Proinsulin C-peptide: Activation of Intracellular Signalling Pathways and Modulation of Transcription Factors in Opossum Kidney Proximal Tubular Cells.

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester.

By

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Abstract

In recent years an increasingly substantial body of data, supports a role for C-peptide in several biological activities. However, the precise molecular mechanisms of C-peptide action are not fully understood. The aim of this thesis was to study the intracellular signalling pathways and the transcription factors that C-peptide activates in proximal tubular cells using opossum kidney cells (OK) as a model.

Using specific inhibitors and phospho-specific antibodies, intracellular signalling pathways activated by C-peptide were examined by kinase assay and Western blotting. The results show that C-peptide is able to activate extracellular signal regulated kinase (ERK), phosphatidylinositol 3-kinase (PI 3-kinase) and PKC-α. ERK activation was attenuated by PKC inhibitor pre-treatment and activation of ERK and PKC-α were abolished in the absence of extracellular Ca^{2+}. Elevations of $[Ca^{2+}]_i$ were examined using confocal microscopy. C-peptide induced transient increase in $[Ca^{2+}]_i$ but the response of cells was variable. Thymidine incorporation assay was used to assess proliferation. C-peptide was found to be a functional mitogen in this cell type stimulating significantly increased cell proliferation.

Proliferator-activated receptor (PPAR) transcriptional activity was measured using a luciferase reporter assay in OK cells. C-peptide induced concentration-dependent stimulation of PPARγ activity. C-peptide also substantially augmented ciglitazone-stimulated PPARγ activity. GW9662, an irreversible PPARγ antagonist, blocked PPARγ activation by ciglitazone, but had no effect on C-peptide-stimulated PPARγ activity. C-peptide stimulation of PPARγ was attenuated by wortmannin pre-treatment, and by expression of a dominant negative PI 3-kinase p85 regulatory subunit (Δp85). C-peptide had no effect on protein expression levels of PPARγ. PPARγ phosphorylation was examined by $[^{32}P]$-orthophosphate labelling of OK cells and immunoprecipitation of phospho-PPARγ. C-peptide-induced PI 3-kinase dependent phosphorylation of PPARγ.

C-peptide is able to protect against tumor necrosis factor-alpha- (TNF-α) induced proximal tubular cells toxicity. Stimulation with 300ng/ml TNF-α for 24 hours resulted in significant reduction of cell viability which was reversed by pre-treatment with C-peptide. TNF-α induced apoptosis was detected by measuring histone associated DNA fragments and DNA nick end-labelling of OK cells. Incubation of cells with 300ng/ml TNF-α for 24 hours induced apoptosis, but C-peptide pr-etreatment protected against TNF-α induced apoptosis. The protective effects of C-peptide were associated with activation of nuclear factor κB (NFκB) and increased expression of TNF receptor-associated factor 2, the product of an NFκB-dependent survival gene. This was dependent upon activation of PI 3-kinase, but not ERK.

All C-peptide effects were abolished by pretreatment with PTX implicating a G-protein coupled receptor (GPCR), to either $G\alpha_i$ or $G\alpha_o$, in the transduction of these events. C-peptide increased $[^{35}S]$-GTPγS binding to $G\alpha_i$ in OK cell membranes. This study has now for the first time demonstrated specifically that $G\alpha_i$ proteins are activated by C-peptide binding to a GPCR.

Despite being ignored for many years it is now clear that C-peptide possesses important biological properties and may potentially protect against diabetic complications.

Publications arising from work described in this thesis:

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ABBREVIATIONS

3-OMG	3-O-methylglucose
A, Ala	Alanine
C, Cys	Cysteine
D, Asp	Aspartic acid
E,Glu	Glutamic acid
F, Phe	Phenylalanine
G,Gly	Glycine
H,His	Histidine
I,Ile	Isoleucine
K,Lys	Lysine
L,Leu	Leucine
M,Met	Methionine
N,Asn	Asparagine
P,Pro	Proline
Q,Gln	Glutamine
R,Arg	Arginine
S,Ser	Serine
T,Thr	Threonine
V,Val	Valine
W,Trp	Tryptophan
Y,Tyr	Tyrosine
Akt	protein kinase B, PKB
AP	alkaline phosphatase
AT1	angiotension receptor1

ATF1	activating transcription factor1
ATP	adenosine triphosphate
BADGE	bisphenyl A diglycidyle ether
BB/Wor	spontaneously Type 1 diabetic rat
β-gal	β-Galactosidase
Bt2cAMP	cAMP analogue
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
C-peptide	connecting peptide
CRE	cAMP response element
CREB	cAMP response element binding
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	egtazic acid
ELISA	enzyme linked immunosorbent assay
eNOS	endothelial oxide synthase
ERK	extracellular signal regulated kinase
FCS	foetal calf serum
fluo-3-AM	Fluo-3-acetoxymethyle ester
GDP	guanosine diphosphate
GFR	glomerular filtration rate
GPCR	G-protein-coupled-receptor
GSK3	glycogen synthase kinase
GTPγS	guanosine 5-[γ-thio]triphosphate

HBA1c	glycosylated haemoglobin
HEPES	N-2-[Hydroxyethyl]-piperazine N-2-ethanesulfonic acid
HPTC	human proximal tubular cells
IGFI, IGFII	insulin like growth factor I/II
IκB-α	inhibitory κB-alpha
IPTG	isopropyl β-D-thiogalactopyranoside
IR	insulin receptor
JNK	c-jun N-terminal kinase
KHB	Krebs-Hepes buffer
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
MTT	Methylthiazoleterazolum
Na^+-K^+-ATPase	sodium-potassium adenosine triphosphatase
NF-κB	nuclear factor κB
NO	nitric oxide
NOS	nitric oxide synthase
OK	opossum kidney
p38K	p38 mitogen-activated protein kinase
p90Rsk	ribosomal S6 kinase
PAGE	polyacrylamide gel electrophoresis
PC1, PC2, PC3	prohormone convertases 1/2/3
pCMX-PPARγ	plasmid encoding mouse PPAR-γ1
PDBU	phorbol ester 12, 13 dibutyrate
PI 3-kinase	phosphatidylinositol 3-kinase
PKC	protein kinase C

PMA	phorbol myristate acetate
pMEK1-CA	plasmid encoding constitutively active MEK-1
pMEK1-KD	plasmid encoding kinase dead mutant of MEK-1
PMSF	phenylmethylsulphonyl fluoride
pNFκB-luc	reporter plasmid of NFκB
pSVβgal	reporter plasmid of β-gal
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferators response elements
pPPRE-TK-luc	reporter plasmid of PPRE
PP1	protein Phosphatase 1
PTC	proximal tubular cells
PTX	pertussis toxin
Rh-C-peptide	rhodamine-labelled C-peptide
SC-peptide	scrambled C-peptide
SDS	sodium-dodecyl-sulphate
STZ	streptozotocin
TCA	Trichloroacitic acid
TEMED	N,N,N`,N`-Tetramethyl-ethylene diamine
TKA	tyrosine kinase activation
TNF-α	tumor necrosis factor alpha
TNF-R	TNF receptor
TRAF2	TNF receptor-associated factor2
TUNEL	terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate-biotin nick end labelling
TZDs	thizolidinediones

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CHAPTER 1
INTRODUCTION

The connecting peptide (C-peptide) is an enzymatic cleavage product derived from proinsulin during the biosynthesis of insulin. Since the discovery of the mode of insulin biosynthesis (Steiner and Oyer, 1967), it has generally been accepted that C-peptide does not possess biological activity of its own. However, accumulating evidence in recent years has challenged this view, indicating that C-peptide may have bioactivity.

1.1. C-peptide structure

C-peptide consists of 30-35 amino acids, depending on the species (**Fig 1.1**). It originates from the mid-portion of proinsulin, corresponding to the segment between the insulin A- and B-chains. It is a highly acidic peptide, with five acidic residues in human and up to seven in other species, without counterbalance from basic. The amino acid sequences of C-peptide from different species are more variable than those of the insulin A and B chains. Some species, including rat, have two isoforms of proinsulin and of C-peptide that also differ see (**Fig 1.1**). However, eight residues in C-peptide are relatively conserved between species. They correspond to the following positions in the human C-peptide: such as four residues at positions 6 (Gln), 12 (Leu), 26 (Leu), 31 (Gln) and residues at positions 1, 3, 11 and 27 all (Glu) (Wahren *et al.*, 2000; Wahren *et al.,* 2004). This type of partial structural conservation suggests that the acidic residues (especially Glu) are important for C-peptide bioactivity. Accordingly, one of these residues, Glu27, has been assigned particular importance on the basis of C-peptide membrane interactions (Pramanik *et al.,* 2001).

Although C-peptide has been thought to lack a stable secondary structure either within the proinsulin molecule or in its intact form, studies have indicated that it is not a random coil, but rather contains detectable ordered structure both when free or attached to insulin in proinsulin (Brems *et al.,* 1990). Moreover, the N-terminal 11 residues of C-peptide exhibit the characteristics of an α-helix in high concentrations of trifluoroethanol (Henriksson *et al.*, 2000), but helical formation is frequent also for other peptides in this solvent (Wahren, 2004). However, apart from these two studies many others have failed to detect ordered structures in the C-peptide structure. Amino acids 13-25, containing the five-glycine residues, have not been reported to have a stable secondary structure and the C-terminal five-residue segment (positions 27-31) lacks secondary structure in trifluoroethanol (Henriksson *et al.*, 2000).

Indeed, random structure in solution is compatible with overall interspecies variability of C-peptide. Thus, like many other small peptides, C-peptide appears to have a random coil structure (Ido *et al.,* 1997). This species variability and the lack of defined conformational properties made it difficult to understand the molecular mechanisms of C-peptide action. Hormones often exhibit structural conservation of their binding segments, which is not the case for C-peptide. Nevertheless, C-peptide is not unique in its variability. Other peptide hormones, including relaxin of the same peptide family as insulin and C-peptide are even more variable than C-peptide (Wahren *et al.,* 2004). Thus the structural variability of C-peptide is large but not exceptional compared to other bioactive-peptides, and does not exclude hormonal action and specific binding of C-peptide to cell membranes (Rigler *et al.,* 1999).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
HUMAN	Glu	Ala	Glu	Asp	Leu	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
MONKEY	Glu	Ala	Glu	Asp	Pro	Gln	Val	Gly	Gln	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Ser	Leu	Glen	Pro	Leu	Ala	Leu	Glu	Gly	Ser	Leu	Gln
HORSE	Glu	Ala	Glu	Asp	Pro	Gln	Val	Gly	Glu	Val	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Leu	Gly	Gly	Leu	Gln	Pro	Leu	Ala	Leu	Ala	Gly	Pro	Gln	Gln
PIG	Glu	Ala	Glu	Asn	Pro	Gln	Ala	Gly	Ala	Val	Glu	Leu	Gly	Gly	Gly	Leu	Gly	---	Gly	---	Leu	Gln	Ala	Leu	Ala	Leu	Glu	Gly	Pro	Pro	Gln
Cow.Lamb	Glu	Val	Glu	Gly	pro	Gln	Val	Gly	Ala	Leu	Glu	Leu	Ala	Gly	Gly	pro	G:y	Ala	Gly	Gly	Leu	----	----	---	---	----	Glu	Gly	Pro	Pro	Gln
RABBIT	Glu	Val	Glu	Glu	Leu	Gln	Val	Gly	Gln	Ala	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Gly	Leu	Glen	Pro	Ser	Ala	Leu	Glu	----	Ala	Leu	Gln
Dog	Glu	Val	Glu	Asp	Leu	Gln	Val	Arg	Asp	Val	Glu	Leu	Ala	Gly	Ala	Pro	Gly	Glu	Gly	Gly	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Gly	Ala	Leu	Gln
RAT I	Glu	Val	Glu	Asp	Pro	Gln	Val	Pro	Gln	Leu	Glu	Leu	Gly	Gly	Gly	Pro	Glu	Ala	Gly	Asp	Leu	Gln	Thr	Leu	Ala	/leu	Glu	Val	Ala	Arg	Gln
RAT II	Glu	Val	Glu	Asp	Pro	Gln	Val	Ala	Gln	Leu	Glu	Leu	Gly	Gly	Gly	Pro	Gly	Ala	Gly	Asp	Leu	Gln	Thr	Leu	Ala	Leu	Glu	Val	Ala	Arg	Gln
GUINA PIG	Glu	Leu	Glu	Asp	Pro	Gln	Val	Gly	Gln	Ala	Asp	Pro	Gly	Val	Val	Pro	Glu	Ala	Gly	Arg	Leu	Gln	Pro	Leu	Ala	Leu	Glu	Met	Thr	Leu	Gln

Fig 1.1. Linear representation of the sequences of proinsulin C-peptide from different mammalian species. The amino acids sequences are numbered as shown (Steiner, 2004).

1.2. Role of C-peptide in insulin biosynthesis

Insulin is a globular protein hormone consisting of an acidic A-chain of 21 residues, and a basic B-chain of 30 amino acids (Steiner *et al.,* 1967) (**Fig 1.2**). The A-chain contains two α-helices (Ile^{A2} –Thr^{A8} and Leu^{A13}-Tyr^{A19}, designated helix 2 and helix 3, respectively), and the B-chain consists of a central α-helix (Ser^{B9} –Cys^{B19}, designated helix 1) with extended N- and C-terminal segments. In its native structure, the N terminus of the A-chain is closely proximate to the C-terminus of the B-chain. The A and B chains are tethered by two interchain disulfide bridges (A7-B7 and A20-B19). The third disulfide bridge (A6-A11) is intra-A-chain disulfide (Hua *et al.*, 2002). The three-disulfide bonds are important in maintaining the native conformation and the biological activities of the insulin molecule (Hua *et al.,* 2001).

Although the functional form of human insulin comprises a double chain, human insulin is synthesed *in vivo* as a single-chain preproinsulin with a signal peptide at the N terminus of the B-chain, and a connecting peptide between the B- and A-chain (Bell *et al.*, 1980). Following cleavage of the N-terminal signal sequence in the endoplasmic reticulum, the nascent polypeptide folds and is packaged into secretory granules as proinsulin. The C terminus of the B-chain is connected to the N-terminus of the A-chain by a 35 amino acids peptide consisting of two bibasic amino acids at each end of the C-peptide of 31 amino acids (Zhi *et al.*, 2003). The amino acid sequence of human proinsulin is shown in (**Fig 1.2**).

