M) induced a similarly rapid and transient increase in PKC activity in VSMCs. *$P < 0.05$ vs. unstimulated VSMCs, $n = 3$ per study.

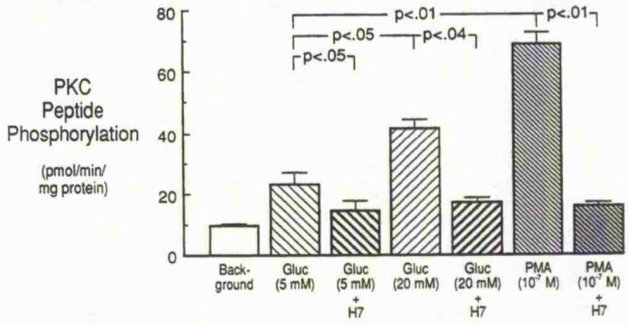

FIG. 9. Effect of H-7 on in situ PKC activity in VSMCs. VSMCs incubated with either control medium (glucose, 5 mM) or high-glucose medium (20 mM) +/− H-7 (5×10^{-5} M) for 48 h. Elevated glucose concentrations (without H-7) significantly enhanced PKC activity. H-7 reduced control PKC activity and prevented glucose-induced PKC activation, $n = 3$. Preincubating VSMCs with H-7 for 10 min also prevented PMA (10 min exposure, 10^{-7} M)-induced PKC activity, $n = 2$.

response to D-glucose, PMA-induced PKC activation was not sustained and downregulated with time, so that after 12 h of continuous exposure to PMA, PKC activity in VSMCs had returned to control levels. The peak PKC response to PMA (10^{-7} M) in VSMCs also occurred after 10 min. This response was lower than that achieved with PMA (10^{-6} M) but was again greater than the maximal response to D-glucose. PKC activity again progressively declined with continued exposure to PMA (10^{-7} M) but at a slower rate than observed with higher PMA concentrations. Thus, the rate of PKC inactivation in VSMCs appears to be related to the previous magnitude of PKC activation, i.e., the higher the peak PKC activity, the greater the rate of PKC inactivation. These observations confirm that the loss of membrane-associated PKC after prolonged exposure to PMA (8,9) corresponds to a loss of in situ PKC activity and therefore represents a true downregulation of this kinase system.

Effects of DiC_8 on in situ PKC activity. In addition to phorbol esters, cell-permeable DAGs are used experimentally to analyze the effects of PKC activation on vascular function (30). DiC_8 (10^{-6} M), a synthetic, cell-permeable DAG, activated PKC in VSMCs (Fig. 8*C*). The use of the in situ PKC assay revealed important differences in the PKC response to DiC_8 compared with PMA. DiC_8 produced a peak PKC response within 3 min and the magnitude of this response was much less than that seen with equimolar doses of PMA. PKC activation by DiC_8 declined rapidly to control values by 20 min. This transient response to diC_8 is most probably because of its rapid metabolism in a manner similar to endogenous diglycerides (31,32).

Effect of AVP and AII on in situ PKC activity in VSMC. Both AVP and AII have been shown to induce the translocation of PKC from cytosol to membrane in VSMC (33). This result has been taken to represent PKC activation but provides little information about the time course or magnitude of the PKC response to these hormones. Using a direct assay of PKC activity in permeabilized VSMCs, Fig. 8*B* and *C* shows the remarkable similarity in the PKC response of VSMCs to equimolar doses of AVP (10^{-7} M) or AII (10^{-7} M). PKC activation by both hormones was maximal within 3–5 min and rapidly declined toward base line by 20 min. The transient nature of the PKC response to these pressor hormones is similar to the reported transience of AVP- or AII-induced PKC translocation in vascular tissue (33). It is also apparent that the magnitude and temporal characteristics of PKC activation by synthetic diglycerides is much more representative of physiological stimulation of PKC than the often-used phorbol esters, suggesting that the latter should be used with caution when attempting to mimic hormonal stimulation of PKC in VSMC.

Effect of H-7 on in situ PKC activity in VSMC. The final series of studies examined the effect of H-7, a relatively specific inhibitor of PKC (34), on the capacity of glucose to induce PKC activity in VSMC. The results are shown in Fig. 9. VSMCs were coincubated with H-7 (5×10^{-5} M in 0.1% DMSO) dissolved in either control medium or high-glucose (20 mM) medium for 48 h before we determined in situ PKC activity. DMSO (0.1%) alone had no effect on basal or glucose-stimulated PKC activity (data not shown). With control medium, H-7 reduced

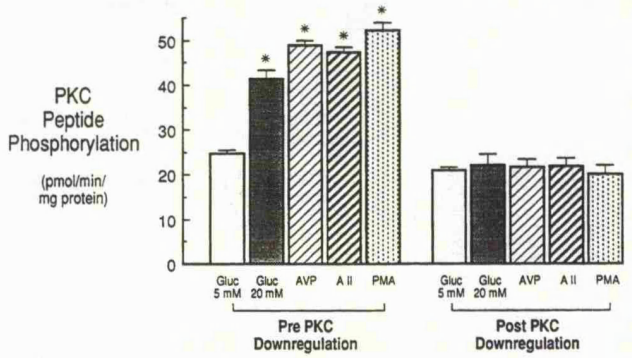

FIG. 10. Effects of PKC downregulation in VSMCs. Before PKC down-regulation, AVP (10^{-7} M), AII (10^{-7} M) for 3 min, PMA (10^{-7} M) for 10 min, or elevated D-glucose concentrations (20 mM) for 10-min exposure activated PKC in VSMCs. *$P < 0.05$ vs. unstimulated VSMCs. However, in VSMC preexposed to PMA (10^{-6} M) for 24 h to down-regulate PKC activity, the same agents failed to activate PKC.

basal PKC activity almost to background counts. When elevated D-glucose concentrations were coincubated with H-7, the latter completely prevented glucose-induced PKC activation. Pretreating VSMCs with H-7 (5×10^{-5} M) for 10 min also prevented PKC activation in response to PMA (10^{-7} M). In additional experiments, VSMCs were pretreated with PMA (10^{-6} M) for 24 h. Our earlier results demonstrate that this maneuver depletes PKC activity in VSMCs (Fig. 7). Figure 10 shows that before PKC downregulation, acute exposure of VSMCs to elevated D-glucose concentrations or various PKC activators promote the phosphorylation of the VRKRTLRRL substrate. However, after PKC downregulation, identical stimuli produced no increase in phosphorylation. That phosphorylation of the peptide substrate used in these studies could be increased by recognized activators of PKC and completely prevented by downregulating PKC activity or by the presence of a specific PKC inhibitor, H-7 confirms that the agonist-induced phosphorylating activity measured in permeabilized VSMCs in this study was caused specifically by PKC.

DISCUSSION

Elevated extracellular glucose concentrations previously have been shown to induce the translocation of PKC from the cytosol to the membrane of cultured retinal endothelial cells and isolated glomeruli in vitro (14,15). This translocation generally has been assumed to represent activation of PKC. Before now, however, no direct measurements of in situ PKC activity have been performed in any cell type, after exposure to elevated glucose concentrations, to either confirm or refute this conclusion. In addition, even though PKC is relatively abundant in VSMCs and VSMCs are a potentially important target for the deleterious effects of hyperglycemia in vivo, before this study, it was unknown whether elevated glucose concentrations actually could influence PKC activity in VSMCs.

This study used a method to assay directly in situ PKC activity. It has been suggested that such methods represent a significant methodological advance in that they allow for the direct and highly specific assay of in situ PKC activity (35). This is in contrast to the more traditional indirect assay, which assumes that the translocation of PKC from the cytosol to cell membrane corresponds to the magnitude of PKC activation. Using the in situ PKC assay, this study demonstrates that elevated extracellular D-glucose concentrations activate PKC in cultured VSMCs. This effect is evident within minutes of exposure to elevated extracellular glucose concentrations and is sustained for up to 48 h of continued glucose exposure. The glucose-induced effect on PKC activity is maximal at a glucose concentration of 15–20 mM, a concentration often attained in poorly controlled diabetic patients. Furthermore, the activation of PKC by glucose in VSMCs is a threshold phenomenon, with no further increase in the magnitude of PKC activation once the extracellular glucose concentration exceeds 20 mM. In addition, this study uniquely demonstrates that restoring extracellular glucose concentrations to normal rapidly restores PKC activity to control values. These results therefore indicate that VSMC PKC activity rapidly responds to both increases and decreases in extracellular glucose concentrations. Moreover, the temporal pattern and characteristics of glucose-induced PKC activation were similar irrespective of whether the studies were performed with quiescent VSMCs (0.5% FCS) or actively proliferating VSMCs in the presence of 10% FCS.

The mechanisms responsible for PKC activation by elevated extracellular glucose concentrations have been explored by others (14,15,17). PKC is a Ca^{2+}- and phospholipid-dependent enzyme that can be activated by increased endogenous synthesis of DAG (8,18). Numerous studies in diabetic animals show that DAG levels are elevated in a variety of tissues, including vascular tissue (14,15,17,36,37). Using cultured retinal endothelial cells, Lee et al. (14) recently showed that elevated extracellular glucose concentrations increase the flux of glucose through a pathway culminating in the enhanced de novo synthesis of DAG and concluded that this in turn activates PKC. The results of this study support the hypothesis that a metabolic product of glucose metabolism is required for PKC activation by demonstrating that elevated extracellular concentrations of a nonmetabolized glucose isomer, L-glucose, do not activate PKC. Moreover, the rapid reversibility of glucose-induced PKC activation in VSMCs suggests that the glucose metabolic pathway involved in PKC activation depends on a continuous extracellular-to-intracellular flux of glucose. The studies with L-glucose and mannitol also exclude an osmotic action of glucose in the activation of PKC, consistent with the finding that similarly elevated concentrations of these agents also fail to promote an increase in membrane-associated PKC in other tissues in vitro (14,15).

With a method to assay accurately in situ PKC activation, we were uniquely able to compare the characteristics of glucose-induced PKC activation in VSMCs with the PKC response to PMA, DiC_8, and pressor hormones in an endeavor to gauge the potential significance of the glucose-induced response. Two important points emerged. First, the glucose-induced effect on PKC ac-

tivity in VSMCs contrasts markedly with the PKC response to PMA. Specifically, the peak response to PMA was much greater than the peak response to glucose (20 mM). Moreover, with continued exposure to PMA, PKC activity in VSMCs progressively downregulated, the rate of downregulation appearing to correlate with the magnitude of previous PKC activation. This finding is consistent with reports of a decrease in membrane-associated PKC after prolonged exposure to PMA (38). In marked contrast, glucose-induced low-grade activation of PKC was sustained for up to 48 h of continued glucose exposure in the presence of either 0.5 or 10% FCS.

Another important observation in this study is that the profile of the PKC responses to pressor hormones in VSMCs also differs markedly from glucose-induced PKC activation. Specifically, PKC activation by pressor hormones was transient and closely resembled the response to synthetic diglycerides. This presumably represents the fact that DAG production in response to hormonal stimulation is reported to be similarly transient (39). It is notable that with a methodology identical to that used in our study, the PKC response to nerve growth factor and epidermal growth factor in cultured PC12 cells was similarly transient (25). Together, these observations emphasize that PKC activation after hormone-receptor interaction is usually transient, returning to base line within minutes. This response is in marked contrast to the chronicity of glucose-induced PKC activation. This temporal pattern of sustained glucose-induced PKC activation also is implied in studies demonstrating the persistence of glucose-induced PKC translocation in cultured endothelial cells in vitro and in a variety of tissues from the diabetic rat in vivo (14–17).

The unique temporal characteristics of PKC activation by glucose in VSMCs could have considerable pathophysiological significance. Changes in regional hemodynamics and vascular morphology are well-recognized developments in diabetes mellitus (1–7). PKC activation is a key event in the initiation and/or regulation of a multitude of cell functions (8,9,40,41). With regard to VSMCs, PKC has been implicated in the regulation of cell-surface hormone-receptor density, intracellular signaling responses to pressor hormones, ion channel activity, intracellular pH, and cell contraction, growth, and differentiation (8–13). Because the hormone-induced PKC activity influencing these various processes appears to be transient, it seems possible that a glucose-induced sustained activation of PKC could have a deleterious effect on VSMC function and thereby contribute to the development of the aberrant vascular growth and contractile responses that are the hallmark of diabetic micro- and macrovascular disease.

In conclusion, by using a highly specific assay for the measurement of in situ PKC activity, this study uniquely demonstrates that elevated extracellular glucose concentrations activate PKC in VSMCs, and that the profile of glucose-induced PKC activation differs markedly from that observed with hormonal or pharmacological stimulation of PKC. Specifically, glucose generates a sustained and low-grade activation of PKC that is rapidly responsive to fluctuations in extracellular glucose concentrations. This capacity of glucose to activate PKC in VSMCs could contribute to the apparent vascular toxicity of elevated glucose concentrations in vivo.

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Chapter 3.

Glucose-Induced Downregulation of Angiotensin II and Arginine Vasopressin Receptors in Cultured Rat Aortic Vascular Smooth Muscle Cells.

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Glucose-induced Downregulation of Angiotensin II and Arginine Vasopressin Receptors in Cultured Rat Aortic Vascular Smooth Muscle Cells

Role of Protein Kinase C

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Abstract

Early diabetes mellitus is characterized by impaired responses to pressor hormones and pressor receptor downregulation. The present study examined the effect of elevated extracellular glucose concentrations on angiotensin II (AII) and arginine vasopressin (AVP) receptor kinetics in cultured rat vascular smooth muscle cells (VSMC). Scatchard analysis of [^{3}H]AVP and ^{125}I-AII binding to confluent VSMC showed that high glucose concentrations (20 mM) similarly depressed AVP and AII surface receptor B_{max} but did not influence receptor K_d. This receptor downregulation was not reproduced by osmotic control media containing either L-glucose or mannitol. Receptor downregulation was maximal at a glucose concentration of 15–20 mM and required 24–48 h for a maximum effect. Normalization of the extracellular glucose concentration allowed complete recovery of AVP and AII binding within 48 h. Receptor downregulation was associated with depressed AVP and AII-stimulated intracellular signaling and cell contraction. High glucose concentrations induced a sustained activation of protein kinase C (PKC) in VSMC, which was prevented by coincubation with H-7. H-7 also markedly attenuated glucose-induced downregulation of AVP and AII receptors on VSMC. This study demonstrates a novel cellular mechanism whereby high extracellular glucose concentrations directly and independently downregulate pressor hormone receptors and their function on vascular tissue via glucose-stimulated PKC activation. (*J. Clin. Invest.* 1992. 90:1992–1999.) Key words: diabetes mellitus • protein kinase C • vascular smooth muscle cells • microangiopathy • vascular injury

Introduction

Early diabetes mellitus in both experimental animals and man is associated with increased blood flow to many tissues, including the kidney, myocardium, retina, skin, muscle, and brain (1–7). These early changes in regional hemodynamics have in turn been strongly implicated in the pathogenesis of the widespread microvascular injury that characterizes this disease (8–10). The principal determinant of this deleterious increase in tissue perfusion is a reduction in arteriolar tone (11). Pressor hormones such as angiotensin II (AII)[1] and arginine vasopressin (AVP) are important modulators of vascular tone (12) and studies of the diabetic rat have demonstrated an early reduction in the aortic contractile response to AII and epinephrine (13). Moreover, glomerular hemodynamic responses to AII are also markedly blunted in diabetic rats (14).

The aforementioned impaired response to pressor hormones could result from either impaired postreceptor actions of the hormone or reduced hormone-receptor binding. In keeping with the latter, reduced glomerular AII binding has been demonstrated in the diabetic rat (15, 16); reduced AVP binding to platelet V_1 receptors has also been observed in patients with diabetes mellitus (17). Additional studies reveal a reduction in the number of beta-adrenergic receptors in experimental diabetes and platelet thromboxane A_2 receptors in human diabetes (18, 19), suggesting that the diabetic state may be associated with a generalized reduction in the expression of a variety of pressor receptors.

In spite of the potential pathophysiological significance of pressor receptor downregulation with regard to the initiation and/or propagation of vascular injury in diabetes, the mechanisms responsible for these marked changes in surface receptor density are unknown. Recent evidence suggests that protein kinase C (PKC), a multifunctional Ca^{2+}- and phospholipid-dependent serine/threonine kinase system, may play an important role in regulating the cell surface density of many receptors (20–22). In this regard, we have shown that hormone-activated PKC is an important component of homologous hormone receptor desensitization in cultured rat vascular smooth muscle cells (VSMC) (23, 24). Others report a similar PKC-dependent downregulation of a variety of receptors in different tissues (25–27). More recent reports suggest that PKC may modulate surface receptor density by regulating receptor mRNA expression and receptor biosynthesis (28). In view of the potentially important role of PKC in the regulation of receptor biosynthesis and expression, it is intriguing that high extracellular glucose concentrations have recently been shown to promote the activation of PKC in numerous tissues, including vascular tissue, both in vivo and in vitro (29–32). Together, these observations raise the possibility that glucose-induced PKC activation could provide a novel biochemical mechanism to account for downregulation of pressor receptors in patients with diabetes mellitus.

The present in vitro study thus examines the hypothesis that elevated extracellular glucose concentrations can directly promote the downregulation of AII and AVP receptors on VSMC and thereby impair the subsequent pressor response of VSMC to these hormones via mechanisms dependent on glucose-induced PKC activation in VSMC.

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1. *Abbreviations used in this paper:* AII, angiotensin II; AVP, arginine vasopressin, DAG, diacylglycerol; PKC, protein kinase C; PSS, physiological saline solution; VSMC, vascular smooth muscle cells.

Methods

Materials. AVP, AII, and H7 were purchased from Sigma Chemical Co. (St. Louis, MO). ^{125}I-AII and [^{3}H]AVP were obtained from New England Nuclear (Wilmington, MA). $^{45}Ca^{2+}$ was obtained from Amersham Corp. (Arlington Heights, IL). The VRKRTLRRL peptide substrate was a generous gift from Drs. Lynn E. Heasley and Gary L. Johnson, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO.

Preparation of cultured rat VSMC. Rat aortic VSMC were isolated and cultured using a modification of the method described by Chamley et al., as previously described from this laboratory (23, 24, 33). Briefly, under sterile conditions, aortas were resected from Sprague-Dawley rats and cleaned of adventitia and connective tissue. The vessels were chopped and incubated for 2 h in Eagle's MEM (Gibco Laboratories, Grand Island, NY) containing 2 mg/ml collagenase. The resulting cell suspension was plated on 35-mm dishes and grown in MEM containing 2 mM L-glutamine, 2 g/liter $NaHCO_3$, 100 IU/ml penicillin, 100 mg/liter streptomycin, and 10% FCS at 37°C in 95% humidified air and 5% CO_2. At confluence, VSMC from second through sixth passage were used.

Test media. To examine specifically the effects of various extracellular D-glucose concentrations on AII or AVP binding to VSMC and the subsequent biological and physiological response to these agents, VSMC were exposed to one of four test media: *a*) control medium: consisted of MEM supplemented with 10% FCS and containing 5 mM D-glucose; *b*) high glucose medium: identical to the control medium except that it was supplemented with D-glucose to increase the glucose concentration up to 30 mM; *c*) L-glucose osmotic control medium: identical to the control medium but supplemented with a cell permeable but poorly metabolized glucose isomer, L-glucose (25 mM); or *d*) mannitol osmotic control medium: identical to the control medium but supplemented with poorly diffusible hexose, mannitol (25 mM).

Receptor binding studies. The experiments examining AVP and AII binding to VSMC were performed using the radioligands [^{3}H]AVP and ^{125}I-AII as previously described (34). To determine AVP binding after preexposure to the test media for various time periods, confluent VSMC monolayers on 35 × 10-mm dishes were washed twice with ice-cold binding buffer (119.2 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 10 mM Hepes, 0.1% BSA, pH 7.4). The cells were then incubated with the same buffer containing 2×10^{-9} M [^{3}H]AVP (specific activity 67.7 μCi/mmol) with or without unlabeled AVP for 90 min at 4°C to prevent receptor internalization and achieve saturation binding conditions. Binding was terminated and the unbound radioligand removed by rapid washing four times with 2 ml ice-cold binding buffer. The cells were then solubilized in 0.1% SDS and 0.1 N NaOH and the cell-associated radioactivity was determined by scintillation counting (Tri-carb 460C; Packard Instrument Co. Inc., Downers Grove, IL). Specific binding of AVP was defined as total binding (2×10^{-9} M [^{3}H]AVP) minus nonspecific binding (2×10^{-9} M [^{3}H]AVP and 10^{-6} M unlabeled AVP). A 50-μl aliquot of the solubilized cells was assayed for protein content by the method of Lowry. AII binding studies were performed using similar conditions except that the composition of the binding buffer was 50 mM Tris-HCl, 100 mM NaCl (pH 7.4), 5 mM $MgCl_2$, supplemented with 0.5 mg/ml bacitracin and 0.2% BSA. Specific ^{125}I-AII binding to VSMC was defined as total binding (^{125}I-AII, 50–100 fmol, specific activity 10^5 dpm/ml) minus nonspecific binding (unlabeled AII [10^{-6} M]). Each binding assay was performed in triplicate and binding constants were determined by Scatchard analysis of binding data fitted to a line.

Measurement of PKC activity in VSMC. For the measurement of in situ PKC activity in VSMC, a modification of the method recently described by Heasley and Johnson (35, 36) was used. VSMC were seeded into flat-bottomed 96-well microtiter plates at a density of 20,000/well. VSMC were maintained in regular growth medium for 1 to 3 d until confluent. The monolayer was then washed with 200 μl of Hanks' balanced salt solution before incubation with one of the four test media described above. After the desired exposure time, the test medium was aspirated and replaced with 40 μl of a buffered salt solution containing 137 mM NaCl, 5.4 mM KCl, 10 mM $MgCl_2$, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 25 mM β-glycerophosphate, 5.5 mM D-glucose, 5 mM EGTA, 1 mM $CaCl_2$ (~ 100 nM Ca^{2+}-free), 100 μM [gamma^{32}P]ATP (~ 5,000 cpm/pmol), 50 μg/ml digitonin, and 20 mM Hepes (pH 7.2, 30°C). In addition, 100 μM of a PKC-specific peptide substrate (VRKRTLRRL) was added to the buffer. This short synthetic peptide is based on the sequence surrounding a major PKC-dependent phosphorylation site within the epidermal growth factor (EGF) receptor (37). This peptide substrate is not phosphorylated by cyclic nucleotide-dependent or Ca^{2+}/calmodulin-dependent protein kinases or S6 kinase and has been extensively characterized to be a highly specific substrate for PKC (35, 36). By permeabilizing VSMC with the digitonin contained in the buffer, the VRKRTLRRL peptide enters VSMC along with [gamma^{32}P]ATP to allow a highly selective and rapid analysis of in situ PKC activity. The concentration of digitonin (50 μg/ml) used in this assay does not modify VSMC morphology or promote cell detachment. Moreover, PKC activity is retained by the monolayer postpermeabilization (PKC activity in the supernatant was < 10% total PKC activity). This latter finding is consistent with the concept that the PKC activity being measured using this assay is tightly associated with the cell membrane as has been proposed for the active form of PKC (20, 38, 39). The kinase reaction was linear with time for up to 20 min and was therefore allowed to proceed for 10 min at 30°C before termination by the addition of 25% (wt/vol) TCA (final TCA concentration 5%). Aliquots (45 μl) of the acidified reaction mixture were spotted onto 2-cm phosphocellulose circles (P81; Whatman Inc., Clifton, NJ) and washed batchwise: three washes with 75 mM phosphoric acid and one wash with 75 mM sodium phosphate (pH 7.5) (500 ml/2-min wash). Because of the basicity of the VRKRTLRRL substrate, it is retained by the phosphocellulose filter at neutral pH, whereas contaminating [^{32}P]ATP is removed. The PKC-dependent phosphorylation of the peptide substrate bound to the filter was quantified by scintillation counting. Results are expressed as PKC-dependent peptide phosphorylation, pmol/min per mg VSMC protein.

Measurement of $^{45}Ca^{2+}$ efflux from VSMC. $^{45}Ca^{2+}$ efflux studies were performed as previously described (24, 40, 41). Briefly, after exposure to the test media, confluent VSMC on 35-mm dishes were washed with physiological saline solution (PSS) and incubated with 1 ml fresh test medium containing 8 μCi $^{45}Ca^{2+}$ (specific activity) for 3 h at 37°C to allow preloading of VSMC with $^{45}Ca^{2+}$. Thereafter, VSMC were rinsed rapidly (10 × 1 ml PSS in 60 s) and then incubated with 1 ml PSS. The PSS was removed and replaced with fresh PSS at 1-min intervals for 6 min. Samples at each time interval were placed into vials for liquid scintillation counting. After 6 min, the PSS was supplemented with either AVP (10^{-7} M) or AII (10^{-7} M) and VSMC were exposed to these agents for 1 min. $^{45}Ca^{2+}$ released by VSMC during the incubation with these agents and for subsequent 5 × 1-min incubations with 1 ml PSS was quantified by liquid scintillation counting. The VSMC were solubilized as described above and the cell-associated radioactivity was counted. Total $^{45}Ca^{2+}$ released after the addition of the effector minus basal release was expressed as a percentage of total available cellular radioactivity at the time of stimulation with the effector.

VSMC shape change studies. The functional response of VSMC to AVP or AII was assessed by quantitation of cell surface area changes using phase-contrast microscopy (IM35; Carl Zeiss, Inc., Oberkochen, Germany) and a computerized digital image analyzer (Zidas, Carl Zeiss Inc.) that had been calibrated using a micrometer scale, as previously reported from this laboratory (23, 24). For these studies, only sparsely plated primary cultures of VSMC in 35 × 10-mm dishes were used because VSMC may lose their contractility after subculture (42). After preexposure to the test media, VSMC were incubated for 20 min in the presence of fresh test media at 37°C. After this equilibration period, the area of individual cells was measured. A 20× objective was used for all studies on groups of 10–15 cells. The microscopic field was chosen randomly and not selected for cell shape or size. AVP (10^{-6} M)

or AII (10^{-6} M) was then added to the medium and the VSMC incubated for a further 15 min at 37°C. Changes in VSMC surface area in the same group of cells in response to AVP or AII was then measured with the image analyzer. Several precautions were incorporated into the protocol. Three measurements of each cell were taken and any group of measurements showing a standard deviation of > 20% of the mean was discarded. Spontaneous cell shape changes due to manipulation of the culture dishes in the absence of effector was measured at < 5% and thus only a value of ≥ 15% change in surface area was considered to be a significant response to AVP or AII. The method has been further validated in this laboratory by comparing the digital image–analyzed assessment of cell surface area changes with the measured area on photographs of the same cells. The results show excellent correspondence between the two methods.

Statistical analysis. Results are expressed as mean±SEM. Statistical analysis was made using the paired or unpaired Student's *t* test or analysis of variance with a Bonferroni correction as appropriate. For each experiment, *n* refers to the number of studies (each in triplicate).

Results

Effect of high extracellular glucose concentrations on AVP and AII binding to VSMC. Studies examining AVP and AII binding to VSMC were performed under conditions at which saturation binding occurs for both ligands and at 4°C to prevent receptor internalization so that only surface binding was measured. Equilibration binding for both radioligands occurred by 60 min, remained stable over the next 30 min, and was identical in the presence of all test media. The binding characteristics of both radioligands were specific, time and protein dependent, saturable, and stable in VSMC up to sixth passage. For both AVP and AII, the nonspecific binding was always < 15% and was not affected by exposure to the different test media.

In the presence of the control medium (D-glucose 5 mM), Scatchard transformation of the binding data for specific [^{3}H]AVP binding to confluent VSMC yielded a linear plot, demonstrating a single class of AVP-binding sites with a maximum number of binding sites (B_{max}) of 1.99×10^{-12} mol/mg cell protein and a K_d of 2.15×10^{-9} mol (Fig. 1). In contrast, however, preexposing VSMC for 48 h to a high glucose medium (D-glucose 20 mM) markedly reduced AVP binding by almost 40% versus control medium, due to a significant decline in B_{max} (1.22×10^{-12} mol/mg cell protein) with no significant change in K_d (2.26×10^{-9} mol) (Fig. 1).

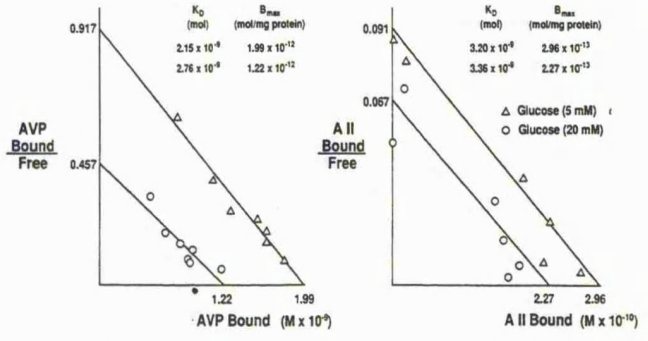

Figure 1. A representative Scatchard analysis comparing the effects of a 48-h exposure to either a normal extracellular glucose concentration (5 mM) (*open triangles*) or a high extracellular glucose concentration (20 mM) (*open circles*) on [^{3}H]AVP- and ^{125}I-AII–specific surface binding to confluent rat VSMC. Similar results were obtained in two additional studies for each peptide.

Similarly, in the presence of control medium, AII binds to a single class of receptors on VSMC with a B_{max} of 2.96×10^{-13} mol/mg cell protein and a K_d of 3.2×10^{-9} mol (Fig. 1). However, preexposure of VSMC to a high glucose medium (D-glucose 20 mM) for 48 h resulted in a marked reduction in AII binding to VSMC. This was similar in magnitude to the effect of the high glucose environment on AVP binding to VSMC and was also due to a significant reduction in AII receptor B_{max} (2.27×10^{-13} mol/mg cell protein) with no significant change in K_d (3.36×10^{-9} mol). Thus exposing VSMC to a high extracellular glucose environment resulted in a significant decrease in AVP and AII binding to VSMC in both cases due to a similar decline in surface density of these pressor receptors with no significant change in receptor affinity. These changes did not reflect changes in VSMC protein content or cell number as both of these parameters were equivalent after a 48-h exposure of confluent VSMC to all test media. Furthermore, the glucose-induced decrease in hormone binding did not represent VSMC toxicity or accelerated cell death because cell detachment rates, percent lactate dehydrogenase release and percent trypan blue exclusion, were also similar after a 48-h exposure to all test media (data not shown).

To examine the possibility that changes in AVP or AII surface binding were related to the increased extracellular osmolality of the high glucose medium, the effects of a 48-h exposure to the two osmotic control media (L-glucose or mannitol) on AVP and AII binding to VSMC was examined. The results indicate that the downregulation of AVP and AII receptors on VSMC was specific for D-glucose and is not reproduced by either of the two osmotic test media (Table I). Moreover, the fact that high concentrations of the poorly metabolized L-glucose isomer did not modify AVP or AII binding to VSMC suggests that intracellular metabolism of D-glucose is necessary for its effects on pressor receptor expression to develop.

Glucose concentration dependency of AVP and AII receptor downregulation in VSMC. The next study examined the relationship between extracellular glucose concentration and depression of AVP and AII binding to VSMC. Confluent VSMC were incubated with different D-glucose concentrations for 48 h before determining the binding of [^{3}H]AVP or ^{125}I-AII to VSMC. The glucose concentration dependency of inhibition of AVP and AII binding to VSMC was a threshold effect with the specific binding of both hormones being significantly depressed at a glucose concentration of 15 mM and maximally depressed between 15 and 20 mM (Fig. 2).

Table I. Effect of a 40-h Incubation with the Different Test Media on the Specific Binding of [^{3}H]AVP and ^{125}I-AII to Confluent VSMC

Test media	AVP	AII
	% specific binding	
Glucose (5 mM)	100	100
L-glucose control	103.9±5.4	101.0±6.1
Mannitol control	98.7±5.2	100.8±7.3
Glucose (20 mM)	61.8±4.9*	66.3±5.5*

Specific binding in the presence of control medium (glucose 5 mM) is designated 100% and results in the presence of the other test media are expressed as percentage of this control. * $P < .01$ vs control.

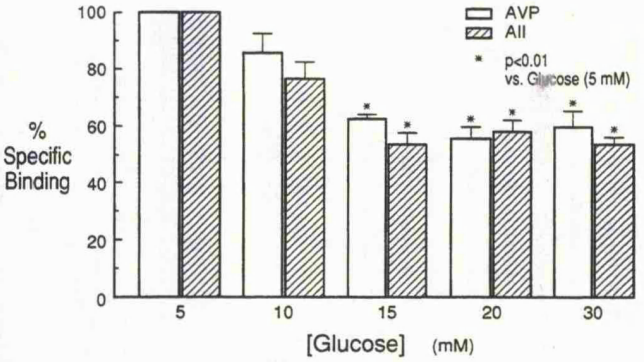

Figure 2. The glucose concentration dependency of AVP- and AII-specific surface binding to confluent VSMC. VSMC were exposed to either control medium (glucose 5 mM) or identical medium supplemented with various concentrations of D-glucose for 48 h before determining the specific surface binding of AVP (*open bars*) and AII (*hatched bars*). AVP or AII binding in presence of control medium is designated 100% and binding in the presence of the other test media is expressed as percent of control. $*P < 0.01$ vs. control binding ($n = 3$).

Time dependency of glucose-induced downregulation of AVP and AII receptors on VSMC. To examine the time dependency of the downregulation of AVP and AII receptors in the presence of a high glucose concentration, VSMC were exposed to a glucose concentration of 20 mM for increasing time periods up to 48 h. Fig. 3 shows that > 12-h exposure to a high D-glucose concentration was required to significantly downregulate either the AVP or AII receptor and between 24 and 48 h was required for maximal glucose-induced downregulation of these receptors on VSMC. The maximum time exposure to the high glucose medium in all studies was 48 h.

Recovery of AVP and AII receptors. Having demonstrated the slow onset of glucose-induced pressor receptor downregulation, the next study examined receptor recovery after restoring the extracellular glucose concentration to normal. VSMC were exposed to a high D-glucose concentration (20 mM) for 48 h to induce maximal downregulation of AVP and AII receptors. The high glucose medium was then replaced with control medium (glucose 5 mM) for various time periods before measuring specific AVP or AII binding to VSMC. The characteristics of AVP and AII receptor recovery were very similar. Both receptors showed signs of recovery within 12 h of normalizing the extracellular glucose concentration but required up to 48 h for full receptor recovery to occur (Fig. 4). These results demonstrate that glucose-induced effects on both AVP and AII surface receptors on VSMC are similarly slow to develop and slow to recover. Furthermore, the complete reversibility of this phenomenon confirms that glucose-induced receptor downregulation does not involve any artifact due to cell death.

Glucose-induced activation of PKC in VSMC. The next series of studies were designed to explore whether PKC activation is involved in the mechanism(s) whereby glucose promoted the downregulation of pressor receptors on VSMC. PKC activation has been implicated in the regulation of hormone receptor biosynthesis and expression. Elevated extracellular glucose concentrations have been shown to induce the activation of PKC in cultured retinal endothelial cells and isolated glomeruli via an increased flux of glucose through an intracellular pathway culminating in the enhanced de novo synthesis diacylglycerol (DAG) (29, 32), which in turn promotes the activation of PKC (20, 38). The next series of studies thus examined whether elevated glucose concentrations activate PKC in VSMC. Fig. 5 shows that after a 3-h exposure to a high D-glucose (20 mM) medium, in situ PKC activity was markedly increased compared with the PKC activity measured in VSMC exposed to control medium (glucose, 5 mM). Of interest, the glucose-induced increase in PKC activity was sustained for up to 48 h, provided that the extracellular glucose concentration remained elevated. In contrast, 48-h exposure to either of the two osmotic control media produced no appreciable activation of PKC, confirming that PKC activation was not osmotically mediated but required the metabolism of glucose (data not shown). Further studies confirmed that PKC activation was glucose-concentration dependent. A threshold glucose concentration of 15 to 20 mM was required to induce a maximal PKC response to glucose (data not shown).

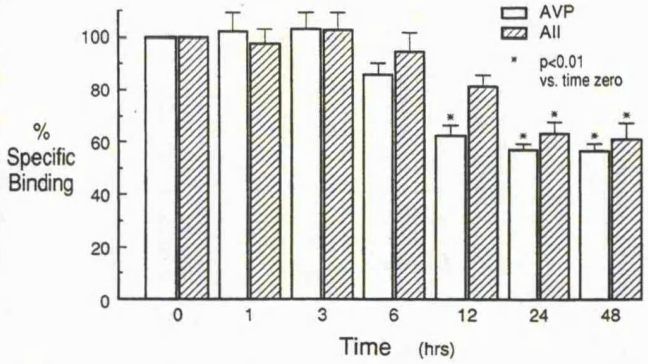

Figure 3. The time dependency of glucose-induced inhibition of AVP and AII binding to VSMC. Confluent VSMC were exposed to a control medium (glucose 5 mM, time 0) or a high extracellular glucose medium (glucose 20 mM) concentration for various time periods. Control AVP- (*open bars*) and AII- (*hatched bars*) specific surface binding (time 0) was designated 100% and peptide hormone binding after varying exposure to the high glucose medium is expressed as percent control. $*P < 0.01$ ($n = 3$).

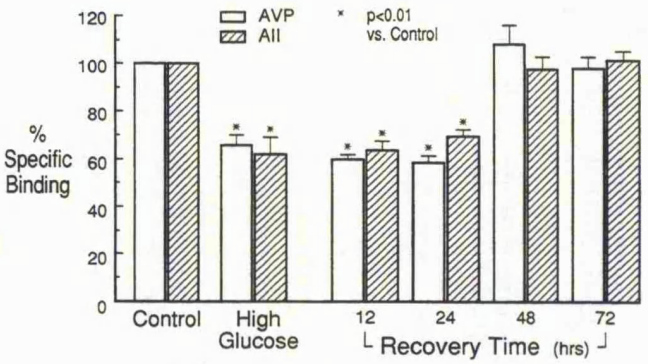

Figure 4. Recovery of AVP and AII surface binding to VSMC after normalization of extracellular glucose concentrations. Confluent VSMC were exposed to control medium or high glucose medium (20 mM) for 48 h to induce AVP and AII receptor downregulation. The culture medium overlying the cells was then replaced with control medium (glucose 5 mM) for varying time periods before determining the recovery of AVP (*open bars*) and AII (*hatched bars*) surface receptor binding. $*P < 0.01$ vs. control binding (glucose 5 mM) ($n = 3$).

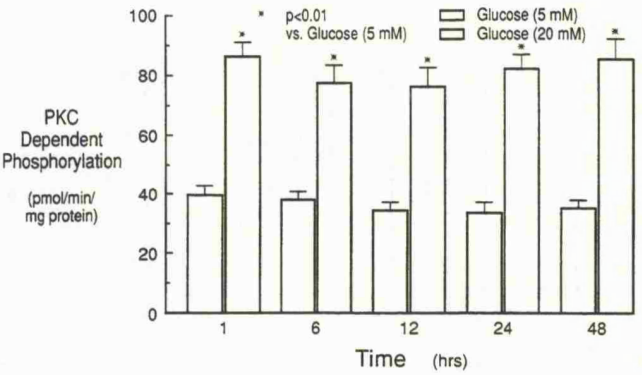

Figure 5. Glucose-induced PKC activity in VSMC. Confluent VSMC were exposed for varying time periods to control medium (glucose 5 mM) (*open bars*) or high glucose medium (glucose 20 mM) (*spotted bars*) before measuring PKC activity. PKC activity was measured in situ in digitonin-permeabilized VSMC by determining the phosphorylation of the PKC-specific VRKRTLRRL peptide substrate. Results are expressed as pmol phosphate transfered to this substrate/min per mg VSMC protein. $*P < 0.01$ vs. the time-matched control ($n = 4$).

To examine the role of glucose-induced PKC activation in mediating pressor receptor downregulation, a means of manipulating PKC activation by glucose was necessary. The next study determined the capacity of H7, a relatively specific inhibitor of PKC activity (43), to prevent glucose-induced PKC activation. VSMC were coincubated with H7 (5×10^{-5} M in 0.1% DMSO) in either control medium or high glucose (20 mM) medium for 48 h before determining in situ PKC activity. DMSO (0.1%) alone had no effect on basal or glucose stimulated PKC activity (data not shown). With control medium, H7 reduced basal PKC activity. Moreover, when high glucose medium was coincubated with H7, glucose-induced PKC activation was almost completely prevented (Fig. 6). The fact that the phosphorylation of the VRKRTLRRL peptide substrate was markedly attenuated by a recognized PKC inhibitor (H7) demonstrates that the phosphorylating activity being measured in the permeabilized VSMC was specific for PKC.

The role of PKC in glucose-induced AVP and AII receptor downregulation in VSMC. After demonstrating that the sustained activation of PKC induced by chronic high glucose exposure could be prevented by coincubation with H7, the same experimental maneuver was used to examine specifically the role of PKC in mediating glucose-induced pressor receptor downregulation in VSMC. Fig. 7 shows that, in the absence of H7, 48-h exposure to high glucose (20 mM) medium resulted in a significant downregulation of AVP and AII receptors on VSMC. In contrast, when the high glucose medium was supplemented with H7 to prevent glucose-induced PKC activation, the downregulation of AVP and AII receptors was almost completely prevented. These results illustrate that glucose-induced downregulation of AVP and AII receptors is strongly dependent on the capacity of glucose to activate PKC in VSMC. Moreover, it is of interest that inhibition of basal PKC activity, in the presence of control medium, increased the expression of both AVP and AII receptors, suggesting that PKC activity is involved in regulating the basal expression of these receptors.