The conversion of proinsulin to insulin, like the processing of many other neuroendocrine precursors, occurs through the combined action of the pro-hormone convertases PC2 and PC1/PC3. These are serine endoproteases having a

high degree of specificity for cleavage of paired basic amino acid. The sites of cleavage are usually identifiable by pairs of basic residues (Lys-Arg and Arg-Arg especially) but mono or polybasic cleavage has also been reported in some species e.g. dog (Kwok *et al.*, 1983; Smeekens *et al.*, 1992; Zhou *et al.*, 1999; Steiner, 2004). Their action is followed by that of carboxypeptidase-E, a carboxypeptidase-B-like enzyme having a high specificity for C-terminal basic residues (Fricker *et al.*, 1986). The latter enzyme removes the C-terminal basic amino acids exposed by the endoproteases, resulting in the generation of native insulin and C-peptide free of any linking basic residues (Steiner, 2004). Under normal conditions, the processing of proinsulin in the secretory granules is highly efficient, yielding over 95% insulin and C-peptide, with only small residual of proinsulin and intermediates (Steiner *et al.*, 2004).

C-peptide plays a key role in insulin biosynthesis by ensuring the efficient and correct pairing of the A and B chains during the folding of proinsulin within the endoplasmic reticulum. Deletions or alanine replacements of the acidic residues at positions 1, 3, and 4 of the human C-peptide impair proinsulin folding *in vitro*. Thus, the highly conserved acidic N-terminal part of C-peptide is very important for insulin precursor folding and C-peptide may have some intermolecular chaperone-like function in the folding of insulin precursor (Chen *et al.*, 2002). Moreover, C-peptide also may facilitate its own excision from proinsulin during the maturation to insulin. The length and flexibility of the C-peptide contributes importantly in this regard (Lipkind and Steiner, 1999). The extended C-peptide, especially after being freed from its B-chain attachment by prior cleavage of this site by PC1/3 (Zhu *et al.*, 2002), may be able to exert sufficient traction on A-chain helix to assist this structural transition, which allows

PC2 to complete the conversion process. Furthermore, C-peptide may play a role in stabilising secretory granules, as it retained in soluble phase in the granules where it may interact with and/or stabilise other secretory products such as islet amyloid polypeptide (Steiner *et al.,* 2004). C-peptide has been reported to retard aggregation of human islet amyloid polypeptide into amyloid fibrils *in vitro* (Westermark *et al.,* 1996). Since there are a large number of soluble molecules, including peptides, nucleotides, amino acids, and ions, within the secretory granules, the C-peptide clearly must maintain compatibility with this environment.

Therefore, the main function of C-peptide in insulin biosynthesis is to facilitate the folding of the proinsulin molecule and the generation of the disulfide bonds between the cysteine amino acid residues of A and B chain of insulin molecule. After cleavage of proinsulin molecule, C-peptide and insulin are released in equimolar quantities into portal circulation along with small amounts of proinsulin and its intermediate forms (Gutman *et al.*, 1972).

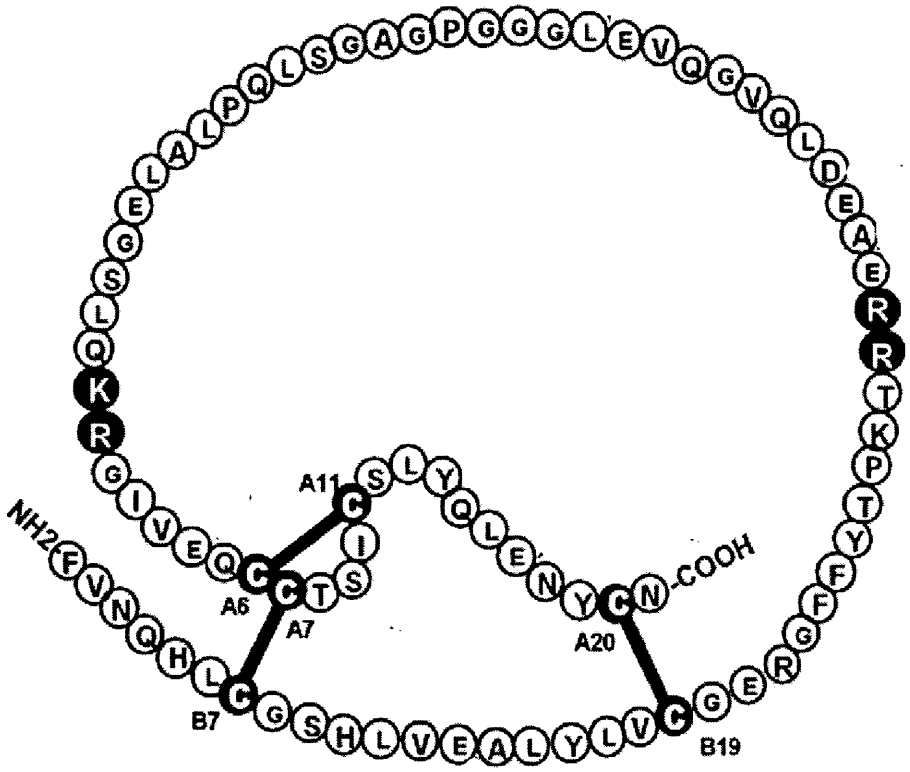


Fig 1.2. Amino acid sequence and disulfide linkage pattern of human proinsulin. Amino acids are shown in one-letter code, and cysteine residues are shown as dark sold circle and two bibasic amino acids at each end of the C-peptide of 31 amino acids are represented as the dark amino acids. Nomenclature of the amino acids in human proinsulin refers to that of insulin when related to the A- and B-chain (Zhi *et al.*, 2003).

1.3. Pharmacokinetics of C-peptide

The pancreatic beta cell not only synthesizes and stores insulin and C-peptide, but is also responsible for degradation of these products if they are not released (Halban, 1991). Such degradation occurs by crinophagy (Orci *et al.*, 1984). This process, common to all secretory cells, involves fusion of secretory granules with lysosomes resulting in the formation of multigranular bodies (Halban, 1991), the classic degradative compartment for granular products. Once insulin and C-peptide have been targeted to this degradative compartment, they are no longer subject to regulated secretion.

Insulin from most animal species exists in crystal form within granules and the relative stability of insulin within multigranular bodies has been attributed to its crystal state (Orci *et al.*, 1984; Halban *et al.*, 1987). This is not the case for C-peptide, which is located in the halo of the secretory granule (Orci *et al.*, 1984), is soluble, and is thus degraded very rapidly once introduced into the lysosome (Orci *et al.*, 1984; Halban *et al.*, 1987). The result of this notable difference in stability of these two molecules is an elevated ratio of insulin to C-peptide in extracts of beta cells (Rhodes and Halban, 1988).

It is commonly assumed that both insulin and C-peptide are equally stable within beta cell secretory granules and that they will thus be secreted in equimolar amounts. However, recent study has shown that this not always the case. In transformed beta cells, C-peptide but not insulin is lost from secretory granules (Neerman-Arbaz and Halban, 1993). Such selective loss results in the disproportionate release of insulin relative to C-peptide. In human insulinoma cells and rat beta cells, C-peptide may actually be partially degraded intracellularly prior to secretion from the beta cell. The mechanism responsible

involves proteolytic cleavage in the C-terminal region of the peptide (Conlon *et al.*, 1995).

In rats, truncation of C-peptide within beta-cell granules has also been reported. This results in the secretion of approximately 10% of total C-peptide as a form without its five C-terminal amino acids (des-[27-31]-C-peptide) along with the other conventional secretory products, proinsulin and insulin (Verchere *et al.*, 1996). In contrast to the situation in rats, des-[27-31]-C-peptide is not a major secretory product of human islets and its contribution to total circulating C-peptide is not increased in Type 2 diabetes or patients with insulinoma (Paoletta *et al.*, 2002).

There is only little information known about the pharmacokinetics of C-peptide's regional distribution and catabolism. In animals (Katz and Rubenstein, 1973; Katz *et al.*, 1975; Oyama *et al.*, 1975) as well as in humans (Kuzuya and Matsuda, 1976), the turn-over of C-peptide is slower than that of insulin. For example, the plasma half-life of C-peptide in humans is ~ 30 minutes (Faber *et al.*, 1978), as compared to no more than 6 minutes for insulin (Horwitz *et al.*, 1975). Unlike insulin, C-peptide is not significantly extracted by the liver. Approximately 30-50% of secreted C-peptide is removed and catabolised by kidneys and about 20% by skeletal muscle (Sjoberg *et al.*, 1991; Polonsky *et al.*, 1983; Bratusch-Marrain *et al.*, 1984, Johansson *et al.*, 1992b). Release of C-peptide in skeletal muscle venous effluent has been observed during physical exercise, suggesting that C-peptide may be reversibly retained by the endothelium (Johansson *et al.*, 1992b). Recent studies of C-peptide biodistribution performed in monkey confirm that the kidneys are the major sites of radioactive C-peptide distribution, less to the heart and to some extent to the liver (Fredriksson *et al.*,

2002). The metabolic clearance rate of C-peptide is independent of the plasma concentration and is similar in healthy subjects and patients with Type 1 diabetes (Faber *et al.,* 1978).

Since C-peptide and insulin are thought to be secreted in equimolar concentrations from the beta cell (Rubenstein *et al.,* 1969), it has been suggested that the peripheral concentration of C-peptide may reflect the portal insulin concentration than does the peripheral insulin concentration. The validity of use of peripheral C-peptide concentration is dependent on the demonstration that its hepatic extraction is consistently negligible, its metabolic clearance rate linear under normal physiological conditions and that its distribution kinetics that allow plasma concentrations to accurately reflect secretion rates (Polonsky *et al.,* 1983). Despite these limitations, peripheral C-peptide concentrations are still very useful for clinical assessments of endogenous insulin secretion.

1.4. Physiological effects of C-peptide

For patients with diabetes mellitus chronic complications can be devastating. Cardiovascular disease is the major cause of morbidity and mortality among these patients, encompassing macrovascular disease, with heart attacks, strokes and limb ischeamia together with microvascular disease, manifest as retinopathy, nephropathy, and neuropathy (Vinik & Vinik, 2003). Persisting residual beta cell function in early stages of diabetes is associated with a lower incidence of microvascular complications (Sjoberg *et al.*, 1987; Kobbah *et al.*, 1988; Kernell *et al.*, 1990). This correlation has been shown to be more marked than that observed between glycemic control and microvascular lesions (Kernell *et al.*, 1990). Despite rigorous efforts to control blood glucose levels with insulin in diabetic patients, the late diabetic complications may be delayed but not completely prevented (Madsbad, 1983). Since the biological effects of exogenous and endogenous insulin are not likely to be different, additional factors are likely involved in progress of these complications. A lack of C-peptide may be one such factor.

In healthy humans, C-peptide infusion has no measurable effects possibly because receptors are saturated at ambient C-peptide concentration (Rigler *et al.*, 1999). Conversely, C-peptide does exert measurable physiological effects in Type 1 diabetes mellitus, a situation with complete insulin and C-peptide deprivation (Samnegard *et al.*, 2001).

1.4.1. Effects on glucose utilization

Early on, C-peptide was investigated for insulin like effects on blood glucose levels and on glucose disposal after glucose loading, but none was established (Hoogwerf *et al.*, 1986; Kitabchi., 1977; Wahren *et al.*, 2000), and the

general view prevailed that C-peptide was without biological effect. However, some animal studies indicated that C-peptide might reduce the insulin and glucagon response after beta cell stimulation with glucose or arginine and that it was able to slightly increase and prolong the hypoglycemic effect of exogenous insulin (Toyota *et al.*, 1975; Wojcikowski *et al.*, 1983).

In vitro studies

These findings are supported by studies that examined the influence of C-peptide on 3-O-methylglucose (3-OMG) transport in skeletal muscle *in vitro* (Zierath *et al.*, 1991; Zierath *et al.*, 1996). C-peptide was capable of stimulating of 3-OMG transport in incubated muscle strips from both healthy subjects and patient with Type 1 diabetes in a concentration-dependent manner. The maximal C-peptide effect, seen at 1nM, was approximately two thirds of the corresponding insulin effect. The mechanism by which this stimulation occurs is not apparent; C-peptide did not displace [^{125}I] insulin binding from partially purified insulin receptor (IR), nor did it activate receptor tyrosine kinase activity (TKA) (Zierath *et al.*, 1996). Furthermore, β-adrenergic receptor stimulation with isoproterenol inhibited the insulin but not the C-peptide-mediated increase in glucose transport (Zierath *et al.*, 1996). In contrast, the cAMP analogue, Bt2cAMP, abolished the stimulation by insulin and C-peptide on glucose transport. Thus, C-peptide stimulated skeletal muscle glucose transport appears to occur via a mechanism that is cAMP-inhibitable, and independent of the IR and of TKA (Zierath *et al.*, 1996).

Furthermore, C-peptide is reported to stimulate glycogen synthesis in isolated human muscle strips (Zierath *et al.*, 1991). This is in agreement with recent data from studies using L6 rat skeletal myoblasts, which show that C-

peptide, at physiological concentrations, mimics insulin effects such as glycogen synthesis and amino acid uptake in rat muscle cells (Grunberger *et al.*, 2001). However, similar effects were not confirmed in mouse soleus muscles (Shashkin *et al.*, 1997).

In vivo studies

Influences of C-peptide on glucose utilization are also seen *in vivo* studies of streptozotocin (STZ) diabetic rats (Li *et al.*, 1999; Wu *et al.*, 1996). Supra-physiological concentrations of human C-peptide were found to elicit marked an increase in whole body glucose utilization, whereas scrambled C-peptide (sC-peptide, a 31 amino acid peptide with its residues arranged in random order) had no effect (Wu *et al.*, 1996). Physiological concentration of rat C-peptides 1 and 2, which differ at two positions 8 and 17 (each consist of 31 amino acids with 21 and 22 amino acids being identical between human C-peptide and rat C-peptide 1 and 2, respectively) were found to be equally potent in stimulating whole body glucose utilization in diabetic animals. Raising the C-peptide concentration two to three fold caused doubling of the basal glucose utilization in diabetic but no effect in healthy rats (Li *et al.*, 1999). A major proportion of the C-peptide–induced stimulation of glucose utilization was blocked by treatment with *N*-monomethyl-L-arginine, suggesting that the influence of C-peptide on glucose utilization may be mediated by nitric oxide (NO) (Li *et al.*, 1999). Furthermore, the effect of five C-peptide fragments and the native peptide on whole-body glucose turnover was studied in STZ diabetic rats (Sato *et al.*, 2004). Rat C-peptide 2 and fragments comparising residues 27-31 and 28-31 were effective in augmenting glucose turnover in diabetic rats from +100% to 150%, while no significant effects were seen for segments 1-26, 11-19 and 11-15. Thus, C-terminal tetra- and

pentapeptides, but not fragments from the middle segment of C-peptide, are as effective as the full-length peptide in stimulating whole-body glucose turnover.