Functional significance of glucose-induced pressor receptor downregulation in VSMC. Spare pressor receptors exist on many tissues in vivo. Thus, the next series of studies examined whether glucose-induced depression of AVP and AII receptor number was biochemically and functionally significant in VSMC. After binding to their specific receptors, both AVP and AII elicit similar intracellular signaling responses in VSMC, which culminate in the mobilization of Ca^{2+} from intracellular stores (34, 44). This release of intracellular Ca^{2+} is associated with an increased rate of extrusion of Ca^{2+} from the cell. The measurement of this Ca^{2+} efflux is, therefore, a measure of the magnitude of the intracellular signaling response to AVP or AII (34). Fig. 8 shows that AVP (10^{-7} M) or AII (10^{-7} M) are efficacious in stimulating Ca^{2+} efflux from VSMC. Similar results were obtained using confluent VSMC that had been preexposed to the osmotic control media (Fig. 8). In contrast, after a 48-h preexposure to a high glucose (20 mM) medium, there was a marked (~ 30% of control) and significant reduction in the amount of Ca^{2+} effluxed from confluent VSMC in response to AVP or AII. The percent of total $^{45}Ca^{2+}$ effluxed from VSMC over a 5-min period under basal, unstimulated conditions was similar after up to 48 h preexposure to each of the test media. Thus, the 30% versus control depression of AVP- or AII-stimulated $^{45}Ca^{2+}$ efflux after preexposing VSMC to a high glucose medium reflects primarily a decrease in the intracellular signaling response to those pressor hor-

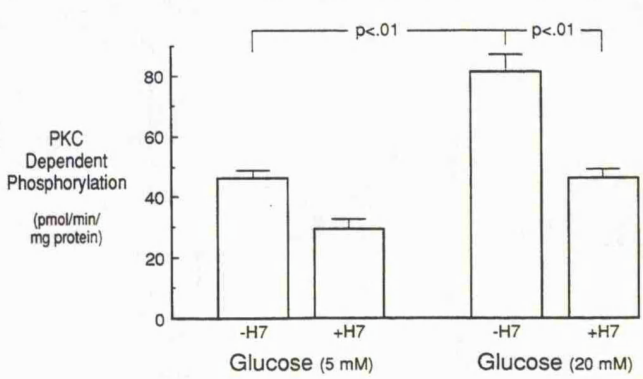

Figure 6. Inhibition of glucose-induced PKC activation by H7. Confluent VSMC were exposed to either control medium (glucose 5 mM) (*open bars*) or high glucose medium (glucose 20 mM) (*spotted bars*) ±H7 (5×10^{-5} M) for 48 h before measuring in situ PKC activity ($n = 3$).

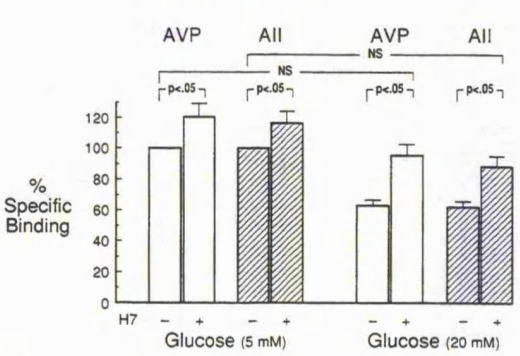

Figure 7. Effect of PKC inhibition on glucose-induced AVP and AII receptor downregulation in VSMC. Confluent VSMC were exposed to either control medium (glucose 5 mM) or high glucose medium (glucose 20 mM) ±H7 (5×10^{-5} M) for 48 h before determining the surface binding of AVP and AII to VSMC. Specific AVP (*open bars*) and AII (*hatched bars*) binding in the presence of control medium (−H7) was designated 100% and binding in the other experimental groups is expressed as percent of control ($n = 3$).

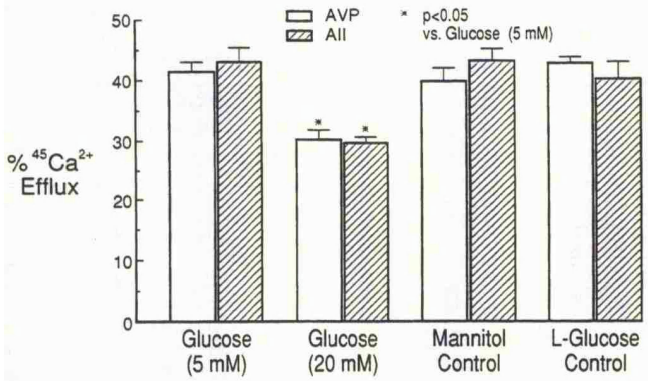

Figure 8. Effect of a high extracellular glucose concentration on AVP and AII induced $^{45}Ca^{2+}$ efflux from VSMC. Confluent VSMC were incubated with either control medium (glucose 5 mM) or high glucose medium (glucose 20 mM) for 48 h and then preloaded with $^{45}Ca^{2+}$. AVP- (10^{-7}) (*open bars*) and AII- (10^{-7} M) (*hatched bars*) stimulated $^{45}Ca^{2+}$ efflux from VSMC was measured over a 5-min period and was expressed as percent total cell $^{45}Ca^{2+}$ available at the time of stimulation. $*P < 0.05$ vs. all of the control media ($n = 4$).

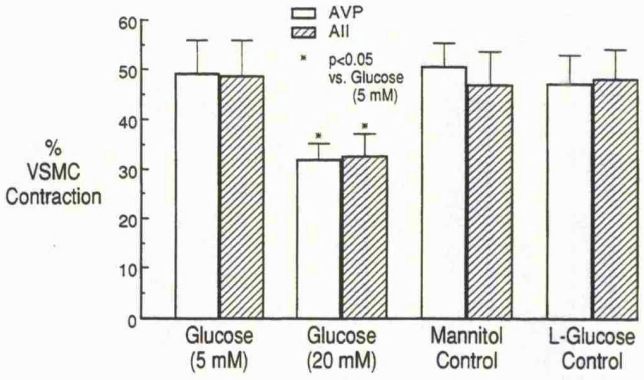

Figure 9. Effect of a high extracellular glucose concentration on the VSMC contractile response to AVP and AII. Sparsely plated primary cultures of VSMC were incubated with either control medium (glucose 5 mM) or high glucose medium (glucose 20 mM) for 48 h before measuring the contractile response of individual VSMC using microscopic digital planimetry after a 10-min exposure to AVP (10^{-6} M) (*open bars*) or AII (10^{-6} M) (*hatched bars*). $*P < 0.05$ vs. all of the control media. Each column represents the mean of measurements made on a total of ~ 100 individual cells in four separate experiments.

mones rather than any change in the basal efflux rate. Furthermore, these data suggest that the magnitude of AVP and AII receptor downregulation is paralleled by a proportionate decrease in intracellular signaling.

The process of Ca^{2+} mobilization in VSMC is closely linked to the intracellular mechanisms regulating cell contraction (44, 45). The next study therefore examined whether the glucose-induced downregulation of AVP and AII receptors on VSMC was also associated with impaired VSMC contractile responses to AVP and AII. Sparsely plated individual primary cultures of VSMC were incubated with one of the four test media for 48 h before examining VSMC contraction in response to AVP or AII (10^{-6} M). Contraction was defined as a > 15% reduction in surface area, measured using microscopic digital planimetry. Spontaneous contraction of cells due to manipulation of the media was the same after preexposure to all test media. Moreover, median cell planar surface area was not significantly different after exposure to different test media, thus contractile responses were measured from a similar baseline surface area in the presence of all test media. In the presence of control medium, almost 50% of VSMC underwent a significant contractile response after a 15-min exposure to either AVP or AII, compatible with data previously published from this laboratory (23, 24, 44). Results in the presence of the mannitol or L-glucose media were equivalent to control. However, after a 48-h exposure to a high D-glucose (20 mM) medium, the contractile response of VSMC to AVP and AII was significantly impaired (Fig. 9). These data complement the signal-transduction studies in suggesting that the PKC-dependent downregulation of AVP and AII receptors on VSMC exposed to a high glucose environment is associated with a significant depression of biochemical and physiological responses of VSMC to these pressor hormones.

Discussion

Several in vivo studies demonstrate that the early diabetic state is associated with a downregulation of diverse classes of pressor receptor on a variety of tissues (15–19). In spite of its potential pathophysiological significance with regard to vascular injury in diabetes mellitus, the factors responsible for receptor downregulation remain undefined. Hyperglycemia is the characteristic metabolic abnormality of diabetes mellitus and some studies demonstrate that an inverse relationship may exist between blood glucose concentration and hormone receptor density (16, 19). Treating rats with insulin to maintain euglycemia prevents glomerular AII receptor downregulation (16). Furthermore, in diabetic patients, there is a significant negative correlation between platelet thromboxane A_2 receptor number and glycosylated hemoglobin $A1_c$ levels (19), the latter being a measure of recent glycemic control. However, because of the multiplicity of factors that can influence the regulation of receptor expression, it has been impossible to examine the specific and independent effect of an elevated extracellular glucose concentration in vivo. To this purpose, the present study used an in vitro cell culture system to exclude other variables and uniquely demonstrates a direct and specific effect of high extracellular glucose concentrations to downregulate AVP and AII receptors on vascular tissue. This effect is not attributable to changes in extracellular osmolality but is dependent on the metabolism of D-glucose. The receptor downregulation was apparent using glucose concentrations compatible with those attained in poorly controlled diabetic patients.

Analysis of receptor-binding kinetics reveals a similar effect of high extracellular glucose concentrations on both AVP and AII binding to VSMC. The specific binding of both pressor hormones to VSMC was depressed due to a significant reduction in surface density of each receptor type rather than any significant change in receptor affinity. Of interest, similar characteristics for pressor receptor downregulation have been observed early in the course of experimental and human diabetes mellitus. In the rat glomerulus, AII receptors are downregulated within 24 h of onset of diabetes mellitus (15, 16) and, in diabetic patients, the AVP (V_1) and the thromboxane A_2 receptor are downregulated on the surface of platelets (17, 19). In each instance, the diminished hormone binding resulted from decreased receptor density without a significant change in re-

ceptor affinity, an identical response in vitro to the effect of glucose on pressor receptors demonstrated in the present study. In addition, the magnitude of receptor downregulation due to diabetes mellitus in vivo (30–58%) is similar to the maximal glucose-induced response of ~ 40%. In each in vivo study, decreased pressor receptor number in diabetic animals and man could not be explained by previous receptor occupancy or homologous desensitization due to reciprocal changes in circulating levels of the relevant hormone (15–17, 19). Moreover, more detailed studies of the AII receptor revealed appropriate AII receptor regulation in diabetic rat glomeruli after pharmacological manipulation of the renin–angiotensin system (15, 16). These latter observations imply a direct effect of a metabolic consequence of the diabetic state on pressor receptor expression. One major consequence is hyperglycemia and the present in vitro study illustrates that a potent and specific effect increased glucose to downregulate AVP and AII receptors on cultured VSMC. This effect is both qualitatively and quantitatively similar to the effects of the diabetic state on these receptors in vivo. Although it is impossible to extrapolate directly from in vitro data to the in vivo situation, this striking correspondence does suggest that the previously documented effects of diabetes on pressor receptor kinetics in vivo may be a direct consequence of an elevated extracellular glucose concentration. The present results also provide information about the cellular mechanism whereby this effect of glucose may be mediated.

PKC may be important in regulating the expression of a diverse population of cell surface receptors in a variety of tissues (25–28). The present study demonstrates that high extracellular glucose concentrations induce a sustained increase in the activity of PKC in VSMC. This observation is compatible with those of others, which show a glucose-dependent activation of PKC in many tissues in vivo and in vitro (29–32). Using isolated glomeruli, Craven and coworkers recently demonstrated that elevated glucose concentrations increase the flux of glucose through an intracellular pathway that culminates in the enhanced de novo synthesis of DAG and concluded that this increase in DAG mass may contribute to glucose-induced activation of PKC (29, 32). Numerous studies in diabetic animals confirm that DAG levels are indeed elevated in a variety of tissues, including vascular tissue (29, 31, 32, 46). The present study lends support to the aforementioned hypothesis of Craven and coworkers by demonstrating that elevated extracellular glucose concentrations of the nonmetabolized glucose isomer, L-glucose, do not activate PKC, implying that a product of glucose metabolism, perhaps DAG, is required for glucose-induced activation of PKC in VSMC.

To explore the relevance of the sustained activation of PKC by glucose to the process of glucose-induced receptor downregulation in vascular tissue, glucose-induced PKC activation was prevented with H7, a relatively specific inhibitor of PKC activation (43). This maneuver almost completely prevented glucose-induced downregulation of the AVP or AII receptor, providing strong evidence that glucose-induced pressor receptor downregulation is dependent on the capacity for glucose to activate PKC in VSMC. It is important to note, however, that H7 is not totally specific for PKC and it therefore remains possible that the concomitant inhibition of other kinase systems could be contributing to the effects of H7 on glucose-induced receptor downregulation, as observed in the present study.

The present study demonstrates a slow onset and slow recovery of glucose-induced AVP and AII receptor downregulation, requiring hours. The recycling time for the AVP (V_1) and AII receptors, however, is rapid in VSMC, ~ 15–20 min (47, 48). An effect of glucose to inhibit this process would have been more immediately apparent. Thus the slow downregulation of AVP and AII receptors by the high glucose environment and their slow recovery is more likely to reflect modulation of receptor biosynthesis. An effect of PKC on epidermal growth factor receptors has suggested this mechanism in the Swiss/3T3 line. It thus seems possible that the PKC dependency of the observed glucose-mediated receptor downregulation may inhibit the expression of receptor mRNA.

Diabetes-induced downregulation of pressor receptors may be relevant to the increased blood flow to many tissues that has been implicated as an important factor in the early pathogenesis of microvascular injury (8–10). The present study thus explored the biochemical and functional significance of glucose-induced AVP and AII receptor downregulation in VSMC. The results show that AVP- and AII-stimulated Ca^{2+} efflux from VSMC and the contractile response of individual VSMC to these agents are markedly attenuated after preexposing VSMC to a high glucose environment for 48 h. As in the effects on receptor density and PKC activation, these effects were specific for the high D-glucose medium. Moreover, the magnitude of glucose-induced AVP or AII receptor downregulation appeared to parallel the glucose-induced defect in AVP- and AII-stimulated intracellular signaling and contractility in VSMC. Although this does not prove causality, recent studies have demonstrated that there are few, if any, spare V_1 or AII receptors on VSMC (47, 49), thus implying that the downregulation of either receptor on VSMC would be associated with a decrease in VSMC response. It should be emphasized that in addition to the role in receptor downregulation, glucose-induced PKC activation may also exert direct downregulatory influences on postreceptor signal transduction mechanisms in VSMC (23, 50–52). Recently, the glucose-induced PKC activation has been characterized in cultured VSMC (53).

In conclusion, the present study uniquely demonstrates that high extracellular glucose concentrations depress the expression of AVP and AII receptors on VSMC. This effect is dependent on glucose-induced PKC activation and is likely to be of physiological significance in view of the associated marked attenuation of VSMC functional responses to AVP and AII. This novel mechanism may contribute to pressor receptor downregulation in early diabetes mellitus, thereby directly implicating hyperglycemia in the pathophysiology of hemodynamically mediated vascular injury in this disease.

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Chapter 4.

Glucose-induced Protein Kinase C Activity Regulates Arachidonic Acid Release and Eicosanoid Production by Cultured Glomerular Mesangial Cells.

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Glucose-induced Protein Kinase C Activity Regulates Arachidonic Acid Release and Eicosanoid Production by Cultured Glomerular Mesangial Cells

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Abstract

Changes in glomerular eicosanoid production have been implicated in the development of diabetes-induced glomerular hyperfiltration and glomerular mesangial cells (GMC) are major eicosanoid-producing cells within the glomerulus. However, the mechanism for the effect of diabetes mellitus on glomerular mesangial eicosanoid production is unknown. The present study therefore examined whether elevated glucose concentrations activate protein kinase C (PKC) in GMC and whether this PKC activation mediates an effect of elevated glucose concentrations to increase the release of arachidonic acid and eicosanoid production by GMC. The percentage of [^{3}H]arachidonic acid release per 30 min by preloaded GMC monolayers was significantly increased after 3-h exposure to high glucose (20 mM) medium (177% vs control medium) and this increase was sustained after 24-h exposure to high glucose concentrations. 3-h and 24-h exposure to high glucose medium also increased PGE_2, 6-keto-$PGF_{1\alpha}$, and thromboxane (TXB_2) production by GMC. High glucose medium (20 mM) increased PKC activity in GMC at 3 and 24 h (168% vs control). In contrast, osmotic control media containing either L-glucose or mannitol did not increase arachidonic acid release, eicosanoid production, or PKC activity in GMC. Inhibiting glucose-induced PKC activation with either H-7 (50 μM) or staurosporine (1 μM) prevented glucose-induced increases in arachidonic acid release and eicosanoid production by GMC. These data demonstrate that elevated extracellular glucose concentrations directly increase the release of endogenous arachidonic acid and eicosanoids by GMC via mechanisms dependent on glucose-induced PKC activation. (*J. Clin. Invest.* 1993. 92:2889–2896.) Key words: glucose • protein kinase C • mesangial cell • arachidonic acid • eicosanoids

Introduction

Diabetes mellitus in experimental animals and humans is associated with qualitative and quantitative changes in PG and TX production by many tissues (1–13). With regard to the kidney, previous studies demonstrate increased production of PGE_2, 6-keto-$PGF_{1\alpha}$, and TXB_2 by glomeruli isolated from rats with early streptozotocin-induced diabetes mellitus (8, 9, 11–13). These changes have in turn been implicated in the pathogenesis of glomerular hemodynamic abnormalities that may play a role in the development of diabetic glomerulopathy (8, 9, 12, 14).

The mechanisms responsible for diabetes-induced changes in glomerular eicosanoid production are unclear but may depend on increased release of arachidonic acid (8, 9), perhaps via diabetes-induced increases in glomerular phospholipase A_2 activity (15). Thus far, however, the factors and mechanisms responsible for these diabetes-induced increases in PLA_2 activity and arachidonic acid release are unknown. Hyperglycemia is the hallmark of diabetes mellitus, thus glucose has to be considered as a potential mediator of increased glomerular eicosanoid production. In this regard, a previous study has shown that when exposed to elevated extracellular glucose concentrations, cultured glomerular mesangial cells (GMC)[1] produce increased quantities of prostaglandin, predominantly PGE_2 (9).

The GMC is a major prostanoid-producing cell within the glomerulus and the rate of endogenous arachidonic acid release is usually rate limiting for GMC eicosanoid synthesis (16). Recent evidence suggests that activation of the calcium/phospholipid-dependent protein kinase C system (PKC) plays a key role in regulating arachidonic acid release by many cell types, including GMC (17–26). Moreover, PKC activation may also increase PGE_2 production by GMC (17, 26, 27). These observations are intriguing because high glucose concentrations have been shown to increase PKC activity in many tissues in vivo and in many cell types in vitro (28–33).

Taken together, the aforementioned observations suggest that glucose may directly influence GMC arachidonic acid release and eicosanoid production via mechanisms dependent on glucose-induced PKC activation. Examining this hypothesis in vivo would be impossible because diabetes mellitus is associated with a myriad of metabolic and hormonal changes that could influence PKC activity and eicosanoid production by GMC. Thus, the present study uses cultured GMC in vitro to test the hypothesis that elevated extracellular glucose concentrations directly increase arachidonic acid release and prostaglandin production by GMC via mechanisms dependent on glucose-induced PKC activation.

Methods

Glomerular mesangial cell culture

GMC were cultured from glomeruli isolated from 200–300-g nondiabetic Sprague Dawley rats as previously described in detail (34, 35). The GMC were grown as monolayers on 35-mm culture dishes in RPMI 1640 buffered with 10 mM Hepes at pH 7.4 and were supplemented with 20% FCS, 5 μg human transferrin, 200 mg/liter

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1. *Abbreviations used in this paper:* DAG, diacylglycerol; GMC, glomerular mesangial cell; PKC, protein kinase C; PLA_2, phospholipase A_2; PSS, physiologic salt solution.

$HaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin. Once confluent, the cells were passaged after incubation with 0.5 ml 0.25% trypsin and 0.01% EDTA for 10 min at 37°C. Studies were performed on GMC monolayers at 4th through 10th passage.

Experimental design

When confluent, GMC monolayers were exposed to one of four test culture media described below.

Control medium. This comprised standard GMC culture medium as described above (D-glucose concentration 5 mM/liter).

High glucose medium. This medium was identical to control medium but with a D-glucose concentration of 20 mM/liter.

Mannitol medium. This was identical to control medium but supplemented with mannitol (D-glucose 5 mM/liter plus mannitol 15 mM/liter), a cell impermeable hexose.

L-Glucose medium. This was identical to control medium but supplemented with L-glucose (D-glucose 5 mM/liter plus L-glucose 15 mM/liter), a cell-permeable but poorly metabolized glucose isomer. The mannitol and L-glucose contained an osmotic load equivalent to the high glucose medium and thus served as osmotic controls. Because L-glucose is poorly metabolized, this medium also served as a metabolic control for the high D-glucose medium.

After exposure to the test media for either 3 or 24 h in an incubator at 37°C, the medium was removed by aspiration and the GMC monolayers were gently washed with 2 ml of a physiologic salt solution (PSS) (37°C, pH 7.4). The monolayers were then incubated for 30 min with 1 ml fresh PSS supplemented with 0.1% BSA and either D-glucose, mannitol, or L-glucose as appropriate to mimic the composition of the test media to which the cells were previously exposed. At the end of this incubation, a 900-μl aliquot of the supernatant was removed and immediately frozen and stored at −20°C until assayed to determine the quantity of eicosanoids released by the GMC monolayers in response to the different media conditions. The GMC monolayers were then solubilized with 0.1% sodium dodecyl sulfate and 0.1 N NaOH and a 50-μl aliquot of the cell solution taken for assay of protein content per dish using the method of Lowry (36).

Assay of prostaglandins and thromboxane

The quantity of PGE_2, 6-keto $PGF_{1\alpha}$ (the stable metabolite of PGI_2 or prostacyclin), and TXB_2 (the stable metabolite of TXA_2) in the GMC supernatants was determined using an ELISA technique as described by Pradelles et al. (37). Briefly, 50-μl aliquots of the GMC supernatant were assayed without prior purification. 50 μl of each sample or buffer (0.1 M potassium phosphate, 0.01% NaN_3, 0.4 M NaCl, 1 mM EDTA, and 0.1% BSA, pH 7.4) was added to each well of a 96-well microliter plate which was previously coated with goat anti-rabbit IgG antibody (2 μg/well). Enzyme tracer (50 μl) consisting of either PGE_2, 6-keto-$PGF_{1\alpha}$, or TXB_2 covalently linked to purified acetylcholinesterase from electric eel was added. Finally, 50 μl of the appropriate eicosanoid-specific antiserum was added and the plates were incubated for 16–24 h at 4°C before washing with 10^{-2} M phosphate buffer, pH 7.4, containing 0.05% Tween 20, using an automatic plate washer (Flow Labs Inc., McLean, VA). The plates were then automatically filled with 200 μl/well of the following medium: 2 μg/ml acetylthiocholine iodide and 2.15 μg/ml of 5-5′-dithiobis(2-nitrobenzoic acid) in 10^{-2} M phosphate buffer. The production of a yellow colored product was measured at 414 nm using an automatic plate reader (Titertek Multiscan MC; Flow Labs). Each sample was assayed in duplicate. Nonspecific binding was determined using an incubation mixture in which the specific antibody was replaced by 50 μl of assay buffer. A standard curve was constructed by plotting B/Bo% (absorbance measured on the bound fraction in the presence of eicosanoid competitor divided by the absorbance in the absence of competitors) vs picograms eicosanoid per well. Fitting of the standard curve and calculation of the quantity of eicosanoid in the sample were performed using a nonlinear curve fitting program (38). Cross-reactivity between each specific antibody and eicosanoids, exogenous arachidonic acid and/or their metabolites was in each case < 1%.

Assay of [^{3}H]arachidonic acid release by GMC

To determine the effects of a 3-h exposure to the various test media on endogenous arachidonic acid release by GMC, the GMC arachidonic acid pool was radiolabeled by exposing GMC monolayers for 24 h to control medium supplemented with 0.5 μCi/ml [^{3}H]arachidonic acid (1, 5, 6, 8, 9, 11, 12, 14, 15-[^{3}H]arachidonic acid, 214 Ci/mmol). The radioactive medium was then aspirated and the GMC monolayers were washed rapidly with 10 × 1 ml PSS supplemented with 0.1% BSA. The [^{3}H]arachidonic acid-labeled GMC monolayers were incubated with the various test media for 3 h at 37°C. Thereafter, the test medium was aspirated and replaced with 1 ml of fresh test medium and incubated for a further 30 min. A 900-μl aliquot of the medium overlying the cells was removed and retained for scintillation counting of the quantity of [^{3}H]arachidonic acid released by GMC into the test medium per 30 min (Tri-carb 460C; Packard, Downer's Grove, IL). The GMC monolayer was washed rapidly (10 × 1 ml PSS plus 0.1% BSA), solubilized, and the protein content was determined as described above. A sample of the solubilized GMC monolayer was retained for scintillation counting of the quantity of [^{3}H]arachidonic acid retained by the GMC. The amount of radioactive arachidonic acid released per 30 min was expressed as a percentage of the total radioactivity available for release (i.e., released plus GMC radioactivity).

To study the effects of 24-h incubation with the test media on the release of arachidonic acid by GMC, a similar protocol was used but the test media was supplemented with 0.5 μCi [^{3}H]arachidonic acid during the 24-h incubation period with the test media to achieve GMC loading. The various test media did not influence GMC loading with [^{3}H]arachidonic acid over the 24-h exposure. After incubation, the GMC monolayers were washed and incubated with fresh test medium for 30 min to determine the impact of prolonged exposure to the test media on the release of [^{3}H]arachidonic acid from GMC as described above.

Effect of test media on [^{3}H]arachidonate reincorporation by GMC

The quantity of [^{3}H]arachidonate measured in the medium overlying the GMC monolayer is determined not only by its rate of release but also by its rate of reincorporation by GMC. To determine whether the test media influenced the rate of [^{3}H]arachidonate incorporation, GMC monolayers were preexposed to the various test media for 24 h. Thereafter, the medium was aspirated and replaced with a similar test medium supplemented with 0.5 μCi [^{3}H]arachidonate for 30 min. The radioactive medium was then removed and retained for scintillation counting. The GMC monolayers were washed rapidly (10 × 1 ml PSS plus 0.1% BSA), solubilized, and an aliquot of the cell solution was taken for counting of the radioactivity incorporated by GMC. The quantity of [^{3}H]arachidonic acid incorporated by GMC per 30 min was expressed as a percentage of total radioactivity available for incorporation (i.e., radioactive supernatant counts plus GMC associated counts).

Measurement of PKC activity in GMC

PKC activity in GMC monolayers was measured using a modification of the method recently described by Heasley and Johnson (39, 40). This method has been validated previously in our laboratory and used to characterize in situ PKC activation in cultured vascular smooth muscle cells (33). GMC were seeded onto flat-bottomed 96-well microtiter plates at a density of ~ 20,000/well and maintained in regular growth medium for 3 d until confluent. GMC monolayers were then incubated with the test media for 3 or 24 h. After incubation, the test medium was aspirated and replaced with 40 μl of a buffered salt solution containing 137 mM NaCl, 5.4 mM KCl, 10 mM $MgCl_2$, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 25 mM β-glycerophosphate, 5.5 mM D-glucose, 5 mM EGTA, 1 mM $CaCl_2$ (~ 100 nM free Ca^{2+}), 100 μM [γ-^{32}P]ATP (~ 5,000 cpm/pmol), 50 μg/ml digitonin, and 20 mM Hepes (pH 7.2, 37°C). In addition, 100 μM of a PKC-specific peptide substrate (VRKRTLRRL) was added to the

buffer. This short synthetic peptide is based on the sequence surrounding a major PKC-dependent phosphorylation site within the epidermal growth factor receptor (41). This peptide substrate is not phosphorylated by cyclic nucleotide-dependent or Ca^{2+}/calmodulin–dependent protein kinases or S6 kinase and has been extensively characterized to be highly specific for PKC (39, 40). By permeabilizing the GMC with the digitonin contained in the buffer, the VRKRTLRRL peptide enters the GMC along with [γ-^{32}P]ATP to allow a highly selective and rapid analysis of in situ PKC activity. Similar to our findings with vascular smooth muscle cells, the concentration of digitonin used (50 μg/ml) did not modify GMC morphology or promote cell detachment. In addition, PKC activity was retained by the monolayer after permeabilization (PKC activity in supernatant was 7.3% of total measured PKC activity). This latter finding is consistent with the concept that the PKC activity being assayed was tightly associated with the cell membrane as has been proposed for the active form of the kinase (42). The kinase reaction was linear for up to 20 min and was thus allowed to proceed for 10 min at 30°C before termination of the assay by the addition of 50 μl ice cold 25% (wt/vol) trichloracetic acid (final TCA concentration 5%). Aliquots (45 μl) of the acidified reaction mixture were then spotted onto 2-cm phosphocellulose paper circles (P81; Whatman Inc., Clifton, NJ) and washed batchwise; three washes with 75 mM phosphoric acid and one wash with 75 mM sodium phosphate (pH 7.5) (500 ml/2-min wash). Due to the basicity of the VRKRTLRRL substrate, it was retained by the phosphocellulose filter at neutral pH, while contaminating [γ-^{32}P]ATP was removed. The PKC-dependent phosphorylation of the peptide substrate bound to the filter was quantified by scintillation counting. Background phosphorylation was assessed in two ways: (*a*) Immediately before the addition of the reaction buffer, 40 μl of ice cold TCA was added to precipitate cellular protein and eliminate kinase activity. The TCA was aspirated, the reaction buffer was added, and the assay was performed as described above to determine the kinase-independent phosphorylation of the VRKRTLRRL substrate. (*b*) To determine background phosphorylation of substrates other than VRKRTLRRL, the assay was performed in the absence of VRKRTLRRL. In both instances, background phosphorylation was always < 0.05% of added cpm and not different in GMC that had been exposed to any of the test media, H-7, or staurosporine. This confirms that the low level of background phosphorylation detected by this assay was constant, was not influenced nonspecifically by the test media, and thus did not influence the interpretation of the final result. Results are expressed as PKC dependent phosphorylation, pmol/min per mg GMC protein.

Statistical analysis

Results are expressed as mean±SEM. Statistical analysis was made using an unpaired Student's *t* test or ANOVA with a Bonferroni correction as appropriate. For each experiment, *n* refers to the number of studies (each in triplicate).

Materials

[^{3}H]Arachidonate and [γ-^{32}P]ATP were purchased from Amersham Corp., Arlington Heights, IL. Goat anti–rabbit IgG antibody was purchased from Pel-Freez Biologicals, Rogers, AR. The VRKRTLRRL substrate was a generous gift from Dr. Lynn E. Heasley and Prof. Gary L. Johnson, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO. The specific PGE_2 and TXB_2 antibodies were a generous gift from Dr. Frank Fitzpatrick, University of Colorado Health Sciences Center, Denver, CO. The specific 6-keto-$PGF_{1\alpha}$ antibody was a generous gift from Dr. Ken Allen, Colorado State University, Fort Collins, CO. All other reagents used were of the highest grade available from Sigma Chemical Co., St. Louis, MO.

Results

Effects of test media on eicosanoid production by GMC. In the presence of control medium (D-glucose 5 mM), GMC produced predominantly PGE_2, with lesser quantities of 6-keto-$PGF_{1\alpha}$ and very small quantities of TXB_2. This eicosanoid production ratio is very similar to that reported by others for cultured rat GMC (9, 43). Exposing GMC to a high glucose medium (glucose 20 mM) for 3 h produced a marked and significant increase in the basal production rate of PGE_2 per 30 min by GMC (359% vs control medium) (Fig. 1). Prolongation of high glucose exposure to 24 h further increased the basal production rates of PGE_2 by GMC (480% vs control medium). Exposure to high glucose medium also increased GMC production of 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2 or prostacyclin) at 3 h (control: 376±19 vs high glucose: 1,108±44 pg/mg protein per 30 min, $P < 0.001$) and 24 h (control: 482±36 vs high glucose: 1,575±60, $P < 0.001$). Relative to PGE_2 and PGI_2, GMC produce trivial quantities of thromboxane (TXA_2), nevertheless, GMC production of TXB_2 (the stable metabolite of TXA_2) was also increased in the presence of a high glucose medium at 3 h (control: 181±17 vs high glucose: 374±28 TXB_2 pg/30 min per mg protein, $P < 0.01$) and 24 h (control: 188±20 vs 386±28, $P < 0.004$). These effects were specific for the high D-glucose medium and not related to changes in extracellular osmolality because they were not reproduced by 24-h exposure to either of the osmotic control media (Fig. 1). Furthermore, the fact that the medium containing the poorly metabolized glucose isomer (L-glucose) did not influence eicosanoid production suggests that the intracellular metabolism of D-glucose was essential for glucose-induced changes in GMC eicosanoid production to occur.

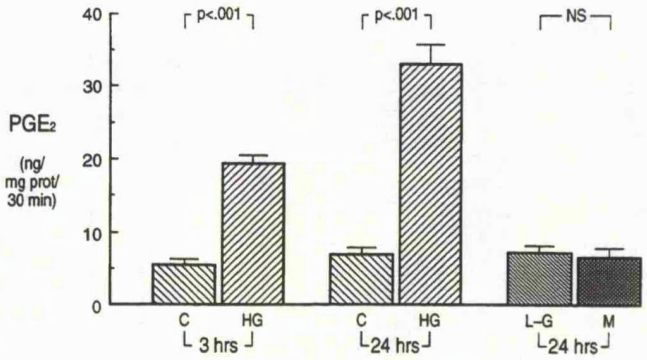

Figure 1. Basal production rates of PGE_2/30 min per mg cell protein by GMC monolayers after 3- or 24-h exposure to control medium (*C*)[D-glucose 5 mM], or high glucose medium (*HG*)[D-glucose 20 mM], and 24-h exposure to either L-glucose osmotic control medium (*L-G*) or mannitol osmotic control medium (*M*) ($n = 3$).

Glucose concentration dependency of changes in GMC eicosanoid production. Fig. 2 shows that glucose-induced increases in GMC-PGE_2 production were glucose concentration dependent up to a threshold glucose concentration of 20 mM. A similar profile of glucose concentration dependence was observed for GMC 6-keto-$PGF_{1\alpha}$ and TXB_2 production (data not shown). The time dependency of glucose-induced increases in GMC eicosanoid production was similar for all three, requiring at least 2-h exposure to the high glucose medium before significant increases in basal eicosanoid production rates occurred.

Effect of exogenous arachidonic acid on PGE_2 production by GMC. The fact that high extracellular glucose concentrations increased the basal production rates of all eicosanoids by a similar order of magnitude implies that glucose acted to in-

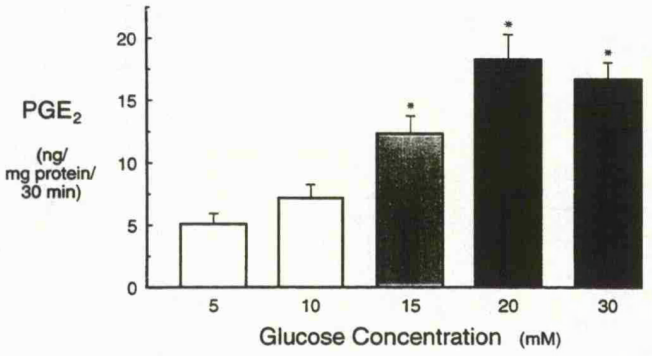

Figure 2. Glucose concentration dependency of PGE_2 production by GMC measured after 24-h exposure to culture media containing various concentrations of D-glucose. * $P < 0.01$ vs glucose 5 mM, $n = 3$.

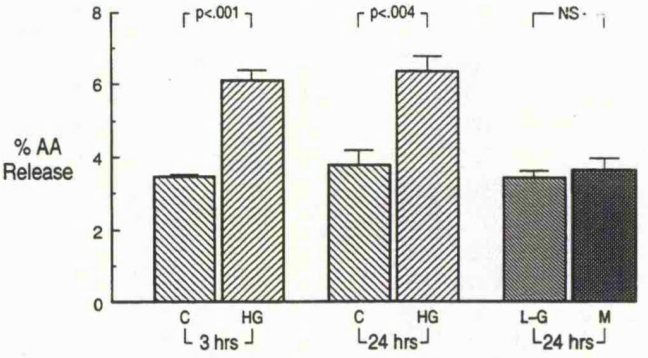

Figure 3. Effects of the various test media on percentage of arachidonic acid release per 30 min by GMC. The GMC monolayers prelabelled with [^{3}H]arachidonic acid were exposed to control (*C*) or high glucose medium (*HG*) for 3 and 24 h, or L-glucose osmotic control medium (*L-G*) and mannitol osmotic control medium (*M*) for 24 h (see Results) ($n = 3$).

crease the availability of a common substrate for their synthesis, notably arachidonic acid. The next series of studies thus examined the effect of elevated extracellular glucose concentrations on endogenous arachidonic acid release by GMC. Table I shows that supplementing the control or high glucose media with exogenous arachidonic acid (30 μM) eliminated the high glucose-induced increase in PGE_2, suggesting that glucose-induced increases in GMC eicosanoid production primarily resulted from enhanced availability of endogenous arachidonic acid.

Effects of test media on [^{3}H]arachidonic acid release by GMC. To examine specifically whether high glucose concentrations directly enhanced the release of endogenous arachidonic acid from GMC, the release of [^{3}H]arachidonate from preloaded GMC was examined. Fig. 3 shows that 3-h incubation with the high glucose medium induced a marked and significant increase in endogenous arachidonic acid release by GMC (177% vs control medium). This effect was sustained with prolonged exposure to the high glucose medium for 24 h. In contrast, 24-h exposure to the osmotic control media did not reproduce the effect of the high glucose medium.

Effect of extracellular glucose concentration on reincorporation of arachidonic acid by GMC. To determine whether glucose stimulated the release of arachidonic acid from GMC, rather than inhibited arachidonic acid reincorporation, GMC were exposed to control or high glucose medium for 24 h. Thereafter, the incorporation of [^{3}H]arachidonic acid by GMC was measured over a 30-min period, a time period identical to that used for the measurement of arachidonic acid release. After exposure to the high glucose medium, the percentage of [^{3}H]arachidonic acid incorporation by GMC was slightly increased (control medium: 16.2±0.2% vs high glucose medium: 17.7±0.4%, $P < 0.05$, $n = 3$). These results confirm that the increased quantities of [^{3}H]arachidonic acid measured in the supernatant of GMC monolayers exposed to high glucose concentrations represented glucose-stimulated release of endogenous arachidonic acid by GMC rather than reduced reincorporation.

Table I. Effect of Exogenous Arachidonic Acid on PGE_2 Production by GMC

	PGE_2 Production	
Experimental group	−Arachidonic acid	+Arachidonic acid
	ng/mg protein per 30 min	
Control	6.4±0.6	76.3±8.1*
High glucose	32.1±3.1*	84.1±4.2*

GMC monolayers were exposed to control or high glucose medium (glucose 20 mM) for 24 h ± exogenous arachidonic acid (30 μM) before the measurement of PGE_2 production. * $P < 0.001$ vs control medium. There was no significant difference in PGE_2 production between control vs high glucose medium in the presence of arachidonic acid ($n = 3$).

Effect of test media on in situ PKC activity in GMC. High extracellular glucose concentrations have been shown to activate PKC in endothelial, vascular smooth muscle, and GMC in culture (30, 32, 33). Furthermore, PKC activation directly via phorbol esters, or via hormonal stimulation, has been shown to augment arachidonic acid release and prostaglandin production by various cell types (17–26). Thus, the next series of studies examined whether PKC activation was involved in the mechanism(s) whereby glucose stimulated increased arachidonic acid release and increased eicosanoid production by GMC. Fig. 4 shows that after 3-h exposure to high D-glucose (20 mM) medium, in situ PKC activity was markedly increased compared to the PKC activity measured in GMC exposed to control medium (D-glucose 5 mM) and was sustained for up to 24 h with continued exposure to the high glucose medium. In contrast, 24-h exposure to the two osmotic control media produced no appreciable stimulation of in situ PKC activity, confirming that PKC activation was not osmotically mediated but did require the increased metabolism of D-glucose (Fig. 4). The glucose concentration dependency of PKC activation in GMC is shown in Fig. 5. Maximal glucose-induced PKC activation was observed at a glucose concentration of 20 mM.

To evaluate the role of PKC activity in mediating glucose-induced increases in arachidonic acid release and eicosanoid production by GMC, two relatively specific but dissimilar inhibitors of PKC were used (H-7 and staurosporine) (44, 45). GMC were coincubated with either H-7 (in 0.1% DMSO) or staurosporine in either control medium or high glucose medium for 24 h before determining PKC activity. DMSO alone

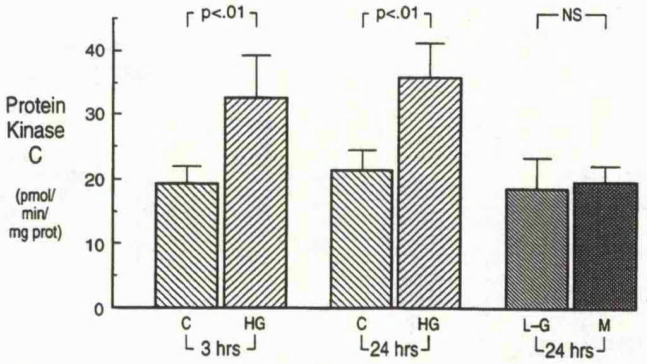

Figure 4. Glucose-induced PKC activity in GMC monolayers. Confluent GMC monolayers were exposed to control (*C*) or high glucose (*HG*) medium for 3 and 24 h, or to L-glucose osmotic control medium (*L-G*) or mannitol osmotic control medium (*M*) for 24 h before measuring PKC activity. PKC activity was measured in situ in digitonin-permeabilized GMC by determining the phosphorylation of a PKC-specific peptide substrate (VRKRTLRRL). Results are expressed as picomoles phosphate transferred to this substrate per minute per milligram GMC protein ($n = 4$).

had no effect on basal or glucose-stimulated PKC activity (data not shown). With control medium, H-7 at a concentration of 50 μM or staurosporine (1 μM) had little effect on basal PKC activity. However, when coincubated with the high glucose medium, both H-7 and staurosporine significantly inhibited glucose-induced PKC activation in GMC (Fig. 6).

The role of PKC in glucose-induced changes in arachidonic acid release and prostaglandin production by GMC. Fig. 7 shows that in the absence of PKC inhibitors, 3- or 24-h incubations with high glucose medium significantly augmented arachidonic acid release by GMC. However, when the high glucose medium was supplemented with either H-7 or staurosporine, glucose-induced increases in arachidonic acid release were prevented, suggesting that glucose-induced increases in arachidonic acid release by GMC are dependent on the capacity of glucose to activate PKC.