The stimulatory influences of C-peptide on whole body glucose appear to be less prominent in humans. Whole-body glucose utilization in patients with Type 1 diabetes increased slightly after intravenous administration C-peptide (Johansson *et al.*, 1992a). In addition, a considerable increase in glucose uptake by exercising muscle was found in patients with Type 1 diabetes during infusion of C-peptide compared to saline infusion. No effect of C-peptide was seen in healthy controls (Johansson *et al.*, 1992b). Moreover, when C-peptide was infused together with insulin in patients with Type 1 diabetes, the resultant fall in blood glucose was more marked than when insulin only is administered (Oskarsson *et al.*, 1997).

The question arises whether the short-term effects of C-peptide on glucose utilization in patients with Type 1 diabetes, observed in some but not all studies (Frost *et al.*, 2002), will result in lower blood glucose level and /or diminished insulin requirements during long-term C-peptide administration. Although there are no direct studies to address this question, this does not seem to be the case. In clinical studies involving subcutaneous injection of C-peptide for 1 or 3 months in patients with Type 1 diabetes, the responses were similar during the two treatment periods. There were no statistically significant differences between the treatment groups with regard to blood glucose, glycosylated hemoglobin A1c (HbA1c) or fructosamine (Johansson *et al.*, 2000). Similarly, subcutaneous injection of C-peptide for 3 months did not affect the level of metabolic control as reflected by HbA1c in patients with Type 1 diabetes and normal subjects (Ekberg *et al.*, 2003). Moreover, more prolonged C-peptide replacement was examined in Type 1

diabetes BB/Wor-rats (Kamiya *et al.,* 2004). C-peptide rat 2 replacements from the onset of diabetes for 8 months had no effect on hyperglycemia.

In summary, there is no clear-cut effect of C-peptide on blood glucose concentrations and it remains to be elucidated whether C-peptide has a clinically relevant metabolic effect in human.

1.4.2. Effects on nerve function

Diabetic neuropathy is a group of disorders and as such the most common chronic complication affecting both Type 1 and 2 diabetes (for review, see Sima, 2003: Li and Sima, 2004). This can affect both the somatic and autonomic nervous systems and is characterized by decreased nerve conduction velocity, abnormal cardiovascular reflexes and vascular dysfuction (Dyck *et al.*, 1986). Most data concerning the early development of diabetic neuropathy have obtained from animal models. Hyperglycemic STZ-induced diabetes in rats or spontaneously Type 1 diabetic BB/Wor rats show, within weeks of onset of diabetes, significant decreases in motor and sensory nerve conduction velocities, increased activity of polyol-pathway, decreased endoneurial blood flow, and impaired neural sodium-potassium adenosine triphosphatase (Na^+-K^+-ATPase) and nitric oxide synthase (NOS) activities (Sima., 2003; Sima and Sugimoto., 1999). The functional deficits at this early metabolic stage of diabetic neuropathy and the associated early structural changes are fully reversible (Li and Sima, 2004).

It is well documented that strict metabolic control will retard but not prevent the development of diabetic neuropathy, indicating that other factors in addition to hyperglycemia are importance (Wahren *et al.,* 2004). No therapy for diabetic neuropathy has yet been established. Several compounds have been

evaluated, most of them directed towards alleviation of the metabolic consequences of hyperglycemia such as aldose reductase inhibitors and agents that inhibit the formation of advanced glycation end products. However, none has been successful so far, due to limited efficacy as well as the occurrence of adverse reaction (Jaspan *et al.,* 1986). Evidence accumulates that replacement of C-peptide in Type 1 diabetes prevents and may improve diabetic neuropathy.

Effects on peripheral nerves

Animal studies

Studies in animal model of Type 1 diabetes, demonstrated that C-peptide in replacement doses has the ability to improve peripheral nerve function and prevent or reverse the development of nerve structural changes. Administration of homologous C-peptide for 2 months starting at the onset of diabetes in BB/Wor rats resulted in significant improvements in caudal motor nerve conduction velocity compared to diabetic control animals (Sima *et al.*, 2001). Administration of homologous C-peptide for 8 months improved caudal motor nerve conduction velocity and reduced early structural abnormalities such as paranodal swelling, axoglial dysjunction and paranodal demyelination of sural nerve fibers (Sima *et al.*, 2001). The introduction of C-peptide treatment after 5 months of diabetes, when neuropathy had become established, led to significant improvement in caudal motor nerve conduction velocity and marked repair of diabetes-induced axonal degeneration and increased fiber regeneration (Sima *et al.*, 2001).

The same effects on nerve function and structure in the diabetic rat could be elicited by human C-peptide but at higher concentration (Ido *et al.,* 1997). Human C-peptide prevented decreased caudal motor nerve conduction velocity, but had no effect on motor nerve conduction velocity in healthy control rats (Ido

et al., 1997). Increasing doses of human C-peptide for 2 months in Type 1 diabetic neuropathy, BB/Wor-rats, resulted in gradually less marked early structural abnormalities and a rising frequency of regenerating sural nerve fibers, demonstrating not only cross-species activity of human C-peptide but also dose dependency of the C-peptide effect (Zhang *et al.,* 2001).

A further recent study examining the effect of C-peptide in sensory and motor nerve function has been evaluated using STZ diabetic rats (Cotter *et al.*, 2003). Infusion of C-peptide for 2 weeks, starting at 6 weeks after induction of diabetes resulted in significant improvements in both motor (sciatic) and sensory (saphenous) nerve conduction velocities. In the same study, co-treatment of STZ-induced diabetic rats with NOS inhibitor N (G)-nitro-L-arginine markedly attenuated the effect of C-peptide on nerve function, suggesting the involvement of NO in C-peptide effects on neuropathy (Cotter *et al.*, 2003).

More recently a study of C-peptide on unmyelinated fiber function in the hind paw, sural C-fiber morphometry, sciatic nerve neurotrophins, and the expression neurotrophic receptor and content of neuropeptides in dorsal root ganglia in Type 1 diabetic BB/Wor-rats was reported (Kamiya *et al.,* 2004). C-peptide replacement from the onset of diabetes significantly prevented progressive thermal hyperalgesia and prevented C-fiber atrophy, degeneration, and loss. These findings were associated with preventive effects on impaired availability of nerve growth factor and neurotrophin 3 in the sciatic nerve and significant prevention of perturbed expression of insulin like growth factor-I (IGF-I), nerve growth factor and neurotrophin 3 receptors in dorsal root ganglion cells. These beneficial effects translated into prevention of the decreased content of dorsal root ganglia

nociceptive peptide such as substance P and calcitonin gene-related peptide (Kamiya *et al.*, 2004).

Patient studies

Beneficial effects of C-peptide on nerve function have also been demonstrated in patients. In a randomized double-blind placebo-controlled study, patients with Type 1 diabetes without overt symptoms of neuropathy and with average diabetes duration of 10 years were given C-peptide or placebo together with their regular insulin regimen for 3 months (Ekberg *et al.*, 2003). At the onset of the study, both sensory and motor nerve conduction velocities were reduced compared to healthy controls. A significant increase in sensory nerve conduction velocity was recorded after 3 months in C-peptide group. Vibration perception thresholds improved but the motor nerve conduction velocity remained unchanged (Ekberg *et al.*, 2003). Similar findings have been reported for a smaller group of patients; in whom lowered thermal perception thresholds were observed after 3 months of C-peptide replacement suggesting improvement of sensory nerve function (Johansson *et al.*, 2000).

Effects on autonomic nervous system

The possible influence of C-peptide on autonomic nerve function (respiratory heart rate variability, acceleration and brake index during tilting) and quantitative sensory threshold determinations, in addition to nerve conduction velocity have been examined in patients with Type 1 diabetes (Johansson *et al.*, 1996). Patients were studied twice under normoglycemic condition and during a 3 hours intravenous infusion of either human C-peptide or saline in a double-blind study. Plasma concentrations of C-peptide during the infusion reached levels within the physiological range. The patients showed amelioration of sensory

neuropathy as evidenced by significantly improved temperature threshold discrimination and heart rate variability during deep breathing (an index of autonomic, primarily vagal, nerve activity (Johansson *et al.*, 1996). A significant improvement was also seen in the brake index during tilting in those patients who showed a reduced index before the study.

Autonomic nerve function has also been evaluated in patients with Type 1 diabetes receiving C-peptide replacement for 3 months in a double-blind cross-over study (Johansson *et al.*, 2000). A significant improvement in heart rate variability was recorded during C-peptide administration, while no change or slight deterioration was observed in the same patients during the control period.

C-peptide influences on sympathetic nerve activity directly or indirectly have been studied in rats (Okamoto *et al.,* 2000). C-peptide administration counteracts the sympathetic nerve-mediated suppression of splenic lymphocyte proliferation in an atropine-sensitive manner. This effect may be through actions of C-peptide on the vagal nerves, but a direct action on central nervous system has been also proposed. In addition, it has been suggested, from studies in STZ-diabetic rats, that insulin-mediated increases in sympathetic nerve activity is attenuated by C-peptide (Rizk and Dunbar, 2004). In agreement with these findings intravenous administration of C-peptide to rats was found to increase the rate of discharge of vagus nerves innervating the stomach and pancreas (Kimura *et al.,* 2005). Moreover, C-peptide increased gastric acid output, which is under the control of vagal nerve (Ohno *et al.,* 1987), and inhibited by atropine (Kimura *et al.,* 2005). These findings could explain, at least in part, the ameliorating effects of C-peptide on impaired cardiac autonomic nerve functions in patients with Type 1 diabetes.

C-peptide and pathogenesis of diabetic neuropathy

C-peptide exerts beneficial effects on diabetic neuropathy but the mechanisms behind these are not fully understood. C-peptide replacement in Type 1 BB/Wor-rat does not influence the polyol-pathway or oxidative stress but ameliorates the Na^+-K^+-ATPase abnormality in a dose-dependent manner (Zhang *et al.,* 2001). Therefore, it is possible that C-peptide's ability to partially correct the reduction in Na^+-K^+-ATPase activity that accompanies experimental diabetes (Ido *et al.,* 1997; Sima *et al.,* 2001) contributes to the C-peptide's positive effects (see section 1.7.1). In addition, direct measurements of sciatic nerve blood flow in STZ-diabetic rats show that C-peptide administration resulted in a significant amelioration of the diabetes-induced decrease in nerve blood flow; this effect was elicited via augmented NO-release in endoneural blood vessels (Cotter *et al.,* 2003) and is probably related to both increased stimulation and expression of endothelial nitric oxide synthase (eNOS) (Kitamura *et al.,* 2003; Wallerath *et al.,* 2003). Moreover, C-peptide replacement in Type 1 diabetic BB/Wor rat prevented oxidative stress and poly (ADP-ribose) polymerase-related apoptotic activities. C-peptide partially ameliorated apoptotic stresses mediated via impaired insulin and IGF activities. These findings were associated with the prevention of increased expression of pro-apoptotic Bcl2 family member (Bax) and active caspase 3 and the frequency of caspase 3-postive neurons (Sima and Li., 2005). Similarly, *in vitro* studies indicate that C-peptide in synergism with insulin stimulated neuroblastoma cell proliferation and neurite outgrowth and decreased glucose-induced apoptosis. C-peptide in presence of insulin also enhanced the expression and translocation of nuclear factor κB (NF-κB) and promoted Bcl2 expression in neuroblastoma cells (Li *et al.,* 2003).

1.4.3. Effects on circulation

Numerous functional and structural microvascular disturbances have been reported in patients suffering from Type 1 diabetes. These microvascular disturbances are thought to be implicated in the development of diabetic complications, such as retinopathy, neuropathy and nephropathy, and the development of foot ulceration. The microcirculation is concerned with the transport and exchange of nutrients and waste products of metabolism, tissue defense and repair (Barbier *et al.,* 1994).

Early Type 1 diabetes is characterised by increased microvascular blood flow, increased shear stress, and tangential pressure on microvascular endothelium (Forst and Kunt, 2004). In consequence, microvascular sclerosis occurs, involving basement membrane thickening as well as arteriolar hyalinosis, which limits the capacity of the microcirculation to dilate at times of increased demand (Tooke, 1995). The etiology of diabetic microvascular dysfunction is not fully understood. However, many pathogenic factors appear to be involved. Functional abnormalities of the microvasculature, such as disturbed neurovascular responses (Forst *et al.*, 1997; Netten *et al.*, 1996), alterations in endothelial function (Johnstone *et al.*, 1993), and increased intracapillary pressure (Sandemann *et al.*, 1992). Furthermore, several haemorheological disturbances have been described in patients with diabetes mellitus. These include increased in leucocyte-endothelial interactions (Kunt *et al.,* 1998), increased blood viscosity (Ernst and Matrai, 1986; Barnes *et al.,* 1977), and changes in the rheologic properties of red blood cells (Finotti and Palatini, 1986; McMillan *et al.*, 1998). These abnormalities may contribute to alterations in blood flow and may precede

structural vascular changes that occur later in the course of the microvascular disease (Rayman *et al.,* 1995; Forst and Kunt, 2004).

Accumulating evidence suggests that proinsulin C-peptide may exert biological effects on the microcirculation. Forearm blood flow in patients with Type 1 diabetes increased in response to intravenous infusion of C-peptide in a concentration dependent manner in the range 0-1nM. No additional circulatory effects of C-peptide could be seen when the concentration was raised above 1nM (Ekberg *et al.,* 2001). These findings are in consistent with a study showed that intravenous administration of C-peptide during exercise increased forearm blood flow by 27% and capillary diffusion capacity by 52% to levels similar to those observed in the healthy controls. Moreover, oxygen uptake by the exercising forearm muscles was increased. No significant changes in blood flow were observed in the healthy controls receiving C-peptide or in the diabetic patients receiving placebo infusions (Johansson *et al.*, 1992b).