Increased arachidonic acid release is often taken to represent PLA_2 activation. It was possible therefore that H-7- and staurosporine-induced inhibition of glucose-stimulated increases in arachidonic acid release represented nonspecific inhibition of PLA_2 by these agents. To examine this possibility,

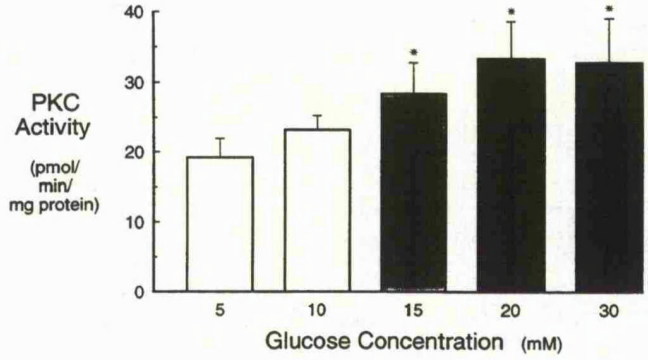

Figure 5. Glucose concentration dependency of PKC activation in GMC exposed to culture media containing various D-glucose concentrations for 24 h. $*P < 0.01$ vs glucose 5 mM, $n = 3$.

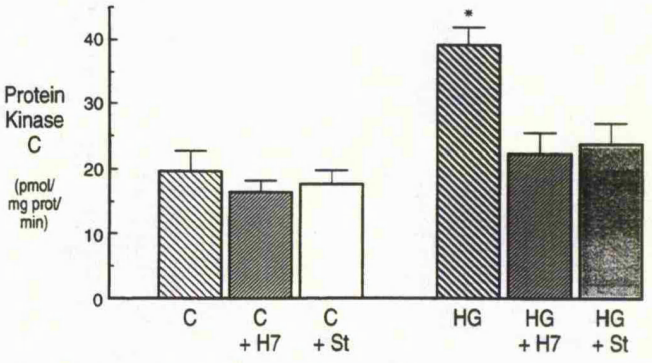

Figure 6. Inhibition of glucose-induced PKC activity by H-7 and staurosporine. Confluent GMC monolayers were exposed to control (*C*) or high glucose medium (*HG*)±H-7 (50 μM) or±staurosporine (St) (1 μM) for 24 h before measuring PKC activity. $*P < 0.01$ vs C and vs C + H-7 and vs C + St ($n = 3$).

GMC were preloaded with [^{3}H]arachidonic acid for 24 h before a 30-min incubation with exogenous PLA_2 (1 U/ml)±H-7 or staurosporine. Table II shows that exogenous PLA_2 significantly increased [^{3}H]arachidonic acid release by GMC. The addition of H-7 or staurosporine did not inhibit PLA_2-induced arachidonic acid release, confirming that the PKC inhibitors used in the present study did not nonspecifically inhibit PLA_2 activity.

The next study examined the effects of H-7 and staurosporine on glucose-induced increases in PGE_2 production by GMC. Fig. 8 shows that exposing GMC to high glucose medium in the presence of either PKC inhibitor markedly attenuates the high glucose-induced increase in PGE_2 production. Together, these data demonstrate that high extracellular glucose concentrations directly increase eicosanoid production by GMC via glucose-induced increases in endogenous arachidonic acid release, which in turn depends on glucose-induced PKC activation in GMC.

Discussion

Mesangial cells are an abundant source of glomerular eicosanoids and the present study demonstrates that elevated extracel-

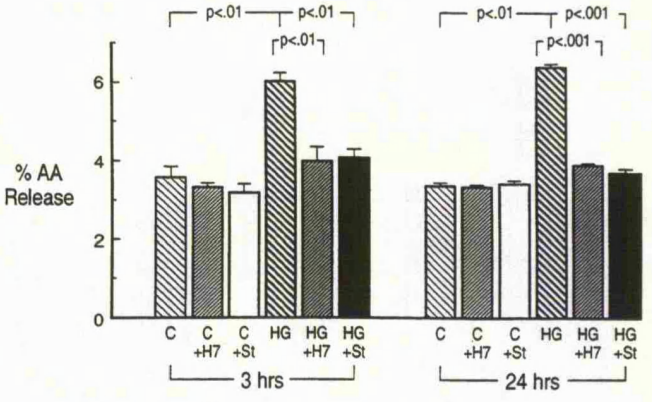

Figure 7. Effect of PKC inhibition on glucose-stimulated arachidonic acid release by GMC. Percentage of [^{3}H]arachidonic acid release by preloaded GMC was measured after 3- and 24-h exposure to control (*C*) or high glucose (*HG*)±H-7 or±staurosporine (St) ($n = 3$).

Table II. Effects of Exogenous PLA_2 on Percentage of [^3H]Arachidonic Acid Release by GMC per 30 min

	Percentage of [^{3}H]arachidonic acid release by GMC
Basal	3.22±0.12
+PLA_2	6.90±0.27*
+PLA_2 + H-7	6.93±0.15*
+PLA_2 + staurosporine	6.88±0.31*

Basal refers to unstimulated GMC in the presence of control medium. PLA_2 (1 U/ml) was added to the culture medium alone, or the presence of H-7 (50 μM) or staurosporine (1 μM) during the 30-min period during which arachidonic acid release was measured. * $P < 0.001$ vs Basal ($n = 3$).

lular glucose concentrations directly and independently increase the basal production rates of PGE_2, PGI_2, and TXA_2 by cultured rat glomerular mesangial cells. This effect is apparent within hours of exposure to high glucose and persists for at least 24 h with continued exposure. These observations are consistent with a previous report demonstrating that chronic exposure to a high glucose medium (30 mM) for 10–14 d increased the production of predominantly PGE_2 by cultured rat GMC (9). Until now however, the mechanism underlying this potentially important action of glucose has remained undefined. In pursuit of such a mechanism, the present study demonstrates that elevated extracellular glucose concentrations directly increase endogenous arachidonic acid release by GMC.

The regulation of endogenous arachidonic acid release by GMC is complex and rate limiting for eicosanoid production (16). Recent evidence suggests an important role for PKC in the regulation of arachidonic acid release (17–26). Specifically, PMA, which binds to and activates PKC, has been shown to simulate arachidonic acid release and eicosanoid production by many cell types including the mesangial cell (17–26). Additional studies support a role for PKC in the hormonal regulation of glomerular arachidonic acid release and PGE_2 production (25–27). The present study demonstrates that elevated

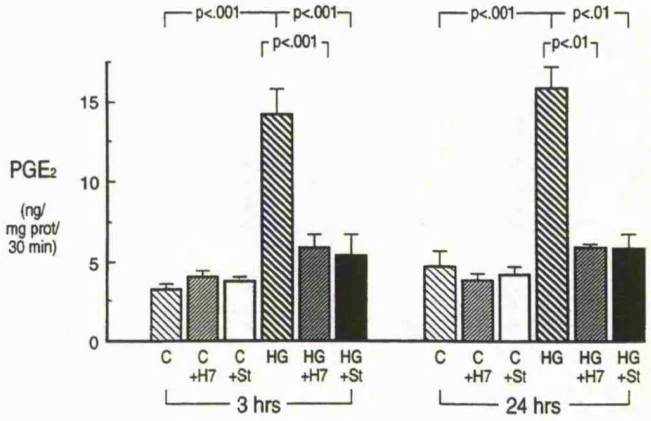

Figure 8. Effect of PKC inhibition on glucose-stimulated PGE_2 release by GMC monolayers. GMC monolayers were exposed to control (*C*) or high glucose (*HG*) medium for 3 and 24 h±H-7 or±staurosporine (St) before determining basal PGE_2 production ng/30 min per mg GMC protein ($n = 3$).

extracellular glucose concentrations induce a sustained activation of PKC in GMC. This result is consistent with a preliminary report showing that glucose-induced PKC activation is sustained for many days in cultured GMC (46). With regard to the mechanism whereby glucose activates PKC in GMC, the studies of Craven et al. (28) recently demonstrated that elevated glucose concentration increase the flux of glucose through an intracellular pathway that culminates in the enhanced de novo synthesis of diacylglycerol (DAG) and concluded that the increase in cellular DAG mass contributes to glucose-induced PKC activation. In support of this conclusion, Ayo et al. (32) recently confirmed that high glucose concentrations (30 mM) for up to 1 wk caused at least a threefold increase in DAG mass in GMC. The present study lends further support to this hypothesis by demonstrating that elevated concentrations of a nonmetabolized isomer, L-glucose, did not activate PKC, implying that a product of glucose metabolism, such as DAG, is required for glucose-induced activation of PKC in GMC.

The glucose concentration required for maximal PKC activation (20 mM) in GMC is compatible with blood glucose concentrations attained in experimental models of diabetes mellitus and poorly controlled diabetic patients. Moreover, the striking similarity between the concentration dependency of glucose-induced changes in PKC activity and GMC eicosanoid production suggests that these two events could be causally related. Concordant with this hypothesis, the present study demonstrates that two chemically dissimilar inhibitors of glucose-induced PKC activation, H-7 and staurosporine, prevented both the glucose-induced increase in arachidonic acid release and eicosanoid production by GMC, suggesting that glucose-induced changes in eicosanoid metabolism may be mediated via glucose-induced PKC activation. However, as neither H-7 nor staurosporine are totally specific in their inhibition of PKC, the role of concomitant inhibition of other kinases by these inhibitors cannot be excluded.

In most cell types, PLA_2 is believed to be the primary effector enzyme for arachidonic acid release (16) and in this regard, [^{3}H]arachidonic acid release rates are often considered to be representative of PLA_2 activity. PKC has been shown to increase PLA_2 activity in many cell types, either by phosphorylation of a 40-kD regulatory protein indistinguishable from lipocortin, and/or direct phosphorylation and posttranslational modification of PLA_2 (47–49). PKC-induced activation of PLA_2 could thus explain the PKC-dependent effect of glucose to increase arachidonic acid release by GMC. In this regard, PLA_2 activity was shown to be increased in glomeruli isolated from diabetic rats, when compared to glomerular PLA_2 activity in control rats (15). Moreover, the diabetic glomeruli also released more arachidonic acid and eicosanoids suggesting that the increased PLA_2 activity was of physiologic significance. The results of the present study complement and extend these observations by indicating that high extracellular glucose concentrations is likely the key mediator of increased glomerular arachidonic acid release and eicosanoid synthesis in these diabetic animals, as a direct consequence of PKC-mediated modulation of PLA_2 activity. These observations do not, however, exclude the possibility that diabetes-induced increases in glomerular eicosanoid synthesis could also occur as a consequence of additional changes in cyclooxygenase activity (8).

Glucose-induced increases in GMC eicosanoid production could have considerable pathophysiologic significance. Early

diabetes mellitus is characterized by the development of glomerular hyperfiltration and these hemodynamic changes have been strongly implicated in the pathogenesis of diabetic glomerulopathy (50–52). Several studies suggest that increased glomerular production of the vasodilator prostaglandins PGE_2 and PGI_2 contributes to the early development of glomerular hyperfiltration in diabetes mellitus (12, 14, 53). Hyperglycemia may be a key mediator of prostaglandin-mediated hyperfiltration, as illustrated by the fact that perfusion of isolated rat kidneys with high glucose concentrations increases GFR via a prostaglandin-dependent mechanism (54). The results of the present in vitro study demonstrate a novel cellular mechanism whereby elevated glucose concentrations could directly increase glomerular prostaglandin production and thus could impact the regulation of GFR.

In addition to their effects on glomerular hemodynamics, PGE_2, PGI_2, and TXA_2 also modify GMC proliferation (55, 56). It is possible therefore, that glucose-induced changes in eicosanoid production by GMC could also have powerful autocrine and paracrine effects on glomerular cell growth and thus could contribute to diabetes-associated glomerular growth abnormalities (57–59).

In conclusion, the present results demonstrate that glucose-induced PKC activation in GMC may provide an important link between the diabetic state and abnormalities in glomerular eicosanoid production. Specifically, we have shown that glucose directly increases arachidonic acid release and eicosanoid production GMC via a PKC-dependent mechanism. On this background, we propose that these effects occur via PKC-induced activation of PLA_2. In so doing, glucose-induced PKC activation provides a novel mechanism that directly implicates hyperglycemia in the dysregulation of glomerular arachidonic acid release and eicosanoid production that may ultimately impact glomerular function and growth and contribute to the development of diabetic glomerulopathy.

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Chapter 5.

Glucose-induced Changes in Na^+/H^+ Antiport Activity and Gene Expression in Cultured Vascular Smooth Muscle Cells

Bryan Williams and Randy L. Howard.

The Journal of Clinical Investigation. 93: 2623-2631, 1994.

Glucose-induced Changes in Na^+/H^+ Antiport Activity and Gene Expression in Cultured Vascular Smooth Muscle Cells

Role of Protein Kinase C

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Abstract

Increased Na^+/H^+ antiport activity has been implicated in the pathogenesis of hypertension and vascular disease in diabetes mellitus. The independent effect of elevated extracellular glucose concentrations on Na^+/H^+ antiport activity in cultured rat vascular smooth muscle cells (VSMC) was thus examined. Amiloride-sensitive $^{22}Na^+$ uptake by VSMC significantly increased twofold after 3 and 24 h of exposure to high glucose medium (20 mM) vs. control medium (5 mM). Direct glucose-induced Na^+/H^+ antiport activation was confirmed by measuring Na^+-dependent intracellular pH recovery from intracellular acidosis. High glucose significantly increased protein kinase C (PKC) activity in VSMC and inhibition of PKC activation with H-7, staurosporine, or prior PKC downregulation prevented glucose-induced increases in Na^+/H^+ antiport activity in VSMC. Northern analysis of VSMC poly A^+ RNA revealed that high glucose induced a threefold increase in Na^+/H^+ antiport (NHE-1) mRNA at 24 h. Inhibiting this increase in NHE-1 mRNA with actinomycin D prevented the sustained glucose-induced increase in Na^+/H^+ antiport activity. In conclusion, elevated glucose concentrations significantly influence vascular Na^+/H^+ antiport activity via glucose-induced PKC dependent mechanisms, thereby providing a biochemical basis for increased Na^+/H^+ antiport activity in the vascular tissues of patients with hypertension and diabetes mellitus. (*J. Clin. Invest.* 1994. 93:2623–2631.) Key words: hypertension • diabetes mellitus • atherosclerosis • hyperglycemia • NHE-1 messenger RNA

Introduction

Diabetes mellitus is a potent risk factor for the development of premature and widespread vascular disease. This risk is greatly accentuated by the coexistence of hypertension. In addition, predisposition to hypertension may be an important determinant in a subset of diabetic patients who subsequently develop complications such as diabetic nephropathy and associated vascular disease (1, 2).

Multiple abnormalities in cell membrane transport have been described in hypertension, the most reproducible being an elevation in Na^+/Li^+ countertransport (3–5). The striking similarities between Na^+/Li^+ countertransport and the physiological Na^+/H^+ antiport have led to the assumption that Na^+/Li^+ countertransport is one mode of operation of the ubiquitous Na^+/H^+ antiport (6, 7). In support of this concept, the activity of the Na^+/H^+ antiport has been shown to be increased in erythrocytes, leukocytes, and platelets from patients with essential hypertension and in lymphocytes and vascular smooth muscle cells (VSMC)[1] from the spontaneously hypertensive rat (8–13).

There are notable similarities in the abnormalities of membrane cation transport from patients with essential hypertension and patients with diabetes mellitus who have developed vascular complications. For example, increased erythrocyte Na^+/Li^+ countertransport and increased leukocyte and fibroblast Na^+/H^+ antiport activity have been demonstrated in type I diabetic patients with hypertension and nephropathy (2, 14–16). The fact that this disturbance in membrane cation transport is common to both hypertension and diabetes has led to the suggestion that increased activity of the Na^+/H^+ antiport may play a role in the pathogenesis of hypertension and vascular disease in patients with diabetes mellitus (2, 14, 15).

The mechanisms responsible for diabetes-induced increases in Na^+/H^+ antiport activity are unknown. Calcium/phospholipid-dependent protein kinase C (PKC) activity plays an important role in the regulation of Na^+/H^+ antiport activity in many tissues (17, 18). Of interest, many of the physical and functional abnormalities demonstrated in cell membranes from hypertensive patients, including Na^+/H^+ antiport activation, can be reproduced by the activation of PKC (11). With regard to diabetes mellitus, more recent studies have shown that the PKC inhibitor staurosporine can restore to normal the previously elevated leucocyte Na^+/H^+ antiport activity of diabetic patients, thereby suggesting that diabetes-induced increases in Na^+/H^+ antiport activity may be dependent on PKC activation (19). Diabetes mellitus is characterized by the development of hyperglycemia, and we have recently shown that elevated extracellular glucose concentrations (20 mM) induce a sustained activation of PKC in cultured VSMC (20, 21). Together, these observations prompt the hypothesis that metabolic factors, in particular, hyperglycemia could directly influence the activity of the Na^+/H^+ antiport in the vasculature of diabetic patients via glucose-induced activation of PKC.

Examining this hypothesis in vivo would be hindered by the fact that the development of hyperglycemia is invariably

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1. *Abbreviations used in this paper:* NMDG, *N*-methyl-D-glucamine; PKC, protein kinase C; VSMC, vascular smooth muscle cell.

associated with many humoral and metabolic changes, each of which could independently influence the activity of PKC and/or the Na^+/H^+ antiport in vascular tissue. The present study thus utilizes an in vitro cell culture system to examine the direct effects of elevated extracellular glucose concentrations on Na^+/H^+ antiport activity and steady-state Na^+/H^+ antiport mRNA levels in cultured VSMC. In addition, the role of glucose-induced PKC activation as a mediator of glucose-induced changes in vascular Na^+/H^+ antiport activity is determined.

Methods

Culture of rat VSMC. Aortic VSMC were isolated from Sprague-Dawley rats and cultured and characterized as previously described in detail (20, 21). For Na^+ uptake experiments VSMC were grown on 35-mm culture plates in MEM (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 2 g/liter $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS in an incubator at 37°C in 95% humidified air and 5% CO_2. Every 5–10 d, the cells were passaged after trypsin EDTA harvesting. For all experiments, second through sixth passaged VSMC were used.

Experimental design. VSMC were grown to confluence in the culture medium described above. The medium was then removed and replaced with one of four "test media" for 3 h (short-term exposure) or 24 h (sustained exposure). The test media comprised:

(*a*) Control medium: standard tissue culture medium as described above, with a normal D-glucose concentration of 5 mM.

(*b*) High glucose medium: this was similar to control medium except that it was supplemented with D-glucose to increase its concentration to 20 mM.

(*c*) L-glucose osmotic control medium: this was similar to control medium but supplemented with the cell permeable but poorly metabolized hexose, L-glucose 15 mM.

(*d*) Mannitol osmotic control medium: this medium was similar to control medium but was supplemented with the relatively impermeable and nonmetabolized hexose, mannitol 15 mM.

The latter two media served as osmotic controls for the high-glucose medium.

Measurement of $^{22}Na^+$ uptake by VSMC. After exposure to the various test media, VSMC monolayers were washed with a bicarbonate and sodium-free balanced salt solution. This "sodium-free buffer" contained choline chloride 135 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgSO_4$ 1 mM, Hepes 5 mM (pH 6.9), ouabain 2 mM, and variable amounts of D-glucose, L-glucose, or mannitol to mimic the composition of the test media to which they had been previously exposed. VSMC were preincubated with this buffer for 30 min at 37°C. This preincubation significantly decreases pH_i in VSMC and thus facilitates the measurement of Na^+/H^+ antiport dependent flux of Na^+ into the cells. VSMC pH_i (see below) after this 30-min preincubation did not significantly differ after 3 or 24 h of exposure to either control or high-glucose medium. After 30 min, the preincubation buffer was removed by aspiration and replaced with a similar buffer except that it contained 125 mM NaCl, 10 mM choline chloride, and 1 μCi/ml $^{22}Na^+$, with or without amiloride 1 mM. The influx of $^{22}Na^+$ into VSMC proceeded for 5 min at 37°C and was terminated by 6 × 1-ml rapid washings of the VSMC monolayers with iced-cold 0.1 M $MgCl_2$. The VSMC monolayer was lysed with warm 0.1 N NaOH and 0.1% SDS. A 50-μl aliquot of the lysate was retained for the measurement of cell protein content by the method of Lowry et al. (22) and $^{22}Na^+$ incorporation into the rest of the cell lysate was determined using a gamma counter (Packard Instrument Co., Inc., Meriden, CT) Na^+ uptake by VSMC was corrected for counter efficiency and cell protein content and is expressed as nanomoles of uptake per minute per milligram of cell protein.

Measurement of intracellular pH in VSMC. VSMC were grown to confluence on 13 × 30-mm glass coverslips and then exposed to the various test media for 24 h before the measurement of pH_i, which was measured using the pH-sensitive dye BCECF [(2,7)-biscarboxyethyl-5(6)-carboxyfluorescein] using a modification of the method described by Chaillet and Boron (23) and Bergman et al. (24). VSMC were loaded with the acetoxymethyl derivative of BCECF (5 μM) for 20 min at 37°C in solution A as noted below (pH 7.4). The cell-coated coverslip was then placed in a plastic cuvette in a Deltascan model 4000 fluorescence spectrometer (Photon Technology International Inc., South Brunswick, NJ) and perfused at 12 ml/min with solutions at 37°C as shown in Fig. 1. All solutions contained 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM D-glucose, 7 mM Hepes, 1 mM KH_2PO_4 and 4 mM K_2HPO_4. Solution A contained 145 mM NaCl and 5 mM KCl. Solution B contained 145 mM *N*-methyl-D-glucamine (NMDG-Cl) and 5 mM KCl. Solution C contained 120 mM NMDG-Cl, 25 mM NH_4Cl, and 5 mM KCl. pH_i was calculated from the ratio of fluorescence at excitation wavelengths 495 and 440 nm and emission at 535 nm. Calibration of the BCECF excitation ratio for each experiment was determined using the K^+/nigericin technique as previously described (24).

VSMC Na^+/H^+ antiport activity was calculated from the initial rate of Na^+-dependent pH_i recovery after an acid load in the absence of CO_2/HCO_3. Typical tracings with and without amiloride (1 mM) are depicted in Fig. 1. The Na^+-dependent pH_i recovery after an acid load was inhibited 90% by 1 mM amiloride. The initial rate of Na^+-dependent pH_i recovery (dpH_i/dt) was calculated from a line drawn tangential to the initial 30-s deflection after return of Na^+ to the perfusate.

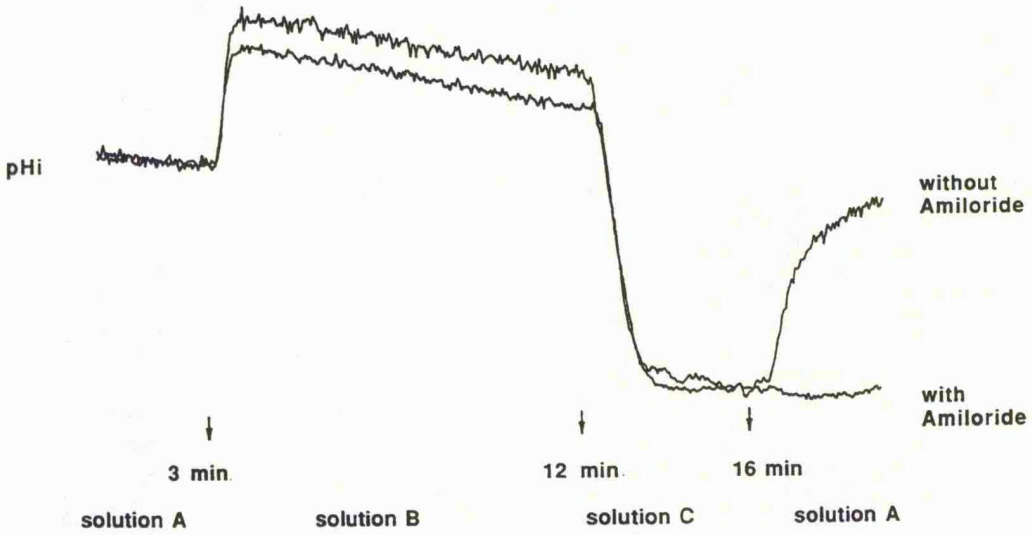


Figure 1. Perfusion schema and effect of 1 mM amiloride on Na^+-dependent recovery of intracellular pH in VSMC monolayers preloaded with BCECF. All solutions contained: 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM D-glucose, 7 mM Hepes, 1 mM KH_2PO_4. Solution A contained 145 mM NaCl and 5 mM KCl. Solution B contained 145 mM NMDG-Cl and 5 mM KCl. Solution C contained 120 mM NMDG-Cl. 25 mM NH_4Cl, and 5 mM KCl. Na^+-dependent recovery of intracellular pH was inhibited by 1 mM amiloride.

Intracellular buffering capacity was determined from the pH_i response to removal of NH_3/NH_4^+ using the formula: Buffering capacity $= [NH_4^+]_i/\Delta\ pH_i$, where $[NH^+]_i$ is the intracellular concentration before NH_3/NH_4^+ removal, calculated as $[NH_4^+]_i = [NH_4^+]_0 \times 10^{(7.4-pH_i)}$ and $\Delta\ pH_i$ is the pH_i change on removal of NH_4^+ (25).

Assay of PKC activity in VSMC. The methods used by our laboratory for the in-situ measurement and characterization of PKC activation in VSMC has been previously described in detail (20, 21). Briefly, VSMC were seeded into 96-well microtiter plates and grown to confluence in control medium. Thereafter, the VSMC were exposed to the test media for 3 or 24 h. The test media was aspirated and replaced with 40 μl of a buffered salt solution containing 137 mM NaCl, 5.4 mM KCl, 10 mM $MgCl_2$, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 25 mM β-glycerophosphate, 5.5 mM D-glucose, 5 mM EGTA, 1 mM $CaCl_2$, 100 μM [τ-^{32}P]ATP, 50 μg/ml digitonin, and 20 mM Hepes (pH 7.2, 30°C). In addition, 100 μM of a PKC-specific substrate (VRKRTLRRL) was added to the buffer. This short synthetic peptide is based on the sequence surrounding a major PKC-dependent phosphorylation site within the epidermal growth factor receptor (26). This peptide substrate is not phosphorylated by cyclic nucleotide-dependent or Ca^{2+}/calmodulin-dependent protein kinases or S6 kinase and has been extensively characterized to be highly specific for PKC (27, 28). By permeabilizing the VSMC with digitonin, the PKC-specific VRKRTLRRL substrate enters the VSMC along with [τ-^{32}P]ATP to allow a selective and rapid analysis of in situ PKC activity. We have previously demonstrated that the concentration of digitonin used (50 μg/ml) did not modify VSMC morphology or promote cell detachment (20). In addition, the measured PKC activity was retained by the monolayer after permeabilization (PKC activity in the supernatant was 6.6% of total measured PKC activity). This latter finding is consistent with the concept that the PKC activity being assayed was tightly associated with the cell membrane as has been proposed for the active form of the kinase (17). The kinase reaction proceeds for 10 min at 30°C before termination by the addition of 10 μl of 25% (wt/vol) trichloroacetic acid. 45-μl aliquots of the reaction mixture are then blotted onto 2-cm phosphocellulose circles (P81, Whatman Inc., Clifton, NJ) and washed batchwise; three washes with 75 mM phosphoric acid and one wash with 75 mM sodium phosphate (pH 7.5). The basic VRKRTLRRL substrate is retained by the P81 filter and its PKC-dependent phosphorylation is quantified by scintillation counting using a Beta Counter (Packard Instrument Co., Inc.). Background phosphorylation was always < 0.05% of added counts per minute and was not influenced by the different test media. VSMC protein content per well was measured in an NaOH/SDS lysate as described above. Results are expressed as PKC-dependent phosphorylation picomoles per minute per milligram cell protein.

Measurement of steady-state Na^+/H^+ antiport mRNA. VSMC were grown to confluence on 100-mm dishes and exposed to the various test media for 3 or 24 h. Thereafter, total cellular RNA was isolated using the acid guanidium thiocyanate-phenol-chloroform method (29). VSMC from 15 dishes were pooled to yield ~ 3 mg of total cellular RNA. Poly A^+ RNA was isolated using an oligo-Dt spin column and reagents as supplied (5Prime 3Prime Inc., Boulder, CO). For Northern blot analysis, Poly A^+ RNA was separated by electrophoresis in 1% agarose/2.2 M formaldehyde gels, transferred to Nitroplus membranes (Micron Separations Inc., Westboro, MA) by capillary diffusion and immobilized by baking at 80°C in a vacuum oven for 2 h. Prehybridization was performed for 8 h at 42°C in a solution containing 5× SSC, 50% formamide, 5× Denhardt's solution, 0.1% SDS, and 200 μg/ml salmon sperm DNA. Hybridization was performed overnight at 42°C in the above solution containing 1×10^6 cpm/ml ^{32}P-random primer-labeled probe. The Na^+/H^+ antiport probe was a 1.8-kb Bam H1 fragment of the human NHE-1 cDNA (30), a generous gift from Jaques Pouyssegur, Nice, France. The β-actin probe was a 2-kb fragment of human cDNA (Clontech Laboratories Inc., Palo Alto, CA). Band intensities were determined from autoradiograms (Kodak XAR-5) using an Seprascan 2001 (Integrated Separation Systems, Hyde Park, MA).

Materials. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Penicillin, streptomycin, culture media, and FCS were obtained from Gibco Laboratories Life Technology Inc. Acetomethyl ester of BCECF was obtained from Molecular Probes Inc. (Eugene, OR). $^{22}Na^+$ was obtained from Amersham Corp. (Arlington Heights, IL).

Statistical analysis. Results are reported as mean±standard error of the mean. Statistical analysis was performed using an unpaired Student's t test or analysis of variance with a Bonferroni correction as appropriate. A P value of < 0.05 was considered significant.

Results

Effect of elevated extracellular glucose concentrations on Na^+/H^+ antiport activity in VSMC. VSMC were exposed to the various test media for 3 h (short-term exposure) or 24 h (sustained exposure) before the measurement of $^{22}Na^+$ uptake. Basal pH_i was significantly reduced but did not differ after 30 min of exposure to the sodium-free preincubation buffer before measurement of $^{22}Na^+$ uptake by VSMC that had undergone short or sustained exposure to either control or high-glucose media. After a 3-h exposure to the high-glucose medium (20 mM), Na^+ uptake by VSMC was significantly increased by 53% when compared to Na^+ uptake by VSMC exposed to control medium (glucose 5 mM) (Fig. 2). The glucose-induced increase in Na^+ uptake by VSMC was sustained with continued exposure to the high-glucose medium for up to 24 h. Na^+/H^+ antiport activity is a major pathway for Na^+ influx in VSMC and this transport system can be inhibited by the addi-

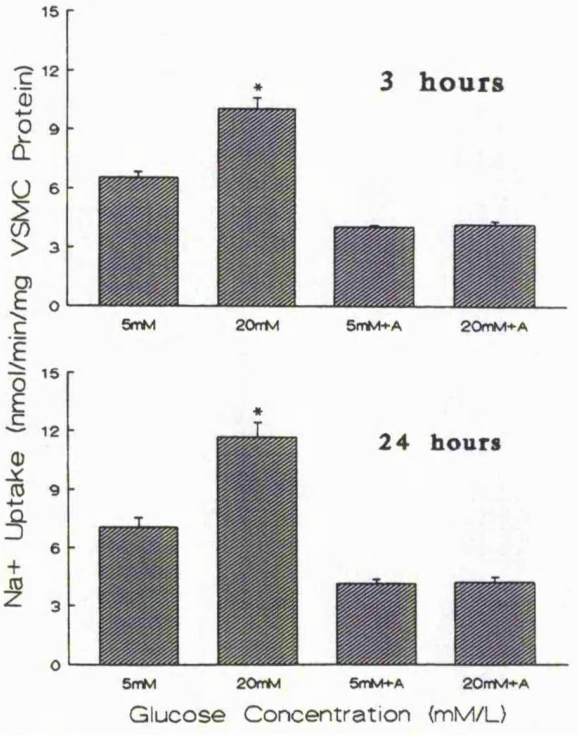


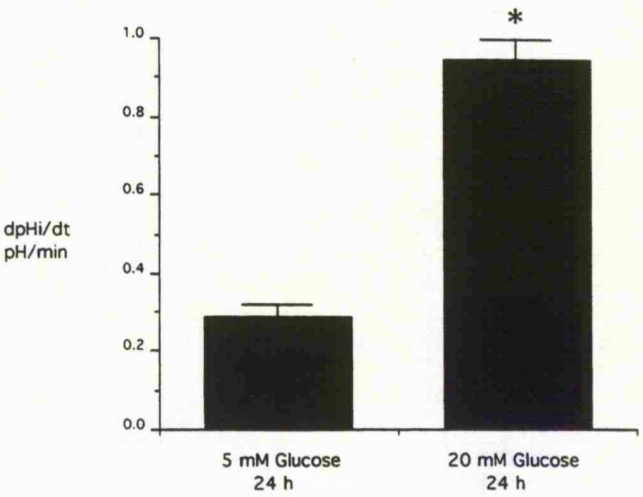
Figure 2. Effect of extracellular glucose concentration on amiloride-sensitive Na^+ uptake by VSMC. VSMC were exposed to control medium (glucose 5 mM) or high-glucose medium (glucose 20 mM) for 3 or 24 h in the absence or presence (+*A*) of amiloride (1 mM) before the measurement of $^{22}Na^+$ uptake. *$P < 0.01$ vs. all other columns; $n = 4$ experiments.

tion of amiloride (31). Amiloride (1 mM) significantly reduced Na^+ uptake by VSMC in the presence of control medium and completely prevented the increase in Na^+ uptake previously observed in the presence of the elevated glucose medium at both 3 and 24 h (Fig. 2). These observations suggest that the glucose-induced increase in Na^+ uptake by VSMC at both 3 and 24 h, was dependent on activation of the Na^+/H^+ antiport.

Osmotic stress is a recognized stimulus for the activation of the Na^+/H^+ antiport in various cell types (32). It was therefore important to determine whether the increased osmolality of the high-glucose medium could account for the glucose-induced activation of the Na^+/H^+ antiport in VSMC. Table I shows that 3 and 24 h of exposure to two osmotic control media (containing concentrations of L-glucose or mannitol designed to mimic the osmolality of the high-glucose medium) had no significant effect on Na^+ uptake by VSMC. This demonstrates that elevated extracellular D-glucose concentrations specifically increased Na^+/H^+ activity in VSMC via mechanisms independent of changes in extracellular osmolality.

Effects of elevated extracellular glucose concentrations on pH_i and intracellular buffering capacity in VSMC. To confirm that elevated glucose concentrations directly and specifically stimulated an increase in Na^+ uptake via the Na^+/H^+ in VSMC, VSMC were exposed to control medium, high-glucose medium, and mannitol osmotic control medium for 24 h before the measurement of Na^+-dependent pH_i recovery from an acid load. Fig. 3 and Table II summarize the results of these experiments. 24 h of preexposure to the high-glucose medium significantly increased dpH_i/dt, approximately threefold, when compared to VSMC incubated with control medium. These differences could not be accounted for by differences in buffering capacity. Moreover, amiloride inhibited the Na^+ dependent recovery in pH_i by 90% in the presence of control medium and eliminated the differences between the control and high-glucose medium, confirming the conclusions of the $^{22}Na^+$ uptake studies, i.e., that elevated glucose concentrations directly activate the Na^+/H^+ antiport in VSMC. Furthermore, Na^+-dependent dpH_i/dt in the presence of the osmotic control medium (mannitol) did not differ from control medium, further confirming that high glucose-induced changes in Na^+/H^+ antiport activity could not be attribute to changes in extracellular osmolality (Table II).

Role of PKC activation in mediating glucose-induced increases in Na^+/H^+ antiport activity in VSMC. PKC has been



	5 mM Glucose 24 h	20 mM Glucose 24 h
Baseline pHi	7.31±0.04	7.31±0.03
Trough pHi	6.34±0.02	6.32±0.02
Buffering Capacity mmol/L x pH	18.4±1.7	17.7±1.8
N	15	16

Figure 3. Effect of the test media on Na^+/H^+ antiport activity in confluent VSMC. VSMC were pretreated for 24 h with the test media shown and then loaded with BCECF. Na^+/H^+ antiport activity was measured as the 30-s recovery from NH_4Cl-induced intracellular acidosis. Buffering capacity was calculated as indicated in Methods. pH_i = intracellular pH, dpH_i/dt = change in intracellular pH with time. $^*P < 0.001$; n, number of experiments.

shown to play an important role in the regulation of Na^+/H^+ antiport activity in many cell types. We have previously demonstrated that elevated glucose concentrations induce a sustained activation of PKC in VSMC (20). Table III shows that a 3-h exposure to high-glucose medium markedly increases PKC activity in VSMC, and that this activity is sustained for at least 24 h providing the extracellular glucose concentrations remain elevated. This effect is specific for D-glucose and is not reproduced by either of the osmotic control media. The addition of two dissimilar PKC inhibitors, H-7 (10^{-5} M) or staurosporine (10^{-6} M) (33, 34), had little effect on basal PKC activity at these concentrations but markedly inhibited glucose-induced

Table I. Effect of Various Test Culture Media on Sodium Uptake by VSMC

Culture media added to cultured VSMC	Sodium uptake by VSMC	
	3-h exposure	24-h exposure
	nmol/min per mg cell protein	
Control	6.53±0.27	6.25±0.17
Mannitol control	6.53±0.28	6.08±0.30
L-Glucose control	6.45±0.34	7.15±0.13
High glucose	10.1±0.50*	10.45±0.39*

Measurement of $^{22}Na^+$ uptake by VSMC after 3 or 24 h of exposure to the various test culture media. $^*P < 0.01$ vs. results with all other test media; $n = 4$ experiments.

Table II. Effect of Mannitol Osmotic Control Medium on Na^+/H^+ Antiport Activity in VSMC

Measurements	Glucose 5 mM	Mannitol control
dpH_i/dt pH/min	0.22±0.03	0.23±0.03
Baseline pH_i	7.24±0.05	7.22±0.07
Trough pH_i	6.31±0.03	6.27±0.02
Buffering capacity	17.1±2.7	18.4±2.5

Confluent VSMC were pretreated with the test media shown and loaded with BCECF. Na^+/H^+ antiport activity was measured as the 30-s recovery from NH_4Cl-induced intracellular acidosis. Buffering capaity = mM × pH. $n = 13$. There were no significant differences in any parameter measured between the 5 mM glucose and mannitol osmotic control media.

Table III. VSMC PKC Activity after Exposure to Various Test Media

Culture media added to VSMC	Protein kinase C activity	
	3-h exposure	24-h exposure
	pmol phosphorylation/min per mg cell protein	
Control	40.0±4.4	42.1±5.3
Mannitol control	38.6±4.9	41.7±6.1
L-Glucose control	41.8±5.1	43.6±4.8
High glucose	79.4±7.7*	83.6±8.3*
Control + H-7	35.8±5.7	33.7±3.9
Control + ST	38.4±6.3	36.5±6.1
High glucose + H-7	49.7±8.2‡	52.1±7.1‡
High glucose + ST	51.6±6.8‡	54.3±7.5‡

PKC activity in VSMC after 3 or 24 h of exposure to the various test media. In some experiments, inhibitors of PKC activity; H-7 (50 μM) or staurosporine (ST) (1 μM) were coincubated with the control or high-glucose media. * $P < 0.01$ vs. control, mannitol, or L-glucose media. ‡ $P < 0.05$ vs. high glucose. There was no significant difference between high glucose + PKC inhibitors vs. all test media except the high-glucose medium alone; $n = 3$.

activation of PKC during the 3- or 24-h incubation with the high-glucose medium. The use of these two PKC inhibitors thus provided a useful experimental tool with which to assess the role of glucose-induced PKC activation in mediating glucose-induced increases in Na^+/H^+ antiport activity in VSMC. To do this, VSMC were exposed to control or high-glucose medium for 3 and 24 h in the absence or presence of either of the two PKC inhibitors, before the measurement of $^{22}Na^+$ uptake by VSMC. Fig. 4 shows that glucose-induced increases in Na^+ uptake by VSMC were almost completely prevented by inhibition of glucose-induced PKC activation with H-7 or staurosporine. Because H-7 and staurosporine are not specific in their inhibition of PKC, further experiments were performed to clarify the relevance of PKC activation in the mediation of glucose-induced changes in VSMC Na^+/H^+ antiport activity. VSMC were preexposed to PMA (10^{-7} M) for 24 h. We have previously demonstrated that this maneuver downregulates PKC activity in VSMC (20). The VSMC were then exposed to either control or high-glucose medium for 3 or 24 h in the continued presence of PMA. As shown in Table IV, basal Na^+/H^+ antiport activity was not significantly affected by PKC downregulation, however, both the short-term and sustained high-glucose–induced increase in Na^+ uptake was significantly attenuated by PKC downregulation. Together, these results suggest that both short-term and sustained glucose-induced increases in Na^+/H^+ antiport activity in VSMC occur via mechanisms dependent on glucose-induced activation of PKC.

Effects of cycloheximide and actinomycin-D on glucose-induced increases in Na^+/H^+ antiport activity. To examine whether gene transcription and protein synthesis were required for the initiation and maintenance of glucose-induced changes in Na^+/H^+ antiport activity, VSMC were incubated with actinomycin-D (10 nM), an inhibitor of gene transcription or cycloheximide (10 μM), an inhibitor of protein synthesis. Confluent VSMC monolayers were exposed to these two inhibitors throughout a 3- or 24-h incubation with control or high-glucose (20 mM) media. Cycloheximide (10 μM) inhibited protein synthesis by ~ 80% (measured by [^{3}H]leucine incorporation into cell protein). Fig. 5 shows that at 3 h the presence of actinomycin-D or cycloheximide had no effect on high-glucose–induced increases in Na^+/H^+ antiport activity in VSMC. These results suggest that short-term (3 h) glucose-induced activation of the Na^+/H^+ antiport in VSMC occurs independent of gene transcription or protein synthesis. In contrast, in-

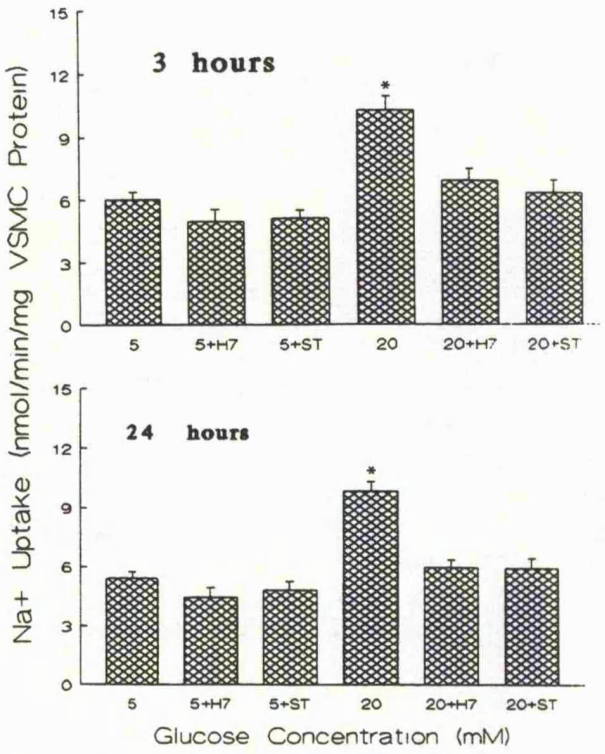


Figure 4. Effect of PKC inhibitors on glucose-induced changes in Na^+ uptake by VSMC. VSMC were exposed to control or high-glucose medium for 3 or 24 h, in the absence or presence of H-7 (50 μM) or staurosporine (ST) (1 μM). * $P < 0.05$ vs. all other columns; $n = 3$ experiments.

Table IV. Effect of PKC Downregulation on Sodium Uptake by VSMC

Culture media added to VSMC	Sodium uptake by VSMC	
	3-h exposure	24-h exposure
	nmol/min per mg protein	
Control (glucose 5 mM)	6.1±0.43	5.96±0.3
Control + PMA	6.95±0.63	6.68±0.57
High glucose (20 mM)	10.69±0.61*	10.92±0.54*
High glucose + PMA	7.19±0.6	7.04±0.66

Measurement of $^{22}Na^+$ uptake by VSMC after 3 or 24 h of exposure to either control or high-glucose culture medium. To down-regulate PKC activity, in some experiments (+PMA) and VSMC were pretreated with PMA (10^{-7} M) for 24 h and during the 3- or 24-h exposure to the control or high-glucose culture medium. * $P < 0.05$ vs. control ± PMA and high glucose + PMA; $n = 4$.

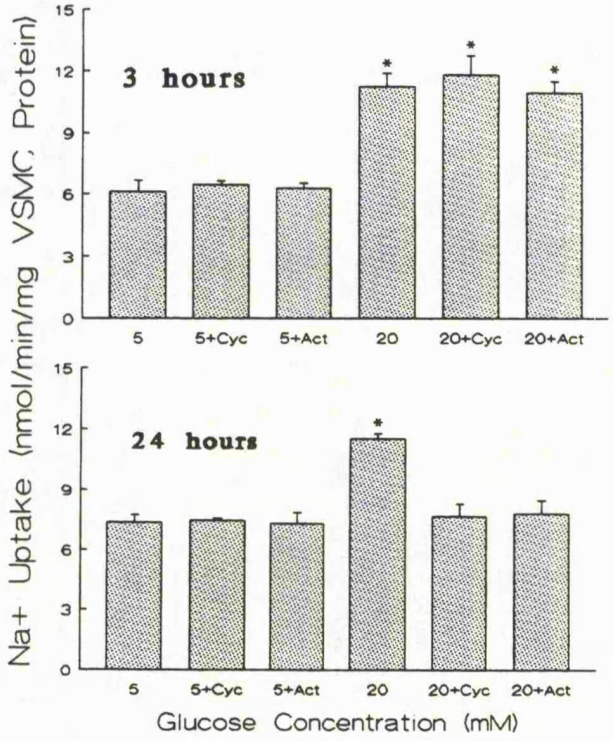


Figure 5. Effect of inhibitors of transcription and translation on glucose-induced changes in Na^+ uptake by VSMC. VSMC were exposed to control or high-glucose medium for 3 or 24 h, in the absence or presence of actinomycin-D (10 nM) or cycloheximide (10 μM) before the measurement of Na^+ uptake by VSMC. $^*P < 0.05$; $n = 3$ experiments.

creased Na^+/H^+ antiport activity in response to sustained exposure to high glucose (24 h) was prevented by coincubation with actinomycin-D and cycloheximide. Neither agent inhibited glucose-induced PKC activation at 24 h and there was no measurable VSMC toxicity as indicated by no change in cell detachment rates, lactate dehydrogenase release, or trypan blue exclusion (data not shown). These results suggest that sustained glucose-induced activation of Na^+/H^+ in VSMC requires gene transcription and translation.

Effect of elevated extracellular glucose on Na^+/H^+ antiport mRNA in VSMC. The above experiments suggested that transcription and translation were involved in the increase in Na^+/H^+ antiport activity observed within 24 but not 3 h of incubation with the high-glucose medium. The effects of high-glucose concentrations on steady-state Na^+/H^+ antiport (NHE-1) mRNA levels in VSMC was thus examined. Northern blot analysis revealed a single band of 4.8–5.0 kb, as previously described for NHE-1 mRNA in cultured VSMC (35). Northern blot analysis revealed an approximately threefold increase in steady-state NHE-1 mRNA concentration normalized for β-actin in VSMC after 24 h of incubation with the high-glucose (20 mM) medium when compared with control medium (glucose 5 mM) (Fig. 6). There was no difference in steady-state levels of β-actin mRNA between the control and high-glucose group; thus the increase in the NHE-1/β-actin ratio was due to a true increase in the abundance of NHE-1 mRNA. There was no difference in steady-state NHE-1 mRNA in VSMC exposed to the control or high-glucose media at 3 h (data not shown).

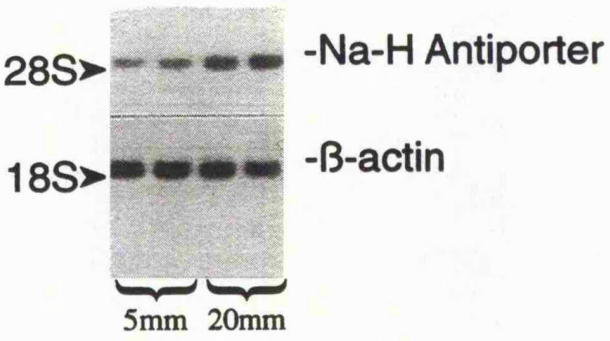


Figure 6. Effect of extracellular glucose concentration on steady-state Na^+/H^+ antiport mRNA after 24 h of incubation. VSMC were treated and Northern blot performed as indicated in Methods. This autoradiogram shows the Na^+/H^+ antiport mRNA in the upper portion of the figure at 4.8–5.0 kb. The lower portion shows the β-actin mRNA from the same membrane. The 28S and 18S markers are shown for reference.

Exposing VSMC to the mannitol osmotic control media for 3 or 24 h did not change NHE-1 mRNA levels vs. glucose 5 mM (not shown) confirming that the high-glucose-induced increase in VSMC NHE-1 mRNA was unrelated to changes in extracellular osmolality.

Discussion

The major finding of the present study is that elevated extracellular glucose concentrations exert a significant effect on the regulation of Na^+/H^+ antiport activity in vascular tissue. Elevated glucose concentrations compatible with those attained in poorly controlled diabetic patients stimulated an increase in the activity of the VSMC Na^+/H^+ antiport within hours and this effect was sustained for up to 24 h providing the glucose concentrations remained elevated. This effect was specific for D-glucose and was unrelated to changes in extracellular osmolality. In addition, both the acute and longer-term activation of the VSMC Na^+/H^+ antiport by high glucose were dependent on glucose-induced PKC activation.

The Na^+/H^+ antiport is a major pathway for the regulation of Na^+ uptake by VSMC (31). Previous studies have emphasized that the activity of Na^+/H^+ antiport in many cell types, including VSMC, is influenced by the activation of PKC (11, 18, 36–42). The present study demonstrates that elevated extracellular glucose concentrations induce a sustained activation of PKC in VSMC. We have previously characterized this effect of glucose in VSMC in detail (20). Both the short-term and sustained high-glucose–induced activation of the VSMC Na^+/H^+ antiport appear to be strongly dependent on glucose-induced PKC activation. This conclusion is based on the fact that two dissimilar inhibitors of glucose-induced PKC activity (H-7 and staurosporine) prevented short-term and sustained glucose-induced Na^+/H^+ antiport activation. The Na^+/H^+ antiport has been shown to be regulated by a number of kinases other than PKC, notably; cAMP-dependent protein kinase and calcium calmodulin-dependent multiprotein kinase (43). Because neither of the PKC inhibitors used in the present study are entirely specific for PKC, the possibility remained that other kinases that are also nonspecifically inhibited by these agents may have contributed to the glucose-induced response.

However, the important role of PKC activation in mediating glucose-induced changes in Na^+/H^+ antiport activity is further emphasized by our observation that prior downregulation of PKC by prolonged exposure to PMA prevents glucose-induced Na^+/H^+ antiport activation.

The finding that short-term (3 h) exposure to high glucose produces a PKC-dependent increase in Na^+/H^+ antiport activity in VSMC which was not blocked by coincubation with actinomycin-D or cycloheximide suggested that this acute effect of high glucose occurred independently of the need for transcription and translation. This conclusion is further supported by the observation that short-term exposure of VSMC to high glucose did not increase the abundance of NHE-1 mRNA. Previous studies of rat proximal tubule cells have demonstrated that acute activation of the Na^+/H^+ antiport after direct PKC activation with phorbol esters, also occurs without the need for transcription or translation (42).

There are numerous potential mechanisms to explain the acute, PKC-dependent, glucose-induced increase in Na^+/H^+ antiport activity in VSMC. One possibility is a phosphorylation-dependent increase in the activity of existing antiporters or the activation of "dormant" membrane-associated antiporters. In support of this concept, Sardet et al. (41) demonstrated that the Na^+/H^+ antiport is rapidly phosphorylated in response to various mitogens and concluded that this phosphorylation of the Na^+/H^+ antiport is temporally correlated with its activation. That the magnitude of phosphorylation could regulate the rate of Na^+/H^+ exchange is also suggested by the finding that vanadate, an inhibitor of phosphatases, activates Na^+/H^+ countertransport in A431 cells (44). Additional experiments support the hypothesis that PKC is one of the kinases responsible for this phosphorylation in VSMC (38–40). It is conceivable, however, that PKC-dependent activation of the Na^+/H^+ antiport could occur via phosphorylation of an ancillary, regulatory protein rather than direct phosphorylation of the antiport itself. An alternative mechanism of Na^+/H^+ antiport activation is that glucose-induced PKC activation could promote the exocytic insertion into the plasma membrane of antiporters previously stored in the cytoplasm. Such a "shuttling" process has been demonstrated for angiotensin II–induced increases in Na^+/H^+ antiport activity in renal proximal tubular cells (45) and many other transporters in various cell types (46–49). Finally, direct PKC activation by PMA has been shown to increase Na^+/H^+ exchange by causing an alkaline shift in the pH_i dependence of the antiport in rat thymic lymphocytes (50) and NHE-1–transfected cell lines (51). This could occur via a PKC-dependent alkaline shift in the pH_i responsiveness of an allosteric modifier site on the cytoplasmic surface of the cell membrane (50, 52–54). There are therefore, numerous potential mechanisms whereby glucose-induced PKC activation could promote an acute increase Na^+/H^+ antiport activity in VSMC.

Continued exposure to high extracellular glucose concentrations for up to 24 h induced a sustained activation of the Na^+/H^+ antiport in VSMC, also via a PKC-dependent process. The kinetic basis for this glucose-induced increase in Na^+/H^+ antiport activity was not specifically examined in the present study. It is important to note, however, that the assays of Na^+ uptake and Na^+-dependent pH_i recovery were performed using extracellular Na^+ concentrations (Na_o^+) of 125 and 145 mM, respectively. These Na_o^+ concentrations greatly exceed the Na^+/H^+ antiport K_m for Na_o^+ ($\sim$ 51 mM) (52); thus it is extremely unlikely that the measured increase in Na^+/H^+ antiport activity using these experimental conditions represents anything other than an increase in the V_{max} of the antiporter. Concordant with this conclusion, in almost all instances, sustained increases in Na^+/H^+ antiport activity in response to a variety of biological stimuli have been associated with an increase in antiporter V_{max}, which in some instances has been associated with increased synthesis of transporters (55–62).

Our observation that sustained high-glucose–induced stimulation of the VSMC Na^+/H^+ antiport was inhibited by coincubation with actinomycin-D or cycloheximide also suggested that transcription and translation were required, perhaps indicating the need for the synthesis of new Na^+/H^+ antiporters to sustain the high-glucose–induced effect. This hypothesis is supported by our finding that sustained exposure to high-glucose medium also caused a threefold increase in the abundance of NHE-1 mRNA in VSMC. Whether this increase in steady-state mRNA was due to glucose-induced changes in transcription rate or message stability has yet to be determined. Our observation that sustained activation of the Na^+/H^+ antiport by glucose requires the induction of transcription and translation is compatible with the studies of Berk et al. (37), who concluded that the long-term regulation of the Na^+/H^+ antiport in VSMC involves alterations in gene expression. Moreover, the response of the Na^+/H^+ antiport to a variety of chronic stimuli in different tissues appears to require gene transcription and translation to sustain an increase in Na^+/H^+ antiport activity (18, 42, 59, 60, 63). It is noteworthy, however, that there may not always be a good correlation between steady-state NHE-1 mRNA abundance and Na^+/H^+ antiport activity (35). Moreover, the results of the cycloheximide and actinomycin-D experiments are also consistent with the possibility that sustained activation of the Na^+/H^+ antiport requires the synthesis of a regulatory protein rather than direct synthesis of antiporters.

The intracellular signaling mechanism responsible for the high-glucose–induced increase in NHE-1 mRNA abundance has not been defined in the present study. Nevertheless, the fact that the sustained increase in VSMC Na^+/H^+ antiport activity is dependent on both glucose-induced PKC activation and gene transcription suggests that glucose-induced PKC activation may signal the increase in Na^+/H^+ mRNA. Concordant with this hypothesis, Horie et al. (42) recently demonstrated that long-term phorbol ester–mediated PKC activation leads to a chronic increase in both Na^+/H^+ antiport activity and mRNA expression in cultured proximal tubular cells. In this regard, PKC has been shown to regulate the expression of numerous different genes. These genes usually express "PKC responsive elements," i.e., various consensus sequences in the 5′ flanking region of the gene that mediate PKC-responsive regulation of gene expression (64–66). In various studies, PKC-induced regulation of these genes has been shown to be due to the binding of AP-1 or fos/jun dimers, to an AP-1 binding site in the regulatory region of the gene (64). With this in mind, it is notable that the 5′ flanking regulatory region of the gene coding for the Na^+/H^+ antiport examined in the present study contains three consensus sequences for AP-1 binding sites (67, 68).

Together the aforementioned observations suggest that glucose-induced PKC activation leads to the activation of the Na^+/H^+ antiport in VSMC via an increase in antiport V_{max}. The acute activation occurs independently of new protein syn-

thesis and gene transcription, whereas more sustained Na^+/H^+ antiport activation by elevated glucose concentrations does require protein synthesis and gene transcription and is associated with a threefold increase in NHE-1 mRNA abundance.

Glucose-induced increases in Na^+/H^+ antiport activity in vascular tissue could have considerable significance with regard to the role of hyperglycemia in the pathogenesis of vascular disease and hypertension in patients with diabetes mellitus. The Na^+/H^+ antiport has been shown to play a key role in the regulation of intracellular pH, cell volume, growth, differentiation, and contractility (7, 35, 69–72). Disordered VSMC growth is a pathologic feature of atherosclerosis and hypertension (73, 74) and increased Na^+/H^+ antiport activity has been implicated in the development of these vascular growth abnormalities (13, 35, 75, 76). It has also been suggested that Na^+/H^+ antiport hyperactivity could explain the relationship between abnormal Na^+ homeostasis and Ca^{2+} metabolism in essential hypertension and thereby contribute directly to enhanced vascular contractility (77). The hypothesis that Na^+/H^+ antiport hyperactivity plays an important role in the pathogenesis of hypertension and vascular disease is supported by reports that both essential hypertension and diabetes mellitus are associated with increased Na^+/H^+ antiport activity in many tissues (2–5, 8–16). It has been suggested that increased Na^+/H^+ antiport activity in these circumstances is genetically determined and that interaction between genetic predisposition, environmental, metabolic and other factors eventually determines the evolution of hypertension and vascular disease in the individual (2, 4–6, 16). For example, it is established that risk for vascular disease in diabetic patients is accentuated by the coexistence of hypertension and poor glycemic control (2). An interaction between genetic influences on Na^+/H^+ antiport activity and our finding of a direct effect of glucose on antiport activity could provide an explanation for this interaction at the cellular level, based on additive stimulation of the antiport. Furthermore, essential hypertension is also associated with abnormal glucose homeostasis (78–80). The findings of the present study provide a novel mechanism whereby metabolic factors, in addition to genetic factors, could directly influence Na^+/H^+ antiport activity in essential hypertension.

In conclusion, hyperglycemia is recognized to be an independent risk factor for the development of vascular disease. Hyperactivity of the Na^+/H^+ antiport has been implicated in the pathogenesis of hypertension and vascular injury. The present study uniquely demonstrates that elevated glucose concentrations induce a sustained activation of the Na^+/H^+ antiport and an increase in NHE-1 mRNA levels in vascular tissue via glucose-induced PKC-dependent mechanisms. These observations suggest a novel cellular mechanism that could explain the apparent synergism that exists between hyperglycemia and genetic factors in the clinical expression of vascular disease and hypertension in patients with abnormal glucose homeostasis.

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Chapter 6.

Effect of Elevated Extracellular Glucose Concentrations on Calcium Ion Uptake by Cultured Rat Vascular Smooth Muscle Cells.

Bryan Williams and Robert W. Schrier.

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Effects of Elevated Extracellular Glucose Concentrations on Transmembrane Calcium Ion Fluxes in Cultured Rat VSMC

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Effect of Elevated Extracellular Glucose Concentrations on Calcium Ion Uptake by Cultured Rat Vascular Smooth Muscle Cells

Key Words
Autoregulation
Calcium ion
Glucose
Vascular smooth muscle
Voltage sensitive ion channels

Abstract
Blood flow autoregulation is impaired in early diabetes mellitus, predisposing the microvasculature to injury. Blood flow autoregulation is in part a myogenic response that is critically dependent on Ca^{2+} uptake via voltage-sensitive calcium channels in vascular smooth muscle cells (VSMC). Recent evidence suggests that impairment of blood flow autoregulation in diabetes may be responsive to variations in glycemic control. The present study thus examined the independent effect of an elevated extracellular glucose concentration on Ca^{2+} uptake by cultured rat VSMC in vitro. A threshold glucose concentration of 15–20 mmol/l markedly and maximally depressed basal and voltage sensitive Ca^{2+} channel activated (BAY K 8644, 10^{-7} *M*) Ca^{2+} uptake. This effect was apparent within 3 h of incubating VSMC with the high glucose medium and was maximal after 48 h incubation. Osmotic control media containing either mannitol or *L*-glucose did not inhibit Ca^{2+} uptake by VSMC, thus confirming the effect was specific for elevated extracellular glucose concentrations and unrelated to changes in extracellular osmolality. Glucose-induced inhibition of basal and voltage-sensitive transmembrane fluxes of Ca^{2+} in VSMC may provide a metabolic mechanism for impaired calcium-dependent blood flow autoregulatory responses in early diabetes mellitus.

Introduction

Early diabetes mellitus in both experimental animals and man is associated with impaired blood flow autoregulation in the microcirculation of many tissues [1–3]. Impaired blood flow autoregulation renders the microcirculation vulnerable to increases in systemic blood pressure [4]. This may at least in part explain the marked predisposition of patients with diabetes mellitus to develop devastating microvascular injury and widespread organ damage when diabetes mellitus and systemic hypertension co-exist.

Impairment of blood flow autoregulation in early diabetes mellitus appears to be responsive to fluctuations in glycemic control. Specifically, Hashimoto et al. [2] have shown, in the streptozotocin-induced diabetic rat, that

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impairment of renal blood flow autoregulation can be prevented by the administration of insulin to prevent hyperglycemia. Moreover, Atherton et al. [5] have shown that retinal blood flow autoregulation is impaired immediately after the infusion *D*-glucose to elevate the circulating glucose level. The rapidity of onset and the reversibility of impaired blood flow autoregulation in early diabetes suggests the development of a functional defect in the tissues responsible for regulating vascular tone.

Blood flow autoregulation is a complex phenomenon that is strongly dependent on the integrity of the intrinsic myogenic response of vascular smooth muscle [6]. The vascular myogenic response is in turn dependent on the uptake of Ca^{2+} by voltage-sensitive Ca^{2+} channels; the so-called L-type channel [7]. This conclusion is supported by the observations that specific pharmacologic inhibition of Ca^{2+} influx via the L-type channel, markedly impairs the blood flow autoregulatory response to increased perfusion pressure in many tissues [8, 9].

Taken together, these observations suggest the possibility that a metabolic consequence of the diabetic state, notably hyperglycemia, may act to functionally impair the cellular mechanism responsible for blood flow autoregulation, i.e. Ca^{2+} uptake by vascular smooth muscle cells. Such a possibility is not without precedent: There is abundant evidence of multiple abnormalities in calcium metabolism in insulin-dependent diabetes in animals and man characterized by defective Ca^{2+} uptake by the renal tubule, the duodenum and bone [10–12]. Despite this evidence of diabetes-induced disturbances in transmembrane Ca^{2+} fluxes in many tissues, few studies have focussed on the possibility that decreased vascular tone in early diabetes may result from defective Ca^{2+} transport into vascular tissue. However, support for the concept that modification of Ca^{2+} influx into vascular tissue in diabetes mellitus may mediate changes in regional perfusion comes from the observation that extracellular Ca^{2+} supplementation was necessary to restore a previously elevated renal plasma flow to normal by insulin infusion in diabetic rats. Moreover, this corrective response was prevented by co-administration of an L-type Ca^{2+} channel blocker, verapamil. The authors concluded that decreased vascular tone in early diabetes may in part be due to defective transmembrane Ca^{2+} flux across vascular smooth muscle cells [13].

Thus, early in the course of diabetes mellitus, various sources of evidence suggest that impairment of vascular reactivity and blood flow autoregulation could relate to an action of a metabolic component of the diabetic milieu to modify Ca^{2+} uptake by vascular tissue, in a manner similar to the aforementioned disturbances in Ca^{2+} transport documented in other tissues. Using an in vivo study, it is impossible to assess the independent impact of a single metabolic variable of the diabetic state, i.e. glucose, on the uptake of Ca^{2+} by vascular smooth muscle because variations in glycemic control are inevitably associated with hormonal and regional hemodynamic changes that may also act to modulate cellular Ca^{2+} flux. The present study thus utilized an in vitro cell culture system to test the hypothesis that high extracellular glucose concentrations may directly and independently depress the transmembrane flux of calcium ions into vascular smooth muscle cells.

Methods

Preparation of Cultured Rat VSMC

Aortic VSMC were isolated from the Sprague-Dawley rat and cultured as previously described in detail [14, 15]. The resulting VSMC were plated on 35-mm culture dishes and grown in Eagle's minimum essential medium (MEM, Gibco) supplemented with 2 m*M* *L*-glutamine, 2 g/l $NaHCO_3$, 60 mg/l penicillin, 135 mg/l streptomycin and 10% fetal calf serum (FCS) in an incubator at 37 °C in 95% humidified air and 5% CO_2. Every 5–7 days, the cells were passaged after trypsin EDTA harvesting. For all experiments, 2nd through 6th passaged VSMC were used. Preliminary studies indicated that calcium ion fluxes were stable and reproducible in VSMC from the 2nd through the 10th passage.

Experimental Design

Confluent VSMC were exposed to one of four 'test' culture media for various time periods prior to determining calcium ion uptake and/or efflux by VSMC. The Ca^{2+} concentration of the test media was 1.4 m*M*/l. (1) Control medium: Regular MEM containing 10% FCS and a normal *D*-glucose concentration of 5 m*M*/l. (2) High glucose medium: Similar to the control medium except that it was supplemented with *D*-glucose to increase the glucose concentration from 10- to 30 m*M*. (3) Mannitol osmotic control medium: Similar to control medium but supplemented with the relatively impermeable hexose, 25 m*M* mannitol, an osmotic control for the high glucose medium. (4) *L*-Glucose osmotic control medium: Similar to control medium but supplemented with the cell permeable but poorly metabolized glucose isomer; *L*-glucose 25 m*M*.

Measurement of Basal $^{45}Ca^{2+}$ Uptake by VSMC

After exposing confluent VSMC to the test media, Ca^{2+} uptake measurements were performed. The test medium was aspirated from the cells and fresh test medium, supplemented with 2 μCi/ml $^{45}Ca^{2+}$ (specific activity 12.3 mCi/mg Ca^{2+}: ICN Radiochemicals, Irvine, Calif.) was added to the VSMC monolayer. The culture dish containing the VSMC was then returned to the incubator for 5 min at 37 °C. After 5 min, the dish was placed on ice to terminate Ca^{2+} uptake, the supernatant aspirated and the cell monolayer

rapidly rinsed 10 times in 1 min with ice-cold Ca^{2+}-free physiologic salt solution (PSS), containing 2 m*M* EGTA. Thereafter, the VSMC were lysed and solubilized in 1 ml of 0.1% sodium dodecyl sulfate (SDS) and 0.1 *N* NaOH and the cell-associated radioactivity determined by scintillation counting (Packard Tri-Carb 460C: Packard, Downer's Grove, Ill.). Prior to counting, a 50-µl aliquot was taken to measure cell protein content using a modification of the method of Lowry et al. [16]. The results are expressed as calcium ion uptake n*M*/min/mg cell protein.

Measurement of Voltage-Sensitive Ca^{2+} Uptake

In some experiments, the effect of BAY K 8644 on $^{45}Ca^{2+}$ uptake by VSMC was determined. BAY K 8644 is a specific activator of the dihydropyridine-sensitive, voltage-dependent (L-type) calcium channel in VSMC [17]. For these experiments, BAY K 8644 (10^{-7} *M*) was added during the 5-min incubation with $^{45}Ca^{2+}$. The effect of BAY K 8644 on calcium ion uptake was then measured as described above.

Measurement of $^{45}Ca^{2+}$ Efflux from VSMC

$^{45}Ca^{2+}$ efflux studies were performed as previously described [15]. Briefly, confluent VSMC monolayers were exposed for 48 h to each of the test media. For the last 3 h of the incubation period, the VSMC were loaded with $^{45}Ca^{2+}$, 10 µCi/ml, incubated at 37 °C. After loading the VSMC with $^{45}Ca^{2+}$ for 3 h, the VSMC monolayer was rapidly rinsed in 10 × 1 ml PSS. 1 ml of the appropriate fresh test media was then added to the monolayer and the VSMC incubated for 5 min at 37 °C. After this incubation, the medium overlying the cells was aspirated and retained for counting the $^{45}Ca^{2+}$ effluxed from the VSMC. The radioactivity retained by the VSMC was measured after solubilizing the VSMC as described above. The $^{45}Ca^{2+}$ effluxed from the VSMC is expressed as a percentage of total radioactivity, i.e. VSMC-retained counts + VSMC-released counts per 5 min.

Statistics

The results are expressed as the mean ± SEM. Statistical analysis was by a two-way analysis of variance with a Bonferroni correction. An 'n' value of 1 refers to the mean of 4 data points. A p value of < 0.05 was considered significant.

Results

Effect of Elevated Extracellular Glucose Concentrations on Basal $^{45}Ca^{2+}$ Uptake by VSMC

Basal Ca^{2+} uptake rates by confluent VSMC in the presence of control medium (glucose 5 m*M*/l) at 37 °C, measured over a 5-min period, were consistently and approximately 3 n*M*/mg cell protein/minute. When VSMC were exposed to an elevated extracellular glucose concentration (20 m*M*/l) for up to 48 h, there was a significant depression of Ca^{2+} uptake by VSMC with increasing time exposure to the high glucose medium, when compared to basal calcium uptake rates in the presence of control medium (fig. 1). Time course studies extended to 72 h incubation with the test media confirmed that the maximal glucose-induced depression of basal Ca^{2+} uptake was observed by 48 h incubation with the high glucose medium. After 48 h exposure to a high extracellular glucose concentration (20 m*M*/l), there was almost a 50% reduction in Ca^{2+} uptake by VSMC compared to basal Ca^{2+} uptake rates in the presence of control medium (fig. 2). Further studies examined the glucose concentration dependency of glucose-induced inhibition of basal Ca^{2+} uptake by VSMC. The results confirmed that an extracellular *D*-glucose concentration of only 15–20 m*M*/l was required to produce maximal glucose-induced inhibition of Ca^{2+} uptake. This appeared to be a threshold phenomenon, with no futher depression of Ca^{2+} uptake with increasing glucose concentrations up to 30 m*M*/l (data not shown).

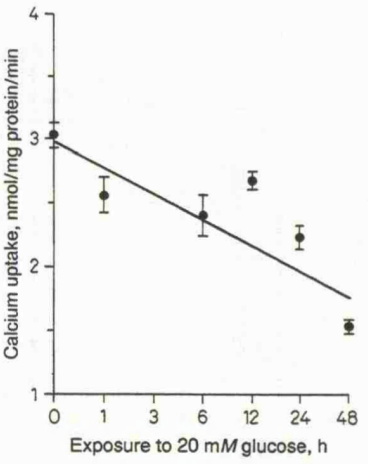

Fig. 1. Time dependency of glucose-induced inhibition of basal Ca^{2+} uptake by VSMC: VSMC were exposed to a high-glucose medium (20 mmol/l) for increasing time periods prior to determining basal $^{45}Ca^{2+}$ uptake. Time zero refers to VSMC exposed to control medium (glucose 5 mmol/l). A linear regression analysis of Ca^{2+} uptake vs. time revealed an 'r' value of -0.92, $p < 0.003$, $n = 4$.

The effect of the high glucose medium to significantly and markedly depress basal Ca^{2+} uptake by VSMC was not reproduced by up to 48 h exposure to either the mannitol or *L*-glucose containing 'osmotic control' media. This confirms that depression of Ca^{2+} uptake by VSMC was specific for elevated *D*-glucose concentrations and was not simply related to changes in extracellu-

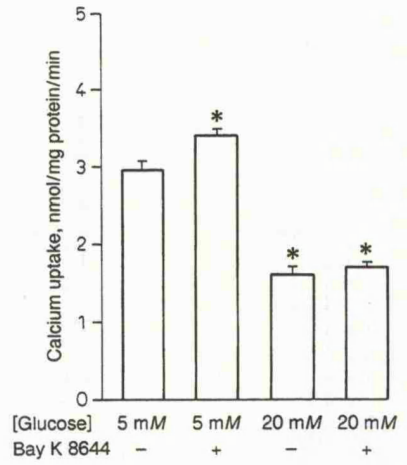

Fig. 2. Effect of glucose and BAY K 8644 on Ca^{2+} uptake by VSMC: VSMC were exposed to either control medium (glucose 5 mmol/l) or high glucose medium (glucose 20 mmol/l) for 48 h prior to determining basal Ca^{2+} uptake and the effect of BAY K 8644 (10^{-7} *M*) on Ca^{2+} uptake by VSMC. *$p < 0.05$ vs. glucose 5 mmol/l, $n = 4$.

lar osmolality. Moreover, normal Ca^{2+} uptake rates in the presence of high concentrations of the poorly metabolized *L*-glucose isomer suggests that the intracellular metabolism of *D*-glucose is necessary for glucose-induced depression of Ca^{2+} uptake by VSMC to occur. Additional studies demonstrated that VSMC viability (as determined by cell detachment rates, % lactate dehydrogenase release and % trypan blue extrusion) was unaffected by up to 48 h exposure to an elevated glucose concentration of up to 30 m*M*/l. Moreover, VSMC monolayer protein content per culture dish was equivalent after 48 h exposure to each of the test media (data not shown).

Effect of the Test Media on $^{45}Ca^{2+}$ Efflux from VSMC

The next study examined the effects of each of the test media on Ca^{2+} efflux rate from pre-loaded VSMC. This was important to determine whether the glucose-induced decrease in Ca^{2+} uptake by VSMC was a true net decrease in Ca^{2+} uptake by VSMC, rather than one compensated for by a simultaneous decrease in Ca^{2+} efflux. After exposing VSMC to the test media for 48 h, percent of total Ca^{2+} effluxed over a 5-min period was not significantly different for any of the test media studied. This confirms that elevated extracellular glucose concentrations did not depress Ca^{2+} efflux and that the glucose-induced decrease in basal Ca^{2+} uptake was a true net decrease in Ca^{2+} flux into VSMC (data not shown). It is of interest, however, that the $^{45}Ca^{2+}$ loading of VSMC over a 3-hour period, after exposure to the high-glucose medium was approximately 30% less than that observed in the presence of the other test media, further confirming the capacity of glucose to limit Ca^{2+} influx into VSMC.

Effect of Elevated Extracellular Glucose Concentrations on $^{45}Ca^{2+}$ Uptake via Voltage-Sensitive Ca^{2+} Channels in VSMC

Ca^{2+} uptake via voltage-sensitive Ca^{2+} channels in VSMC can be stimulated by the specific Ca^{2+} channel activator, BAY K 8644. As indicated in figure 2, when VSMC are exposed to BAY K 8644 (10^{-7} *M*) for 5 min, in the presence of control medium, there is a significant increase in Ca^{2+} uptake (approximately 120% vs. basal uptake). In marked contrast, however, after 48 h exposure to a high *D*-glucose medium (glucose 20 m*M*/l), basal Ca^{2+} uptake by VSMC was significantly depressed compared to control medium and BAY K 8644 failed to produce any significant augmentation of Ca^{2+} uptake. This demonstrates that in the presence of a high extracellular glucose medium, voltage-sensitive Ca^{2+} channel activation is markedly inhibited and almost completely prevented. To define whether the high glucose medium modified the threshold for Ca^{2+} channel activation by BAY K 8644, the study was repeated using BAY K 8644 concentrations of 10^{-6} and 10^{-5} *M*. Even at these higher concentrations, BAY K 8644 failed to significantly increase Ca^{2+} uptake by VSMC in the presence of a high glucose medium. In contrast, after 48 h exposure to either the mannitol or *L*-glucose 'osmotic control' media, Ca^{2+} uptake in response to BAY K 8644 was similar to that seen in the presence of medium containing glucose 5 m*M*/l (data not shown). These results demonstrate that elevated extracellular *D*-glucose concentrations depress both basal and voltage-sensitive Ca^{2+} uptake by VSMC, in vitro. Moreover, the mechanism is unrelated to changes in extracellular osmolality, is specific for *D*-glucose and is probably dependent on its intracellular metabolism.

Discussion

The entry of Ca^{2+} into most cell types, including VSMC, occurs mainly via voltage-sensitive channels [18, 19]. At least for types of channel (L, T, N and P) have now been characterized on the basis of their single-channel conductance, time course of inactivation and specific pharmacology [19, 20]. The most thoroughly studied voltage sensitive Ca^{2+} channel is the long-lasting or L-type channel which is characterized by a high activation threshold, a slow inactivation time and high sensitivity to pharmacologic agents, notably the dihydropyridine related classes of 'Ca^{2+} entry blocking drugs'. In addition, the L-type channel is selectively activated in many cell types by BAY K 8644, an agent that is structurally similar to the dihydroprydine class of Ca^{2+} entry blocking drugs [17, 20]. L-type Ca^{2+} channels play a crucial role in excitation-contraction coupling in cardiac and vascular smooth muscle and the myogenic component of blood flow autoregulation [7–9, 18, 21, 22]. The present study demonstrates that elevated extracellular glucose concentrations markedly inhibit basal and BAY K 8644 stimulated Ca^{2+} uptake by VSMC. This effect is specific for *D*-glucose and occurs at a glucose level compatible with those attained in poorly controlled diabetic patients.

Glucose-induced inhibition of voltage-sensitive Ca^{2+} flux could have considerable pathophysiologic relevance with regard to disordered cardiovascular function in diabetes mellitus. For example, disproportionate losses in cardiac output have been demonstrated in isolated diabetic rat hearts, compared to control hearts, upon lowering extracellular Ca^{2+} in the perfusate [23]. In studies of the diabetic dog, the L-type channels blocking drug, verapamil, was reported to precipitate cardiovascular collapse [24], an effect thought to represent increased cardiovascular sensitivity to Ca^{2+} channel blockade. In support of this conclusion, studies of the effect of diltiazem and verapamil on isolated hearts, using a constant flow model, confirmed a marked increase in the sensitivity of diabetic versus control hearts to L-type Ca^{2+} channel blockade [25]. In other studies, coronary vessels, mesenteric arteries, isolated aortas and thoracic aortic rings from diabetic animals have all been shown to exhibit increased sensitivity to the vasodilator action of L-type Ca^{2+} channel-blocking drugs when compared to the response in nondiabetic animals [23–28]. Together these observations strongly suggest that the diabetic state disturbs normal transmembrane Ca^{2+} transport and thereby renders the vasculature more susceptible to Ca^{2+} channel blockade.

The present study suggests that the enhanced vascular sensitivity of diabetic animals and man to extracellular Ca^{2+} depletion or L-type Ca^{2+} channel blockade may relate to a glucose-induced depression of both basal and voltage-sensitive Ca^{2+} uptake by vascular tissue. This direct extrapolation from the in vitro situation is supported by in vivo data demonstrating that the impairment of retinal blood flow autoregulation in the nondiabetic cat can be specifically induced within minutes of infusing *D*-glucose to elevate the extracellular glucose concentration [5]. Of interest, this effect was not reproduced by an isosmotic mannitol infusion, suggesting an effect on blood flow autoregulation that was specific for *D*-glucose [5]. Moreover, in recently diagnosed diabetic patients, elevations in renal plasma flow, which are predominantly due to afferent arteriolar vasodilatation, can be reversed by strict glycemic control. Finally, studies directly examining renovascular responses in early diabetes mellitus have concluded that changes in vascular tone in early diabetes may, at least in part, be due to defective transmembrane Ca^{2+} fluxes across vascular smooth muscle cells [13]. The present study demonstrates that such a defect may be due to a specific action of elevated extracellular glucose concentrations to depress transmembrane Ca^{2+} fluxes in vascular tissue, thereby perhaps explaining the aforementioned evidence of enhanced vascular sensitivity to extracellular calcium depletion or pharmacologic manipulation of L-type Ca^{2+} channel activity in diabetic animals or man. Whether these observations also lend insight into a cellular mechanism whereby adverse glycemic control contributes to disordered regional hemodynamics and the subsequent predisposition of the diabetic population to microvascular injury, requires further evaluation.

Acknowledgements

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Effect of elevated extracellular glucose concentrations on transmembrane calcium ion fluxes in cultured rat VSMC

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Effect of elevated extracellular glucose concentrations on transmembrane calcium ion fluxes in cultured rat VSMC. Blood flow autoregulation is impaired in early diabetes mellitus, predisposing the renal microcirculation to injury. These hemodynamic changes have been strongly implicated in the development and progression of diabetic glomerulopathy. Blood flow autoregulation is predominantly a myogenic reflex which is strongly dependent on Ca^{2+} uptake by vascular smooth muscle cells (VSMC). Because impaired blood flow autoregulation may be responsive to glycemic control, the present study examined the effects of elevated extracellular glucose concentrations on basal, voltage sensitive and receptor operated Ca^{2+} uptake by VSMC. Confluent cultured rat VSMC were exposed to: (1) control medium (CM; 5 mM glucose); (2) high glucose medium (HGM; 10 to 30 mM glucose); or (3) osmotic control medium (OCM; glucose 5 mM + L-glucose 25 mM or mannitol 25 mM). A threshold glucose concentration of 15 mM markedly and maximally depressed basal Ca^{2+} uptake by VSMC (HGM 52% vs. CM). In addition, HGM significantly depressed voltage sensitive Ca^{2+} uptake by VSMC as determined by responses to BAY K 8644 (10^{-7} M) or high extracellular [K^+] (65 mM, HGM 50% vs. CM). HGM similarly depressed pressor hormone-stimulated Ca^{2+} uptake (AVP or Ang II 10^{-7} M) by VSMC. The effects of HGM on Ca^{2+} uptake were time exposure dependent and reversible. Ca^{2+} uptake by VSMC in the presence of OCM did not differ from CM. Elevated extracellular glucose concentrations thus exert a direct and profound effect on basal, voltage sensitive and receptor operated Ca^{2+} uptake by VSMC. These observations may provide a biochemical basis for glucose-induced dysregulation of regional blood flow autoregulation in early diabetes mellitus.

Diabetes mellitus is characterized by the premature development of vascular disease. Hemodynamic factors have been strongly implicated in the primary pathogenesis and propagation of this vascular injury [1–3]. The marked susceptibility of the diabetic microcirculation to injury when exposed to moderate elevations of systemic blood pressure implies the breakdown of mechanisms such as blood flow autoregulation, that normally act to protect the microcirculation from excessive barotrauma. Numerous recent studies confirm that blood flow autoregulatory responses to increased systemic perfusion pressures are impaired in many tissues early in the course of both experimental and human diabetes [4–8].

Blood flow autoregulation is a complex phenomenon that is critically dependent on a local pressure-activated myogenic reflex generated within the vascular smooth muscle of arterioles and some larger arteries [9–12]. This reflex can be modulated by the action of circulating pressor hormones such as angiotensin II (Ang II) and arginine vasopressin (AVP) as well as the local release of prostaglandins, thromboxanes and endothelial derived factors [13–15]. Importantly, the intrinsic myogenic reflex in vascular smooth muscle and the cellular action of factors modulating the autoregulatory response are all dependent on an increased transmembrane flux of calcium ions (Ca^{2+}) into the vascular smooth muscle cell [16–19]. This conclusion is supported by the observations that the pharmacological inhibition of Ca^{2+} uptake by vascular smooth muscle completely inhibits blood flow autoregulatory responses to increased perfusion pressure in many tissues and inhibits the pressor actions of both Ang II and AVP *in vivo* [17, 19, 20–23].

The factors responsible for impairment of blood flow autoregulation in diabetes mellitus remain undefined. However, numerous observations suggest that metabolic factors, in particular hyperglycemia, may be important. Specifically, impairment of renal blood flow autoregulatory responses to increased perfusion pressures in the diabetic rat, which is demonstrable within days of onset of hyperglycemia, can be prevented by the administration of insulin to maintain euglycemia [24]. More direct evidence implicating hyperglycemia comes from the observation that retinal blood flow autoregulation is impaired in the cat within minutes of infusing D-glucose to induce hyperglycemia [25]. Although these findings implicate hyperglycemia in the pathogenesis of impaired blood flow autoregulation in diabetes mellitus, they provide no insight into the underlying mechanism.