In aortic endothelial cells C-peptide had the capacity to stimulate eNOS activity via a calcium-dependent mechanism (Wallerath *et al.,* 2003). This effect has also been confirmed *in vivo* where infusion of C-peptide into the brachial artery of patients with Type 1 diabetes resulted in an immediate blood flow increase of approximately 35% (Johansson *et al.,* 2003). The stimulatory effect of C-peptide was abolished when an eNOS inhibitor was coinfused. C-peptide-induced improvement in blood flow has been reported not only for forearm tissue, which is mostly muscle (Johansson *et al.*, 1992b; Ekberg *et al.,* 2001; Johansson *et al.*, 2003) but also for kidney (Johansson *et al.,* 1992a) and nerve (Cotter and Cameron, 2001).

Skin blood flow is found to be altered early after diagnosis of diabetes mellitus (Tooke *et al.,* 1995; Flynn and Tooke, 1992). Although total skin blood flow is increased in diabetes mellitus, nutritional capillary skin blood flow was shown to be reduced in diabetic patients (Jorneskog *et al.,* 1990; Jorneskog *et al.,* 1995). Forst *et al* (1998) demonstrated that C-peptide increased skin microcirculation in patients with Type 1 diabetes. Infusion of C-peptide resulted in redistribution of skin microvascular blood flow to levels comparable to those in healthy subjects.

C-peptide influences on endothelial function of large arteries has been examined in Type 1 diabetes, using ultrasound measurements of brachial artery blood flow and vascular diameter during reactive hyperemia. C-peptide administration resulted in augmented basal blood flow but the response to reactive hyperemia was unchanged, indicating that C-peptide exerts its effect primarily on the distal resistance vessels (Fernqvist-Forbes *et al.*, 2001).

Recently, the effects of C-peptide on myocardial blood flow and function were assessed in patients with Type 1 diabetes without signs of heart disease in the basal state (Hansen *et al.*, 2002) and during adenosine-induced myocardial hyperemia (Johansson *et al.,* 2004). Short-term replacement of C-peptide improved impaired myocardial function and increased myocardial blood flow in the basal state and during adenosine-induced myocardial hyperemia. Both studies also showed that C-peptide-induced improvements in left ventricular performance as reflected by augmented rates ventricular contraction and relaxation (Hansen *et al.*, 2002), and increased left ventricular ejection fraction and stroke volume (Johansson *et al.,* 2004).

C-peptide's circulatory effects also involved rheological factors. (Pre-incubation of diabetic blood samples with various concentrations of human C-peptide for 8 hours restored the deformability of erythrocytes towards that of control samples (Kunt *et al.,* 1999). Ouabain inhibited this effect, thus C-peptide influences on erythrocyte may be mediated by the restoration of Na^+-K^+-ATPase activity, known to be attenuated in diabetic patients (Raccah *et al.*, 1992).

In conclusion, the available evidence indicates that in animal models of diabetes and in Type 1 diabetic patients, C-peptide stimulates eNOS and local release of NO, resulting in augmented blood flow in several tissues. Improved rheological conditions may also contribute to the flow augmentation.

1.4.4. Renal effects of C-peptide

Effects on renal function

Diabetic nephropathy is one of the major causes of end-stage renal disease in Western world. About 40% of all Type 1 diabetic patients develop diabetic nephropathy (Borch-Johnson *et al.,* 1985). Several large studies have shown that the progression of diabetic nephropathy may be reduced, but not prevented by strict metabolic control and antihypertensive treatment (Lehmann and Schleicher, 2000). Therefore, a comprehensive understanding of the pathophysiology of diabetic nephropathy is necessary to develop innovative therapeutic approach to prevent its development and progression. Although initially thought that renal injury in diabetic nephropathy is mainly caused by haemodynamic alterations such as hyperfiltration and hyperperfusion, there is now evidence that these changes are only one aspect of a complex series of pathophysiological alterations caused by disturbed glucose homeostasis (Wolf, 2004).

In patients with Type 1 diabetes, glomerular hyperfiltration is common in early stage of their disorder (Mogensen *et al.*, 1975). Adequate insulin therapy does not fully correct this phenomenon (Sandahl *et al.*, 1981). These considerations prompted studies of possible C-peptide effects on renal function and structure in diabetic nephropathy both in animal models of diabetes and in patients with Type 1 diabetes with focus on early stage of the disorder.

Animal studies

The influences of C-peptide on glomerular hyperfiltration, functional reserve capacity, and renal protein leakage have been examined in STZ diabetic rats (Sjoquist *et al.*, 1998). Acute administration of a human C-peptide bolus of 6nmol/kg followed by continuous infusion of 30nmol/kg per hour for 60 minutes lowered glomerular hyperfiltration and proteinuria and improved renal functional reserve. The specificity of the C-peptide effect was confirmed by the finding that sC-peptide had no effect. Moreover, acute infusion of C-peptide and captopril for 60 minutes was examined in STZ-induced diabetic rats (Samnegard., 2004). Each agent lowered diabetes-induced glomerular hyperfiltration to a similar extent, but the influence of captopril on blood flow was greater than that of C-peptide, suggesting different mechanisms of action. No additive effects of C-peptide and captopril were shown in this study.

The influence of more prolonged C-peptide administration on renal function and morphology has also been examined. Intravenous infusion of C-peptide or saline for 14 days was studied in STZ diabetic rats (Samnegard *et al.*, 2001). C-peptide infusion prevented the development of glomerular hyperfiltration, decreased glomerular hypertrophy, preserved the available renal functional reserve and prevented urinary albumin leakage. Moreover,

morphological studies showed that C-peptide replacement partially normalized the diabetes-induced augmentation of glomerular volume (Samnegard *et al.*, 2001). In the C-peptide treated rats glomerular volume exceeded that of the control by no more than 23% compared with 63% in untreated diabetic group. When increasing doses of human C-peptide was used in diabetic rats, C-peptide dose-dependently lowered the increased glomerular filtration rate (GFR) and albumin excretion (Huang *et al.*, 2002). Renal function in healthy animals was unresponsive to C-peptide administration (Huang *et al.*, 2002).

Morphological changes during C-peptide treatment have been studied by the same group (Samnegard *et al.*, 2005). C-peptide administration in replacement dose to STZ rats served to prevent the glomerular hypertrophy and the mesangial matrix expansion seen in the post-hyperfiltration phase of early diabetic nephropathy.

Patient studies

The possible effects of C-peptide on renal function have been examined in patients with Type 1 diabetes. The short-term effects of C-peptide administration on renal function have been studied in young patients with Type 1 diabetes without signs of renal disease (Johansson *et al.*, 1992a). During C-peptide intravenous infusion GFR decreased by 7%, renal plasma flow increased slightly and renal filtration fraction decreased from 19 to 17%.

These observations have been extended over more prolonged administration of C-peptide. In patients with Type 1 diabetes with hyperfiltration and low level urinary albumin excretion, both insulin and human C-peptide administration by subcutaneous pump infusion for 2 and 4 weeks revealed that GFR decreased by 6% and urinary albumin excretion by 40% and 55%,

respectively, while GFR remained unchanged in diabetic patients treated with insulin alone (Johansson *et al.*, 1993). Combined treatment with insulin and human C-peptide for 3 months was found to reduce urinary albumin excretion in patients with Type 1 diabetes mellitus (Johansson *et al.*, 2000).

C-peptide and the pathogenesis of diabetic nephropathy

As described earlier C-peptide has been shown to exert beneficial effects on both renal function and morphology of diabetic nephropathy (Sjoquist *et al.*, 1998, Samnegard *et al.*, 2001, Huang *et al.*, 2002). These findings are supported by the finding that pancreas transplantation induces reversal of diabetic nephropathy in Type 1 diabetes (Fioretto *et al.*, 1998), and by recent evidence that patients with Type 1 diabetes with kidney and islet transplants have a better renal prognosis than patients transplanted with kidney alone (Fiorina *et al.*, 2003). Successful islet transplantation is associated with improvement of kidney graft survival rates, restoration of Na^+- K^+-ATPase activity, reduction of natriuresis, and improvement of urinary albumin excretion among patients with Type 1 diabetes mellitus and kidney graft. Thus, it could be hypothesized that islet transplantation exerts its beneficial effects by restoring endogenous C-peptide secretion in addition to providing insulin secretion and glyco-metabolic control.

The mechanism(s) underlying the beneficial effects of C-peptide on renal function and structure in diabetes are not yet completely understood.

1.5. C-peptide binding to cell membranes

The first study describing interaction between C-peptide and cell membranes appeared in 1986 (Flatt *et al.*, 1986). Specific binding of the C-peptide was evaluated using cultured rat pancreatic islet tumor cells, predominantly composed of insulin secreting beta cells, using a radio ligand binding technique. Synthetic rat C-peptide 1 and iodinated tyrosylated rat C-peptide 1, exhibited 54 ± 6% specific binding to tumor beta cells. Displacement of tracer increased with increasing concentrations of unlabeled rat C-peptide 1 and the specificity of binding was substantiated by reduced displacement with human C-peptide. The demonstration of specific C-peptide binding to insulin-secreting beta cells provides support for the earlier observations that C-peptide modulated the function of these cells (Wojcikowski *et al.*, 1983). In contrast, however, human C-peptide was reported not to bind to crude cell membranes prepared from skeletal muscle, using the same technique (Zierath *et al.*, 1996).

Human C-peptide has been recently shown to bind to cell membranes from human renal tubular cells, skin fibroblasts, and saphenous vein endothelial cells using fluorescence correlation spectroscopy (Rigler *et al.*, 1999). Measurements of ligand-membrane interactions at single-molecule detection sensitivity showed specific binding of fluorescently labelled C-peptide to these human cell types. Full saturation of the C-peptide binding to the cell surface is obtained at low nanomolar concentrations.

The C-terminal pentapeptide also displaces C-peptide bound to cell membranes, indicating the binding occurs at this segment of the ligand. Non-native D-C-peptide and a randomly sC-peptide did not compete for binding with the labelled C-peptide. Proinsulin, which includes the pentapeptide segment,

failed to displace of bound C-peptide, suggesting that the free COOH-terminal end of the segment is required for binding. Likewise, addition of insulin, IGF I, IGF II, or neuropeptide (NPY) was not accompanied by displacement of bound C-peptide, indicating absent cross-reaction with these hormones and their membrane receptors. Labelled insulin bound to the cell membranes was not displaced by excess concentration of C-peptide.

The estimated association constant for C-peptide was approximately $3x10^9$ M^{-1}. Binding curves indicated that saturation of binding occurred already at a concentration of approximately 0.9nM, which is within the physiological concentration range. This finding may help explaining why no effects of exogenous administered C-peptide have been demonstrated in healthy individual or animal (Wojcikowski *et al.,* 1983; Hoogwerf *et al.,* 1986; Johansson *et al.,* 1992b; Sima *et al.,* 2001). Thus, receptor saturation in healthy subjects or animals probably occurs already at the ambient C-peptide concentration, so no further physiological effects are to be expected from higher concentrations.

Pre-treatment of the cells with pertussis toxin (PTX), known to modify G proteins (G_i, G_o), abolishes the binding. Thus, it was suggested that C-peptide bound to a G protein-coupled–receptor (GPCR) in human cell membranes (Rigler *et al.,* 1999).

Using the same technique, the binding of rhodamine-labelled human C-peptide (Rh-C-peptide) to intact human skin fibroblasts and to detergent-solubilised extracts of fibroblasts has also been studied (Henriksson *et al.*, 2001). C-peptide was found to bind to the cell membranes of intact fibroblasts with an association constant of $3x10^9M^{-1}$, giving full saturation at about 0.9nM, close to the physiological C-peptide plasma concentration (0.5-1.5) nM. In the same study,

the detergent CHAPS released C-peptide-binding macromolecules from cells, suggesting that binding components may be purified and characterized (Henriksson *et al.*, 2001).

Bioactive peptides generally exert their cellular effects via specific binding of a limited region of the ligand molecule to the receptor. The C-terminal pentapeptide of human C-peptide (EGSLQ) competitively displaces the bound, full-length peptide (Rigler *et al.,* 1999), indicating the involvement of the C-terminal segment as an 'active site' in the binding process. Furthermore, Glu 27 of C-peptide is critically involved in binding to cellular targets (Pramanik *et al.*, 2001). Fluorescence correlation spectroscopy was used to investigate the structural requirements of the pentapeptide region for C-peptide binding. All pentapeptide residues, E_{27}GSLQ, individually were replaced with Ala and the capacity of the resulting peptides to displace rhodamine-lablled full-length human C-peptide from human renal tubular cell membranes was studied. These experiments showed that Glu27 is essential for displacement, while replacement of Gly28 with Ala has little effect. Moreover, free Glu displaces full-length C-peptide to about 50%, while free Ala, C-peptide (1-26), and the truncated pentapeptide, corresponding to tetrapeptide $G_{28}SLG_{31}$ have no displacing capacity. The peptide EVARQ (corresponding to the rat C-terminal pentapeptide) and ELGGGPGAG (corresponding to positions 11-19 of human C-peptide) do not displace human C-peptide. These findings indicated that Glu27 is important for C-peptide binding.

In summary, C-peptide binding data provide new insight into C-peptide membrane interactions. There is evidence of direct binding of C-peptide to cell surface and solubilised cell components. Association constants, binding curves,

and displacement characteristics have been established at physiological relevant concentrations. However, identification of functional receptor requires further evaluation for detailed understanding.

1.6. Non-receptor interactions

Cellular C-peptide effects may be mediated by a mechanism that defies the general rule of ligand-receptor interaction (Johansson *et al.*, 2002). As such human C-peptide mediated beneficial effects on vascular and neural dysfunction induced by experimental diabetes in rats were also seen with reverse–sequence C-peptide (Ido *et al.*, 1997). These authors proposed that C-peptide effects may be independent of chirality, the peptide binding directly to cell membranes and exerting effects via mechanisms similar to those of amphipathic antimicrobial peptides. The authors suggested that the glycine–rich segment in the central region of C-peptide (position 13-17) was important for such biological activity. This conclusion agreed with findings from fragment studies involving determination of Na^+-K^+-ATPase activity (Ohtomo *et al.*, 1996).