The strong dependence of blood flow autoregulation on Ca^{2+} uptake by vascular smooth muscle raises the possibility that hyperglycemia could inhibit autoregulation via an adverse effect on Ca^{2+} uptake by vascular smooth muscle cells. Such a possibility is not without precedent. In patients with diabetes mellitus, there is abundant evidence of depressed transmembrane Ca^{2+} transport by some tissues including the renal tubule, duodenum and bone [26–28]. If similar disturbances in Ca^{2+} transport existed in vascular tissue then this could provide a cellular mechanism whereby glucose might adversely affect excitation:contraction coupling in vascular tissue and thereby impair blood flow autoregulation. Heretofore, however, no

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study has specifically examined the effects of elevated extracellular glucose concentrations on Ca^{2+} uptake by vascular tissue. The present study thus utilizes an *in vitro* cell culture system to examine specifically the independent effects of high extracellular glucose concentrations on basal, voltage sensitive and pressor hormone stimulated Ca^{2+} uptake by vascular smooth muscle cells (VSMC).

Methods

Preparation of cultured rat vascular smooth muscle cells

Aortic VSMC were isolated from the Sprague Dawley rat and cultured as previously described in detail [29, 30]. The resulting VSMC were plated on 35 mm culture dishes and grown in Eagle's minimum essential medium (MEM, Gibco) supplemented with 2 mM L-glutamine, 2 g/liter $NaHCO_3$, 60 mg/liter penicillin, 135 mg/liter streptomycin and 10% fetal calf serum (FCS) in an incubator at 37°C in 95% humidified air and 5% CO_2. The Ca^{2+} concentration of the culture medium was 1.4 mM/liter. Every five to seven days, the cells were passaged after trypsin-EDTA harvesting. For all experiments, second through sixth passaged VSMC were used. Preliminary studies indicated that Ca^{2+} fluxes were stable and reproducible in VSMC from the second through tenth passage.

Experimental design

Confluent VSMC were exposed to one of four "test" culture media for various time periods prior to determining Ca^{2+} uptake and/or efflux by VSMC.

1. Control medium: Regular MEM containing 10% FCS and a normal D-glucose concentration of 5 mM/liter.
2. High glucose medium: Similar to the control medium except that it was supplemented with D-glucose to increase its concentration from 10 to 30 mM/liter.
3. Mannitol osmotic control medium: Similar to control medium but supplemented with the relatively cell impermeable hexose, mannitol (25 mM/liter), which served as an osmotic control for the high glucose medium.
4. L-glucose osmotic control medium: Similar to control medium but supplemented with L-glucose (25 mM/liter), a cell permeable but poorly metabolized glucose isomer. This medium served as a second osmotic control for the high glucose medium. In addition, it also served as a metabolic control, helping to define whether the intracellular metabolism of glucose was necessary for any of the observed effects of the high glucose medium to develop. When the incubation period with the test media extended beyond 12 hours, the medium was replaced every 12 hours to ensure stability of the extracellular glucose concentration and to prevent fluctuations in extracellular pH.

Measurement of basal $^{45}Ca^{2+}$ uptake by VSMC

After exposing confluent VSMC to the various test media, Ca^{2+} uptake measurements were performed. The test medium overlying the cells was aspirated and replaced with 1 ml of fresh test medium supplemented with 2 μCi/ml $^{45}Ca^{2+}$ (specific activity 12.3 mCi/mg Ca^{2+}: ICN Radiochemicals, Irvine, California, USA). The culture dish containing the VSMC was then returned to the incubator for five minutes at 37°C. After five minutes, the dish was placed on ice to terminate Ca^{2+} uptake; the supernatant was aspirated and the cell monolayer rapidly rinsed 10 times in one minute with ice-cold Ca^{2+}-free physiologic salt solution (PSS) containing 2 mM EGTA. Thereafter, the VSMC were lysed and solubilized in 1 ml of 0.1% sodium dodecyl sulfate (SDS) and 0.1 N NaOH and the cell associated radioactivity determined by scintillation counting (Packard Tri-Carb 460C: Packard, Downer's Grove, Illinois, USA). Prior to counting, a 50 μl aliquot was taken to measure cell protein content using a modification of the method of Lowry. The results are expressed as Ca^{2+} uptake, nM/min/mg cell protein.

Reversibility of glucose-induced depression of basal Ca^{2+} uptake by VSMC

To determine whether glucose-induced depression of Ca^{2+} uptake by VSMC was reversible, VSMC were first exposed to high glucose medium (20 mM/liter) for 48 hours to induce depression of Ca^{2+} uptake. The medium overlying the cells was then changed to control medium (glucose 5 mM/liter) and basal Ca^{2+} uptake rates were determined at different time intervals after normalizing the extracellular glucose concentration.

Measurement of voltage sensitive Ca^{2+} uptake by VSMC

The uptake of Ca^{2+} by VSMC is markedly increased by the activation of voltage sensitive Ca^{2+} channels. These channels can be activated experimentally, either by depolarizing the cell membrane or more specifically by the use of agents such as BAY K 8644 [31–34]. To depolarize the cell membrane, the test medium was supplemented with 65 mM KCl during the five minute incubation period with $^{45}Ca^{2+}$. To more specifically activate the voltage sensitive channel, the test medium was supplemented with BAY K 8644 (10^{-7} M) during the five minute incubation period with $^{45}Ca^{2+}$. BAY K 8644 binds specifically to the dihydropyridine binding site within the L type voltage sensitive Ca^{2+} channel and has been shown to activate this channel in a variety of tissues, including VSMC [32, 33]. The effects of both of these experimental maneuvers on Ca^{2+} uptake by VSMC was then measured as described above.

Measure of AVP and Ang II-stimulated Ca^{2+} uptake by VSMC

Ca^{2+} uptake by VSMC is an important component of the intracellular signaling response of vascular tissue to pressor hormones [35]. To determine the effects of the various test media on pressor hormone-stimulated Ca^{2+} uptake by VSMC during the five minute incubation period with $^{45}Ca^{2+}$, the test medium was supplemented with either AVP (10^{-7} M) or Ang II (10^{-7} M). Thereafter, Ca^{2+} was measured as described above.

Measurement of $^{45}Ca^{2+}$ efflux from VSMC

To examine the effects of the various test media on Ca^{2+} efflux rates from VSMC, $^{45}Ca^{2+}$ efflux studies were performed as previously described [30]. Briefly, confluent VSMC monolayers were exposed for 48 hours to each test medium. For the final three hours of the incubation period, $^{45}Ca^{2+}$ (10 μCi/ml) was added to the test media. After loading the VSMC with $^{45}Ca^{2+}$ for three hours, the VSMC monolayers were rinsed rapidly 10 × 1 ml PSS. One milliliter of the appropriate fresh test medium was then added to the VSMC monolayer and incubated at 37°C for five minutes. After this incubation, the

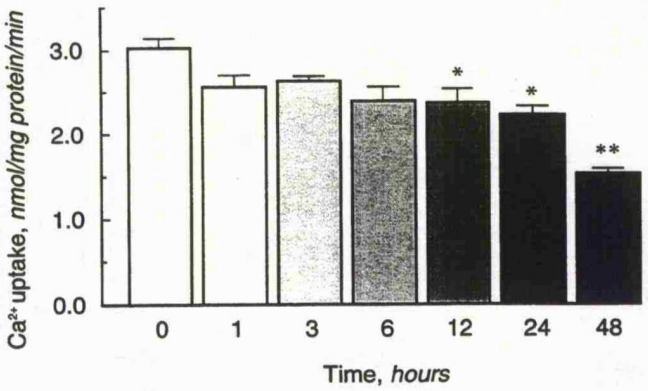

Fig. 1. *Time dependency of glucose-induced inhibition of basal Ca^{2+} uptake by VSMC.* Time 0 refers to VSMC exposed to control medium (glucose 5 mM) for 48 hours. 1-48 hours represents time exposure to high glucose medium (glucose 20 mM). $*P < 0.05$, $**P < 0.001$ vs. time 0, $N = 4$.

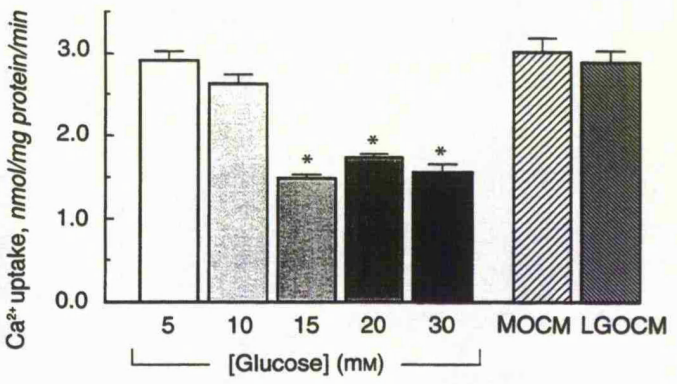

Fig. 2. *Glucose concentration dependency of the inhibition of basal Ca^{2+} uptake by VSMC.* VSMC were exposed for 48 hours to culture medium containing various glucose concentrations or mannitol osmotic control medium (MOCM) or L-glucose osmotic control medium (LGOCM), prior to measuring basal Ca^{2+} uptake rates. $*P < 0.05$ vs. glucose 5 mM, $N = 4$.

test medium overlying the cells was aspirated and retained for counting the $^{45}Ca^{2+}$ that had effluxed from the VSMC. The radioactivity retained by the VSMC was measured after solubilizing the cells as described above. The $^{45}Ca^{2+}$ effluxed from the VSMC is expressed as a percentage of total $^{45}Ca^{2+}$ available for efflux, that is: $^{45}Ca^{2+}$ effluxed counts/($^{45}Ca^{2+}$ effluxed + retained counts)%.

The effects of the various test media on AVP, Ang II, BAY K 8644 and KCl stimulated $^{45}Ca^{2+}$ efflux was measured using the same protocol. After washing the $^{45}Ca^{2+}$ loaded VSMC, 1 ml of fresh test supplemented with one of the aforementioned effectors was added to the VSMC monolayer and $^{45}Ca^{2+}$ efflux was measured over the subsequent five minutes as described above.

Statistics

The results are given as means ± standard error of the mean. Statistical analysis is by a two-way analysis of variance with a Bonferroni correction. An N value of 1 refers to a mean of four data points. A P value of <0.05 was considered significant.

Results

Effect of elevated extracellular glucose concentrations on basal Ca^{2+} uptake by VSMC

Basal Ca^{2+} uptake rates by confluent VSMC measured in the presence of control medium were consistent and approximated 3 nM/mg cell protein/minute. The test media contained 10% FCS which provided a background stimulation of Ca^{2+} uptake, illustrated by the fact that Ca^{2+} uptake was 1.9 nM/mg cell protein/min after 48 hours exposure to control medium (glucose 5 mM) containing only 0.5% FCS ($N = 3$). During a timed exposure to an elevated glucose concentration (20 mM/liter), Ca^{2+} uptake by VSMC was significantly depressed when compared to basal Ca^{2+} uptake rates in the presence of control medium (Fig. 1). When the concentration of FCS in the test media was reduced to 0.5% for 48 hours, the effect of the elevated extracellular glucose concentration to depress Ca^{2+} uptake persisted (Glucose 5 mM/liter: 1.9 ± 0.08 vs. glucose 20 mM: 1.3 ± 0.05, $P < 0.05$, $N = 3$). All subsequent results refer to studies performed in the presence of 10% FCS. The effect of glucose on Ca^{2+} uptake became more pronounced with increasing time exposure to the high glucose medium, Ca^{2+} uptake by VSMC being significantly depressed within 12 hours. Further studies, during which VSMC exposure to the test media was extended to 72 hours, confirmed that the maximal glucose-induced depression of basal Ca^{2+} uptake occurred by 48 hours. At this time point, there was a 50% reduction in basal Ca^{2+} uptake by VSMC when compared to basal Ca^{2+} uptake rates in the presence of control medium.

Glucose concentration dependent inhibition of Ca^{2+} uptake by VSMC

To examine the glucose concentration dependency of inhibition of Ca^{2+} uptake by VSMC, the VSMC monolayers were exposed to different glucose concentrations (5 to 30 mM) for 48 hours. This time point was selected because a maximal effect of glucose on Ca^{2+} uptake was observed by this time. Figure 2 shows that basal Ca^{2+} uptake was significantly inhibited once the glucose concentration had been increased to 15 mM/liter. This appeared to be a threshold phenomenon because there was no further depression of calcium uptake with increased glucose concentrations up to 30 mM/liter.

Effects of osmotic control media on Ca^{2+} uptake by VSMC

The effects of the high glucose medium on basal Ca^{2+} uptake by VSMC were not reproduced by up to 48 hours exposure to either the mannitol or L-glucose containing osmotic control media (Fig. 2). This confirms that glucose-induced depression of Ca^{2+} uptake by VSMC was specific for D-glucose, and not simply related to changes in extracellular osmolality. Moreover, the lack of any effect of the high L-glucose medium on Ca^{2+} uptake by VSMC suggests that the intracellular metabolism of D-glucose is necessary for glucose-induced depression of Ca^{2+} uptake by VSMC to occur.

Reversal of glucose-induced depression of basal Ca^{2+} uptake by VSMC

Having demonstrated glucose-induced depression of Ca^{2+} uptake by VSMC, further studies were performed to determine

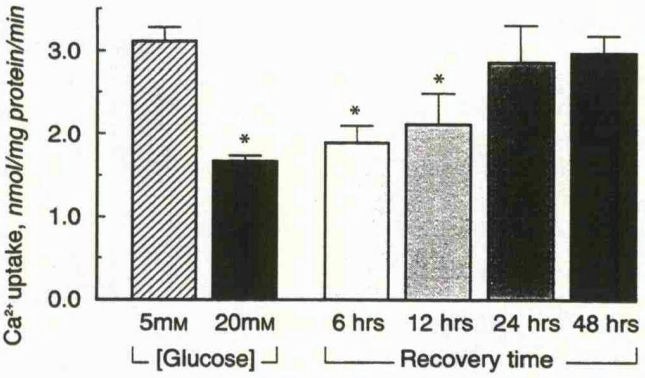

Fig. 3. *Time dependent recovery from glucose-induced inhibition of basal Ca^{2+} uptake by VSMC.* Recovery time refers to VSMC time exposure to control medium (glucose 5 mM) after a 48 hour pre-exposure to high glucose medium (glucose 20 mM). $^*P < 0.05$ vs. glucose 5 mM, $N = 3$.

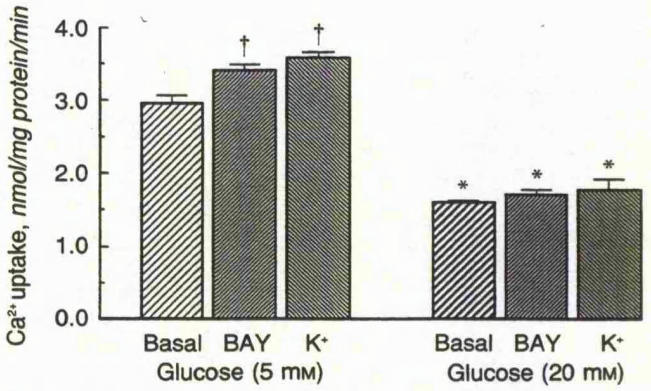

Fig. 4. *Glucose-induced inhibition of voltage sensitive Ca^{2+} uptake by VSMC.* VSMC were pre-exposed to control medium (glucose 5 mM) or high glucose medium (glucose 20 mM) for 48 hours prior to a 5 minute exposure to similar media containing either BAY K 8644 (10^{-7} M) (BAY) or 65 mM KCl (K^+). $+P < 0.05$, $^*P < 0.01$ vs. basal (glucose 5 mM), $N = 4$.

whether this effect of glucose was reversible. Figure 3 shows that following a 48 hour exposure to high glucose medium (20 mM), there was a time dependent recovery of basal Ca^{2+} uptake rates by VSMC once the extracellular glucose concentration was restored to normal. The effects of glucose on Ca^{2+} uptake by VSMC were thus reversible but required up to 24 hours of a normal glucose environment before Ca^{2+} uptake rates were restored to normal. The reversibility of glucose-induced depression of Ca^{2+} uptake confirms that these effects cannot be explained by irreversible cell toxicity. Additional studies demonstrated that VSMC viability (as determined by cell detachment rates, % lactate dehydrogenase release and % trypan blue extrusion) was unaffected by 48 hours exposure to elevated glucose concentrations up to 30 mM/liter (data not shown). These conclusions are supported by the fact that cell toxicity is invariably associated with increased rather than depressed Ca^{2+} uptake rates [36].

Effect of elevated glucose concentrations on Ca^{2+} uptake via voltage sensitive Ca^{2+} channels in VSMC

The myogenic component of blood flow autoregulation is critically dependent on the activation of L type voltage sensitive Ca^{2+} channels in VSMC [16–18]. To mimic this action *in vitro*, these same channels were activated either by membrane depolarization using high extracellular potassium concentration or by direct stimulation with BAY K 8644. In the presence of control medium supplemented with KCL (65 mM), Ca^{2+} uptake by VSMC significantly increased (121% vs. basal, Fig. 4). Previous studies have confirmed that this effect is due to the activation of voltage sensitive Ca^{2+} channels [31, 37]. This response is lower than that observed when studies are performed utilizing a simple buffer (approximately: 150% vs. basal). This reflects the fact that Ca^{2+} uptake by VSMC is augmented by the presence of 10% FCS (see above). In contrast, after 48 hours pre-exposure to a high glucose medium (20 mM) there was a marked decrease in basal Ca^{2+} uptake and a failure of the high K^+ concentration to augment Ca^{2+} uptake. These observations suggest that high extracellular glucose concentrations, in addition to depressing basal Ca^{2+} uptake, also inhibit the activation of voltage sensitive Ca^{2+} channels in VSMC. To confirm this hypothesis, studies were performed in which the L type voltage sensitive channel was more specifically activated using BAY K 8644. Studies in the presence of control medium confirmed that BAY K 8644 (10^{-7} M) significantly increased Ca^{2+} uptake by VSMC (Fig. 4). This increase in Ca^{2+} uptake in response to BAY K 8644 is not observed in all cell types under basal conditions. Previous studies, however, confirm that rat aortic vascular smooth muscle cells are responsive to BAY K 8644 in the resting state [38]. The response to BAY K 8644 confirms that cultured VSMC express voltage sensitive Ca^{2+} channels and that even under basal conditions, a proportion of these channels are active [39]. The magnitude of the response to BAY K 8644 (115% vs. basal) was similar to that observed with membrane depolarization. In marked contrast, after pre-exposure to high glucose medium (20 mM) for 48 hours, BAY K 8644 failed to increase the already significantly depressed basal Ca^{2+} uptake by VSMC. To determine whether the high glucose medium modified the threshold for voltage sensitive channel activation by BAY K 8644, the study was repeated using BAY K 8644 concentrations of 10^{-6} M and 10^{-5} M. Even at these higher concentrations, BAY K 8644 failed to augment Ca^{2+} uptake in the presence of high glucose medium. In contrast, results obtained after pre-exposure to both of the osmotic control media confirmed that there was no inhibition of Ca^{2+} uptake in response to high [K^+] or BAY K 8644 (data not shown). Together, these observations demonstrate that high glucose concentrations inhibit Ca^{2+} uptake via voltage sensitive Ca^{2+} channels in VSMC.

Effect of elevated extracellular glucose concentrations on receptor operated Ca^{2+} channels in VSMC

The next study examined the effects of the various test media on pressor hormone stimulated Ca^{2+} uptake by VSMC. Figure 5 shows that the pressor hormones Ang II and AVP (10^{-7} M) both increased Ca^{2+} uptake by VSMC in the presence of control medium (glucose 5 mM). Similar results were obtained for VSMC pre-exposed to the osmotic control media for 48 hours (data not shown). In contrast, after 48 hours pre-exposure

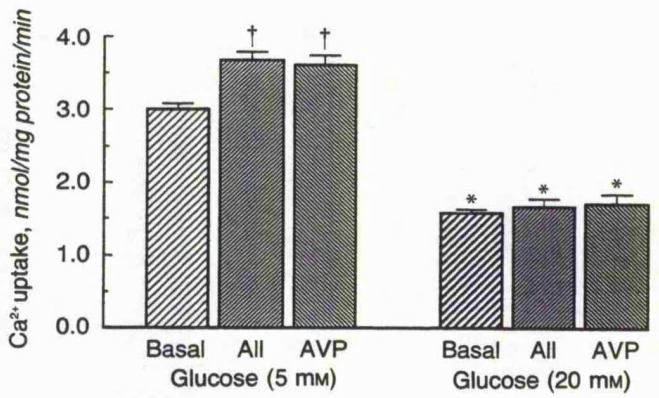

Fig. 5. *Glucose-induced inhibition of pressor hormone-stimulated Ca^{2+} uptake by VSMC.* VSMC were pre-exposed to control medium (glucose 5 mM) or high glucose medium (glucose 20 mM) for 48 hours prior to a 5 minute exposure to AVP (10^{-7} M) or Ang II (10^{-7} M). $+P <$ 0.05, $*P < 0.01$ vs. basal (glucose 5 mM), $N = 4$.

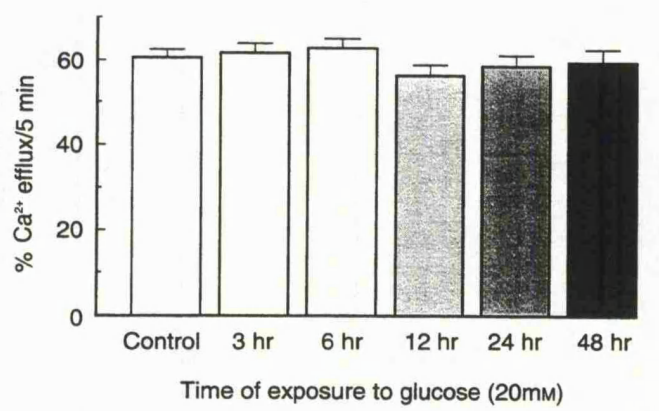

Fig. 6. *Effect of extracellular glucose concentration on % basal Ca^{2+} efflux from VSMC.* VSMC were pre-exposed to control medium (glucose 5 mM) for 48 hours or high glucose medium (glucose 20 mM) for various time periods. There was no statistical difference in % basal Ca^{2+} efflux rate vs. control at any time point, $N = 3$.

to high glucose medium (20 mM), basal Ca^{2+} uptake was markedly depressed and both AVP and Ang II failed to enhance Ca^{2+} uptake by VSMC. These results demonstrate that high extracellular glucose concentrations also inhibit the activation of pressor receptor operated Ca^{2+} channels in VSMC.

Effect of elevated extracellular glucose concentrations on Ca^{2+} efflux from VSMC

The effect of exposing VSMC to high glucose medium (20 mM) for up to 48 hours on % basal Ca^{2+} efflux rates was then studied. Figure 6 shows that % basal Ca^{2+} efflux rates were unaffected by prolonged exposure to the high glucose medium.

AVP or Ang II (10^{-7} M) increased $\%Ca^{2+}$ efflux/5 min from VSMC that had been pre-exposed for 48 hours to control medium or either of the two osmotic control media (Table 1). In contrast, 48 hours pre-exposure to the high glucose medium (glucose 20 mM) significantly inhibited the VSMC Ca^{2+} efflux response to the pressor hormones. K^+ or BAY K 8644 did not significantly increase the Ca^{2+} efflux response versus basal in the presence of any of the test media (Table 1). These results confirm that the glucose-induced depression of Ca^{2+} uptake by VSMC represents a true net decrease in the transmembrane flux of Ca^{2+} which is not compensated for by an associated change in Ca^{2+} efflux rate. Together, these results demonstrate that elevated extracellular glucose concentrations have a powerful depressive effect on basal, voltage sensitive and receptor operated mechanisms regulating Ca^{2+} uptake by VSMC.

Table 1. % $^{45}Ca^{2+}$ Efflux from VSMC/5 minutes

Stimulus	Glucose *5 mM*	Glucose *20 mM*
Basal	56.3 ± 5.0	54.1 ± 6.2
Ang II 10^{-7} M	71.4 ± 6.2[a]	57.4 ± 4.9
AVP 10^{-7} M	70.8 ± 4.1[a]	59.3 ± 5.9
KCl *65 mM*	60.8 ± 6.1	56.5 ± 5.8
BAY K 8644 10^{-7} M	59.7 ± 5.6	55.3 ± 5.4

VSMC monolayers were exposed to control (glucose 5 mM) or high glucose (20 mM) medium for 48 hours prior to measurement of basal Ca^{2+} efflux or the Ca^{2+} efflux response to 1 minute stimulation with Ang II, AVP, KCl or BAY K 8644.

[a] $p < 0.05$ vs. Basal, $N = 3$.

Discussion

Ca^{2+} entry is crucial for excitation:contraction coupling in vascular tissue and plays a major role in the regulation of vascular tone [35, 37, 40, 41]. Ca^{2+} entry into most cell types, including VSMC, occurs predominantly via voltage sensitive channels that are activated by membrane depolarization [37]. At least four types of channel (L, T, N & P) have been defined on the basis of their electrophysiologic characteristics [34, 41]. The predominant voltage sensitive Ca^{2+} channel in VSMC is the "L" type voltage sensitive channel which is characterized by a high activation threshold, slow inactivation time, and high sensitivity to dihydropyridines [34, 37].

Activation of the L type channel in VSMC plays a pivotal role in blood flow autoregulatory responses. The application of transmural pressure to resistance sized arteries causes membrane depolarization which activates L type Ca^{2+} channels; these in turn increase Ca^{2+} uptake by VSMC and thereby enhance vascular tone [8, 37, 42–44]. The relevance of this pressure activated Ca^{2+} uptake to autoregulatory responses is illustrated by the fact that the spontaneous development of myogenic tone in various resistance sized arteries can be prevented by extracellular Ca^{2+} depletion, the administration of drugs that inhibit L type Ca^{2+} channels, or by prior membrane hyperpolarization [43–46].

Early diabetes mellitus is characterized by impaired blood flow autoregulatory responses to increased perfusion pressures in many organs [4–8]. In view of the fact that autoregulation is so dependent on Ca^{2+} uptake by vascular smooth muscle, the present study examined the independent effects of elevated extracellular glucose concentrations on Ca^{2+} uptake by VSMC. In order to mimic the action of transmural pressure-induced voltage sensitive channel activation, *in vitro*, these channels were activated by exposing VSMC to a high extracellular K^+ concentration. This maneuver has been previously shown to depolarize the cell membrane, increase Ca^{2+} uptake and increase vascular tone [31, 37]. The results of the present study

demonstrate that elevated extracellular glucose concentrations impair the capacity of membrane depolarization to augment Ca^{2+} uptake by VSMC. Because the effect of glucose on membrane polarity was not measured, it is impossible to exclude the possibility that glucose indirectly inhibited the activation of voltage sensitive Ca^{2+} channels by rendering the cell membrane more resistant to depolarization. This explanation is, however, unlikely because the direct activation of the L type voltage sensitive channel by BAY K 8644 was also inhibited by a high glucose medium. Whether this direct glucose-induced inhibition of L type Ca^{2+} channel activation in VSMC may result from either a reduction in Ca^{2+} channel number or reduced ion conductance by individual channels is currently being evaluated.

The possibility that our results reflect variations in cell membrane bound Ca^{2+} rather than Ca^{2+} uptake by VSMC must also be considered. This explanation is, however, most unlikely because the increase in $^{45}Ca^{2+}$ uptake measured in the presence of AVP, Ang II, K^+ and BAY K 8644 cannot be attributed to increased membrane binding of $^{45}Ca^{2+}$, thereby validating our conclusions.

The myogenic component of the autoregulation response is modulated by the action of circulating pressor hormones such as AVP and Ang II. The vascular action of pressor hormones is strongly dependent on Ca^{2+} uptake by VSMC [19, 35, 40]. The present study demonstrates that elevated extracellular glucose concentrations inhibit this pressor hormone-induced Ca^{2+} influx. The mechanism whereby pressor hormones increase Ca^{2+} entry into VSMC is unclear. One hypothesis proposes hormonal activation of a distinct group of "receptor operated Ca^{2+} channels" [47]. More recent evidence, however, suggests that the receptor operated Ca^{2+} channel is indistinguishable from the voltage sensitive L type channel, the main difference being a more indirect route of activation, perhaps via the generation of intracellular second messengers [37]. If the latter explanation is correct, then glucose-induced inhibition of pressor hormone activated Ca^{2+} uptake could be a manifestation of a generalized glucose-induced depression of L type Ca^{2+} channel activity. There is, however, another potential mechanism to explain glucose-induced depression of pressor hormone stimulated Ca^{2+} uptake by VSMC. We have recently shown that high extracellular glucose concentrations (20 mM) cause a marked down-regulation of AVP and Ang II receptors on VSMC and depress intracellular signaling and contraction in response to these hormones [48]. Thus, the decrease in pressor receptor mediated Ca^{2+} influx could be secondary to glucose-induced pressor receptor down-regulation in addition to any direct action of glucose to depress the L type Ca^{2+} channel.

In the present study, one of the most striking effects of elevated glucose concentrations was the depression of basal Ca^{2+} uptake by unstimulated VSMC. It is well recognized that even during resting conditions, there is a large basal transmembrane influx of Ca^{2+} into VSMC [49]. As the molecular basis for this basal Ca^{2+} "leak" is poorly understood, it is difficult to define a mechanism whereby glucose may modify this process. Nevertheless, such an action of glucose could have a significant effect on basal vascular tone because the resting tone of resistance sized arteries and systemic vessels can be abolished by depleting extracellular Ca^{2+} [49, 50], thus indicating the importance of basal Ca^{2+} influx in the maintenance of vessel tone.

The present study therefore demonstrates a hitherto unreported potent *in vitro* effect of elevated extracellular glucose concentrations to depress both the basal and dynamic mechanisms regulating Ca^{2+} entry into VSMC. This effect is specific for D-glucose and occurred at glucose concentrations compatible with those seen in patients with poorly controlled diabetes mellitus.

The present study has focused on the effects of glucose on net Ca^{2+} uptake by VSMC rather than the intracellular compartmentalization of Ca^{2+}. In this regard, it is the uptake of Ca^{2+} that has been convincingly demonstrated to be relevant to the cellular mechanisms regulating the myogenic component of blood flow autoregulation [16–19]. Nevertheless, it seems likely that glucose-induced reductions in Ca^{2+} uptake by VSMC could also influence the intracellular compartmentalization of Ca^{2+}, in particular the free intracellular Ca^{2+} concentration. As the latter is critical in regulating many aspects of VSMC function [40], further work is necessary to define whether elevated extracellular glucose concentrations also influence the intracellular compartmentalization of Ca^{2+} in vascular tissue.

If the action of glucose to depress Ca^{2+} fluxes is relevant to the *in vivo* situation, then one would predict increased sensitivity of diabetic vascular tissue to either a decrease in extracellular Ca^{2+} concentrations or pharmacologic inhibition of L type Ca^{2+} channel. Numerous sources of evidence suggest that this is indeed the case. Disproportionately large decreases in cardiac output have been demonstrated in isolated diabetic rat hearts when extracellular Ca^{2+} concentrations were reduced [51]. Further studies of the effects of diltiazem or verapamil on isolated hearts confirmed a marked increase in the sensitivity of the diabetic versus control hearts to L type Ca^{2+} channel blockade [52]. In addition, coronary vessels, mesenteric arteries, isolated aortas and thoracic aortic rings from diabetic animals all exhibit increased sensitivity to pharmacological inhibition of L type Ca^{2+} channels or extracellular Ca^{2+} depletion when compared to responses in non-diabetic vessels [53–55]. Furthermore, studies of the effects of Ca^{2+} supplementation on renovascular responses in diabetic rats suggest that decreased vascular tone in early diabetes may in part be due to defective Ca^{2+} flux into VSMC [56]. Additional evidence supporting the hypothesis that decreased vascular tone in early diabetes results from decreased transmembrane Ca^{2+} transport by vascular tissue comes from the observation that four weeks after the onset of alloxan-induced diabetes, isolated rat aortas show significant contractile subsensitivity to $CaCl_2$ as well as K^+ and Ang II [53]. More recently others have demonstrated similar findings using isolated resistance vessels obtained from diabetic patients [57]. That these effects may relate to hyperglycemia is supported by the finding that aortic rings from non-diabetic rats develop impaired contractility when exposed to elevated glucose concentrations (30 mM) *in vitro* [58].

Together, the aforementioned observations provide strong evidence to support the hypothesis that some aspect of the diabetic milieu acts to depress Ca^{2+} uptake by vascular tissue. The findings of the present study complement earlier reports by producing the first evidence that the link between the diabetic state and impaired Ca^{2+} uptake by vascular tissue may be a

direct and independent effect of elevated glucose concentrations to inhibit basal, voltage sensitive and receptor operated Ca^{2+} uptake. In so doing, we propose that this action of glucose could disturb at least two key components of the Ca^{2+} dependent process of blood flow autoregulation: notably, the myogenic response and pressor receptor modulation of this response. The findings of the present study thus suggest a biochemical mechanism to implicate directly hyperglycemia in the pathogenesis of disturbed regional blood flow that ultimately predisposes diabetic patients to severe and premature vascular injury.

Acknowledgments

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Chapter 7.

Glucose-induced Vascular Smooth Muscle Dysfunction: The Role of Protein Kinase C.

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REVIEW

Glucose-induced vascular smooth muscle dysfunction: the role of protein kinase C

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Keywords: Diabetes, vascular, protein kinase C.

Introduction

Diabetes mellitus is characterized by the premature development of microvascular and macrovascular disease [1]. It is widely accepted that the development of vascular disease in diabetic patients is greatly accelerated by the coexistence of hypertension. Moreover, patients with diabetes are more commonly hypertensive than the non-diabetic population. For these reasons evaluating the mechanisms underlying the development of vascular injury in the diabetic patient is relevant to the understanding of the pathogenesis of cardiovascular disease in a significant proportion of hypertensive patients.

Previous [2] and more-recent [3] clinical trials have emphasized that hyperglycaemia is an independent risk factor for the development of cardiovascular disease. The fact that glucose uptake by vascular cells is largely insulin-independent renders vascular cells vulnerable to glucose-induced injury when the extracellular glucose concentration is elevated. The mechanism by which a high extracellular–intracellular flux of glucose contributes to the pathogenesis of vascular injury has not been confirmed, although numerous possibilities have been explored, including glucose-induced increases in intracellular sorbitol accumulation [4], the formation of advanced glycosylation end-products [5] and, more recently, glucose-induced changes in cellular redox state [6].

Attention has also been focused on a potential role for protein kinase C as a mediator of diabetes-induced vascular injury [7–14]. In this regard, protein kinase C is an attractive candidate, because it plays a pivotal role in the regulation of many biological functions within various cell types [15–17]. Moreover, vascular smooth muscle is an abundant source of protein kinase C, and its activity has been strongly implicated in the regulation of vascular smooth muscle cell contraction, growth and differentiation, intracellular signalling, ion-channel activity and matrix protein expression by vascular smooth muscle cells [18–21]. The recent demonstration that elevated extracellular glucose concentrations activate protein kinase C in vascular smooth muscle cells [7] provides a mechanism by which glucose-induced changes in vascular smooth muscle cell protein kinase C activity could have profound effects on a spectrum of vascular smooth muscle cell functions, and thereby provide a final common biochemical pathway for vascular smooth muscle cell dysfunction in diabetic patients. The availability of cell culture techniques have allowed a direct examination of the independent effects of elevated extracellular glucose concentration on protein kinase C activity and the resultant changes in the biological function of vascular smooth muscle cells. This review examines the evidence available to support the hypothesis that glucose-induced protein kinase C activation may be an important mediator of diabetes-induced vascular dysfunction and injury.

Protein kinase C activity and vascular smooth muscle

Protein kinase C is a serine–threonine kinase that was first described by Nishizuka [15]. It has since been established that protein kinase C is not a single molecular entity, but consists of a family of closely related isoenzymes that differ in their structure, cofactor requirement and substrate specificity [16,17,22,23]. At present the known mammalian protein kinase C family consists of

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12 different polypeptides: α, β_I, β_{II}, γ, δ, ε, ζ, η, θ, ι, λ and μ. These isoenzymes have been identified in different species, tissues and cell lines, and more are likely to be discovered (Fig. 1). The protein kinase C family has been subdivided into the conventional protein kinase C enzymes, comprising protein kinase C α, β_I, β_{II} and γ, which are calcium-dependent enzymes that require diacylglycerol and membrane phospholipid (usually phosphatidylserine) for activation. The novel protein kinase C enzymes protein kinase C δ, ε, η, θ and μ also require diacylglycerol and membrane phospholipid for activation, but are calcium-independent. An additional group has been classified as the atypical protein kinase C enzymes (protein kinase ζ, λ and ι) because, unlike the others, they are not activated by diacylglycerol or phorbol esters. The differential tissue expression of the various protein kinase C isoforms, together with the subtle variation in their activation conditions and substrate specificity, may explain how a protein initially perceived to be a single ubiquitous enzyme has subsequently been shown to play a pivotal role in so many biological functions.

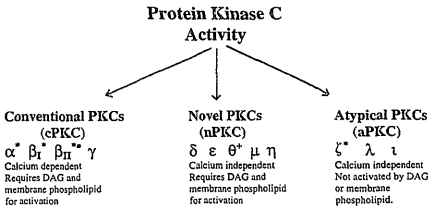

Fig. 1. The various isoforms of the ever-expanding protein kinase C (PKC) family. *PKC isoforms that have been definitively identified to be present in vascular smooth muscle. +PKC θ is a major PKC isoform expressed in skeletal muscle, and is also expressed by cardiac and airway smooth muscle. Its presence and significance with regard to vascular smooth muscle awaits confirmation. ⊙Confirmed to be activated in vascular tissues (heart, blood vessels and retinal tissues) of diabetic animals. DAG, diacylglycerol.

In vascular smooth muscle protein kinase C is present in relatively high concentrations, supporting the concept that it plays an important role in the regulation of smooth muscle function [21]. Using antibodies specific to the various protein kinase C isoforms, α, β, ε and ζ isoforms have so far been identified in vascular smooth muscle [21], and more are likely to exist, although the substrate specificity and specific function of each isoform within the vascular smooth muscle await clarification.

The mechanism of protein kinase C activation has been best elucidated for the calcium-dependent group of isoenzymes [24]. Classically, this involves receptor-mediated activation of phospholipase C, resulting in generation of diacylglycerol and inositol(1,4,5)trisphosphate (IP_3) from membrane-associated phosphotidyl inositol(4,5)-bisphosphate. IP_3 stimulates the release of intracellular calcium, which then binds to the second conserved region of the protein kinase C enzyme and promotes its translocation from the cytosol to the plasma membrane [24]. Protein kinase C is activated at the plasma membrane after complexing with diacylglycerol and membrane phospholipid. Kinetic analysis indicates that even small increases in cellular diacylglycerol level markedly increase the affinity of protein kinase C for calcium by reducing the receptor affinity for calcium to the micromolar range [24]. Thus, small increases in diacylglycerol synthesis can fully activate the conventional protein kinase C enzymes without any change in phosphoinositide turnover or calcium mobilization. As discussed below (Mechanism of glucose-induced protein kinase C activation), this mechanism of activation is relevant to the actions of glucose to activate protein kinase C.

Diabetes-induced protein kinase C activation

Interest in a possible role for protein kinase C in the development of diabetic vascular complications developed from the observation that its activity is increased in several tissues from diabetic animals. These tissues include the aorta [10,11], heart [11], retinal cells [10], renal glomeruli [25], renal proximal tubular cells [26] and hepatocytes [27]. In each case protein kinase C activation was measured as a significant translocation of its activity from the cytosolic to the membrane fraction of the respective tissue, or as enhanced *in situ* phosphorylation of specific endogenous protein substrates for protein kinase C, or both. The diabetes-induced increase in protein kinase C activity in vascular tissue (heart and aorta) is characterized by its chronicity, occurring within days (perhaps hours) of onset of hyperglycaemia, and persisting for as long as the diabetic state is maintained [10]. This contrasts with protein kinase C activation in response to normal physiological stimuli (i.e. hormone stimulation), which peaks within minutes and is often very transient [7]. Differential activation of protein kinase C isoforms has been observed in hepatocytes of streptozotocin-induced diabetic rats, suggesting that the measured increase in activity is accounted for by translocation of some but not all protein kinase C isoforms [27]. With regard to vascular tissue, the use of protein kinase C isoform-specific antibodies has also revealed differential activation of isoforms in the heart and aorta of diabetic animals *in vivo* [10,11]. Although these tissues most abundantly express protein kinase C α, only the β_{II} isoform undergoes significant cytosol–membrane translocation (indicative of protein kinase C activation) in the vascular tissues of diabetic animals. It is noteworthy, however, that the repertoire of antibodies used were incapable of detecting the protein kinase C ε and ζ isoforms, so it is not presently known whether these isoenzymes are also activated in the vasculature of diabetic animals and humans.

Mechanism of diabetes-induced protein kinase C activation: the role of glucose

A multitude of factors could potentially influence the activity of protein kinase C in the vascular tissues of diabetic animals and humans. A prime candidate is hyperglycaemia, and, in support of this hypothesis, elevated extracellular glucose concentrations have been shown to activate protein kinase C *in vitro* in isolated glomeruli [25,28], cultured mesangial cells [28–30], endothelial cells [10,31], adipocytes [32] and pancreatic islet cells [33]. We recently characterized the effect of elevated extracellular glucose concentrations on protein kinase C activity in cultured vascular smooth muscle cells [7,8]. Protein kinase C activity increased within minutes of elevating the extracellular glucose concentration from 5 to 20 mmol/l (Fig. 2). The increase in protein kinase C activity was sustained for as long as the extracellular glucose concentration remained elevated [7]. This further emphasizes the chronicity of the cellular protein kinase C response to elevated extracellular glucose concentrations. Protein kinase C activation was maximal at glucose concentrations of 15–20 mmol/l, a concentration often observed in patients with poorly controlled diabetes. Furthermore, the activation of protein kinase C by glucose in vascular smooth muscle cells is a threshold phenomenon, with no further increase in the magnitude of activation once the extracellular glucose concentration exceeds 20 mmol/l. Restoring the extracellular glucose concentration to normal rapidly restores protein kinase C activity to control values, indicating that in vascular smooth muscle cells the protein kinase C activity is rapidly responsive to both increases and decreases in extracellular glucose concentrations. Protein kinase C activation is not observed in vascular smooth muscle cells incubated with equimolar elevations of mannitol or L-glucose, confirming that protein kinase C activation is specific for D-glucose and excluding osmotic activation of protein kinase C. Moreover, because L-glucose is cell-permeable but poorly metabolized, the absence of an effect of elevated L-glucose concentrations on protein kinase C activity implies that the intracellular metabolism of glucose is essential for protein kinase C activation in vascular smooth muscle cells [7].