C-peptide lacks traditional pore forming peptide characteristics such as self-association. Also, C-peptide is hydrophilic, negatively charged (Steiner and Rubenstein, 1997) and does not associate in a stable manner with lipid membranes or micelles (Henriksson *et al.*, 2000; Johansson *et al.*, 2002). All of these findings argue against the concept of direct C-peptide membrane interactions.

1.7. Cellular effects of C-peptide

1.7.1. Effects on Na^+, K^+-ATPase

Na^+-K^+-ATPase, sodium pump, is a ubiquitous membrane-associated protein complex that is expressed in most eukaryotic cells. The "pump" transduces energy from the intracellular hydrolysis of adenosine triphosphate (ATP) to the active countertransport of sodium and potassium across the cell membrane (Clausen *et al.,* 1991). It is known that Na^+-K^+-ATPase activity is impaired in the cell membrane of many tissues obtained from diabetic individuals or animals and this defect may play a role in the development of the complication of diabetes (Vague *et al.,* 2004).

Renal tubular cells reabsorb a huge amount of sodium in part through Na^+-K^+-ATPase activity. Accordingly, they are a rich source of Na^+-K^+-ATPase. The basic function of the Na^+-K^+-ATPase is to maintain high sodium and potassium gradients across the plasma membrane of cells (for review, see Feraille and Doucet, 2001). C-peptide has been shown to stimulate the activity of Na^+-K^+-ATPase of rat renal tubule segments as measured by hydrolysis of [^{32}ATP] (Ohtomo *et al.*, 1998). Rat C-peptide 1 and 2, were largely equipotent in stimulating Na^+-K^+-ATPase activity in rat renal tubular segment (Ohtomo *et al.*, 1998). The same study showed that sC-peptide had no effect on Na^+-K^+-ATPase activity.

In rat tubular collecting duct cells, stimulation of Na^+-K^+-ATPase was reported to be mediated via PKC-α (Tsimaratos *et al.,* 2003). Similarly, studies using human tubular cells in primary culture and oubain-sensitive uptake of 86Rb+ as a marker of Na^+-K^+-ATPase activity, have confirmed a concentration dependent effect of human C-peptide on Na^+-K^+-ATPase activity at physiological

concentrations via activation of extracellular regulated kinase (ERK) (Zhong *et al.,* 2004). Pre-treatment of the tubular segments or cells with PTX or a specific inhibitor of calcineurin inhibited the C-peptide effect on Na^+-K^+-ATPase activity (Ohtomo *et al.,* 1996). The C-terminal pentapeptide has been found to be as potent as the full-length C-peptide in stimulating Na^+-K^+-ATPase activity (Ohtomo *et al.*, 1998).

A similar stimulatory effect of C-peptide on Na^+-K^+-ATPase was obtained by *in vivo* administration. Replacement of C-peptide in Type 1 diabetic rats for periods of 2-8 months resulted in partial restoration of sciatic nerve Na^+-K^+-ATPase activity (Ido *et al.*, 1997; Sima *et al.,* 2001). Moreover, red blood cell Na^+-K^+-ATPase activity is reduced in patients with Type 1diabetes (Finotti and Palatini, 1986, McMillan *et al.,* 1978). The reduction is proportional to the simultaneous decrease in C-peptide plasma concentration (Dufayet de la Tour *et al.,* 1998) and can be corrected by C-peptide administration (Forst *et al.,* 2000).

1.7.2. Effects on mitogen-activated protein kinases (MAPK)

The MAPK cascade consists of three signalling modules; ERK, c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38K). The ERK enzymes are activated by extracellular stimuli such as growth factors, while p38K and JNK respond to environmental stress and inflammation (Whitmarsh and Davis, 1996). ERK constitutes a major signalling module conserved throughout evolution that is activated in mammalian cells via stimulation of receptor tyrosine kinases, G-protein coupled receptors and integrins (Widmann *et al.*, 1999). These cell surface signals converge towards activation of the small G-protein Ras that recruits the serine/threonine kinase, MAPK kinase kinase (RAF) to the membrane where it is fully activated (Kerkhoff and Rapp, 2001). The signal is amplified via

two downstream kinases, MAPK kinase (MEK) and ERK that are uniquely activated since MEK is dually phosphorylated on two serine residues by RAF, and then ERK is dually phosphorylated on a tyrosine and threonine residue by MEK (Hallberg *et al.*, 1994).

In mouse embryonic fibroblast cells line (Swiss 3T3), human, rat (1 and 2) C-peptide has been found to stimulate ERK phosphorylation in a concentration dependent manner. Interestingly, these authors failed to observe the same effect in other cells such as 3T3-L1 fibroblasts and HepG2 hepatoma, emphasizing the cell specificity of ERK activation by C-peptide. In Swiss 3T3 fibroblasts, all-D-amino acid C-peptide and reverse-sequence C-peptide did not stimulate ERK phosphorylation (Kitamura *et al.*, 2001). Pre-treatment of the cells with PTX or MEK1 inhibitor abolishes the stimulatory effect on ERK phosphorylation (Kitamura *et al.*, 2001; Zhong *et al.*, 2005). Furthermore, human C-peptide has been found to stimulate p38K and ERK in mouse lung capillary endothelial cells (LEII), but failed to activate JNK (Kitamura *et al.*, 2002). In LEII, C-peptide induced p38K activation caused phosphorylation of cAMP response element binding (CREB) and activating transcription factor1 (ATF1) and their binding to CRE (Kitamura *et al.*, 2002). Thus, C-peptide may promote CRE-dependent gene expression in capillary endothelial cells. In latter study, effects of human C-peptide were replicated using rat C-peptide (1and 2); neither retro-sequenced nor all D-amino acid human C-peptide induced phosphorylation of these kinases. These data provide evidence that MAPK activation is involved in C-peptide signal transduction in several cell types.

1.7.3. Effects on intracellular Ca^{2+} $[Ca^{2+}]_i$

Several lines of evidence suggest that $[Ca^{2+}]_i$ functions as second messenger in the C-peptide transduction. In primary cultures of rat tubular cells, exposure of the cells to homologous C-peptide in physiological concentrations range resulted in a prompt elevation of the $[Ca^{2+}]_i$ concentration (Ohtomo *et al.*, 1996). In human renal tubular cells, human C-peptide and its C-terminal pentapeptide position (27-31, EGSLQ), but not the des-(27-31) C-peptide, or randomly sC-peptide, elicited a transient increase in $[Ca^{2+}]_i$ (Shafqat *et al.*, 2002). Addition of a calcium chelator to the medium abolished the effect of C-peptide, indicating that the effect is elicited via an influx of extracellular Ca^{2+} rather than by release from intracellular stores (Ohtomo *et al.*, 1996; Wallerath *et al.,* 2003). Addition of PTX to the medium blocked the effects of C-peptide and the pentapeptide segment on intracellular Ca^{2+} concentrations (Shafqat *et al.,* 2002). Thus, C-peptide elevates intracellular Ca^{2+} through a PTX sensitive G-protein-coupled receptor.

1.7.4. Effects on eNOS

ENOS is constitutively expressed in vascular endothelial cells, and is activated by an agonist-induced increase of intrcellular Ca^{2+}, followed by a complex formation of Ca^{2+}-calmodulin and eNOS (for review see, Venema *et al.,* 1996).

An *in vitro* study showed that C-peptide increased the endothelial release of NO in concentration and time dependent manner (Wallerath *et al.,* 2003). The effect was abolished in the presence of a NOS inhibitor, and no effect was found when calcium removed from incubation medium (Wallerath *et al.,* 2003). Likewise, C-peptide, in the presence of small amounts of insulin, elicited NO-mediated dilation of rat skeletal muscle arterioles (Jensen and Messina, 1999).

Although increased expression of eNOS mRNA levels in lung tissue have been reported in rats injected with C-peptide (Scalia *et al.,* 2000), no such effect was apparent in bovine aortic endothelial cells (Wallerath *et al.,* 2003). Furthermore, studies in rat aortic endothelial cells have demonstrated that C-peptide augmented NO production by enhancing eNOs expression through MAPK-dependent transcriptional activation (Kitamura *et al.,* 2003).

These data clearly indicate that induction of NO release is one of the intracellular signalling effects of C-peptide.

1.7.5. Interaction with ligand-gated ion channels

Due to ability of free glutamic acid to partially displace C-peptide from cell membrane, and the importance of N-terminal Glu in intact C-peptide and its C-terminal pentapeptide in binding and biological activity (Pramanik *et al.*, 2001). Johansson *et al* (2002) suggested that C-peptide might interact with a ligand-gated ion channel coupled to glutamate receptor.

1.8. Cross-talk with insulin signalling pathway

Recent studies suggested that C-peptide signal transduction may cross-talk with the insulin-signalling pathway at the level of the IR (Li *et al.*, 2001; Grunberger *et al.*, 2001; Grunberger *et al.*, 2004).

Physiologic effects of insulin are initiated by insulin binding to the extracellular domain of the IR. Binding results in activation of the IR tyrosine kinase, followed by phosphorylation of intracellular substrates, propagating receptor signal throughout the cell. These include the Ras/MAPK cascade and the PI 3-kinase/Akt (protein kinase B, PKB) system, which are thought to play key roles in mitogenic and metabolic arms of insulin signalling, respectively (Holman and Kasuga, 1997: Virkamaki *et al.*, 1999). C-peptide inhibits protein tyrosine phosphatase activity in skeletal muscle myoblasts (Li *et al.*, 2001). Autophosphorylation of IR plays a crucial role in insulin action. Protein tyrosine phosphatases dephosphorylate the IR, insulin receptor substrates, and reduce TKA, therefore attenuating insulin action (Goldstein *et al.*, 1998). Thus, C-peptide, by inhibiting activity of protein tyrosine phosphatases, could shift the balance to increased phosphorylation and thus increased IR signalling activity.

C-peptide, at physiological concentrations mimics insulin effects such as glycogen synthesis and amino acid uptake in rat muscle cells and L6 myoblasts (Grunberger *et al.*, 2001). Stimulation of glycogen synthase by insulin is mediated by dephosphorylation through the activation of protein Phosphatase 1 (PP1) and inhibition of glycogen synthase kinase (GSK3), which in turn, is inhibited by phosphorylation. There are at least three plausible kinases which could phosphorylate GSK3 or PP1: p70S6 kinase, ribosomal S6 kinase (p90Rsk) and Akt (Shepherd, 1995). In L6 myoblasts, C-peptide increased insulin receptor

TKA, PI 3-kinase, MAPK phosphorylation, GSK3 phosphphrylation and p90Rsk (Grunberger *et al.*, 2001). An exception occurred in the case of Akt where C-peptide had no effect. Akt is believed to be necessary for insulin-induced activation of glycogen synthesis in L6 myoblasts (Takata, *et al.*, 1999). Whether stimulation of glycogen synthesis by C-peptide was mediated by a p70S6k and/or p90Rsk-dependent and Akt-independent pathway (s) is need further investigation.

Grunberger and Sima, (2004), suggested four distinct possibilities for explaining insulinomimetic effects of C-peptide. The first hypothesis was that C-peptide effects resulted from direct binding to and activation of a specific C-peptide receptor as supported by binding studies (Rigler *et al.*, 1999) and PTX inhibition of C-peptide effects (Maestroni *et al.*, 2005; Zhong *et al.*, 2005; Walcher *et al.*, 2004; Marx *et al.*, 2004; Shafqat *et al.*, 2002; Kitamura *et al.*, 2001; Ohtomo *et al.*, 1996; Grunberger *et al.*, 2001). The second hypothesis was that C-peptide activates the IR. The lack of C-peptide stimulation of glycogen synthesis in the parental rat 1 fibroblasts (which contain few IR) but robust effects in HIRcB (overexpressing human IR) support a role of IR in mediating the C-peptide effect. Given the structural differences between insulin and C-peptide, the interaction with IR would need to occur with an α-subunit domain distinct from that responsible for insulin binding to IR. Alternatively, a plasma membrane perturbation caused by C-peptide could lead indirectly to a conformational change of the IR, leading to activation of its β-subunit and its autophosphorylation and activation of tyrosine kinase. The fact that only submaximal concentrations of C-peptide and insulin are additive (Grunberger *et al.*, 2001), suggests sharing of at least some elements of the signalling pathways used by these ligands. The third hypothesis was a combination of the two above interaction of C-peptide with its

cognate membrane receptor and with a specific domain of the IR. The fourth hypothesis was an interaction of C-peptide with another cells surface receptor such as the insulin-like growth factor (IGF)-1 receptor. IGF-1 and C-peptide may share structural domains, which could form the basis for cross talk between their signalling pathways. Some investigators have already shown that C-peptide replacement normalized both IGFI and IGF-II receptor expression (Sima and Li, 2005; Sima *et al.,* 2001).

Once the C-peptide receptor is cloned and sequenced, details of the molecular interaction between the insulin and C-peptide signalling pathways will be clearer.

1.9. Summary

The potential role of C-peptide as a biological active peptide has been controversially discussed in the past on the basis of studies being contradictory concerning the impact of this cleavage product of insulin. However, recent publications derived from studies of both animals and human with diabetes, now supports a role for C-peptide in glucose homeostasis, amelioration of the consequences of hyperglycemia, and abrogation of diabetic complications (Wahren *et al.,* 2000; Ekberg *et al.*, 2003; Hansen *et al.*, 2002). Neverthless, the exact molecular effects of C-peptide are not fully characterized. The data to date support the existence of C-peptide signaling pathways are illustrated in (**Fig 1.3**).

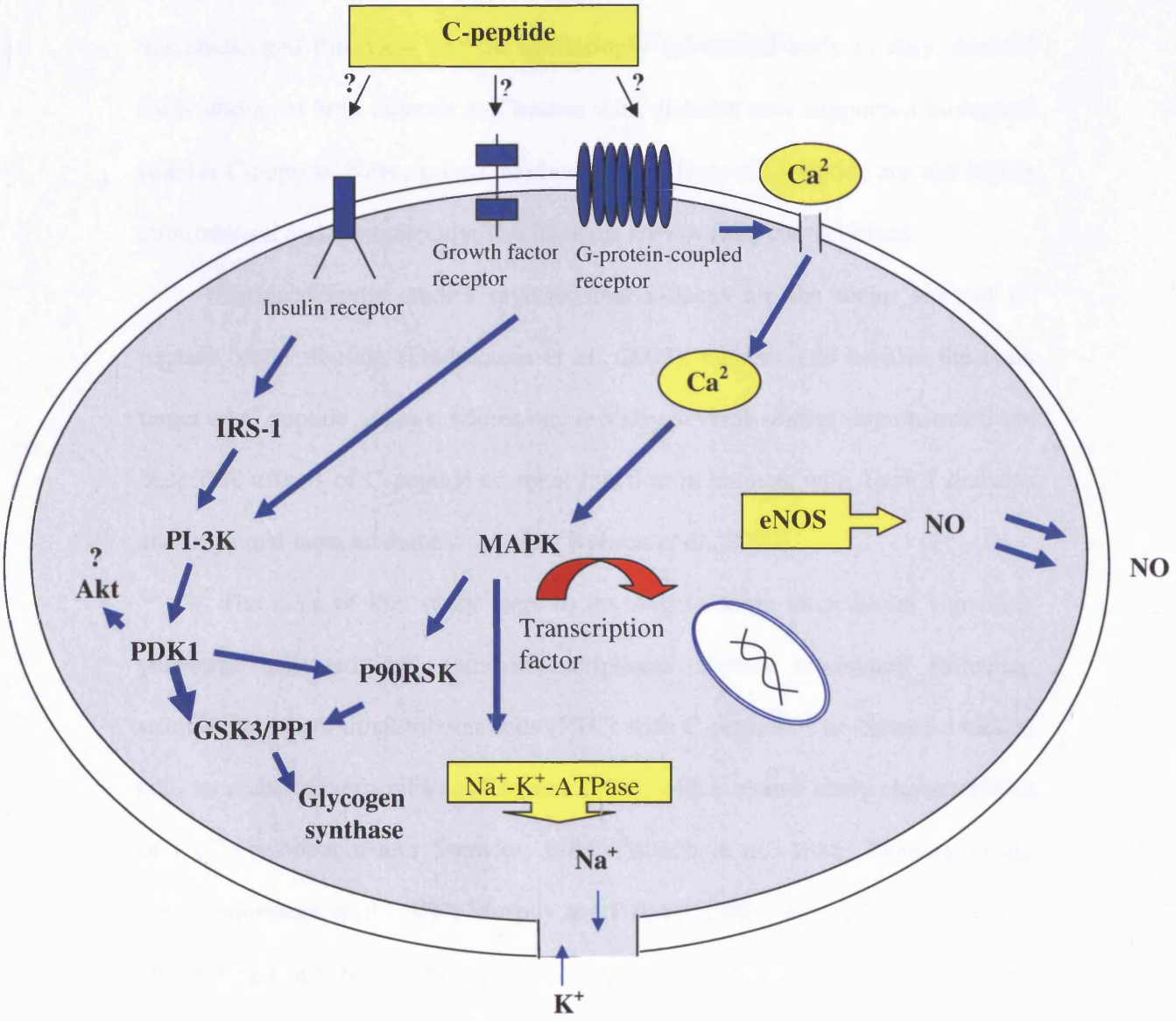


Fig 1.3. Possible mechanisms of C-peptide effects (Wahren, 2004; Grunberger and Sima, 2004). This pattern involves interaction of the peptide with a membrane receptor, possibly GPCR, and activation of Ca^{2+}-dependent intracellular-signalling pathways. The latter include phosphorylation of specific isoforms of PKC and activation of the MAPK system, eliciting increased activity of Na^+-K^+-ATPase and eNOS. In addition, there is activation and DNA-binding of transcriptional factors, causing increased expression of e.g. eNOS. Some studies also indicate that C-peptide may interact with insulin or IGFI receptors.

1.10. Aims of the thesis

For many years C-peptide itself has been regarded as biologically inert with no significant physiological purpose. In the past decade, a trickle of publications has challenged this view and an increasingly substantial body of data, derived from studies of both animals and human with diabetes now supports a biological role for C-peptide. Nevertheless, the biological effects of C-peptide are still highly controversial and the molecular mechanisms are not fully characterised.

Pharmacokinetic studies revealed that kidneys are the major sites of C-peptide biodistribution (Fredriksson *et al.*, 2002), which could be also the main target of C-peptide actions. Moreover, recently several studies demonstrated the beneficial effects of C-peptide on renal function in patients with Type 1 diabetes and in animal induced diabetes model (Wahren *et al.*, 2000).

The aims of this study were to investigate some intracellular signalling pathways activated and some transcriptional factors modulated following stimulation of proximal tubular cells (PTC) with C-peptide. The opossum kidney cell, an immortalized cell line (Koyama 1978), which retains many characteristics of PTC (Teitelbaum and Strewler, 1984; Pollock *et al.*, 1986; Bandary *et al.*, 2005; Guimaraes *et al.*, 1997; Murphy and Bylund, 1989), was utilised as a model of proximal tubular epithelial cells. One of its major strengths being the endogenous expression of the IR (Yagil *et al.*, 1988a; Yagil *et al.*, 1988b; Yagil *et al.*, 1988C) because C-peptide receptor is not yet characterized and that C-peptide may cross-talk with insulin signaling pathways at level of IR (Grunberger and Sima, 2004).

The initial aim of this project was to determine whether C-peptide possessed the capacity to signal in PTC, focusing especially on pathways involved

in cell growth and potentially the development of diabetic nephropathy. Subsequently analysis of functional responses in PTC as a consequence of activation of signalling pathways by C-peptide was a major goal. The eventual aim was to determine functional and phenotype responses of PTC to C-peptide application.

The ultimate goal was to establish the potential for C-peptide as a therapeutic agent in diabetes and diabetic nephropathy.

CHAPTER 2
MATERIALS AND METHODS

2.1. Chemicals and materials

Standard chemical reagents of analytical grade were obtained from Sigma (Poole, UK) and Elgastat purified water was used throughout. DMEM-F12, RPMI 1640 medium, penicillin/streptomycin, L-glutamine, foetal calf serum and all tissue culture materials were from Invitrogen Life Technologies (Paisley, UK).

Human 31 amino acid (aa) C-peptide, and a 31 aa sC-peptide were generously provided by Dr. J. Wahren, Karolinska Institute (Stockholm, Sweden). These peptides were prepared as 250μM stock solutions in 50μM glacial acetic acid and stored frozen at -20°C.

Wild-type OK cells were obtained from Dr. J. Caverzasio, University Hospital (Geneva, Switzerland). Mutant OK cells inducibly expressing a dominant negative form of the p85 regulatory subunit of the class 1A p85/p110 PI 3-kinase (Δp85), or the wild type p85, both under control of LacSwitch, have been previously described (Brunskill *et al.,* 1998). Human proximal tubular cells (HPTC) from the normal pole of nephrectomy specimens from patients with renal cell carcinoma, Leicester General Hospital (Leicester, UK). The human monocytic cell line THP-1 was obtained from ATCC (Manssas VA, USA).

Wortmannin, pertussis toxin (PTX) and carbobenzoxyl-L-leucyl-leucyl-L-leucinal (MG-132) were purchased from Calbiochem (Nottingham, UK). Ciglitazone was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). GW9662 and bisphenyl A diglycidyle ether (BADGE) were purchased from Cayman Chemical Co (Nottingham, UK). Bovine insulin, TNF-α

and IPTG were obtained from Sigma (Poole, UK). Fluo-3-acetoxymethyle ester (fluo-3-AM) from molecular Probes (Cambridge, UK). 3-(4,5- dimethyl-2-thiazol-2-yl) 2,5-diphenyltrazolium bromide from sigma.

ERK substrate, a peptide (RRELVEPLTPSGEAPNQALLR) derived from the cytoplasmic tail of the epidermal growth factor (EGF) receptor, was synthesized by the Protein and Nucleic Acid Chemistry Laboratory University of Leicester (Leicester, UK). Protein A sepharose CL-4B was from Pharmacia Biotech (Uppsala, Sweden).

γ- [^{32}P] ATP was purchased from Perkin Elmer Life Sciences (Cambridge, UK), [^{3}H] thymidine was obtained from Amersham Life Sciences (Buckinghamshire, UK), [^{32}P]-orthophosphate and [^{35}S]-GTPγS were obtained from Amersham Pharmacia Biotech (Little Chalfont, England). Ultma Gold and Emulsifier-Safe Scintillation cocktail were supplied by Packard (Pangbourne, UK). Adenosine triphosphate (ATP), guanosine diphosphate (GDP) and unlabelled GTPγS were obtained from Sigma (Poole, UK)

25mm diameter glass coverslips were obtained from Chance Proper (UK) and Lab-Tek 4 chamber slides for transferase-mediated deoxyuridine triphosphate-biotin nick end labeling assay (TUNEL assay) from Sigma (Poole, UK).

For details of all primary antibodies, please refer to **Table 2.1**. All antibodies purchased from Santa Cruz Biotechnology Inc, Upstate Biotechnology Inc, or Transduction Laboratories (USA).

Protein electrophoresis gel equipment was from Bio-Rad (Hercules, USA). Nitrocellulose (0.4μM pore size) for protein transfer was supplied by Schleicher

and Schuel (London, UK). Hyperfilm-ECL and chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Little Chlfont, UK).

The plasmid, pCMX-PPARγ encoding mouse PPAR-γ1 was kindly provided by Dr. R. Evans, Salk Institute (San Diego, CA). The reporter plasmid pPPRE-TK-luc was kindly provided by Dr. M. Lazar, University of Pennsylvania (Philadelphia, PA). The plasmid pFC-MEK1 encoding constitutively active MEK-1 (pMEK1-CA), and the kinase dead mutant of MEK-1 in pCMV5 (pMEK1-KD) were provided by Dr. J. Blank, University of Leicester (Leicester, UK). The reporter plasmid pNFκB-luc and plasmid β-Galactosidase (pSVβgal) were obtained from Stratagene (La Jolla, CA, USA). Fugene 6 transfection agent was purchased from Roche Diagnostics (Lewes, UK). Luciferase assays were performed using the LucLite kit from Packard (Pangbourne, England). β-Galactosidase assay kits were obtained from Promega (Madison, WI).

Cell death detection (ELISA) and *in situ* cell death detection kit were from Roche (Lewes, UK). Fast Red, levamisole and hematoxylin were from Sigma (Poole, UK).

2.2. Cell culture

Wild type OK cells were maintained in DMEM-Ham's F-12 mix (DMEM-F12) supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, 100u/ml penicillin, 100μg/ml streptomycin, and 10mM N-2-[Hydroxyethyl]-piperazine N-2-ethanesulfonic acid (HEPES). Cells were grown in 80-cm^2 flasks and passaged every 5-6 days when confluent. Cells were rinsed with serum free medium and detached from the flask by incubation with 0.25% trypsin/ 1mM ethylenediaminetetraacetic acid (EDTA). Trypsin then was neutralized by addition of 10-15ml medium containing supplements and cells were pelleted by

centrifugation (1000g for 5 minutes). The cells in these experiments were used from passage 67-90.

Mutant OK cells expressing Δp85 and wild type p85 under the control of the LacSwitch (Brunskill *et al.*, 1998) were maintained in the above media with addition of 200μg/ml hygromycin and 300μg/ml G418. Cells were maintained under similar conditions as wild type. Expression of p85 and Δp85 proteins was induced by treating OK cell monolayers with 5mM β-D-thiogalactopyranoside (IPTG) in culture medium overnight before stimulation with other agents. Successful induction of recombinant p85/Δp85 was confirmed by Western blotting with specific anti-PI3-kinase p85-α (see section 2.3.3, and 2.3.4).

HPTC were maintained in the same conditions as wild type OK cells with addition of 36ng/ml hydrocortisone, 5μg/ml 3,3,5 Triiodo-L-thyronine and 5μg/ml insulin transferring sodium selenite. These cells used up only until passage 3.

The human monocytic cell line THP-1 was cultured in RPMI 1640 medium containing 10% foetal calf serum, 100U/ml penicillin, and 0.1mg/ml streptomycin and passaged every 7 days when confluent.

All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air and culture medium was changed three times a week. In all experiments, cells were serum starved overnight before being subject to experimental intervention to ensure as far as possible that cells were not exposed to mitogenic agents or C-peptide derived from FCS.

2.3. Electrophoresis and immunoblotting

2.3.1. Preparation of samples for electrophoresis

After treatment with C-peptide or other test reagents, cells were rinsed twice with 1ml ice- cold PBS. Cells were then lysed in Laemmli's buffer (50mM Tris, PH 6.8, 10% glycerol, 2% sodium-dodecyl-sulphate (SDS), 100mM dithiothreitol (DTT) and 0. 1% bromophenol blue) and incubated on ice for 10 minutes. Aliquots were denaturated by heating at 99° C for 3-5 minutes.

2.3.2. Polyacrylamide gel electrophoresis

Samples (see section 2.3.1) were electrophoresed using 10% SDS polyacrylamide gel electrophoresis (PAGE) running gels (4ml dH2O, 3.3ml 30% acrylamide mix, 2.5ml 1.5M Tris (PH 8.8), 0.1ml 10% SDS (w/v), 0.1ml 10% ammonium persulfate (w/v) and 4µl N,N,N`,N`-Tetramethyl-ethylene diamine (TEMED) in a total volume of 10ml) and stacking gels (3.4 ml dH2O, 0.83 ml 30% acrylamide mix, 0.63ml 1.0M Tris (PH 6.8) 0.05ml 10% SDS (w/v), 0.05ml 10% ammonium persulfate (w/v), and 4µl TEMED in a total volume of 5ml) at 200V for approximately 45 minutes submerged in running buffer (25mM Tris-base, 250mM glycine, 0.1% SDS (w/v)) according to method of Laemmli (1970). At least one lane of each gel was reserved for the separation of pre-stained molecular weight standards.