Mechanism of glucose-induced protein kinase C activation

The mechanisms responsible for glucose-induced protein kinase C activation have not been fully elucidated in vascular smooth muscle cells. Nevertheless, a considerable body of evidence obtained in other tissues suggests that glucose-induced protein kinase C activation is dependent on increased diacylglycerol production [10–13,30,32,34]. This hypothesis is supported by the observation that cellular diacylglycerol mass is increased substantially, both in aortic and in cardiac

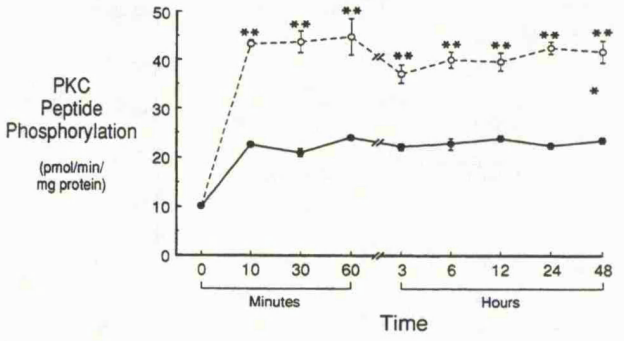

Fig. 2. Time course of glucose-induced protein kinase C (PKC) activity in vascular smooth muscle cells. Vascular smooth muscle cells were exposed to control medium (5 mmol/l glucose; ●) or a high-glucose medium (20 mmol/l; ○) for up to 48 h before measurement of *in situ* PKC activity. PKC activity was measured as the PKC-dependent phosphorylation of a PKC-specific peptide substrate (VRKRTLRRL). PKC activity in vascular smooth muscle cells increased in the presence of high glucose within minutes, and remained activated with continued exposure to high-glucose medium. $^{**}P<0.01$, versus control glucose. Published by permission [7].

tissue, in animals with experimental diabetes, and that the increase in diacylglycerol mass is paralleled by an increase in protein kinase C activity [10–13]. The direct role of glucose in modulating diacylglycerol levels in vascular cells was confirmed by studies showing that total diacylglycerol levels are significantly increased in cultured bovine aortic endothelial cells and vascular smooth muscle cells when exposed to elevated glucose concentrations (22 mmol/l) [10,11].

The glucose-induced increase in diacylglycerol production could be derived either from inositol phosphate turnover or *de novo* synthesis from glucose-derived precursors. In various tissues elevated glucose concentrations increase diacylglycerol levels and activate protein kinase C with no change in inositol phospholipid turnover, which implies that glucose-induced increases in cellular diacylglycerol mass are derived from *de novo* synthesis [10,30,34]. Evidence from previous studies [30,34] supports the hypothesis that diacylglycerol levels are increased in cells exposed to high concentrations of glucose through *de novo* synthesis. The *de novo* synthetic pathway involves the progressive stepwise acylation of glycolytic intermediates, ultimately forming phosphatidic acid and subsequently diacylglycerol [34]. The diacylglycerol species thus derived are likely to be heterogeneous, because their structure is determined largely by the cellular profile of available fatty acids for incorporation into the 1 and 2 positions. This is unlike diacylglycerol derived from phosphoinositide turnover, which produces a single diacylglycerol species (1-stearoyl-2-arachidonyl-diacylglycerol). *In vitro* studies using purified rat brain protein kinase C indicate that a variety of naturally occurring diacylglycerol species containing different unsaturated fatty acids in the 2-position are as potent as 1-stearoyl-2-arachidonyl dia-

cylglycerol with respect to protein kinase C activation [10,11,30,32,34]. The magnitude of the glucose-induced increase in diacylglycerol mass both *in vivo* and *in vitro* would be sufficient to function as an endogenous activator of protein kinase C in vascular smooth muscle cells without any alteration in intracellular calcium [24].

It is therefore evident that elevated concentrations of extracellular glucose, compatible with those observed in patients with poorly controlled diabetes mellitus, activate protein kinase C in vascular smooth muscle cells. It is proposed that this results from a glucose-induced increase in *de novo* synthesis of diacylglycerol in sufficient quantities to activate protein kinase C directly. The differential expression of protein kinase C isoforms in vascular tissue *in vivo* suggests that the measured increase in protein kinase C activity is restricted to the β_{II} isoform. A recent study [35] strongly supports the concept that preferential activation of the β_{II} isoform in diabetic vascular tissue *in vivo* is likely to be due to elevated glucose concentrations. Kunisaki *et al.* [35] recently demonstrated that elevated glucose concentrations (22 mmol/l) sufficient to activate protein kinase C in a variety of cultured cells, including vascular smooth muscle cells *in vitro*, promoted the preferential translocation of protein kinase C β_{II} to the membrane (indicative of protein kinase C activation) of vascular smooth muscle cells *in vitro*. The mechanism underlying this glucose-induced preferential activation of protein kinase C β_{II} in vascular tissue is not clear. If this profile of activation was due solely to the glucose-induced increase in diacylglycerol mass, then this should have resulted in the activation of both protein kinase C α and β_{II}, as both of these isoforms have similar affinities for the diacylglycerol moieties generated in the presence of high glucose concentrations. Thus, the mechanism responsible for the specific activation of protein kinase C β_{II} by elevated glucose levels in vascular smooth muscle cells is unclear, because the study of the differential regulation of protein kinase C isoform activity is very new. One possible explanation for the differential response to glucose is that the various protein kinase C isoforms are compartmentalized intracellularly, restricting the access of some isoforms to the increased levels of diacylglycerol, as has been shown in some cells.

Functional consequences of glucose-induced protein kinase C activation in vascular smooth muscle cells

Protein kinase C activation by hormonal stimulation is characteristically transient, peaking within seconds and declining to baseline values within minutes. This contrasts with the chronicity of protein kinase C activation in the vascular tissues of diabetic animals *in vivo* and the effects of elevated glucose on isolated vascular smooth muscle cells *in vitro* [7–10]. Protein kinase C is a prominent component of intracellular 'crosstalk' between different converging and diverging biochemical cell signalling pathways, leading to the accentuation of some cellular responses and the inhibition or desensitization of others [36]. The pivotal role of protein kinase C in the regulation of a myriad of cell functions and the capacity of elevated glucose concentrations to disturb the regulation of protein kinase C in vascular cells reveals its enormous potential to modify various key aspects of vascular cell function significantly. The potential significance of sustained protein kinase C activation with regard to vascular smooth muscle cell dysfunction in diabetes mellitus is considered below.

Modification of hormone receptor expression

Early diabetes mellitus, both in experimental animals and in humans, is associated with increased blood flow to many tissues, including the kidney, myocardium, retina, skin, muscle and brain. These disturbances in regional blood flow regulation are accentuated by systemic hypertension, and have been strongly implicated in the pathogenesis of microvascular injury [37]. The principal determinant of this deleterious increase in tissue perfusion is a reduction in arteriolar tone, which relates partly to a reduction in vascular responsivity to pressor hormones such as angiotensin II and adrenaline [38,39]. These impaired responses to pressor agents could be caused by reduced expression of pressor receptors on vascular smooth muscle cells, or attenuation of their intracellular signalling mechanism. Concordant with the former hypothesis, there is reduced glomerular angiotensin II binding in the diabetic rat and reduced arginine vasopressin binding to the platelets in patients with diabetes mellitus [38–40]. Other studies [41,42] have revealed a reduction in the density of β-adrenoceptor receptors and endothelin I receptors in experimental diabetes and platelet thromboxane angiotensin II receptors in human diabetes. Together, these observations suggest that the early diabetic state is associated with a downregulation of a diverse spectrum of pressor receptors on a variety of tissues.

Hyperglycaemia has been implicated in the development of this receptor downregulation, because there is an inverse relationship between blood glucose concentration or glycosylated haemoglobin A_{1c} and hormone receptor density *in vivo*. Using cultured vascular smooth muscle cells *in vitro*, we have demonstrated [8] a direct and specific effect of high extracellular glucose concentrations (20 mmol/l) to downregulate arginine vasopressin and angiotensin II receptor expression. Analysis of receptor binding kinetics revealed that, in the presence of elevated glucose concentrations, the specific binding of both pressor hormones to vascular smooth muscle cells *in vitro* was depressed because of a significant reduction in the surface density of receptors rather than because of any significant change in receptor affinity. Similar changes in receptor kinetics have been observed early in the course of experimental and human diabetes [40,41,43,44]. The magnitude of receptor downregulation caused by diabetes mellitus *in vivo* (30–58% decrease) is also similar to the maximal effect of elevated glucose on pressor

receptor downregulation on vascular smooth muscle cells *in vitro* (40% decrease).

As indicated above, the cellular response to several vasoactive agents may be modulated by protein kinase C activation. For example, the stimulation of protein kinase C by phorbol esters impairs the action of many hormones, including angiotensin II, noradrenaline, adrenaline, arginine vasopressin, acetylcholine, glucagon and endothelin I, via a variety of mechanisms which include receptor uncoupling from intracellular signalling pathways and receptor downregulation (see below) [36,45–49]. We have shown [8] that protein kinase C inhibition, sufficient to prevent glucose-induced protein kinase C activation, almost completely prevents glucose-induced downregulation of arginine vasopressin or angiotensin II receptors on vascular smooth muscle cells, suggesting that this is a protein kinase C-dependent process. Consistent with this conclusion, others have since confirmed [47,48] that sustained protein kinase C activation is also involved in the downregulation of thromboxane A_2 and endothelin I receptors in experimental diabetes. The mechanisms responsible for protein kinase C-induced downregulation of pressor receptors in response to glucose have not been defined, but recent reports (for review [36]) emphasize a variety of mechanisms by which protein kinase C activation could modulate receptor responsivity to hormonal stimulation. For example, direct protein kinase C-dependent phosphorylation of surface receptors can lead to uncoupling of receptors from their intracellular signalling pathways. In this regard, protein kinase C-induced phosphorylation of the β_{II}-adrenergic receptor on its cytoplasmic domain leads to uncoupling. Moreover, uncoupling of receptors from G-proteins also occurs in response to protein kinase C activation; for example, the glucagon receptor (GR2) when phosphorylated by protein kinase C, uncouples from its G_s protein, attenuating intracellular signalling. A similar pattern of protein kinase C-induced uncoupling of receptors from their G_s proteins has also been reported for prostaglandin E_1, parathormone, luteinizing hormone and vasoactive intestinal peptide, among others [36]. However, our results have suggested that glucose-induced attenuation of angiotensin II- and arginine vasopressin-induced intracellular signalling in vascular smooth muscle cells is not caused by receptor uncoupling, but relates to a protein kinase C-dependent decrease in the number of angiotensin II and arginine vasopressin receptors. This decrease in receptor number could occur as a consequence of either receptor internalization or decreased receptor synthesis. Protein kinase C phosphorylation has been shown to promote the internalization of various receptors including the transferrin receptor and the epidermal growth factor receptor (for review [36]). Receptor internalization is usually a rapid process, occurring within minutes, leading to rapid desensitization, whereas decreased receptor synthesis usually takes longer to manifest as a decrease in receptor binding. Our results have shown that the glucose-induced downregulation of angiotensin II and arginine vasopressin receptors is slow to develop (i.e. in 48 h), suggesting that it is consequent on a decrease in receptor synthesis. Consistent with such a mechanism, it has been shown that chronic protein kinase C overexpression in fibroblasts leads to the downregulation of epidermal growth factor receptors by decreasing receptor messenger RNA expression and receptor synthesis [49]. There are thus multiple mechanisms by which glucose-induced protein kinase C activation could lead from impaired vascular responses to a variety of pressor hormones and growth factors.

The magnitude of receptor downregulation induced by diabetes *in vivo* and reproduced by exposure to high glucose concentrations *in vitro* is likely to be of functional significance, because the contractile responses of individual vascular smooth muscle cells to angiotensin II or arginine vasopressin *in vitro* are markedly attenuated after previous exposure of vascular smooth muscle cells to a high-glucose environment for 48 h [8]. The magnitude of glucose-induced pressor receptor downregulation paralleled the glucose-induced defect in arginine vasopressin- and angiotensin II-stimulated intracellular signalling and contractility in vascular smooth muscle cells [8]. Although this does not prove causality, it is known that there are few (if any) spare arginine vasopressin or angiotensin II receptors on vascular smooth muscle cells, implying that downregulation of either receptor on vascular smooth muscle cells would be associated with a decrease in the response of the cells. Recent evidence lends further support to the concept that glucose-induced protein kinase C activation might be an important determinant of abnormal blood flow in the microcirculation. Shiba *et al.* [12] showed that the abnormal retinal circulation observed in diabetic rats is associated with increased retinal diacylglycerol levels and activation of retinal protein kinase C β_{II}. Moreover, these diabetes-induced abnormalities in retinal blood flow could be prevented by intravitreous injection of protein kinase C inhibitors [12].

Modification of ion-channel activity

In addition to disturbances in microcirculatory blood flow, systemic hypertension is also more common in patients with diabetes mellitus. Alterations in the transport of ions, particularly calcium and sodium ions, across vascular smooth muscle cell membranes have repeatedly been implicated in the pathogenesis of hypertension or diabetic vascular diseases, or both [50–54]. Most attention has been focused on the ubiquitous sodium–proton antiporter, the activity of which is increased both in hypertensive patients and in diabetic patients with complications [50–53].

Protein kinase C activity is involved in the regulation of many plasma membrane ion-transport systems, and has been shown to play an important role in the regulation of the sodium–proton antiporter in a variety of tissues [55–59]. Many of the physical and functional abnormalities demonstrated in cell membranes from hypertensive patients, including increased sodium–proton antiport ac-

tivity, can be reproduced by protein kinase C activation [60]. With regard to diabetes mellitus, protein kinase C inhibitors have been shown [61] to normalize the previously elevated leucocyte sodium–proton antiport activity of diabetic patients, suggesting a role for protein kinase C in modulating sodium–proton antiport activity in diabetic patients. We therefore examined the effects of elevated extracellular glucose concentrations on sodium–proton antiport activity in vascular smooth muscle cells, and demonstrated that sodium–proton antiport activity in these cells was significantly increased (by two- to threefold) after acute (3 h) or longer-term (24 h) exposure to elevated glucose concentrations (20 mmol/l) when compared with control medium (5 mmol/l glucose) [9]. Both the acute and the sustained glucose-induced increase in sodium–proton antiport activity were prevented by inhibition of glucose-induced protein kinase C activation with protein kinase C inhibitors or by prior protein kinase C downregulation, suggesting that glucose-induced activation of the sodium–proton antiport in vascular smooth muscle cells is mediated via mechanisms dependent on glucose-induced protein kinase C activation.

The acute increase in sodium–proton antiport activity in vascular smooth muscle cells was not dependent on gene transcription or translation, whereas the chronic effect of glucose (24 h) on sodium–proton antiport activity was prevented by inhibitors of gene transcription and translation, and was associated with a threefold increase in sodium–hydrogen exchanger messenger RNA isoform NHE-1 in vascular smooth muscle cells [9]. This is consistent with the hypothesis that long-term regulation of the sodium–proton antiporter in vascular smooth muscle cells involves alterations in gene expression [59]. Although not confirmed, it seems likely that protein kinase C activation plays a role in upregulating the expression of the NHE-1 gene in response to glucose. Long-term phorbol ester-mediated protein kinase C activation, similar to that observed with glucose, leads to a chronic increase both in sodium–proton antiport activity and in messenger RNA expression in cultured proximal tubular cells [59]. Moreover, protein kinase C has been shown to regulate the expression of numerous different genes expressing 'protein kinase C responsive elements' (i.e. consensus sequences in the 5'-flanking region of the gene that mediate protein kinase C-responsive regulation of gene expression) [62,63]. Protein kinase C-induced regulation of these genes has been shown to be caused by the binding of AP-1 of *fos–jun* dimers to an AP-1 binding site in the regulatory region of the gene [62]. It is notable that the 5'-flanking regulatory region of the gene coding for NHE-1 contains three consensus sequences for AP-1 binding [64,65].

The observation that glucose can increase sodium–proton antiport activity in vascular tissue could have considerable significance with regard to the role of hyperglycaemia in the pathogenesis of vascular disease and hypertension in patients with diabetes mellitus. The sodium–proton antiporter has been shown to play a key role in the regulation of intracellular pH, cell volume, growth, differentiation and contractility [66–71]. It has been suggested [72,73] that increased sodium–proton antiport activity in diabetes and hypertension is genetically determined. The data mentioned above provide a cellular mechanism by which metabolic factors could interact with genetic predisposition to determine the evolution of hypertension and vascular disease in individuals.

The effects of glucose on the activity of other ion channels in vascular smooth muscle cells is less clear, but protein kinase C influences the function of many ion channels, and in preliminary studies [74,75] we have demonstrated that elevated glucose concentrations significantly influence voltage-sensitive and receptor-operated calcium channel activity in vascular smooth muscle cells *in vitro*. Furthermore, elevated glucose concentrations have also been shown [76] to reduce the expression of calcium-channel messenger RNA by 73% in rat pancreatic β-cells. The potential role of protein kinase C in regulating glucose-induced changes in calcium channel activity or channel gene expression, or both, in vascular smooth muscle cells warrants and awaits further investigation.

Increased extracellular matrix protein expression

In addition to the above clinical and experimental evidence of abnormalities in vascular function in diabetes, there are also significant and important changes in the mechanical properties and structure of blood vessels [77–79]. The mechanical properties of blood vessels are determined largely by structural factors, particularly the smooth muscle of the inner intima and media, and the composition of the vascular extracellular matrix [80,81]. There is premature and increased deposition of matrix (fibronectin, collagen and elastin) in the vasculature of experimental animals and humans with diabetes [82]. This increase in vascular matrix promotes a reduction in vascular compliance, which in turn modifies the characteristics of the pulse wave, leading to an increased pulse-wave velocity [83]. These changes in vascular structure also play a key role in the development of impaired autoregulatory responses which accentuate microvascular injury in the presence of hypertension in diabetic patients.

There is considerable evidence to suggest that protein kinase C plays an important role in regulating the production of various extracellular matrix proteins by a variety of cell types [84]. This has been studied most extensively in the cultured mesangial cell [28]. These mesangial cell studies have particular relevance to vascular biology, because the mesangial cell is both phenotypically and functionally similar to the vascular smooth muscle cell. Elevated glucose concentrations (20–30 mmol/l) increase the production by cultured mesangial cells of fibronectin, type IV collagen and laminin [30,85–87]. This increased matrix protein production is associated with an increase in extracellular matrix protein messenger RNA expression, suggesting that glucose-induced matrix protein

production is regulated at the level of gene transcription or messenger RNA stabilization, or both [85,87]. Similarly to in vascular smooth muscle cells, glucose increases the diacylglycerol content and activates protein kinase C in mesangial cells [30,85], and several observations [30,85,88,89] have indicated that glucose-induced protein kinase C activation might signal the increase in matrix protein production. For example, agents that activate protein kinase C to a similar degree as glucose also increase matrix protein synthesis, including phorbol esters, stable analogues of diacylglycerol, angiotensin II and low-density lipoproteins. Moreover, the action of elevated glucose concentrations to stimulate matrix protein synthesis in mesangial cells is blocked by inhibitors of protein kinase C or by its prior downregulation [85].

Much less is currently known concerning the action of elevated extracellular glucose concentrations on matrix production by vascular smooth muscle cells. In a preliminary report [90] a high glucose level alone (25 mmol/l) was shown to stimulate a significant increase in cell associated and released fibronectin. It is not clear whether these changes represented increased production of fibronectin or its reduced degradation. Nevertheless, most reports in other cell types have shown that glucose-induced increases in matrix protein expression result primarily from an increase in matrix protein synthesis, rather than from a significant change in degradation [91,92]. It therefore seems likely that elevated glucose concentrations could play a key role in modifying vascular structure, and in particular the production of vascular matrix. Because of the functional significance of changes in vascular compliance resulting from altered matrix protein deposition, further work is essential to elucidate the role of glucose-induced protein kinase C activation in regulating this process.

In conclusion, an overwhelming body of scientific evidence confirms that elevated extracellular glucose concentrations, compatible with those found in patients with diabetes mellitus, can activate protein kinase C, probably exclusively the β_{II} isoform, in vascular smooth muscle cells. It is proposed that this sustained increase in protein kinase C activity results from a glucose-driven increase in *de novo* synthesis of diacylglycerol. However, further studies are needed in isolated vascular tissues and cells, to confirm and define more precisely the role of the *de novo* diacylglycerol synthetic pathway in regulating glucose-induced protein kinase C activation. Several studies have indicated that various vascular abnormalities identified in diabetic animals can be normalized by inhibition of glucose-induced protein kinase C inhibition. However, the potential use of protein kinase C inhibition as a therapeutic approach to target specifically a glucose-driven metabolic pathway of vascular dysfunction and injury is hindered by the toxicity and lack of specificity of protein kinase C inhibitors *in vivo*. Nevertheless, the characterization of protein kinase C isoforms and the recognition that the protein kinase C β_{II} isoform is selectively activated in vascular tissue by high glucose concentrations raises the possibility that the development of isoform-specific protein kinase C inhibitors may reduce the toxicity and improve the specificity of this potential therapeutic strategy. An alternative and potentially less toxic approach to the inhibition of glucose-induced protein kinase C activation is suggested by the intriguing observation by Kunisaki *et al.* [35], who recently showed that an active form of vitamin E (D-α tocopherol) inhibits glucose-induced protein kinase C β_{II} activation in vascular smooth muscle cells *in vitro* and diabetes-induced increases in rat aortic protein kinase C activity *in vivo*. The mechanism of inhibition of glucose-induced protein kinase C activation by D-α tocopherol is not clear, but does not appear to be due to direct non-specific inhibition of protein kinase C itself, but rather to the inhibition of glucose-induced diacylglycerol accumulation.

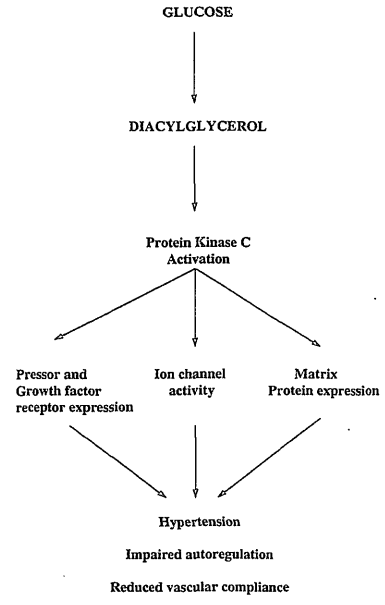

Fig. 3. Mechanism for glucose-induced vascular smooth muscle cell dysfunction. Elevated glucose concentrations stimulate an increase in the *de novo* synthesis of diacylglycerol. This increase in diacylglycerol mass is sufficient to activate protein kinase C. Sustained protein kinase C activation can modulate pressor hormone receptor expression and ion-channel activity in vascular smooth muscle cells. Together, these changes could contribute to the development of hypertension and impaired autoregulatory response in diabetic patients, leading to increased tissue perfusion and microvascular injury. Glucose-induced protein kinase C activation could also increase extracellular matrix protein production by vascular smooth muscle cells, and thereby adversely influence the mechanical properties of blood vessels in diabetic patients.

This review has emphasized that sustained activation of protein kinase C activity induced by glucose has profound effects on the biological function of vascular smooth muscle cells (Fig. 3). The changes so far described are unlikely to be exhaustive, and further work is necessary to explore the pathophysiological significance of glucose-induced protein kinase C activation with regard to the structural and functional changes that occur in blood vessels in diabetic patients. Moreover, it is possible that a better understanding of the mechanisms regulating glucose-induced protein kinase C activation will allow the development of a variety of potent and specific therapeutic agents designed to target this potentially injurious pathway in vascular tissues.

Finally, this review has focused on vascular smooth muscle cells, but the effects of glucose are not restricted to those cells within blood vessels. Considerable evidence has accumulated which demonstrates that elevated extracellular glucose concentrations also lead to sustained activation of protein kinase C in endothelial cells *in vitro* [10,14,93,94]. Furthermore, glucose-induced protein kinase C activation in endothelial cells has been implicated in the development of increased endothelial permeability to macromolecules, changes in endothelin-1 and prostanoid production, gene expression and changes in endothelial responses to circulating pressor hormones [10,14,93–96]. These observations suggest that protein kinase C activation by glucose could provide a final common path for vascular smooth muscle cell and endothelial cell dysfunction in diabetes, and in so doing provide a novel biochemical mechanism by which hyperglycaemia could contribute directly to various manifestations of vascular dysfunction in diabetic patients.

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Chapter 8.

Serum and Platelet-Derived Growth Factor-induced Expression of Vascular Permeability Factor mRNA by Human Vascular Smooth Muscle Cells, *in vitro*.

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Serum and platelet-derived growth factor-induced expression of vascular permeability factor mRNA by human vascular smooth muscle cells *in vitro*

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1. Endothelial dysfunction and vascular smooth muscle cell (VSMC) proliferation are key events in the pathogenesis of atherosclerosis. Vascular permeability factor (VPF), an endothelial-cell-specific multifunctional cytokine, was recently described, and has the potential to contribute to the development of endothelial dysfunction. The present study determines whether cultured human VSMCs express mRNA for VPF and whether VPF mRNA expression is influenced by human VSMC proliferation.
2. A 204 bp cDNA fragment, specific for all known variants of VPF mRNA, was cloned and used to demonstrate that human VSMCs express abundant quantities of VPF mRNA, whereas human endothelial cells do not. VPF mRNA levels were markedly diminished in non-proliferating human VSMCs. In contrast, when human VSMCs were stimulated to proliferate by exposure to serum, there was a rapid 6.6-fold increase ($P < 0.01$ versus time 0 h) in VPF mRNA expression, which was maximal at 3 h and persisted beyond 24 h. The magnitude of the VPF mRNA response in human VSMCs was dependent on the serum concentration.
3. Platelet-derived growth factor also increased VPF mRNA expression by human VSMCs, thus confirming that recognized growth factors for VSMCs also potently influence the VPF gene.
4. In conclusion, VPF mRNA is expressed by human VSMCs, the magnitude of VPF expression being temporally related to the proliferation of human VSMCs and the potency of the growth-promoting stimulus. We propose that VPF produced by proliferating human VSMCs could act as a paracrine hormone to powerfully influence the permeability and growth of the overlying vascular endothelium. We thus report a novel mechanism whereby the stimulation of VSMC proliferation could potently and directly contribute to the development of endothelial dysfunction and the pathogenesis of vascular disease.

INTRODUCTION

Abnormalities in endothelial cell function and vascular smooth muscle cell (VSMC) growth are prominent in the pathogenesis of atherosclerosis [1]. Disturbed endothelial function during the initiation and early propagation of the atherosclerotic lesion is characterized by an increase in vascular permeability to circulating macromolecules. This increase in vascular permeability occurs at sites of morphologically intact endothelium, which implies the development of endothelial dysfunction [1]. Considerable attention has focused on the pathogenesis of endothelial dysfunction, but, to date, the factors and mechanisms responsible remain incompletely defined.

A potent vascular permeability factor (VPF) has recently been described, which is expressed and secreted at high levels by various cells of human and animal origin [2]. VPF is also a potent mitogen for microvascular and macrovascular endothelial cells, but does not stimulate the proliferation of a wide variety of other cell types. Owing to its mitogenic potential, VPF has also been named vascular endothelial cell growth factor or VEGF [2–4]. VPF is a 34–42 kDa heparin-binding, dimeric, disulphide-bonded glycoprotein that binds to two high-affinity receptors, each with tyrosine kinase domains, predominantly located on vascular endothelium [2, 5–8]. Alternative splicing of mRNA yields four different VPF transcripts encoding polypeptides of 206, 189, 165 and 121 amino acids [9, 10]. VPF is among the most potent vascular-permeability-enhancing factors so far identified, and, on a molar basis, it is 50 000 times as potent as histamine [11]. This potent action of VPF makes it an attractive candidate as a mediator of normal and pathological changes in vascular endothelial permeability. In this regard, a logical site for VPF expression in man would be VSMCs,

Key words: atherosclerosis, endothelium, vascular smooth muscle.
Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; SSC, 0.15 mol/l NaCl–0.15 mol/l sodium citrate; SSPE, 0.15 mol/l–10 mmol/l sodium phosphate (pH 7.4)/1 mmol/l EDTA; VPF, vascular permeability factor; VSMC, vascular smooth muscle cell.
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which are in close proximity to the endothelium in blood vessels and would allow VPF to act as a paracrine regulator of vascular function. Until now, however, the potential significance of VPF with regard to vascular biology has not been known.

Early in the pathogenesis of atherosclerosis, simultaneous with the development of endothelial dysfunction, VSMC proliferation occurs [1, 12]. Various mechanisms have been described whereby endothelium-derived factors could influence the process of VSMC proliferation [1]. Nevertheless, little attention has focused on the possibility that VSMC-derived factors might contribute simultaneously to the development of endothelial dysfunction. We reasoned that if VSMCs produced VPF, then it is conceivable that, during VSMC proliferation, VPF production could be increased and thereby act as a paracrine hormone to increase endothelial permeability directly. The present study used cultured human VSMCs to determine whether human VSMCs express VPF mRNA and whether the stimulation of human VSMC growth increases VPF mRNA expression. Such a finding would indicate a novel mechanism whereby stimuli that modify VSMC growth could simultaneously and powerfully influence vascular endothelial function.

METHODS

Human VSMC culture

VSMCs were cultured from human aortic tissue which was obtained fresh from cadavers at the time of procurement of kidneys for renal transplantation. Ethics committee approval and relatives' consent were obtained. The vascular smooth muscle layer was identified by dissection, and then diced and digested in collagenase (Type I; Worthington Biochemicals, Freehold, NJ, U.S.A.) for 2 h as described previously [13, 14]. The resulting cell suspension was centrifuged, resuspended in Ham's F12 medium (Sigma Co.) supplemented with 20% foetal bovine serum (serum) (Gibco, Life Technology), 0.5% chick embryo extract (Gibco, Life Technology), 2 mmol/l L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, and cultured in 100 mm tissue culture plates in humidified air supplemented with 5% CO_2. The cell monolayers were extensively characterized to be well-differentiated VSMCs on the basis of light and electron microscopic morphology and immunohistochemistry. The VSMCs during primary culture initially had thin elongated cell bodies with dense arrangements of contractile filaments and focal densities, typical of the contractile VSMC phenotype. At this stage, the VSMCs expressed abundant quantities of smooth muscle actin (detected by the use of an anti-α-smooth muscle actin monoclonal antibody). As the primary cultures of VSMCs approached confluence, and in passaged VSMCs, the cells were larger, with decreased numbers of actin filaments and increased abundance of rough endoplasmic reticulum and Golgi apparatus, indicating a phenotypic shift to a synthetic VSMC population. VSMC monolayers were passaged every 6–7 days after trypsinization using 0.1% trypsin–EDTA, and used for experiments from the second to the sixth passage. Thus, the human VSMCs studied were of the synthetic rather than contractile phenotype.

Human umbilical vein endothelial cell culture

Endothelial cells were isolated from human umbilical vein as described previously [15]. Briefly, umbilical veins were cannulated at either end and filled with 5 ml of 0.1% collagenase solution (CLS I; Worthington Biochemicals) and incubated in humidified air supplemented with 5% CO_2 for 15 min. The vein was flushed with 20 ml of Minimum Essential Medium and the resulting cell suspension was centrifuged at 300 *g* for 10 min. The endothelial cell pellet was resuspended in 5 ml of culture medium comprising: Medium 199, 20% serum, 100 units/ml penicillin and 100 μg/ml streptomycin, 20 mmol/l Hepes, 2 mmol/l glutamine, 1 mmol/l pyruvate, 15 μg/ml endothelial cell growth factor (all Sigma Co.) and plated in 100 mm culture dishes. Cells were subcultured as described above and used for experiments from the second to the fourth passage. Endothelial cell identity was confirmed by morphological appearance at microscopy and positive immunohistochemical staining for von Willebrand factor.

Polymerase chain reaction (PCR) amplification and cDNA probes

To detect VPF mRNA, a 204 bp cDNA fragment was generated from human kidney RNA using two oligonucleotide primers, which were based on the human VPF cDNA sequence [9]:

1 (forward):
5′-CGCGGATCCAGGAGTACCCTGATATGAG-3′
2 (reverse):
5′-CCGGAATTCACATTTGTTGTGCTGT-3′

The primers have built in restriction sites (*Bam*HI in primer 1 and *Eco*RI in primer 2) at their 5′ ends to facilitate subcloning. For PCR amplification, 0.1 μg of total RNA from human kidney was annealed with random hexanucleotides and reverse transcribed for 30 min at 42°C using 100 μg of reverse transcriptase (Gibco, Life Technology) in a volume of 20 μl. The reaction mixture was heated to 95°C for 10 min before the addition of the PCR primers and Taq polymerase (Biotaq; Bioline Corporation), in a final volume of 100 μl. PCR amplification was performed on 100 μl samples using a DNA thermal cycler (Perkin Elmer 480). Amplification was carried out for 30–35 cycles (1 min at 94°C to separate, 1 min at 55°C to anneal and 1 min at 72°C to extend). The resulting 204 bp cDNA fragment was subcloned into the polylinker region of

pBluescript II SK(+). The identity of the cloned human insert was confirmed by Sanger dideoxy-DNA sequencing and found to be identical with the previously reported human VPF sequence. The 204 bp fragment generated in this way is common to all known VPF splicing variants.

Northern analysis

The human VPF cDNA insert from the pBluescript II SK(+) construct was radiolabelled with [α^{32}-P]dCTP (Amersham) to a specific activity of approximately 2×10^9 c.p.m./μg DNA using the random primer labelling system (Gibco, Life Technology). Typically, 20 ng of labelled probe was used for each 70 cm^2 filter. Total cellular RNA was prepared from cultured human VSMCs, and human endothelial cells using the one-step guanidinium–phenol–chloroform purification method [16]. RNA samples (15–25 μg/lane) were size-fractionated on 1.2% agarose containing 6% formaldehyde and blotted on to Hybond nylon membranes (Amersham). Hybridization was carried out for 18 h at 42°C in 50% formamide, 5 × SSPE, 5% Denhardt's solution, 0.5% SDS and 6% (w/v) polyethylene glycol 6000 (Fisons). Final washes were carried out at high stringency (0.1 × SSC, 0.1% SDS at 65°C). The blots were exposed to Kodak XAR2 film with an intensifying screen at −72°C for 24 h. To control for total mRNA content and lack of degradation, the blots were subsequently stripped and hybridized with a cDNA fragment for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (no. 9805/1; Clontech). The resulting autoradiographs were subjected to densitometric analysis (LKB Gelscan) to quantify the ratio of human VPF/GAPDH mRNA.

RESULTS

Human VSMCs but not human endothelial cells express VPF mRNA

Northern blots of RNA isolated from confluent human VSMCs grown in complete medium containing 20% serum, were probed with the 204 bp VPF cDNA fragment which is specific and complementary to all known variants of VPF mRNA. In human VSMCs, this radiolabelled cDNA probe hybridized with a single 4.2 kb VPF transcript, which is compatible with the reported size of VPF mRNA [9]. In contrast, when Northern blots of similar quantities of RNA isolated from human endothelial cells were probed with the 204 bp cDNA probe, no VPF transcript was detected (Fig. 1). These results demonstrate that human VSMCs express abundant quantities of VPF mRNA, whereas human endothelial cells do not express VPF mRNA. The absence of VPF mRNA in endothelial cells was a repeated finding, irrespective of whether the cells were exposed to 20% serum or potent endothelial mitogens, such as endothelial cell growth factor (Sigma Co.). This latter result is consistent with previous reports that, although the vascular endothelium is the principal target for VPF, it does not produce VPF.

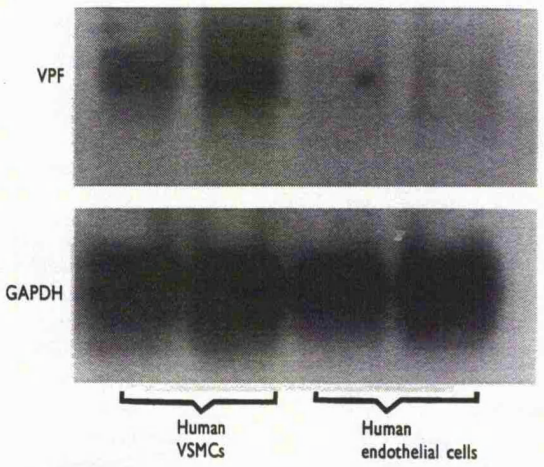

Fig. 1. VPF mRNA expression by human vascular smooth muscle and endothelial cells. This autoradiograph from a Northern blot is representative of four experiments. Each lane contains 20 μg of total RNA from confluent human VSMCs (two left-hand lanes) and confluent human endothelial cells (two right-hand lanes). The radiolabelled VPF-specific cDNA hybridized with a single 4.2 kb transcript in human VSMCs, but no VPF mRNA was detected in endothelial cells. There was equivalent loading of total RNA to each lane, as indicated by the consistent expression of GAPDH (1.4 kb) mRNA in each lane.

Effect of serum withdrawal on VPF mRNA expression by human VSMCs. The presence of 20% serum in the culture medium provides a potent stimulus for cell growth, whereas reducing the serum concentration to a minimum (1%) renders human VSMCs quiescent within 48 h [17, 18]. To determine whether there was a relationship between human VSMC growth and VPF mRNA expression, the effect of reducing the serum concentration to 1% on VPF mRNA expression by human VSMCs was examined. Fig. 2 shows that reducing the serum concentration to 1% results in a progressive decrease in VPF mRNA abundance, so that, by 48 h of serum depletion, VPF mRNA was not detectable in human VSMCs. It is notable that this progressive reduction in steady-state VPF mRNA levels after serum depletion corresponds temporally to the progressive induction of human VSMC quiescence.

Time course of serum-induced VPF mRNA expression by human VSMCs. Confluent human VSMCs were rendered quiescent by serum depletion (1%) for 48 h. As described above, this manoeuvre markedly reduces VPF mRNA expression. Thereafter, the quiescent human VSMCs were exposed to culture medium containing 20% serum for various time periods. Fig. 3 shows that the addition of 20% serum to quiescent human VSMCs stimulated a rapid increase in VPF mRNA expression, first observed within 1 h and maximal by 3 h. Thereafter, with continued exposure to 20% serum, there was a progressive decline in VPF mRNA levels; nevertheless, they remained markedly elevated at 24 h when

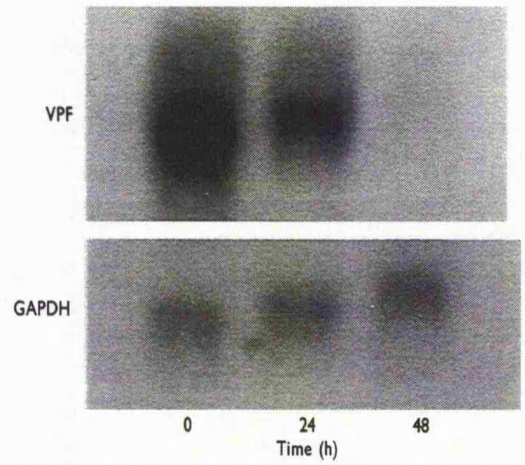

Fig. 2. Effect of serum withdrawal on VPF mRNA expression by human VSMCs. This autoradiograph from a Northern blot is representative of four experiments, and shows that the steady-state abundance of VPF mRNA decreased 24 h after reducing the serum concentration of the culture medium from 20% to 1%. By 48 h after serum depletion, VPF mRNA expression was markedly diminished. The GAPDH expression confirms that mRNA loading was equivalent in all lanes.

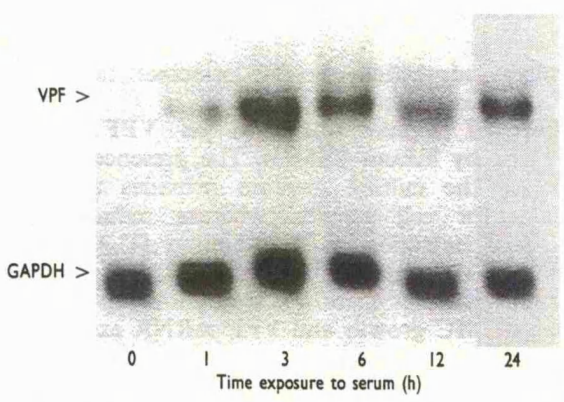

Fig. 3. Time course of serum-induced VPF mRNA expression by human VSMCs. This autoradiograph of a Northern blot is representative of five experiments and shows VPF mRNA expression by confluent VSMCs that had been exposed to serum-depleted culture media for 48 h before exposure to media containing 20% serum for various times. VPF mRNA was maximally induced at 3 h and remained elevated with continued exposure to 20% serum. Analysis of GAPDH mRNA levels confirmed equivalent loading of mRNA to each lane.

compared with serum-deprived, quiescent human VSMCs. Densitometric analysis of autoradiographs from repeated time course experiments ($n=4$) revealed a consistent response to 20% serum (VPF mRNA/GAPDH mRNA ratio at $t=0$ h 1.4 ± 0.3 and at $t=3$ h 9.3 ± 1.1, $P<0.01$, means $\pm$ SEM, statistics by analysis of variance with Bonferroni correction). Further studies confirmed that this increase in VPF mRNA levels persisted as long as VSMCs continued to be exposed to 20% serum, for up to 2 weeks (data not shown).