2.3.3. Immunoblotting of proteins

Separated proteins were transferred from polyacrylamide gels to nitrocellulose membranes, pre soaked in transfer buffer composed of (39mM glycine, 48mM Tris base, 0.037% SDS (w/v) and 20% methanol (v/v)). The transfer was performed by using a semi-dry blotter and application of current of 0.65mA/cm^2 for 60-70 minutes at room temperature. The nitrocellulose membranes were then blocked either overnight at 4°C or 1 hour at room

temperature with 5% (w/v) non-fat milk in TTBS (20mM Tris-base, 100mM NaCl and 0.05%Tween 20, pH 7.5). The membranes were then incubated at room temperature with primary antibodies (see **Table 2.1** for working concentrations), diluted in blocking solution (5% (w/v) non-fat milk in TTBS), for 1 hour. After washing the membranes in TTBS three times for five minutes each, the membranes were incubated, again at room temperature with appropriate peroxidase conjugated secondary antibodies diluted in blocking solution for one hour. The membranes then were washed in TTBS three times for five minutes each and then immunoreactive bands were detected by enhanced chemiluminescence (ECL) reagent (five minutes incubation) and exposure to photographic film. Immunoblots were quantified using Scion Image analysis software (version 4.0.2 Scion Corporation, Frederick, Maryland, USA). Local background subtraction was applied to images and data were normalized to basal (non-stimulated cells).

2.3.4. Stripping and re-probing nitrocellulose membranes

The nitrocellulose membranes were stripped to remove primary and secondary antibodies to allow immunoblotting of a second protein. Membranes were submerged in stripping buffer (100M 2-Mercaptoethanol, 2% SDS, and 62.5mM Tris-HCl, pH 6.7) and incubated at 50°C for 30 minutes, washed 3 times for 10 minutes in large volume of TTBS, and then blocked in 5% (w/v) non-fat milk in TTBS either overnight at 4°C or 1 hour at room temperature. Immunoblotting was then repeated in order to determine the total protein of interest.

2.4. ERK imunoprecipitation and assay

2.4.1. ERK immunoprecipition

Confluent monolayers of OK cells in 6-well plates were serum starved for overnight prior to addition of agonists. After stimulation, the cells were washed once with 1 ml ice- cold PBS and then incubated for 10 minutes on ice with 300µl lysis buffer1 (20mM Tris-HCl, PH 8.0, 0.5% Nonidet P-40, 250mM NaCl, 3mM (EDTA), 3mM (EGTA), 1mM PMSF, 2mM Na_3vo_4 and 1mM DTT) and then were clarified by 10 minutes of centrifugation at 14000-x g, 4°C. 200µl Lysates were incubated for 2 hours at 4°C with a 1: 100 dilution of rabbit polyclonal anti-ERK-1 antiserum (200µg/ml). Immune complexes were incubated with 100µl of a 15% (v/v) slurry of protein A-sepharose for 30 minutes at 4°C. Immunoprecipates were collected by 3 minutes of centrifugation at 5000-x, 4°C, then washed twice in 200µl lysis buffer and twice in 200µl kinase buffer (20mM HEPES, PH 7.2, 20mM β-glycerophosphate, 10mM $MgCl_2$, 50µM ATP, 1mM DTT and 50µM Na_3vo_4) each time the immunoprecipitates were collected by 3 minutes of centrifugation at 5000-x g, 4°C.

2.4.2. *In vitro* kinase assay

A 40µl aliquot of the above kinase buffer containing 20µM (γ-^{32}P)-ATP (2.5µCi/nmol) and 200µM of a synthetic peptide substrate corresponding to amino acids 662 to 681 of the EGF receptor was added to immunoprecipitates. The mixture were incubated for 20 minutes at 30°C and shaken every 5 minutes. The reactions were then terminated by addition of 10µl of 25% (w/v) Trichloroacitic acid (TCA). The mixtures were centrifuged at 14000-x g for 2 minutes and 40µl spotted onto squares of P81 cation exchange paper (Whatman) numbered according to each sample then were left for 2 minutes. Papers were washed 3 times for 5 minutes in 0.5% (v/v) orthophosphoric acid and rinsed once with

acetone, dried and then counted by adding 5ml scintillation fluid (Ultma Gold) to each sample. These were counted using a Packard 1900 CA Tri- Carb liquid scintillation counter. To correct for nonspecific phosphorylation, levels of phosphate incorporation were measured in the absence of substrate and were subtracted from values measured in the presence of substrate.

2.5. Confocal imaging

2.5.1. Imaging of Rhodamine-labelled human C-peptide (Rh-C-peptide) binding to cells

Cells were seeded onto 25mm diameter non-coated glass coverslips and cultured for 24-48 hours. Serum-starved cells were washed with Krebs-Hepes buffer (KHB) (10mM HEPES, 4.2mM $NaHCO_3$, 11.7mM D-glucose, 1.18mM $MgSO_4.7H_2O$, 1.18mM KH_2PO_4, 4.69mM KCl, 118mM NaCl, 1.29mM CaCl2.$2H_2O$, pH 7.4) prior to coverslips being mounted in a chamber on the stage of an Olympus 1X70-SIF inverted microscope with 40X oil emersion objective. The chamber was pre-heated to 37°C with a peltier unit and was perfused with KHB at rate of 5ml/min. Using an UltrasVIEW confocal imaging system (PerkinElma Life Science, Cambridge, UK), cells were excited at λ569 nM and emitted light collected with a broad band RGB emission filter. Rh-C-peptide was added via 250μl bath application at concentration of 100nM and images were taken intermittently.

2.5.2. Imaging of $[Ca^{2+}]_i$

OK cells were plated onto sterile 25mm diameter non-coated glass coverslips and allowed to adhere for 24 hours. Cells were washed twice with KHB and then incubated in KHB containing 6μM fluo-3-AM at room temperature for 45-90 minutes. Cells were then maintained in 2ml KHB to equilibrate at room

temperature. Cover slips were mounted onto the stage of an UltraVIEW microscope as described in section 2.5.1. The chamber was perfused with KHB, with or without 5nM C-peptide or 5nM sC-peptide, at 5ml per minute. The intracellular fluorescence, as an index of intracellular [Ca^{2+}], was determined by using an ultraview confocal imaging system. Cells were excited with a krypton/argon laser at λ488 nM and emitted light collected above λ510 nM with a narrow band pass filter.

2.6. Assessment of activation of PKC

For measurement of the cytosol to membrane translocation of PKC as an index of activation and detection of proteins predominately localised in cytosol such as inhibitory of NFκB (IκB-α) and TNF receptor-associated factor2 (TRAF2), OK cells were separated into cytosolic and membrane fractions as previously described (Willars *et al.*, 1999). Serum-starved cells were stimulated with C-peptide or other test reagents at room temperature and then lysed in 200μl ice-cold lysis buffer2 (20mM Tris-HCl, 5mM egtazic acid (EGTA), 2mM EDTA, 1mM DTT, 0.5mM phenylmethylsulphonyl fluoride (PMSF), 10μM iodoacetamide, pH 7.4) and then incubated on ice for 30 minutes. Samples were then centrifuged (20,800-x g, 4°C) for 15 minutes to separate cytosolic and membrane fractions. A volume of 150-180μl of supernatant (cytosolic fraction) was removed and the insoluble membrane fraction was re-suspended in similar volume of solubilization buffer1 (9.2mM Tris, 0.92mM EGTA, 150mM NaCl, 0.1%(v/v) SDS, 1% (v/v) ethylphenylpolyethylene glycol (Nonidet P-40), 0.5% (w/v) deoxycholate, 0.5mM PMSF, 10μM benzamidine hydrochloride and 5μM iodoacetamide, pH 7.4). An equal volume of Laemmli's buffer (100mM Tris, 2% SDS, 0.1% bromophenol blue, 10% (v/v) glycerol, 100mM DTT) was added to

the membrane and cytosolic fractions. Proteins were separated by SDS PAGE (see section 2.3.2), transferred to nitrocellulose and immunoblotted for PKC isoforms or other proteins in cytosol fraction as described in section 2.3.3. For the antibody concentration, see **Table 2.1**.

2.7. Assessment of $G\alpha_i$ activation by [^{35}S]- guanosine 5-[γ-thio]triphosphate GTPγS binding assay

Activation of Gαi was assessed by a [^{35}S]-(GTPγS) binding/ immunoprecipitation assay as previously described (Akam *et al.*, 2001). This assay measures GDP/ [^{35}S]-(GTPγS) exchange on Gα subunits.

2.7.1. Membrane preparation

OK cells were grown in 175cm^2 flasks to 90% confluency. Cells were serum starved overnight and then washed once and incubated with 10ml lifting buffer (10mM HEPES, 0.2% EDTA, 0.9% NaCl, pH 7.4) at room temperature for 10 minutes with gentle agitation to dislodge. OK cells were then centrifuged at 2000-x g for 5 minutes at 4°C. Pellets were homogenised for 20 seconds in hypo-osmotic lysis buffer3 (10mM HEPES, 10mM EDTA, pH 7.4) using a polytron homogeniser and the homogenate was centrifuged at 18000-x g for 15 minutes at 4°C. Final pellets were homogenised in freezing buffer (10mM HEPES, 0.1mM EDTA). Protein concentration was determined by method of Bradford (see section, 2.14.), and adjusted to 2mg/ml and aliquots frozen at -80°C.

2.7.2. [^{35}S]-GTPγS binding after C-peptide stimulation

Frozen aliquots of OK cell membrane preparation were diluted in assay buffer (10mM HEPES, 100mM NaCl, 10mM MgCl, pH 7.4) to give a final protein concentration of 25μg/50μl. Membranes (25μg) were added to 50μl of assay buffer containing (final concentrations) 1nM [^{35}S]-GTPγS (1250Ci/mmol)

diluted in 10mM tricine, 10mM DTT, 10μM GDP, 5nM C-peptide or 5nM sC-peptide and where appropriate 10μM unlabelled GTPγS to determine non-specific binding and incubated at 37°C for 1, 3, and 5 minutes. Incubation was terminated by addition of 900μl ice-cold assay buffer, and all subsequent procedures were performed at 4°C. Cell membranes were pelleted by centrifugation (20000-x g, 4°C) for 6 minutes and solubilised by addition of 50μl ice-cold solubilisation buffer2 (100mM Tris, 200mM NaCl, 1mM EDTA, 1.25% Igepal, 0.2% SDS, pH 7.4). Following solubilisation of pellets, a further 50μl solubilisation buffer2, without SDS, was added to each sample.

2.7.3. Immunoprecipitation of [^{35}S]-GTPγS-bound Gαi proteins

Solubilised OK cells membranes were pre-cleared by adding of 13μl normal rabbit serum (diluted 1:10 with assay buffer) and 30μl protein sepharose A beads (protein sepharose A suspension 30% w/v in TE buffer, 10mM Tris-base, 1mM EDTA, pH 7.4) with rotating incubation at 4°C for 90 minutes. Protein sepharose A beads and any insoluble material were cleared by centrifugation (20000-x g, 4°C) for 6 minutes and 100μl of supernatant of each sample was transferred to tube containing 5μl specific anti-$G\alpha_i$ (1:1000 dilution with sterile H_2O). Samples were mixed and incubated with rotation at 4°C overnight. 70μl of a 30% (w/v) protein sepharose A was added to each sample then mixed and incubated in rota for 90 minutes at 4°C. The protein sepharose A beads were then pelleted (16000-x g, 4°C) for 6 minutes and supernatant removed by aspiration. Beads were then washed using 500μl solubilisation buffer2 without SDS then mixed and pelleted (16000-x g, 4°C) for 6 minutes for a total of 3 times. Following the final wash, the protein sepharose A beads were mixed with 1.1ml

scintillation fluid (SafeFluor) and radioactivity counted by standard liquid scintillation methods.

2.8. [^{3}H]-Thymidine incorporation

Proliferation of OK cells in response to C-peptide was assessed by [^{3}H]-thymidine incorporation. Cells were plated in 24-well plates and grown to approximately 80% confluence. Cells were serum-starved overnight and then were exposed to various concentrations of C-peptide, 10% FCS or serum-free media (as a control). After 22 hours of incubation at 37°C, culture medium was replaced with serum-free medium containing 1μCi of [^{3}H]-thymidine. After a further 2 hours of incubation, cells were washed 3 times with 1ml ice-cold PBS and fixed at 4°C in 50% methanol and 10% glacial acetic acid for 30 minutes. Cells then were washed twice with 1ml ice cold PBS and then incubated with 0.5ml per well 10% TCA acid for 10 minutes, and then washed 3 times with 1ml ice cold PBS and air-dried for 30 minutes. Cells were then solubilised overnight in 250μl per well 100mM NaCl containing 0.1% SDS. [^{3}H]-thymidine incorporation was determined by adding 4.5ml scintillation fluid (Ultma Gold) to the samples. These were counted using a Packard 1900 CA Tri- Carb liquid scintillation counter.

2.9. Luciferase reporter studies

2.9.1. Transfection studies

OK cells were transfected with reporter plasmid consisting of peroxisome proliferator-activated response element upstream of a thymidine kinase promoter and the luciferase reporter gene pPPRE-TK-luc, NFκB dependent luciferase plasmid pNFκB-luc reporter and/or various combinations of plasmid encoding full length mouse PPARγ1 pCMX-PPAR-γ, plasmid encoding constitutively active

MEK-1 pMEK1-CA, plasmid encoding kinase dead mutant of MEK-1 pMEK1-KD. As a control of transfection efficiency, the plasmid pSVβgal, which contain a beta galactosidase gene that is driven by a Rous Sarcoma virus promoter and enhancer, was co-transfected with each construct. All transient transfection studies were performed in fully supplemented media using Fugene 6 transfection reagent. Briefly, 25,000 cells per well plated in 24-well plates were grown overnight to approximately 50% confluency. Cells were then washed once with serum free-media and then supplemented with fresh 1ml fully supplemented media.

The plasmid DNA was mixed with Fugene in the ratio of 1μg DNA: 3μl Fugen and diluted in serum free-media. Fugene/DNA mixtures were incubated for 20 minutes at room temperature, and then a volume of 25μl was added directly to each well of cells. For 24 well plates 0.25μg of each plasmid DNA was used per well, and in experiments involving the transfection of multiple plasmids, equivalent concentrations of DNA were maintained by addition the appropriate empty vector. The cells were then incubated as normal for a further 24 hours following which the medium/DNA mixture was removed and replaced with 1ml fresh serum free medium per well overnight before stimulation with C-peptide or other agents.

Where appropriate native and recombinantly expressed proteins were detected in cell lysates by western blotting using appropriate antibodies as described in sections 2.3.3 and 2.3.4.