Concentration dependency of serum-induced VPF mRNA expression by human VSMCs. The time

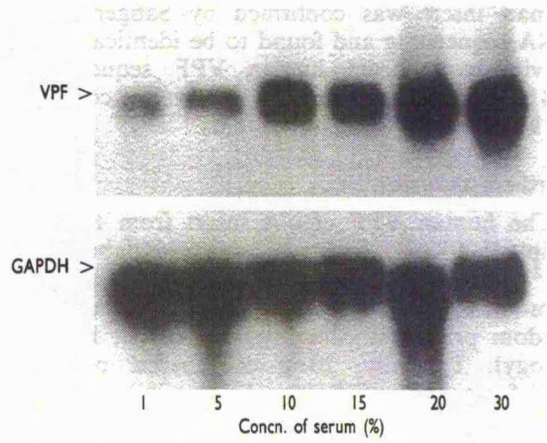

Fig. 4. Concentration dependency of serum-induced VPF mRNA expression by human VSMCs. This autoradiograph of a Northern blot is representative of four experiments, and shows VPF mRNA expression by human VSMCs that had been exposed to serum-depleted (1%) media for 48 h before the addition of media containing various serum concentrations (1–30%) for 3 h. There is a serum-concentration-dependent increase in VPF mRNA expression, maximal at serum concentrations of 20%. Analysis of GAPDH mRNA levels confirms consistent RNA loading to each lane.

point of maximal response to 20% serum (3 h) was chosen to examine the dependency of serum-induced VPF mRNA expression on serum concentration. Confluent human VSMCs, rendered quiescent by 48 h exposure to culture medium containing 1% serum, were exposed to culture media containing various serum concentrations for 3 h. Fig. 4 shows a serum-concentration-dependent increase in VPF mRNA expression, maximal at a serum concentration of 20%. In a second series of studies, a different batch of more potent serum was used, which maximally stimulated human VSMC growth at a serum concentration of 10–15%. Using this serum, VPF mRNA expression by human VSMCs was also dependent on serum concentration; however, the maximal response was observed using a serum concentration of 10–15% (data not shown). These results suggest that the dependency of human VSMC growth on serum concentration equates with the potency of serum-induced VPF mRNA expression.

Effect of platelet-derived growth factor (PDGF) on VPF mRNA expression by human VSMCs

PDGF is a physiologically important regulator of human VSMC growth [19, 20]. PDGF exists as an approximately 30 kDa dimer of two different polypeptide chains (A and B) that can generate three isomers: AA, AB and BB. Two isoforms of the PDGF receptor exist (α and β). PDGF BB binds to both receptor isoforms and is a potent VSMC mitogen. Human VSMC monolayers were rendered quiescent by exposure to culture medium containing 1% foetal calf serum for 48 h. The addition of

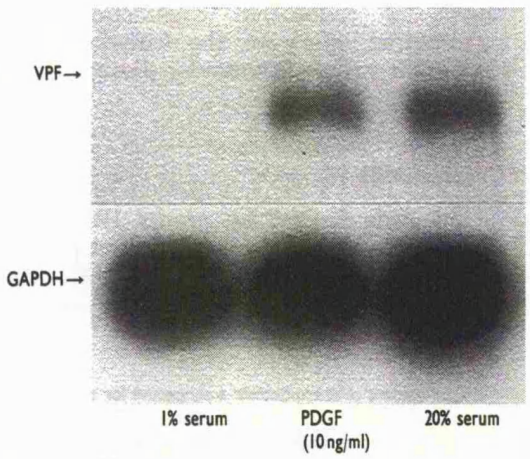

Fig. 5. PDGF-BB-induced VPF mRNA expression by human VSMCs. This autoradiograph of a Northern blot is representative of three experiments and shows the effect of incubating quiescent human VSMCs (1% serum for 48 h) with control medium (1% serum), PDGF-BB (10 ng/ml) or 20% serum for 3 h. Both PDGF and 20% serum markedly increased steady-state VPF mRNA levels in human VSMCs at 3 h. Analysis of GAPDH mRNA levels confirms consistent RNA loading to each lane.

10 ng/ml PDGF BB (Sigma Co.) to the serum-deplete medium markedly increased VPF mRNA expression by human VSMCs (Fig. 5). The effect of PDGF on VPF mRNA levels in human VSMCs was concentration-dependent and showed a time course similar to that seen for 20% serum (data not shown). These results demonstrate that naturally occurring growth-regulating peptides modulate the expression of VPF mRNA by human VSMCs.

DISCUSSION

Accelerated VSMC growth plays a fundamental role in the formation and propagation of atherosclerotic lesions in man [1, 12]. Early in the development of an atherosclerotic lesion, after a variety of diverse initiating events, VSMCs migrate from the medial layer of blood vessels to the intima, triggering abnormal smooth muscle cell proliferation. The present study uniquely demonstrates that stimulating human VSMC growth potently induces the expression of VPF mRNA by human VSMCs, the magnitude of the response being directly related to the concentration of the growth-inducing stimulus. Moreover, removal of the stimulus for cell growth resulted in a progressive decline in VPF mRNA expression so that, in quiescent cells, VPF mRNA was undetectable. These observations demonstrate that the expression of VPF mRNA by human VSMCs is temporally related to the growth phase of the cell and proportional to the potency of the growth-promoting stimulus.

Serum, as used in the present study, is widely used to promote cell growth in cell culture systems *in vitro* to mimic the induction of cell growth *in vivo* [17]. With regard to VSMCs, the addition of serum to quiescent cells in culture leads to the induction of mRNA pools for proto-oncogenes such as c-*fos* and c-*myc*, which rapidly reach peak levels (15–30 min for c-*fos* and 3 h for c-*myc*). Thereafter, as cells progress through the cell cycle, c-*myc* continues to be expressed at a constant high level, whereas c-*fos* mRNA can no longer be detected after the initial burst [18]. As the transition from quiescence to proliferation progresses, the cells enter S-phase after 12 h [17, 18]. These molecular events, induced by serum in human VSMCs, are qualitatively and quantitatively similar to those occurring in response to angiotensin II, PDGF and other mitogens implicated in the pathogenesis of VSMC growth abnormalities in atherosclerosis [21, 22]. Thus, serum is a valid and appropriate stimulus to use to study the relationship between the induction of human VSMC growth and VPF mRNA expression. It is intriguing that the pattern of VPF mRNA expression by human VSMCs in response to growth induction mimics the previously reported temporal pattern of c-*myc* mRNA induction in the same tissue, perhaps suggesting common mechanisms of intracellular regulation. In this regard, work is in progress to determine whether the serum-induced increase in VPF mRNA occurs via an increase in gene transcription and/or stabilization of the message.

Two distinct phenotypes of VSMC have been described, contractile and synthetic, which represent the extremes of a continuous spectrum of VSMC phenotypic expression [12, 17]. Culturing VSMCs beyond one passage promotes a change from the contractile to synthetic phenotype [23], as confirmed by our morphological characterization of the cells. The present studies were thus performed using human VSMCs of the synthetic phenotype. This has considerable relevance to vascular disease, as the synthetic VSMC phenotype is dominant in the media and neointima surrounding human atheromatous plaques [12, 24, 25].

This study has focused on the regulation of VPF mRNA levels in human VSMCs, and VPF peptide production in response to serum was not measured. Nevertheless, in previous studies in which steady-state VPF mRNA levels are increased, there has been a corresponding increase in VPF peptide production [2]. This implies that the abundant expression of VPF mRNA in human VSMCs, and its potent modulation by growth, is likely to be biologically significant.

The observations reported herein have important implications for the role of VPF in the normal physiology of blood vessels and the pathogenesis of vascular injury. It is conceivable that during VSMC proliferation in the development of the early atherosclerotic lesion, VPF mRNA expression and peptide production would be increased. This would allow VPF to act as a paracrine hormone and thereby directly influence the permeability and function of the overlying endothelium. This novel concept suggests a mechanism whereby a variety of

stimuli for VSMC growth, including PDGF, could directly influence endothelial function. Moreover, it also suggests a mechanism whereby significant endothelial dysfunction could develop during the pathogenesis of atherosclerosis, independent of direct trauma or injury to the endothelium. Such a hypothesis is consistent with morphological data, which demonstrate that the dysfunctional endothelium is intact in the developing atherosclerotic lesion [1].

In addition to its potent effects on vascular permeability, VPF is also a powerful endothelial-cell-specific mitogen *in vitro* and is expressed in a variety of highly vascularized tissues, where its expression is temporally and spatially related to angiogenesis [2–4, 26–28], suggesting an important role for VPF as a physiological mediator of angiogenesis *in vivo*. VPF is the only mitogenic factor that acts exclusively on the vascular endothelium, and our demonstration that VPF mRNA is abundantly expressed by human VSMCs suggests that VPF could play an important role in the normal growth and repair of the overlying vascular endothelium *in vivo*. Indeed, the close anatomical and functional relationship between VSMCs and endothelial layers within blood vessels requires coordinated growth of these two cell types in response to injury. After denuding injury such as angioplasty, VSMC proliferation occurs, generating a neointima [12]. Our results suggest a mechanism whereby this VSMC proliferative response to injury could generate VPF locally, which could then act as a paracrine endothelial-cell-specific mitogen to promote endothelial regeneration and healing of the endothelium. Furthermore, PDGF has been widely implicated in the regulation of VSMC growth [19, 20]. The present study suggests a novel mechanism whereby PDGF could also contribute to the regulation of endothelial growth and function, via the induction of VPF production by vascular smooth muscle.

In addition to the aforementioned changes in endothelial permeability and growth, VPF has been reported to promote the release of von Willebrand factor and tissue factor, generating a procoagulant state on the endothelial surface [29, 30], and also to induce monocyte activation and migration to the endothelium [30]. All of these events are recognized as being important in the initiation of endothelial dysfunction and the early pathogenesis of atherosclerosis [1]. The present study therefore suggests additional mechanisms whereby stimuli for VSMC proliferation could contribute to a spectrum of endothelial dysfunction that could ultimately play a role in the pathogenesis of vascular disease in a variety of disease states.

In conclusion, we report that human VSMC growth induces the expression of abundant VPF mRNA. This novel multifunctional cytokine has an activity profile on the endothelial cell that suggests it could play a key role in regulating normal endothelial functions in health and the pathogenesis of endothelial dysfunction during growth-promoting injury to the vascular smooth muscle of blood vessels in vascular disease.

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Chapter 9.

Angiotensin II Increases Vascular Permeability Factor Gene Expression by Human Vascular Smooth Muscle Cells

Bryan Williams, Ann Quinn-Baker, Barbara Gallacher, David Lodwick

Hypertension. 25: 913 - 917, 1995.

Rapid Communication

Angiotensin II Increases Vascular Permeability Factor Gene Expression by Human Vascular Smooth Muscle Cells

Bryan Williams, Anne Quinn Baker, Barbara Gallacher, David Lodwick

Abstract Angiotensin II (Ang II) has been implicated in the pathogenesis of the vascular injury associated with hypertension and diabetes mellitus. Increased vascular permeability is an important early manifestation of endothelial dysfunction and the pathogenesis of atherosclerosis. How Ang II contributes to endothelial dysfunction and promotes an increase in vascular permeability is unknown but is classically attributed to its pressor actions. We demonstrate that human vascular smooth muscle cells express abundant mRNA for vascular permeability/endothelial growth factor. Vascular permeability factor is a 34- to 42-kD glycoprotein that markedly increases vascular endothelial permeability and is a potent endothelial mitogen. Ang II potently induced a concentration-dependent (maximal, 10^{-7} mol/L) and time-dependent increase in vascular permeability factor mRNA expression by human vascular smooth muscle cells that was maximal after 3 hours and diminished by 24 hours. Ang II–induced vascular permeability factor mRNA expression by human vascular smooth muscle cells was inhibited by the specific Ang II receptor antagonist losartan (DuP 753), confirming that this is an Ang II receptor subtype 1–mediated event. These results describe a new action of Ang II on human vascular smooth muscle, notably the induction of vascular permeability factor mRNA expression. The wide spectrum and potent activity of vascular permeability factor suggest a novel mechanism whereby Ang II could locally and directly influence the permeability, growth, and function of the vascular endothelium independent of changes in hemodynamics. (***Hypertension.* 1995;25:913-917.)**

Key Words ◦ angiotensin II ◦ capillary permeability ◦ endothelial growth factors ◦ muscle, smooth, vascular ◦ gene expression

An early and important event in the pathogenesis of atherosclerosis is the development of endothelial dysfunction, a prominent manifestation of which is an increase in endothelial permeability to circulating macromolecules.[1] It has long been proposed that in hypertensive states, increased vascular permeability occurs as a direct consequence of pressure-mediated mechanical injury to the endothelium.[2] Morphological studies, however, are not consistent with this conclusion, having shown that the dysfunctional endothelial cell layer is intact.[1] This has prompted the search for additional factors that may adversely influence endothelial permeability. In this regard, it has been proposed that a humoral permeabilizing factor exists that acts in concert with elevated blood pressure to increase vascular permeability.[1,3] Consistent with this hypothesis, Asscher and Anson[4] reported that the injection of renal extracts caused serous effusions and vascular lesions in rats that they attributed to a renal-derived "vascular permeability factor" (VPF). Subsequently, the active component of the renal extract was shown to be renin, and the permeabilizing actions of the extract were reproduced in animals by infusion of angiotensin II (Ang II).[5,6] It was thus concluded that Ang II increases vascular permeability and injury via its pressor action.[6]

Increasing evidence supports a role for Ang II in the pathogenesis of vascular injury via mechanisms that are independent of its pressor activity. Ang II induces the expression of a wide spectrum of genes in vascular tissue, suggesting a key role for Ang II in regulating vascular structure and function.[7,8] Whether the action of Ang II to increase vascular permeability could also be attributed to Ang II–induced vascular expression of VPFs is unknown.

A potent VPF has recently been described that is expressed and secreted at high levels by various cells of human and animal origin.[9] VPF is also a powerful endothelial cell–specific mitogen and also named vascular endothelial cell growth factor, or VEGF (referred to herein as VPF).[9-11] VPF is a 34- to 42-kD heparin-binding, dimeric, disulfide-bonded glycoprotein that binds to two high-affinity receptors each with tyrosine kinase domains, predominantly located on vascular endothelium.[9,12-15] Alternative splicing of mRNA yields four different VPF transcripts encoding polypeptides of 206, 189, 165, and 121 amino acids.[16,17] VPF is among the most potent vascular permeability–enhancing factors thus far identified, and on a molar basis, it is 50 000 times as potent as histamine.[18] This potent action of VPF makes it an attractive candidate as a mediator of normal and pathological changes in vascular permeability. In this regard, a logical site for VPF expression in humans would be vascular smooth muscle cells (VSMCs), which are in close proximity to the endothelium in blood vessels and would allow VPF to act as a

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paracrine regulator of vascular function. However, the potential significance of VPF with regard to vascular biology and its potential modulation by vasoactive peptides such as Ang II has remained undefined.

The present study tests the hypothesis that human VSMCs express VPF mRNA and that Ang II acts as an independent humoral modulator of VPF mRNA expression by human vascular smooth muscle. Such a finding would describe a new action of Ang II and provide a novel mechanism whereby Ang II could powerfully influence vascular endothelial permeability independent of its pressor activity.

Methods

Human VSMC Culture

VSMCs were cultured from human aortic tissue obtained fresh from cadavers at the time kidneys were procured for renal transplantation. The vascular smooth muscle layer was identified by dissection, diced, and digested in collagenase for 2 hours as previously described.[19,20] The resulting cell suspension was centrifuged; resuspended in Ham's F-12 medium (Sigma Chemical Co) supplemented with 20% fetal bovine serum (FBS) (Life Technologies), 0.5% chick embryo extract (Life Technologies), 2 mmol/L L-glutamine, and antibiotics; and cultured in 100-mm tissue culture plates in humidified air supplemented with 5% CO_2. The cell monolayers were extensively characterized to be well differentiated VSMCs on the basis of light and electron microscopic morphology and immunohistochemistry. When confluent, VSMC monolayers were passaged every 6 to 7 days after trypsinization and were used for experiments from the second to sixth passages.

Polymerase Chain Reaction Amplification and cDNA Probes

For detection of VPF mRNA, a 204-bp cDNA fragment was generated from human kidney RNA with the use of two oligonucleotide primers that were based on the human VPF cDNA sequence[16]: (1) (forward): 5′-CGCGGATCCAGGAG-TACCCTGATATGAG-3′ and (2) (reverse): 5′-CCGGAAT-TCACATTTGTTGTGCTGT-3′. The primers have built-in restriction sites, *Bam*HI in primer 1 and *Eco*RI in primer 2, at their 5′ ends to facilitate subcloning. For polymerase chain reaction (PCR) amplification, 0.1 μg total RNA from human kidney was annealed with random hexanucleotides and reverse transcribed for 30 minutes at 42°C with the use of 100u reverse transcriptase (Life Technologies) in a volume of 20 μL. The reaction mixture was heated to 95°C for 10 minutes before the addition of the PCR primers and *Taq* polymerase (Biotaq, Bioline Corp) in a final volume of 100 μL. PCR amplification was performed on 100-μL samples with the use of a DNA thermal cycler (Perkin-Elmer 480). Amplification was carried out for 30 to 35 cycles (1 minute at 94°C to separate, 1 minute at 55°C to anneal, and 1 minute at 72°C to extend). The resulting 204-bp cDNA fragment was subcloned into the polylinker region of pBluescript II SK(+). The identity of the cloned human insert was confirmed by Sanger dideoxy-DNA sequencing and found to be identical to the previously reported human VPF sequence. The 204-bp fragment generated in this way is also common to all known VPF splicing variants.

Northern Analysis

The human VPF cDNA insert was radiolabeled with [α-^{32}P]dCTP (Amersham) to a specific activity of approximately 2×10^9 cpm/μg DNA using the random primer labeling system (Life Technologies). Typically, 20 ng of labeled probe was used for each 70-cm^2 filter. Total cellular RNA was prepared from cultured human VSMCs with the one-step guanidinium–phenol chloroform purification method.[21] RNA samples (15 to 25 μg per lane) were size-fractionated on 1.2% agarose containing 6% formaldehyde and blotted onto Hybond nylon membranes (Amersham). Hybridization was carried out for 18 hours at 42°C in 50% formamide, 5% SSPE, 2.5% Denhardt's solution, 0.1% sodium dodecyl sulfate, and 10% dextran sulfate. Final washes were carried out at high stringency (0.1× SSC and 0.1% sodium dodecyl sulfate at 65°C). The blots were exposed to Kodak XAR2 film with an intensifying screen at −72°C for 24 hours. To control for total mRNA content and lack of degradation, the blots were subsequently stripped and hybridized with a cDNA fragment for human GAPDH (No. 9805/1, Clontech). The resulting autoradiographs were subjected to densitometric analysis (LKB Gelscan, Pharmacia) to quantify the ratio of VPF to GAPDH mRNA. Statistical differences in the VPF-GAPDH ratio were defined using ANOVA with a Bonferroni correction. Results are given as mean±SD unless indicated otherwise; a value of $P<.05$ was considered significant.

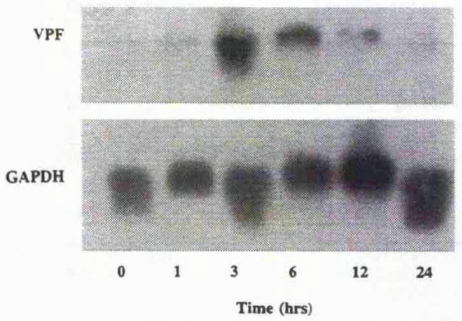

FIG 1. Northern blots show time dependency of angiotensin II–induced vascular permeability factor (VPF) mRNA expression in human vascular smooth muscle cells (VSMCs). Angiotensin II (10^{-7} mol/L) added to fetal bovine serum–deprived culture medium (1% fetal bovine serum) stimulated a marked increase in steady-state VPF mRNA expression (approximately 4.2 kb) in human VSMCs in a time-dependent manner. Total RNA loaded onto each lane was equivalent, as indicated by the consistent expression of GAPDH (1.4 kb) mRNA at each time point.

Results

Effect of Ang II on VPF mRNA Expression by Human VSMCs

Northern blots of RNA isolated from confluent human VSMCs were probed with the 204-bp VPF cDNA fragment that is specific for and complementary to all known variants of VPF mRNA. In human VSMCs, this radiolabeled cDNA probe hybridized with a single 4.2-kb VPF transcript. In preliminary experiments, we demonstrated that the abundance of VPF mRNA expressed by human VSMCs was strongly influenced by the FBS concentration in the overlying culture medium, VPF mRNA being less abundant when human VSMCs were rendered quiescent by 48 hours of exposure to serum-deprived (1% FBS) culture medium.[22] The effect of Ang II on steady-state VPF mRNA levels was thus examined in confluent monolayers of quiescent human VSMCs. Supplementation of serum-deprived culture medium with Ang II (10^{-7} mol/L) resulted in a rapid, marked, and significant increase in VPF mRNA expression (Fig 1). This effect of Ang II was maximal after 3 hours and progressively diminished with increasing time exposure up to 24 hours. Densitometric analysis of autoradiographs from repeated experiments (n=4) revealed a consistent response to Ang II (VPF mRNA–GAPDH mRNA ratio: 1.9±0.2 versus 8.0±1.4, 0 versus 3 hours, $P<.01$, mean±SEM).

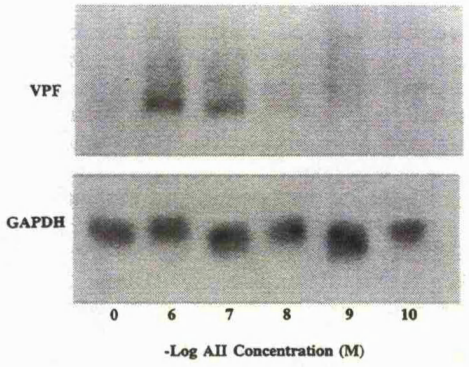

FIG 2. Northern blots show concentration dependency of changes induced by angiotensin II (Ang II; AII in figure) in vascular permeability factor (VPF) mRNA expression by human vascular smooth muscle cells (VSMCs). Steady-state abundance of VPF mRNA from human VSMCs was increased in an Ang II concentration-dependent manner after 3 hours of exposure to fetal bovine serum–deprived culture medium (1% fetal bovine serum) supplemented with various Ang II concentrations. Maximal increases in VPF mRNA level were observed at Ang II concentrations of 10^{-6} to 10^{-7} mol/L. GAPDH expression confirms that mRNA loading was equivalent in all lanes.

Concentration Dependency of Ang II–Induced VPF mRNA Expression

Maximal Ang II–induced VPF mRNA expression occurred at 3 hours; therefore, the 3-hour time point was used to define the concentration dependency of Ang II–induced VPF mRNA expression by human VSMCs. Fig 2 shows that at this time point, VPF mRNA expression was Ang II concentration dependent and maximally stimulated by Ang II concentrations of 10^{-6} to 10^{-7} mol/L. Densitometric analysis of the VPF mRNA–GAPDH mRNA ratio (n=4) revealed a consistent, maximal, and significant increase in VPF mRNA expression with Ang II concentrations of 10^{-7} mol/L (VPF mRNA–GAPDH mRNA ratio: 1.3±0.3 versus 7.2±0.9, control versus 10^{-7} mol/L Ang II, $P<.01$).

Identifying the Ang II Receptor Subtype Responsible for Ang II–Induced Increases in VPF mRNA Expression

At least two subtypes of the Ang II receptor have been identified in human tissues, AT_1 and AT_2.[23,24] The AT_1 receptor is by far the most abundant on human VSMCs, where it is responsible for all of the recognized actions of Ang II.[24] The function of the AT_2 receptor in human VSMCs is unclear. The AT_1 receptor can be selectively and specifically inhibited by the nonpeptide imidazole derivative losartan (DuP 753).[23] Fig 3 shows that supplementation of FBS-deprived culture medium with losartan (10^{-5} mol/L) alone had no effect on the basal expression of VPF mRNA by human VSMCs. However, losartan did inhibit the Ang II–induced increase in VPF mRNA expression by human VSMCs. Densitometric analysis of autoradiographs from further experiments (n=4) confirmed a consistent, complete inhibition of the Ang II–induced increase in VPF mRNA expression by losartan in human VSMCs (VPF mRNA–GAPDH ratio: 1.5±0.3 versus 7.9±1.7 and 2.3±0.9, control versus Ang II alone [$P<.01$] and Ang II plus losartan [$P<.8$]).These results confirm that the action of Ang II to stimulate an increase in VPF mRNA abundance

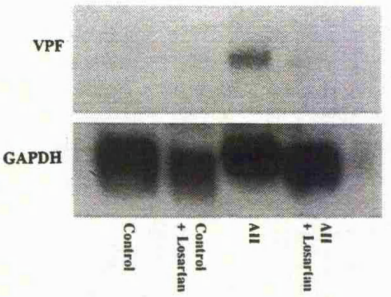

FIG 3. Northern blots show effects of a specific angiotensin II type 1 (AT_1) receptor antagonist (losartan) on angiotensin II–induced vascular permeability factor (VPF) mRNA expression by human vascular smooth muscle cells (VSMCs). Three hours of exposure to losartan (DuP 753) (10^{-5} mol/L), a specific antagonist of the AT_1 receptor, did not influence basal steady-state levels of VPF mRNA in human VSMCs cultured in fetal bovine serum–deprived medium (1% fetal bovine serum) (lane 2). Three hours of exposure to angiotensin II (10^{-7} mol/L) alone markedly increased VPF mRNA expression by human VSMCs (lane 3). Three hours of exposure to angiotensin II (10^{-7} mol/L) in the presence of losartan (10^{-5} mol/L) completely prevented the angiotensin II–induced increase in VPF mRNA expression (lane 4). Analysis of GAPDH mRNA levels confirmed equivalent loading of mRNA to each lane. AII indicates angiotensin II.

in human VSMCs is receptor mediated and occurs via the AT_1 receptor.

Discussion

The present study demonstrates a novel action of Ang II on human vascular tissue, notably a direct and potent regulation of VPF gene expression. Ang II–induced VPF mRNA expression in human VSMCs is rapid and maximal within 3 to 6 hours and diminishes by 12 hours. The rapidity of this response is consistent with previous reports of Ang II–induced gene expression in VSMCs; for example, Ang II induces platelet-derived growth factor A–chain mRNA expression in VSMCs, which is maximal at 9 hours.[7] The mechanism underlying the progressive diminution of Ang II–induced VPF mRNA expression over time is unclear. It may represent tachyphylaxis to continuous Ang II stimulation or reflect progressive peptidase-mediated neutralization of Ang II, which is known to occur in cell culture media. Further studies are required to establish the precise mechanism. The Ang II concentration required to maximally induce VPF mRNA expression (10^{-7} mol/L) in vitro is considerably higher than normal circulating levels of Ang II in vivo. Nevertheless, these concentrations are consistent with those required to reproduce the physiological actions of Ang II in VSMCs in vitro.[20] Moreover, it is likely that tissue levels of Ang II are considerably higher than circulating levels.[25]

The present study has not defined the mechanism whereby Ang II regulates VPF gene expression. The Ang II–induced increase in steady-state VPF mRNA levels could relate to either an increase in VPF gene transcription or Ang II–induced stabilization of VPF mRNA. Studies are ongoing to determine the relative importance of each mechanism in regulating VPF mRNA levels in human VSMCs in response to Ang II and a variety of other stimuli.

This study has focused on the regulation of VPF mRNA levels in human VSMCs, and VPF peptide

production in response to Ang II was not measured. Nevertheless, as expected for a secreted protein, in all previous reports in which steady-state VPF mRNA levels are increased, there has been a corresponding increase in VPF peptide production.[9] This implies that the abundant expression of VPF mRNA in human VSMCs and its potent modulation by Ang II are likely to be biologically significant.

The observations reported herein have important implications for the role of Ang II in the normal physiology of blood vessels and the pathogenesis of vascular injury. Although Ang II has been classically defined as an endocrine substance acting on blood pressure regulation, many tissues express endogenous renin-angiotensin system activity, implying that locally generated Ang II is involved in complex autocrine/paracrine regulatory mechanisms.[25] Human blood vessels contain all components of the renin-angiotensin system[25-27]; it is thus conceivable that locally generated Ang II could act as an autocrine hormone to regulate VPF production within the vascular smooth muscle of human blood vessels, thereby allowing VPF to act as a paracrine hormone to regulate the permeability of the overlying endothelium. This novel concept suggests a mechanism whereby Ang II could directly influence vascular permeability independent of its actions on blood pressure or microcirculatory hemodynamics.

In addition to its potent effects on vascular permeability, VPF is also a powerful endothelial cell–specific mitogen in vitro and is expressed in a variety of highly vascularized tissues where its expression is temporally and spatially related to angiogenesis.[9-11,20-30] Furthermore, the application of VPF to biological membranes such as the chorioallantoic membrane promotes neovascularization,[9] suggesting an important role for VPF as a physiological mediator of angiogenesis in vivo. The abundant expression of VPF mRNA by human VSMCs suggests that Ang II–induced VPF production could play an important role in the normal growth and repair of the overlying vascular endothelium in vivo.

The capacity of VPF to function simultaneously as a potent vascular permeabilizing agent and mitogen suggests that Ang II–induced increases in VPF production could also play a key role in the vascular injury that complicates disease states such as diabetes mellitus. Increased vascular permeability and neovascularization are prominent features of the microvascular complications of diabetes mellitus. Recent studies have shown that elevated plasma prorenin levels identify diabetic subjects who are at high risk for the development of diabetic microvascular complications such as proliferative retinopathy and nephropathy.[31] Prorenin levels are also markedly increased in the vitreous fluid extracted from the eyes of patients with proliferative retinopathy.[32] It is intriguing that in a recent report, VPF levels were markedly elevated in the vitreous fluid of eyes from diabetic patients with proliferative retinopathy compared with the vitreous fluid from the eyes of diabetic patients without active retinopathy.[33] We propose that these observations suggest a novel mechanism whereby increased circulating or local production of Ang II could directly influence vascular permeability and neovascularization in diabetic patients via Ang II–induced VPF production.

In addition to the aforementioned changes in endothelial permeability and growth, VPF has been reported to promote the release of Von Willebrand factor and tissue factor, generating a procoagulant state on the endothelial surface,[34,35] and also to induce monocyte activation and migration to the endothelium.[35] Each of these events is recognized to be important in the initiation of endothelial dysfunction and the early pathogenesis of atherosclerosis.[1] The present study thus suggests an additional mechanism whereby increased systemic or local Ang II production could contribute to a spectrum of endothelial dysfunction that could ultimately play a role in the pathogenesis of vascular disease in a variety of disease states.

Acknowledgments

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Chapter 10.

Glucose-induced Protein Kinase C Activation Regulates Vascular Permeability Factor mRNA Expression and Peptide Production by Human Vascular Smooth Muscle Cells.

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Glucose-induced Protein Kinase C Activation Regulates Vascular Permeability Factor mRNA Expression and Peptide Production by Human Vascular Smooth Muscle Cells.

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Abstract:

Diabetes mellitus is characterised by the development of two important markers of disturbed endothelial function, notably; increased endothelial permeability to circulating macromolecules and endothelial proliferation leading to neovascularisation. Vascular permeability factor (VPF)(also termed vascular endothelial growth factor) is 34-42kDa that is produced by vascular smooth muscle and markedly increases endothelial permeability and is a potent endothelial cell mitogen that can induced angiogenesis. Hyperglycaemia is a major risk factor for the development of diabetic microvascular disease; hence we hypothesised that glucose may directly stimulate VPF production by human vascular smooth muscle cells (human VSM cells). High glucose concentrations (20mM) markedly increased VPF mRNA expression by human VSM cells (230% vs glucose 5mM, $p<0.01$), maximal after 3 hours exposure and also significantly increased VPF peptide production by human VSM cells, maximal after 12 hours exposure to high glucose. These effects of high glucose were not reproduced by osmotic control media containing elevated mannitol or L-glucose concentrations. Protein kinase C (PKC) activation increases VPF mRNA and peptide production by human VSM cells, moreover high glucose activates PKC in human VSM cells. PKC inhibition (H7 or chelerythrine chloride) and downregulation (prior exposure to phorbol myristate acetate 10^{-7}M for 24 hours) each prevented glucose-induced VPF gene expression by human VSM cells. These studies demonstrate that high glucose concentrations directly increase VPF gene expression and peptide production by human VSM cells, via a PKC-dependent mechanism. These observations suggest a novel cellular mechanism whereby hyperglycaemia could directly contribute to the development of endothelial dysfunction and neovascularisation in diabetes mellitus.

Key Words: **Glucose, vascular permeability factor, diabetes, protein kinase C, vascular disease.**

Running Title: **Williams et al. Glucose and vascular permeability factor.**

INTRODUCTION

Diabetes mellitus is characterised by the premature and widespread development of macrovascular disease and microangiopathy and endothelial dysfunction has been implicated in the pathogenesis of diabetic vascular complications [1-6]. One of the earliest events in the pathogenesis of atherosclerosis is an increase in endothelial permeability to circulating macromolecules such as albumin and lipids [7]. Increased vascular permeability to macromolecules has also been documented in humans and experimental animals with diabetes [1,8-10]. The mechanism accounting for increased endothelial permeability in diabetes is not clear, however, ultrastructural studies suggest that there is little endothelial denudation and that the permeability changes are a manifestation of endothelial dysfunction rather than injury [11]. This diabetes-induced increase in vascular permeability is often improved, at least in its earliest stages, by improvements in glycemic control [9]. This latter observation suggests that elevated glucose concentrations *per se*, may play a role in regulating endothelial permeability.

Neovascularisation due to stimulation of endothelial cell proliferation is another manifestation of endothelial dysfunction in diabetes that contributes to the development of microangiopathy and ultimately retinopathy, nephropathy and perhaps also diabetic neuropathy and cardiomyopathy. The results of the recent diabetes control and complications trial (DCCT) suggests that the development of microangiopathy is delayed by strict glycaemic control [12]. This observation confirmed a long-held belief that hyperglycemia is an important independent stimulus for the development of neovascularisation and microvascular disease.

The mechanism whereby an elevated glucose concentration might promote changes in endothelial permeability and abnormal angiogenesis has been source of much speculation, but remains unknown. We have previously proposed that the induction of a recently discovered closely related group of cytokines with a potent capacity to increase endothelial permeability and induce endothelial growth, may be involved in the development of endothelial dysfunction in hypertension and diabetes [13]. This cytokine family is known as vascular permeability factor (VPF) and also known as vascular endothelial growth factor (VEGF) or vasculotropin [14-16]. VPFs are 34-42kDa homodimeric, heparin binding glycoproteins that are produced by a variety of cells of human origin, including vascular smooth muscle cells (VSM cells) [13-17]. Unlike many other vascular growth factors such as basic fibroblast growth factor and platelet derived factor, VPF possesses a hydrophobic signal sequence which governs its secretion from its site of synthesis [18]. Consistent with this observation, we and others have confirmed that VPF is secreted by cultured VSM cells [19,20]. Five different VPF peptides have now been described [21,22], each formed by alternative mRNA splicing of a single eight exon gene [21,23].

VPF binds almost exclusively to the vascular endothelium via two specific receptor tyrosine kinases. The Flt-1 (*fms*-like tyrosine kinase) and the KDR (kinase-insert-domain-containing receptor) receptor proteins both bind VPF with high affinity [24-27]. Upon binding to its receptors, VPF is the most potent vascular permeability-enhancing factor thus far identified and on a molar basis it is 50,000 times as potent as histamine [28]. In addition to its dramatic effects on endothelial permeability, VPF is also a powerful

endothelial cell specific mitogen with the capacity to induce angiogenesis independently of other growth stimuli [16,29]. These potent actions of VPF make it an attractive candidate to function as a regulator of key aspects of endothelial function and growth. Moreover, the close proximity of vascular smooth muscle to the endothelium makes VSM cells a logical site for VPF production within blood vessels. It is thus conceivable, that abnormalities in the regulation of VPF production by VSM cells, in particular increased VPF production, could lead to pathological increases in vascular permeability and accelerated angiogenesis in disease states such as diabetes mellitus.

We have shown that various vasoactive peptides such as angiotensin II, arginine vasopressin and platelet derived growth factor can markedly increase VPF mRNA expression and peptide production by cultured human VSM cells [13,17,19]. We have also shown that arginine vasopressin-induced VPF mRNA expression is dependent on protein kinase C (PKC) activation [19]. This latter observation is of particular interest because we have also shown that elevated glucose concentrations can activate PKC in VSM cells [30-33]. Together, the aforementioned observations prompts the hypothesis that elevated glucose concentrations *per se*, may be sufficient to stimulate an increase in VPF mRNA expression and VPF peptide secretion by human VSM cells via an intracellular signalling mechanism that is dependent on PKC activation. Such a finding would describe a novel biological mechanism whereby elevated glucose concentrations could directly contribute to endothelial dysfunction and neovascularisation in diabetes mellitus.

METHODS

Human VSM Cell Culture

VSM cells were cultured from macroscopically normal venous tissue obtained from varicose veins removed at surgery. The VSM layer was identified by dissection and digested in collagenase for two hours as previously described [32,33]. The resulting cell suspension was centrifuged, re-suspended in Ham's F-12 medium (Sigma Chemical Company) supplemented with 20% foetal bovine serum (FBS) (Life Technologies), 0.5% chick embryo extract (Life Technologies), 2mmol/L l-glutamine and antibiotics. The cells were cultured in 100mm tissue culture plates in humidified air supplemented with 5% CO_2. The cell monolayers have been extensively characterised to be well differentiated VSM cells on the basis of light and electron microscopic morphology and immunohistochemistry. When confluent, VSM cell monolayers were passaged every six to seven days after trypsinization and were used for experiments from the second to the sixth passage.

Polymerase Chain Reaction Amplification and cDNA probes

To detect VPF mRNA, a 204-bp cDNA fragment was generated from human VSM cells RNA with the use of two oligonucleotide primers based on the human VPF cDNA sequence as previously described from our laboratory [13,17]. The primers have built in restriction sites, *Bam*HI in primer I and *Eco*RI in primer II, at their 5' ends to facilitate

cloning. For polymerase chain reaction amplification,, 0.1μg total RNA from human VSM was annealed with random hexanucleotides and reverse transcribed for 30 minutes at 42^0C with the use of 100u reverse transcriptase (Life technologies) in a volume of 20μL. The reaction mixture was heated to 95^0C for 10 minutes before the addition of the PCR primers and *Taq* polymerase (Biotaq, Bioline Corp.) in a final volume of 100μL. PCR amplification was performed on 100μL samples using a DNA thermal cycler (Perkin-Elmer 480). Amplification was carried out for 30 to 35 cycles as previously described (hypertension paper). The resulting 204-bp cDNA fragment was sub-cloned in the polylinker region of p-Bluescript II SK(-). The identity of the cloned human insert was confirmed by Sanger Dideoxy-DNA sequencing and found to be identical to the previously reported human VPF sequence [23]. Five different VPF mRNA transcripts formed by alternative splicing of an 8 exon gene have now been identified in various tissues [21,22]. The 204-bp cDNA probe used in these studies is common to all known VPF splicing variants.

Northern Analysis

The human VPF cDNA insert was radiolabeled with [α-^{32}P]dCTP (Amersham) to a specific activity of approximately $2x10^6$ cpm/μg DNA using the random primer labelling system (Life technologies). Total cellular RNA was prepared from cultured human VSM cells using the one-step guanidinium-phenol chloroform purification method [34]. RNA samples (approximately 20μg per lane) were size-fractionated on a 1.2% agarose containing 6% formaldehyde and blotted on to Hybond nylon membranes (Amersham). Hybridisation was carried out for 18 hours at 42^0C and high stringency washes were

carried out as previously described [13,17]. To control for total mRNA content and lack of degradation, the blots were subsequently stripped and re-hybridised with a cDNA fragment for human GAPDH (9805-1, Clontech). The resulting autoradiographs were subjected to densitometric analysis (LKB Gelscan, Pharmacia) to quantify the ratio of VPF to GAPDH mRNA.

VPF Peptide Assay

VPF peptide was assayed in the cultured media overlying the hVSMC using a solid phase ELISA. This assay specifically assays VPF_{165} which is recognised to be the main peptide synthesised and secreted by VSM cells [20]. The primary antibody used in this assay is a polyclonal antibody raised against recombinant human VPF_{165} expressed from an insect cell *sf*-21 (R&D Systems). A commercial murine monoclonal antibody specific for VPF (R&D Systems) was also used to coat the microtiter plates and generate the conditions necessary for a quantitative sandwich enzyme immunoassay. During the assay, the immobilised monoclonal antibody binds to any VPF in the sample of cell culture supernatant. The unbound proteins are then washed away and the immobilised VPF is exposed to the second VPF specific, polyclonal antibody which is conjugated to the horseradish peroxidase enzyme. Following this incubation, any unbound antibody-enzyme reagent is washed away and a substrate for horseradish peroxidase is added to the well (stabilised hydrogen peroxide and stabilised chromogen; tetramethylbenzidine) and a yellow-brown colour develops within the well, in proportion to the amount to VPF initially trapped by the monoclonal antibody. The colour density is then read by an automated microtiter plate reader (Dynatech, MRX, Dynatech, UK). A standard curve is

simultaneously prepared by plotting the optical density of "standard wells" against the known concentration of the recombinant VPF_{165} protein that has been added to the "standard wells". The concentration of VPF in the cell supernatant samples can be calculated by comparing their optical density to the standard curve.

Prior to assay, the cell culture supernatants were collected, centrifuged at 1000 rpm. to remove any particulate matter and stored at -20^0C until assayed. An intra-assay precision of 4.8% was calculated by assaying 5 samples of known concentration in a single assay. An inter-assay precission of 5.8% was a calculated by assaying the same 10 samples in three separate assays. The recovery of VPF from cell culture supernatant was greater than 90%. This was calculated by "spiking" the media with pure VPF_{165} peptide and assaying its recovery. The assay is highly specific for VPF and the assay has shown no cross reactivity when tested commercially against 86 different cytokines.

Experimental Design.

Human VSM cells were grown to confluence in 100mm tissue culture plastic tissue culture plates in the media described above containing 5mM D-glucose and 20% fetal bovine serum (FBS). 48 hours prior to exposing the human VSM cells to the various test media (see below), the cells were cultured in serum-deprived media containing 5mM D-glucose and 1% FBS. This manoeuvre was designed to render the cells quiescent and minimise the influence of FBS because we have previously shown that serum is a potent stimulus for VPF mRNA expression by human VSM cells [17]. The human VSM cells were then exposed to one of four test culture media described below:

Control Medium: This comprised the standard culture medium described above and containing 1% FBS and 5mM/liter D-glucose.

High Glucose Medium: This was identical to control medium but supplemented with D-glucose to increase its concentration up to 30mM/liter.

Mannitol Osmotic Control Medium: This was similar to control medium except that it was supplemented with mannitol, a non permeable hexose (D-glucose 5mM/liter + Mannitol 15mM/liter), which served as an osmotic control medium for the high glucose medium.

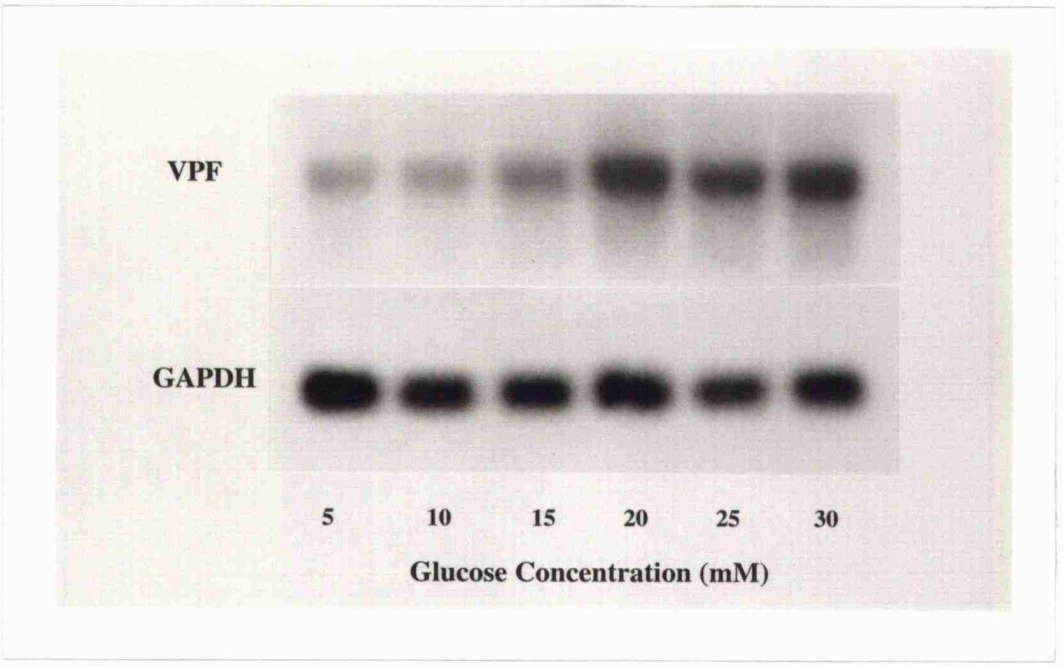
L-Glucose Osmotic Control Medium: This was identical to control medium but supplemented with L-Glucose, a cell permeable but poorly metabolized glucose isomer (D-glucose 5mM/liter + L-glucose 15mM/liter), which also served as an osmotic control for the high glucose medium. In addition, because L-glucose is so poorly metabolized, this medium is also served as a metabolic control for the high glucose medium.

RESULTS

Effect of elevated glucose concentrations on VPF mRNA expression by human VSM cells.

In preliminary experiments, human VSM cells were exposed to control media or high glucose medium containing various D-glucose concentrations 10-30mM/liter for 3 hours. This time point was chosen because we have previously shown that VPF mRNA expression in response to a variety of stimuli is maximal at this time point [13,17,19]. In human VSM cells, the VPF cDNA probe hybridized with a single 4.2 kb transcript which is compatible with the previously reported size of VPF mRNA and a previous report, other than our own, demonstrating that VSM cells express a single VPF transcript [20,23]. As shown in figure 1, VPF mRNA expression by human VSM cells was markedly increased when the extracellular glucose concentration was increased to 20mM/liter. This appeared to be a threshold phenomenon because further increases in the extracellular glucose concentration (up to 30mM/liter) failed to induce a further increase in steady state VPF mRNA expression. In additional experiments, the time course of glucose-induced VPF mRNA expression by human VSM cells was examined by exposing cells to high glucose medium containing 20mM/liter glucose for up to 48 hours. The glucose-induced increase in VPF mRNA expression was consistently observed to be maximal after 3-6 hours exposure to the high glucose media and diminished thereafter, despite continued exposure to high glucose (data not shown). Nevertheless, even after 48 hours of continuous exposure to high glucose, VPF mRNA expression remained markedly higher than that observed in the presence of control medium.

Figure 1.



Glucose concentration-dependency of glucose-induced VPF mRNA expression by human VSM cells. This autoradiograph is representative of 4 experiments. Each lane contains 20μg of total RNA that has been hybridized with VPF cDNA to reveal a single 4.2 kb transcript. The VPF mRNA expression was markedly increased in the presence of elevated extracellular glucose concentrations (20-30mM/liter). There was equivalent loading of total RNA to each lane of the gel as indicated by consistent expression of the "housekeeping gene" GAPDH (1.4 kb.) in each lane.

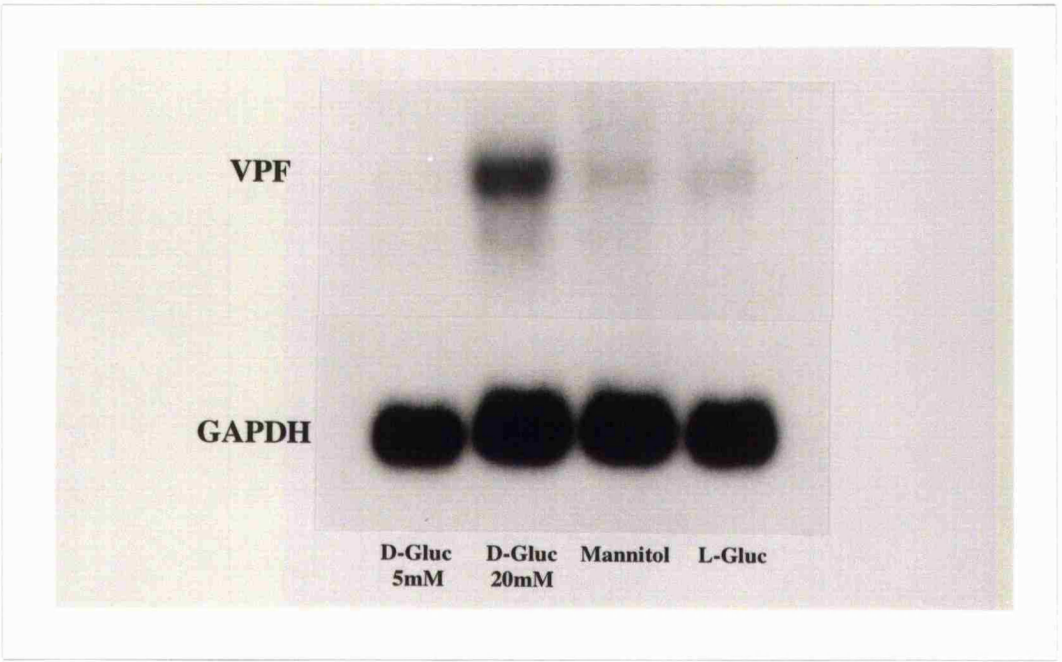
Effect of Osmotic Control Media on VPF mRNA expression by Human VSM cells.

Human VSM cells were exposed to control medium, high glucose medium (glucose 20mM/liter), and the mannitol and L-glucose osmotic control media for 3 hours prior to the measurement of VPF mRNA expression. As shown in figure 2, exposure to high glucose media stimulated a marked increase in VPF mRNA expression whereas the osmotic control media did not. These observations suggest that the observed increase in VPF mRNA expression is specific for D-glucose and is not simply related to changes in extracellular osmolality. Moreover, the absence of any effect of the medium supplemented with L-glucose (the cell permeable but non-metabolized glucose isomer) implies that the intracellular metabolism of glucose is essential for glucose-induced increases in VPF mRNA expression to occur.

Effect of Elevated Glucose Concentrations of VPF Peptide Production by Human VSM Cells.

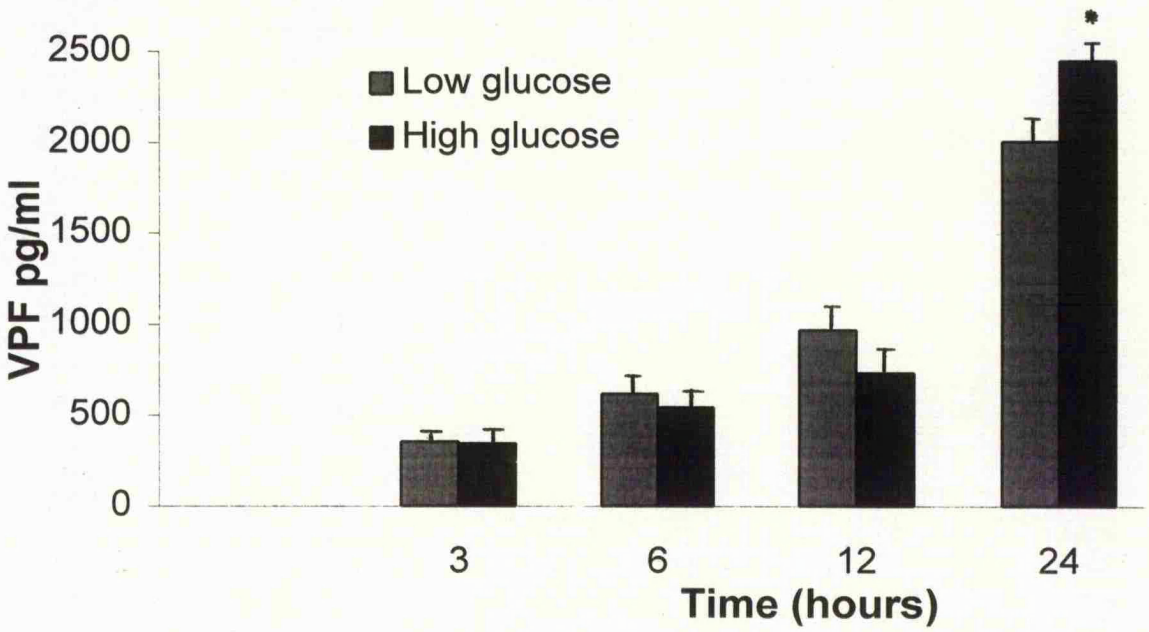
Having demonstrated that elevated glucose concentrations stimulate an increase in VPF mRNA expression, it was necessary to confirm that this increase in VPF mRNA expression was translated into increased VPF peptide synthesis and secretion by human VSM cells. Human VSM cells were exposed for 24 hours to control or high glucose medium (D-glucose 20mM/liter). Aliquots (500μL) of the cell supernatant were taken at various time points to assay the time-related change in VPF secretion by human VSM cells. As shown in figure 3, human VSM cells continuously secrete VPF into the culture media overlying the cells. In the presence of high glucose medium, the VPF concentration

Figure 2.



Effects of osmotic control media on VPF mRNA expression by human VSM cells. This autoradiograph is representative of 3 experiments which showed that media containing an elevated extracellular D-glucose concentration (20mM/liter) increased VPF mRNA expression whereas the mannitol and L-glucose osmotic control media did not. The expression of GAPDH confirms equivalent loading of total RNA to each lane.

Figure 3.



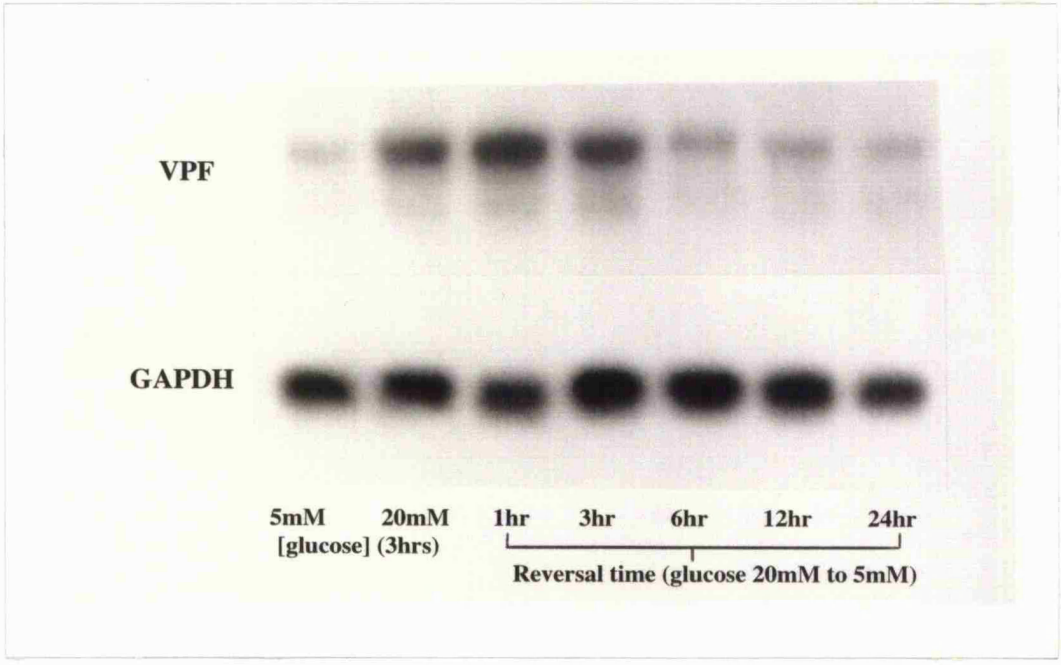
The effect of elevated glucose concentrations on VPF peptide production by human VSM cells. VPF peptide concentrations were measured at various time points after exposing human VSM cells to control (glucose 5mM/liter) or high glucose (glucose 20mM/liter) media for up to 24 hours. After 12 hours, high glucose induced a significant increase in VPF peptide secretion by human VSM cells into the cell culture media, * = $p<0.01$, n = 3 separate experiments, each assaying 3 separate tissue culture plates/ glucose concentration at each time point.

appears to decline within a few hours of changing to the high glucose medium. This was a consistent trend in all of our studies. However, after 12 hours exposure to the high glucose medium, there was a dramatic and significant increase in VPF secretion from the human VSM cells. So much so, that by 24 hours, the VPF content of the media from cells exposed to high glucose was significantly greater than that from cells chronically exposed to the control media. The glucose-induced increase in VPF peptide production by human VSM cells was not accounted for by a glucose-induced increase in VSM cell mass because after 24 hours incubation with the high glucose media, the VSM protein content per cell culture media was no different in the presence of all test culture media. These observations thus confirm that glucose-induced increases in VPF mRNA expression by human VSM cells are followed by a marked increase in VPF peptide secretion by these cells.

Reversibility of Glucose-induced Changes in VPF mRNA Expression by Human VSM Cells.

To determine whether the high glucose-induced increase in VPF mRNA expression was reversible, human VSM cells were exposed to a high glucose medium (20mM/L) for 3 hours to induce the VPF gene. The culture media was then changed to one containing D-glucose 5mM/liter for up to 24 hours to define how long it took for VPF gene expression to return to normal after normalisation of the extracellular glucose concentration. Figure 4 shows that within 6 hours of normalizing the extracellular glucose concentration, the previously elevated of VPF mRNA expression was restored to normal. These results demonstrate that VPF mRNA expression by human VSM cells is rapidly responsive to

Figure 4.



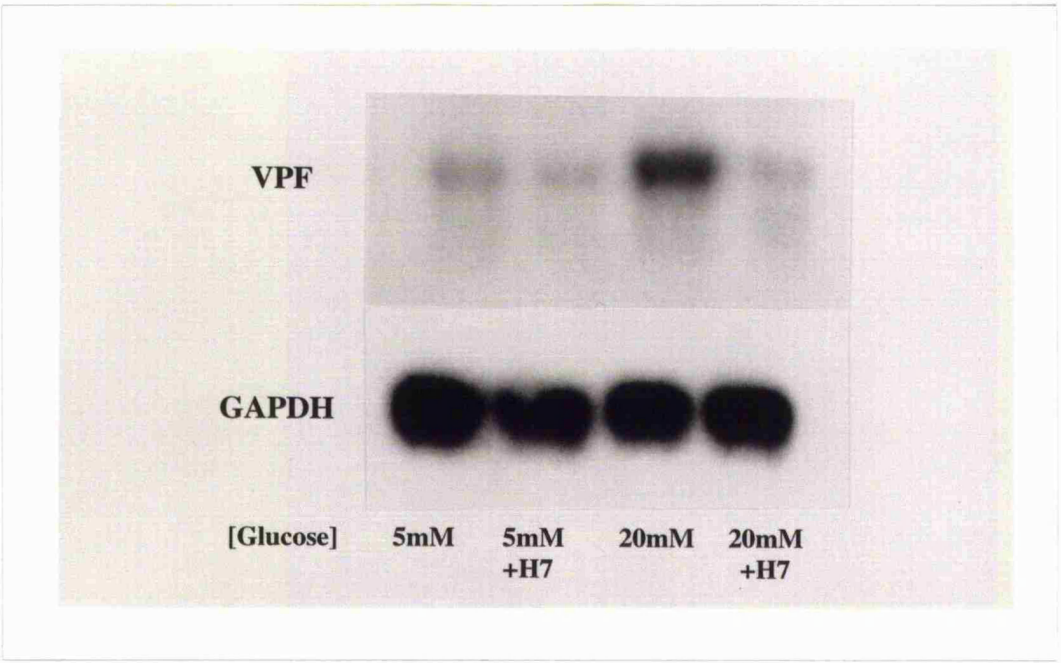
The reversibility of the glucose-induced increase in VPF mRNA expression by human VSM cells. This autoradiograph of a northern blot is representative of 3 experiments in which the cell monolayers were first exposed to high glucose medium (20mM/liter) for 3 hours, which induced an increase in VPF mRNA expression when compared to control medium (glucose 5mM/liter). Normalizing the extracellular glucose concentration by changing the high glucose medium back to control medium was associated with a rapid decline in VPF mRNA expression, back to baseline within 6 hours.

fluctuations in the extracellular glucose concentration. Moreover, the fact that the glucose-induced increase in VPF mRNA expression by human VSM cells is so rapidly reversed excludes the possibility that these effects of glucose relate to cellular injury.

Role of Protein Kinase C in Mediating the Glucose-induced increase in VPF mRNA Expression by Human VSM cells.

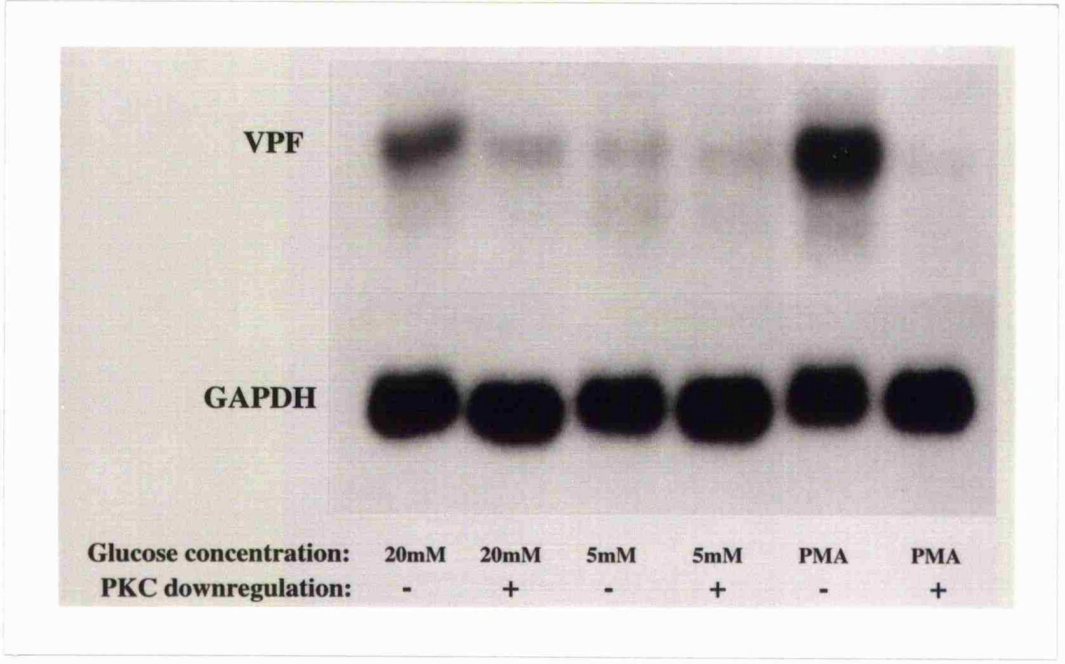
We have previously shown that VPF mRNA expression and peptide production by human VSM cells can be induced the exposure to phorbol myristate acetate (PMA) a direct activator of protein kinase C (PKC) in VSM cells [19]. We have also shown that elevated glucose concentrations induce PKC activation in human VSM cells [30-33]. This prompted the hypothesis that the glucose-induced increase in VPF mRNA expression may be dependent on glucose-induced PKC activation. To test this hypothesis we used two manoeuvres to inhibit PKC activity. The first involved the use of two dissimilar PKC inhibitors. The second utilized the capacity of prolonged exposure to PMA (PMA 10^{-7}M for 24 hours) to downregulate cellular PKC activity and render it refractory to further stimulation. Figure 5 shows that H-7(Sigma Chemical Corp.) (100μM), a PKC inhibitor prevents the high-glucose-induced increase in VPF mRNA expression. We have previously confirmed that this dose of H-7 is sufficient to inhibit a glucose-induced increase in PKC activation [30]. Although H-7 is acknowledged to be an inhibitor of PKC, it is not very specific in this regard and inhibits other kinase systems. We thus used a second PKC inhibitor, chelerythrine chloride (Sigma Chemical Corp.) which is much more specific for PKC. Compared to H-7, we found chelerythrine chloride was much more toxic to human VSM cells and unsuitable for prolonged incubations. Nevertheless,

Figure 5.



Effect of PKC inhibition of glucose-induced VPF mRNA expression by human VSM cells. This representative autoradiograph (3 experiments) shows VPF mRNA expression by human VSM cells which were previously incubated with control medium (glucose 5mM/liter) or high glucose medium (20mM/liter) ± H-7 (100μM) for 3 hours. High glucose alone markedly increased VPF gene expression. H-7 blocked the glucose-induced increase in VPF mRNA expression.

Figure 6.



Effect of PKC downregulation on glucose or PMA-induced VPF mRNA expression by human VSM cells. This autoradiograph of a northern blot is representative of 3 experiments which showed the effect of downregulating PKC activity on glucose-induced VPF mRNA expression (see text for details). In the absence of PKC downregulation, both high glucose and PMA (10^{-7}M) increase VPF mRNA expression. In contrast, after PKC downregulation, high glucose media and PMA both failed to increase VPF mRNA expression.

at shorter incubations times (less than 12 hours), chelerythrine chloride (50μM) was not obviously toxic to human VSM cells but did inhibit the glucose-induced increase in VPF expression (data not shown).

To overcome the problems of specificity and cellular toxicity with PKC inhibitors, we used an additional experimental manoeuvre frequently employed to assess the role of PKC in mediating a cellular event. Prior to exposure to high glucose media, human VSM cells were incubated with control media supplemented with PMA 10^{-7}M for 24 hours. We have previously shown that such a prolonged exposure to VSM cells to PMA leads to downregulation of PKC activity and renders the cells refractory to further PKC stimulation [33]. Figure 6 shows that before PKC downregulation, high glucose induced an increase in VPF mRNA expression, but following PKC downregulation it did not. Figure 6 also shows that PKC activation was blocked by this manoeuvre because prior to PKC downreguation, PMA markedly increased VPF mRNA expression by human VSM cells, whereas after PKC downregulation, it did not. Together, these results demonstrate that glucose-induced increases in VPF mRNA expression by human VSM cells and thus the resulting increase in VPF peptide secretion are dependent on glucose-induced PKC activation.

DISCUSSION

The present study demonstrates a novel action of elevated glucose concentrations on human VSM cells, notably, a glucose-induced increase in VPF mRNA expression and VPF_{165} peptide secretion. This glucose-induced effect was observed using glucose concentrations which are compatible with those observed in patients with poorly controlled diabetes and was specific for D-glucose as it was not reproduced by elevated concentrations of mannitol or L-glucose. Moreover, VPF mRNA expression by human VSM cells was rapidly responsive to fluctuations in the extracellular glucose concentration.

The increase in mRNA expression does not necessarily imply peptide synthesis and secretion. VPFs possess a hydrophobic sequence typical of that observed in other secreted peptides [18]. Thus unlike many other endothelial mitogens such as fibroblast growth factor [35] or platelet-derived endothelial growth factor [36], VPF has the potential to be released by intact cells, without the need for postulating cell death or specialized transport mechanisms. We have recently demonstrated that arginine vasopressin-induced increases in VPF mRNA expression by human VSM cells *in vitro,* is associated with increased VPF peptide secretion by these cells. The present study shows that glucose-induced VPF mRNA is associated with increased secretion of VPF peptide by human VSM cells. The ELISA assay used to detect VPF peptide was specific for VPF_{165} which is also detectable in human serum (unpublished observations) and presumably corresponds to the single 4.2

kb. VPF mRNA transcript identified in human VSM cells. These conclusions are consistent with data from a previous report [20] which demonstrated a similar single VPF mRNA transcript in cultured bovine aortic VSM cells, which also secreted VPF_{165}. Further studies using specific antibodies against the other VPF peptides will be necessary to define whether other additional VPF species are secreted by human VSM cells.

The intracellular signalling pathways regulating VPF mRNA expression and peptide secretion by VSM cells have not been elucidated. Hypoxia is known to induce VPF mRNA expression by many cells including rabbit VSM cells [37]. A recent study showed that the hypoxic induction of VPF mRNA expression by u87 glioma cells *in vitro,* was dependent on the activation of tyrosine kinases and in particular $pp60^{c\text{-}Src}$ [38]. In addition, we have previously shown that PKC activation by PMA induced VPF mRNA expression and peptide secretion by human VSM cells and that arginine vasopressin-induced VPF mRNA expression by human VSM cells can be prevented by PKC inhibition [19]. These early studies suggest that multiple intracellular signalling mechanism may be involved in the regulation of VPF mRNA expression and that one of the mechanisms involves PKC activation. The present study confirms that direct PKC activation by PMA was sufficient to potently induce VPF mRNA expression by human VSM cells (figure 6). With regard to glucose, we have previously demonstrated that elevated glucose concentrations activate PKC in VSM cells and that glucose-induced PKC activation can be prevented by H-7 or prior PKC downregulation after prolonged exposure to PMA [30-33]. The present study demonstrates that glucose-induced VPF mRNA expression is dependent on PKC activation. The PKC inhibitors used in this study are not completely specific for PKC

(especially H-7). Nevertheless, the fact that two dissimilar PKC inhibitors and the PKC downregulation manoeuvre, each prevented the glucose-induced increase in VPF mRNA expression by human VSM cells, is strong evidence to support the hypothesis that glucose-induced PKC activation is a necessary component of the signalling pathway culminating in increased VPF mRNA expression and peptide production by human VSM cells.

PKC is not a single molecular entity, but consists of a family of closely related isoenzymes that differ in their structure, cofactor requirement and substrate specificity [31]. There is differential activation of PKC isoforms in various tissues from diabetic animals. In vascular tissue, although PKCα is the most abundant, only the PKC β_{II} isoform undergoes significant cytosol:membrane translocation, indicative of activation *in vivo*, in diabetic animals [40]. In addition, PKCβ_{II} appears to be preferentially translocated in VSM cells, in response to elevated extracellular glucose concentrations *in vitro* [39]. Whether PKCβ_{II} is the PKC isoform responsible for the glucose-induced increased in VPF mRNA expression and peptide production by human VSM cells awaits confirmation.

VPF is an attractive candidate to function as a mediator of endothelial dysfunction in diabetes mellitus. Increased vascular permeability, neovascularisation and markers of endothelial activation such as increased Von Willebrandt factor release are prominent features of vascular disease in diabetic patients [1-6]. In addition to its potent capacity to increase endothelial permeability and angiogenesis, VPF also promotes the endothelial release of Von Willebrandt factor, induces the development of a procoagulant state on the

endothelial surface and stimulates the migration of monocytes to the endothelium [40]. All of these events are recognised to be important in the initiation of endothelial dysfunction and the pathogenesis of macrovascular and microvascular disease [7]. The levels of VPF peptide produced by human VSM cells, in the present study, *in vitro*, approximate to 200pg per mg. VSM cell protein per hour and are more than sufficient to induce biological effects on endothelial cells. VPF levels of only 400 pg/L are required to increase intracellular free calcium in endothelial cells *in vitro* [40], and the half maximal effects of VPF on endothelial mitogenesis are achieved at 2-3ng/L [14]. It is thus conceivable that glucose-induced increases in VPF synthesis and release could play a role in the development of such complications.

Although we have proposed that VSM-derived VPF acts as a paracrine factor to directly influence endothelial function, VPF is also detectable in the circulation. It is thus possible that VSM cells are a major source of circulating VPF levels, thereby allowing any glucose-induced increase in VPF to influence tissues distal to its site of production ie. microvascular endothelial function. Moreover, elevated glucose concentrations are capable of activating PKC in other cell types such as mesangial cells [41,42] and retinal pericytes. It is thus possible that elevated glucose concentrations may increase the local production of VPF and leading to tissue specific increases in permeability to macromolecules and angiogenesis. Consistent with this possibility, was recently shown that VPF levels are markedly elevated in the ocular vitreous fluid of patients with proliferative diabetic retinopathy [43].

In conclusion, the present study shows that elevated glucose concentrations increase VPF mRNA expression and VPF peptide secretion by human VSM cells via a PKC-dependent mechanism. In so doing, we demonstrate a novel biological mechanism whereby elevated glucose concentration could directly contribute to the development of a wide spectrum of endothelial dysfunction in diabetes mellitus.

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Chapter 11.

Conclusions

Conclusions

This thesis contains my published work extending from my initial characterisation of glucose-induced PKC activation in vascular smooth muscle (VSM) cells, through subsequent published works in which I explored the potential significance of glucose-induced PKC activation on cell function. Subsequently my studies focused on vascular permeability factors (VPF) as a potential mediator of endothelial dysfunction in diabetes, culminating in my most recent work which discovered that glucose-induced PKC activation may account for increased VPF mRNA expression and VPF peptide production by human VSM cells. I will briefly review the key points to be derived from the published works contained in this thesis and how the novel cellular mechanisms described herein may provide a basis for the development of novel therapeutic strategies for diabetic vascular disease.

In chapter 2, I reported the first detailed characterisation of glucose-induced PKC activation in any cell type. To do this, it was necessary to improve on the existing PKC assay which was cumbersome and utilised a non-specific substrate. I was fortunate that new methods for detecting in-situ phosphorylation were being developed in a nearby laboratory at the University of Colorado Health Sciences Center, Denver, USA. I utilised these methods and a PKC-specific substrate to improve the specificity of the assay for PKC and improve the assay's flexibility to enable multiple time point measurements etc. to be made (for details see chapter 2). This was an important advance because previously, glucose-induced PKC activation had only been implied from the observation that elevated glucose concentrations promoted the translocation of the PKC enzyme system from the

cell cytosol to membrane. Although this usually indicates PKC activation, it provides no information regarding the actual level of PKC activity within the cell. The publication of our results in *Diabetes* represented the first confirmation that elevated glucose concentrations not only promoted PKC translocation but also increased intracellular PKC activity. Moreover, the methods we had developed enabled me confirm that commercially available PKC inhibitors could prevent glucose-induced PKC activation, thereby providing and validating an invaluable experimental tool to test the hypothesis that glucose-induced PKC activation may contribute to a wide spectrum of cellular dysfunction.

The demonstration of glucose-induced PKC activation in VSM cells led to much speculation surrounding its potential significance with regard to vascular disease but no evidence existed to support these assumptions. Chapter 3 comprises a publication from the *Journal of Clinical Investigation* in which I described how a basic cellular event such as pressor receptor turnover and intracellular signalling could be profoundly disturbed by chronic glucose-induced PKC activation. This observation, to my knowledge, was the first demonstration that glucose-induced PKC activation could disturb a key aspect of VSM cell function.

The glomerular mesangial cell is phenotypically very similar to the VSM cell. It was important to determine whether elevated glucose concentrations were also capable of enhancing PKC activity and modifying the function of cells other than those derived from vascular smooth muscle. This was a critical experiment because it defined whether the

glucose-induced PKC hypothesis was potentially applicable to other cell types. Moreover, the mesangial cell was an obvious choice for these experiments because its function disturbances to its function are thought to play a key role in the pathogenesis of diabetic nephropathy. Chapter 4 contains a study, published in the *Journal of Clinical Investigation*, in which I reported a detailed characterisation of glucose-induced PKC activation in mesangial cells and confirmed that the characteristics of PKC activation in these cells were very similar to that previously observed in VSM cells. Moreover, I demonstrated that glucose-induced PKC activation was once again associated with a disturbed biological response in these cells, notably; increased arachidonic acid release and changes in prostaglandin and thromboxane synthesis. The similarities in the response of mesangial and VSM cells to elevated glucose concentrations implied that abnormal biological responses to glucose-induced PKC activation in one cell type may be generally applicable to other cell types.

In chapter 5, I returned to the study of VSM cells, the major focus of my work. At the time this study was conceived, there was tremendous interest in the possibility that increased activity of the Na^+/H^+ antiporter may be genetically pre-determined and predispose some, but not all diabetic patients to vascular complications. Simultaneously, there was an accelerated growth in our knowledge of how such membrane transporters are regulated at the cellular level. Some preliminary evidence emerged to suggest that PKC may be involved in the regulation of Na^+/H^+ antiport activity. I thus examined the effect of elevated glucose concentrations on the activity of this important membrane transport system in VSM cells. The results of this work were published in the *Journal of*

Clinical Investigation and revealed that elevated glucose concentrations *per se*, directly increased Na^+/H^+ antiport mRNA expression and antiport activity in VSM cells via a PKC dependent mechanism. Moreover, when the mechanism of antiporter regulation in response to glucose was further examined, I discovered a complex time-dependent regulatory system. Following short term exposure to elevated glucose concentrations (3 hours), the increase in Na^+/H^+ antiporter activity occurred without the need for gene transcription or protein synthesis. This suggested a PKC-dependent membrane recruitment of existing antiporters from within the cell, or a phosphorylation dependent increase in the activity of Na^+/H^+ antiporters already established within the membrane. In contrast, the Na^+/H^+ antiporter response to more prolonged exposure to elevated glucose concentrations (24 hours) was dependent on protein synthesis and gene transcription, perhaps suggesting the need for new transporter synthesis to sustain the glucose-induced response. In addition to revealing another glucose-induced PKC dependent mechanism whereby VSM cell function and growth might be profoundly disturbed, this study provided the first evidence of the capacity of elevated glucose concentrations to directly influence the expression of a gene (Na^+/H^+ mRNA) that would not ordinarily have been associated with glucose metabolism. Furthermore, this study suggested that even if genetic factors are a pre-determinant of VSM cellular Na^+/H^+ activity, the prevailing glucose concentration has the capacity to modify this response.

The uptake of calcium ions by VSM cells is a pivotal event in the regulation of VSM cell contraction and thus vascular tone. The early stages of diabetes mellitus are characterised by impaired regulation of vascular tone, in particular, disturbed blood flow autoregulation

in the microcirculation. Such a disturbance allows abnormally high pressures experienced in the systemic circulation to be transmitted directly to the microcirculation, thereby contributing to microvascular injury. Blood flow autoregulation is predominantly a myogenic response to increased perfusion pressure within the vascular smooth muscle cells of the afferent arterioles. The myogenic response is in turn dependent on the stretch-activated influx of calcium ions and is fine tuned by pressor and vasodilator hormones . I hypothesised that elevated glucose concentrations could influence transmembrane calcium ion fluxes in VSM cells and this may in turn act to inhibit the myogenic response. Chapter 6 contains two published works in which this hypothesis has been tested and reported. The results of these experiments demonstrated that elevated glucose concentrations were capable of inhibiting VSM cell transmembrane calcium ion fluxes in response to a variety of stimuli. At the time, it was not possible to test whether glucose-induced PKC activation was involved in this response because the various PKC inhibitors available produced a variety of non-specific effects on calcium ion fluxes which made it impossible to draw consistent and confident conclusions. However, new isoform specific PKC inhibitors are now being developed that may improve the specificity of such experiments. For example, it now appears that elevated glucose concentrations predominantly activate PKC β_{II} in VSM cells (see chapter 7). As new isoform specific PKC inhibitors continue to be developed it should soon be possible to selectively inhibit glucose-induced increases in PKC β_{II} activity in VSM cells and confirm or refute the hypothesis that glucose-induced changes in calcium ion

transmembrane fluxes in VSM cells are mediated via yet another PKC dependent mechanism.

It has been remarkable how the glucose and PKC hypothesis has grown over the past five years and it is likely that additional aspects of cellular functions will be shown to be modified by glucose-induced PKC activity. In chapter 7, I have summarised these developments with regard to VSM cells in a recently review article published in *the Journal of Hypertension.*.

One of the most consistent features of diabetic vascular disease is the development of endothelial dysfunction. This manifests as increased vascular permeability and increased angiogenesis leading to neovascularisation. In the late 1980's a glycopeptide, variously known as vascular permeability factor (VPF), vascular endothelial growth factor (VEGF) or vasculotropin (for details see chapters 8-10), was described. It had unusual properties, not only was it a powerful endothelial cell specific growth factor, it was also the most potent vascular permeabilising agent ever identified. Much attention was then being focused on the potential role of VPF in the angiogenesis and permeability changes associated with malignancy. It seemed possible, however, that VPF may also play a role in the normal regulation of endothelial function in health and the pathogenesis of endothelial dysfunction in diabetes mellitus.

I developed a programme of research into VPF. Although this may initially appear to be something of a digression from the original theme of this thesis, the early work was

designed to learn more about VPF production by VSM cells before defining whether glucose-induced PKC activation may be involved in the regulation of VPF production by VSM cells. Simultaneously, I decided the time had come to focus our future studies on cultured cells of human origin.

In my laboratory in Leicester, we synthesised and sequenced a cDNA probe for VPF and examined whether human VSM cells and human endothelial cells expressed VPF mRNA. Human VSM cells expressed abundant quantities of a single transcript of VPF mRNA , whereas human endothelial cells did not. This to my knowledge was the first demonstration that human VSM cells expressed VPF mRNA. Moreover, this result was consistent with the concept that human VSM cells are a major source of VPF synthesis and we proposed that VPF released from these cells could act in a paracrine fashion to regulate endothelial function and growth. Chapter 8 contains the results of these studies, published in *Clinical Science*. This publication also contained the results of experiments which showed that VPF mRNA expression by human VSM cells was increased by exposure to growth factors such as foetal bovine serum and platelet-derived growth factor.

Encouraged by these early results, I next examined the effects of angiotensin II on VPF mRNA expression by human VSM cells. Angiotensin II has been widely implicated in the pathogenesis of the renal and vascular complications of diabetes mellitus. Chapter 9 comprises the results of these studies which were published as a "rapid communication" in the journal; *Hypertension*. These results revealed a novel action of angiotensin II,

notably its capacity to markedly increase VPF mRNA expression by VSM cells. We proposed that angiotensin II-induced VPF mRNA expression could account for many of the actions of angiotensin II on vascular permeability and endothelial dysfunction that had previously been attributed to the pressor actions of this peptide hormone.

These early studies had strengthened my belief that VPF may be playing a heretofore unrecognised role in regulating vascular endothelial function. The observation that angiotensin II could increase VPF mRNA expression by human VSM cells was particularly intriguing because both of this peptide hormone utilises an intracellular signalling pathway that involves PKC activation (see chapter 2). To examine whether PKC may be involved in the regulation of VPF mRNA expression, I conducted a simple study in which human VSM cells were exposed to phorbol myristate acetate (PMA), a phorbol ester that directly activates protein kinase C. The PMA markedly increased VPF mRNA expression suggesting that the VPF gene was PKC-responsive.

The aforementioned observations revealed the potential of elevated glucose concentrations to increase VPF mRNA expression via a PKC-dependent mechanism. Chapter 10 comprises the results of my latest studies which have been submitted to *the Journal of Clinical Investigation.* These studies confirmed that elevated glucose concentrations increase VPF mRNA expression via a PKC dependent mechanism. Furthermore, these studies also showed that increased VPF mRNA expression was associated with an increase in VPF peptide synthesis. This represents, to my knowledge, the first demonstration of VPF peptide synthesis by human VSM cells. Based on these

results, in chapter 10, I have proposed that glucose-driven increases in VPF production could provide an important mechanism for diabetes-induced endothelial dysfunction. Moreover, these results reveal another potentially important and novel cellular mechanism for glucose-induced vascular injury which is mediated via glucose-induced PKC activation.

The results contained in this thesis support the hypothesis that glucose-induced PKC activation may provide a final common path for a wide spectrum of VSM cell dysfunction and perhaps the dysfunction of other cell types as well. It would be easy to conclude that if this were true, then it should be possible to prevent diabetic complications by administering inhibitors of PKC activation to diabetic patients. The PKC inhibitors currently available are unsuitable for such an application. The majority of such agents are not PKC isoform specific and indiscriminately inhibit PKC activity. The fact that PKC is so ubiquitous and plays such a pivotal role in the regulation of so many cellular events suggests that indiscriminate PKC inhibition is likely to be lethal. Nevertheless, elevated glucose concentrations appear to activate a specific PKC isoform (PKC β_{II}) in vascular tissue (see chapter 7). The repertoire of cellular activities regulated by this particular isoform are unknown at present but should become clearer in the not too distant future when the PKC isoform specific inhibitors of PKC that are currently being developed are made available. It is conceivable that highly specific PKC inhibitors may ultimately provide a therapeutic approach, designed to target the pathological consequences of hyperglycaemia, rather than hyperglycaemia itself.

Our observation that elevated glucose concentrations also induce the expression of VPF mRNA and increase VPF peptide production suggests further therapeutic possibilities. Two receptors for VPF have now been cloned (see chapter 10) various biotechnology companies have programmes dedicated to the development of specific VPF receptor antagonists. If VPF is a major factor in the development of vascular permeability changes and neovascularisation in diabetes, the use of VPF receptor antagonists may provide an additional adjunctive therapy to limit diabetes-induced macrovascular and microvascular disease in the not too distant future.

In conclusion, the work contained in this thesis has highlighted a novel biochemical pathway via which elevated glucose concentrations could induce a wide spectrum of cellular dysfunction and injury. The introduction to this thesis has already emphasised the other glucose-induced mechanisms that have also been implicated in the pathogenesis of cellular injury. Glucose-induced PKC activation could be the sole explanation for some cellular disturbances but most likely forms part of a wider conspiracy in which all, or most, of the aforementioned glucose-induced mechanisms play a role at various stages in the evolution of the final pathology of vascular disease in diabetic patients.

Appendix I

Local and National Awards

in recognition of the research work contained in this thesis

- **1993; Doctor of the Year Award.** Awarded by the BUPA Medical Foundation, Ltd. "In recognition of an outstanding scientific contribution to the understanding of vascular disease in diabetes mellitus"

- **1994; The Frank May Medal and Lectureship.** Awarded by the Faculty of Medicine, University of Leicester School of Medicine.

- **1994; The Linacre Medical and Lectureship.** Awarded by the Royal College of Physicians of London.