2.9.2. Luciferase reporter assays

After appropriate treatments in transfected cells, media was removed and cells lysed in lysis buffer4 containing of (500mM HEPES, 2% Triton N101, 1mM

$CaCl_2$, 1mM $MgCl_2$, pH 7.8). Cell lysis was allowed to proceed for 10 minutes and luciferase activity in lysates was measured using the LucLite assay kit in a LumiCount luminometer (Packard, Pangbourne, England). In all experiments a 50µl aliquot of lysate was removed for β-galactosidase (β-gal) assay as described bellow and luciferase activity was normalized to β-gal content.

2.9.3. β-Galactosidase (β-gal) assay

β-gal assay was performed according to the manufacturer's instructions. An aliqute of 50µl of cell lysates from each well was transferred to a 96 well plate then mixed with 50µl of assay buffer provided with the kit. All samples were mixed well by pipetting the well contents. Then the plate was incubated in the dark at 37°C for 30 minutes until a faint yellow shade developed. The reaction was stopped by adding 150µl of 1M ice-cold sodium carbonate to each well. Absorbance was measured at 420 nm using a spectrophotometric plate reader.

2.10. [^{32}P]-orthophosphate labelling of OK cells and immuno-precipitation of phospho-PPARγ

This assay was performed as previously described (Chana *et al.*, 2004). Confluent wild-type OK cells in 6-well plates were serum-starved overnight, incubated with or without 100nM wortmannin for 30 minutes then washed three times with phosphate-free DMEM F-12. Cells were then incubated with serum-free medium containing 200µCi/ml [^{32}P]-orthophosphate and 5nM C-peptide or 100nM insulin for 4 hrours at 37°C. Cell monolayers were then washed three times with 1ml ice-cold PBS pH 7.4, and then incubated for 30 minutes with 500µl ice-cold lysis buffer5 composed of (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 200µM Na_3vo_4), made up in PBS pH 7.4. The lysates were clarified by 5 minutes of centrifugation at 11000-x g and the supernatant was

pre-cleared with 50µl protein A-Sepharose for 45 minutes at 4°C then centrifuge at 13000-x g for 1 minute. 550µl of supernatant was incubated with 10µl of anti-PPARγ overnight at 4°C. Immune-complexes were collected using 70µl protein A-sepharose beads per sample and incubated for 90 minutes at 4°C. The beads were then washed 3 times with lysis buffer and then 35µl of Laemmli's buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 100mM DDT, and 0.1% bromphenol blue) was added. Immunoprecipitated proteins were then separated on 12% polyacrylamide gels that consist of 12% SDS PAGE running gels (4ml dH2O, 3.3ml 30% acrylamide mix, 2.5ml 1.5 M Tris (PH 8.8), 0.1ml 10% SDS (w/v), 0.1ml 10% ammonium persulfate (w/v) and 4µl TEMED in a total volume of 10ml) and stacking gels (3.4 ml dH2O, 0.83 ml 30% acrylamide mix, 0.63ml 1.0M Tris (PH 6.8) 0.05ml 10% SDS (w/v), 0.05ml 10% ammonium persulfate (w/v), and 4µl TEMED in a total volume of 5ml), as described in section 2.3.3 and 2.3.4. Gels were then stained for 25 minutes at room temperature in methanol: H2O 1/1 (v/v), 10% glacial acetic acid and 2.5mg/ml Coomassie brilliant blue. The gels were destained in methanol: H2O 1/1 (v/v), 10% glacial acetic acid for 1 hour at room temperature and then dried for 1 hour at 80°C. Radiolabeled proteins were detected by autoradiography.

2.11. THP-1 cell differentiation experiments

THP-1 cells were plated in 6 well plates in serum free medium containing potential stimulators of differentiation, including as a positive control 1µM phorbol myristate acetate (PMA), for either 24 or 48 hours. Cells were collected by centrifugation at 5000-x, 4°C for 1 minute, washed twice with 200µl serum free medium, and then lysed in 200µl lysed in Laemmli's buffer (50mM Tris, PH 6.8, 10% glycerol, 2% SDS, 100mM (DTT) and 0.1% bromophenol blue) and

incubated on ice for 10 minutes. Aliquots were denaturated by heating at 99° C for 3-5 minutes. Whole cell lysates were then separated on 12% polyacrylamide gels (see sections 2.3.3. and 2.9.). Expression of the differentiation marker CD 36 (Huh *et al.,* 1996; Nicholson, 2004) was examined by western blotting using anti-β-actin as a loading control as described in section 2.3.4.

2.12. Methylthiazolteterazolum (MTT) assay

Cell viability was measured using MTT assay. This assay is based upon the reduction of soluble yellow salt 3-(4,5- dimethyl-2-thiazol-2-yl) 2,5-diphenyltrazolium bromide to purple formazan by viable cells. A high correlation between the viable cell number and the formazan product has been reported. The MTT assay was performed as previously described (Chana *et al.,* 2004). OK cells were seeded at $2.5x10^4$ / well in 24 well plates for 24 hours and then pre-incubated with or without 5nM C-peptide or 100nM insulin for 24 hours in serum-free media. Cells then were stimulated with different concentrations of human tumour necrosis factor alpha (TNF-α) (50-300 ng/ml) for 24 hours. Cells were incubated with 50μl of 2mg/ml MTT solution for 1 hour. The upper medium was removed carefully and the intracellular formazan was solubilised by adding 200μl dimethyl sulfoxide to each well. The absorbance of produced formazan was measured at 540 nm with spectrophotometric plate reader.

2.13. Apoptosis assay

2.13.1. Cell death detection assay, enzyme linked immunosorbent assay (ELISA)

This assay used the presence of cytoplasmic histone associated DNA fragments (mononucleosome and oligonucleosomes) to identify apoptotic cells by use of an enzyme-linked immunosorbent assay. Cells were cultured in 24 well

plates for 24 hours and then pre-incubated with or without 5nM C-peptide or 100nM insulin for further 24 hours in 1ml serum-free media. The cells were then stimulated with 300ng/ml TNF-α or other agents for 24 hours. C-peptide and insulin remained in the media during stimulation with TNF-α and other agents. In some experiments, cells were incubated with an NFκB inhibitor for 1 hour prior to incubation with C-peptide or insulin. After stimulations cells were washed twice with 1ml PBS then lysed using 200μl of a lysis buffer provided with the kit, for 30 minutes at 15-25°C. Lysates were then centrifuged 200-x g for 10 minutes and 20μl of supernatant was transferred carefully into a streptavidin-coated microplate. Immunoreagent was prepared by mixing of 1/20 volume anti-DNA-POD, peroxidase conjugated antibody for the binding of the DNA components of the nucleosomes, and 1/20 volume Anti-histone-biotin, for the binding of histone component of the nucleosomes, with 18/20 volume incubation buffer and mixed thoroughly. 80μl of the immunoreagent was then added to each well, the microplate covered with adhesive foil, and incubated with gentle shaking (300 rpm) for 2 hours at 15-25°C. The solution then removed thoroughly by suction and each well was washed three times with 200μl of incubation buffer for 3 minutes. Finally 100μl of substrate solution was added to each well and the microplate incubated on a shaker at 300 rpm for 15 minutes at room temperature to develop colour sufficient for photometric analysis. The end-product colour was measured at λ405 nm against substrate solution as a blank using a spectrophotometric plate reader.

2.13.2. DNA nick end-labelling of OK cells

The method of terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate-biotin nick end labelling (TUNEL) was performed

using an *in situ* cell death detection kit manufacture. OK cells were plated into sterile Lab-Tek 4 chamber slides and allowed to adhere for 24 hours. The cells were pre-incubated with or without 5nM C-peptide or 100nM insulin for 24 hours in serum-free media prior to stimulation with 300ng/ml TNF-α for 24 hours. C-peptide and insulin remained in the media during stimulation with TNF-α. After stimulations cells were rinsed twice for 5 minutes with 1ml PBS and fixed with 200µl 4% paraformaldehyde freshly prepared in PBS, pH, 7.5 for 30 minutes. Cells were then washed 3 times for 5 minutes with 1ml PBS and incubated in 1ml permeabilisation solution 0.1% Triton X-100 freshly prepared in PBS for 8 minutes at room temperature. Cells were washed 2 times for 5 minutes with 1ml PBS and air dried for 3 minutes. The TUNEL reaction mixture was prepared by adding 50µl terminal deoxynucleotidyl transferase from calf thymus to 450µl nucleotide mixture in reaction buffer. A total of 50µl TUNEL reaction mixture was added to cover the cells and the slides were then incubated in a humidified chamber for 60 minutes at 37°C. Cells were washed three times for 5 minutes with 1ml PBS, and incubated with 50µl converter-alkaline phosphatase (AP) ,antifluorescein antibody, for 30 minutes at 37°C in a humidified chamber. Cells were washed with 1ml PBS and were incubated with 50µl substrate buffer with Fast Red and 1mM levamisole to block endogenous AP activity for 10 minutes at room temperature, and then washed three times for 5 minutes with 1ml PBS, counterstained with hematoxylin, and mounted under glass coverslips with drop of glycerol and specific staining of apoptotic cells was visualised with a light microscopy (original magnification X 400). For quantification of apoptotic cells, 50 cells from 6 random microscopic fields of each slide were counted and the mean percentage of apoptotic cells was represented graphically.

2.14. Bradford protein assay for protein determination

Protein concentrations were determined as described by (Bradford, 1976). Briefly, duplicate protein standards were created by serial dilution of 1mg/ml BSA from 0-50μg/ml. The total liquid volume in each tube was adjusted to 1ml by the addition of deionised water. The unknown samples with an approximate concentration of protein between 1-50μg/ml were prepared and the total volume also adjusted to 1ml with deionised water. Thereafter, to each tube, 2ml of Bradford's reagent (0.0035% (w/v) Coomassie brilliant blue, 3.5% (v/v) ethanol and 7.5% (v/v) phosphoric acid) was added and mixed. Both standards and samples were incubated at room temperature for 5 minutes. The absorbance of developed colour was measured at λ595 nm using a spectrophotometer.

The net absorbance versus the protein concentration of each standard was plotted and the protein concentration of the unknown sample was determined from the standard curve plotted using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

2.15. Data analysis

All data given in text are mean ± SEM. Unpaired, two-tailed, Student's *t* test was used for comparisons between two groups. For multiple comparisons, one-way ANOVA with Tukey's correction was used. P values of less than 0.05 were considered statistically significant. The analysis was conducted using Prism software.

Table 2.1: Antibody working dilution

Antibody	Source	Working concentration µg/ml	Supplier
Phospho-ERK1/2	mouse	0.2	Santa Cruz Biotechnology
Total ERK1/2	rabbit	0.2	Santa Cruz Biotechnology
Phosp-Akt1/PKBα	sheep	0.9	Upstate
Total Akt1/PKBα	sheep	0.9	Upstate
PKCα	mouse	0.1	Transduction Laboratories
PKCε	mouse	0.5	Transduction Laboratory
PKCι	mouse	0.5	Transduction Laboratory
PPAR-α	rabbit	0.2	Santa Cruz Biotechnology
PPAR-β	rabbit	0.2	Santa Cruz Biotechnology
PPAR-γ	rabbit	0.2	Santa Cruz Biotechnology

PI3-kinase p85-α	rabbit	0.2	Santa Cruz Biotechnology
TRAF2	rabbit	0.2	Santa Cruz Biotechnology
Gαi	rabbit	0.2	Santa Cruz Biotechnology
IκB-α	rabbit	0.2	Santa Cruz Biotechnology
B-actin	mouse	0.05	Santa Cruz Biotechnology

CHAPTER 3
C-PEPTIDE SIGNALLING IN PROXIMAL TUBULAR CELLS

3.1. INTRODUCTION

Diabetic nephropathy remains a serious complication of diabetes, and is characterised initially by renal growth. Hypertrophy of glomerular and tubular cells is prominent together with thickening of basement membranes, but hyperplasia is absent (Ziyadeh *et al.,* 1993; Mogensen and Andersen., 1973; Seyer-Hansen., 1976). Later, accumulation of matrix components occurs in the glomerulus and tubulo-interstitium with progressive sclerosis. Tubular atrophy with almost complete loss of PTC is evident, and clearly implies major disturbances in growth and survival of this cell type. Interestingly there does not appear to be substantial differences in the pathogenesis of nephropathy in types 1 and 2 diabetes (Ritz and Stefanski, 1996; Ibrahim and Hostetter 1997; Wirta and Pasternack 1995).

Signal transduction pathways activated in response to persistent homeostatic disturbances in diabetes that may initiate and/or propagate diabetic renal pathophysiology have been subject to much study. Activation of PKC in the kidney occurs within days after the onset of experimental diabetes (Derubertis and Craven., 1994), and this may in turn stimulate phospholipase A2, leading to release of membrane–bound arachidonate (Derubertis and Craven., 1994; Craven *et al.,* 1988). Another consequence of renal PKC activation is stimulation of MAPK (Ziyadeh *et al.,* 1995). Activation of MAPK in glomeruli obtained from rats with STZ-induced diabetes, and mesangial cells grown in high glucose is mediated by PKC and contributes crucially to cell hypertrophy (Haneda *et al.,* 1997).

Although C-peptide has an important clinical role as a surrogate marker of insulin release and hence pancreatic beta cell function, for many years C-peptide itself has been regarded as biologically inert with no significant physiological purpose. In recent years a trickle of publications has challenged this view and an increasingly substantial body of data, derived from studies of both animals and human with diabetes, now supports a role for C-peptide in glucose homeostasis, the amelioration of the consequences of hyperglycaemia, and the abrogation of diabetic complications (Zierath *et al.*, 1991; Zierath *et al.*, 1996; Ido *et al.,* 1997, Sjoquist *et al.,* 1998, Johansson *et al.,* 2000, Ekberg *et al,* 2003) for review (see section 1.4). Despite this accumulating evidence, the concept of C-peptide as a bioactive molecule continues to struggle to gain wide acceptance. Nonetheless, the observation that C-peptide can alter cell function suggests that it may, similarly to insulin, exert endocrine actions and possess signalling activity.

Loss of PTC during the development of Type 1 diabetic nephropathy may reflect sever defects in the proliferation and/or survival programs of these cells (Ortiz *et al.,* 2001), possibly related to lack of periodical stimulation by C-peptide and activation of downstream targets. Therefore, in this results chapter, it was chosen to study effects of C-peptide in kinases with well defined role in regulation of cells growth. Activation of ERK is reported to result in cell proliferation (Gutkind, 2000), and anti-apoptotic role of Akt stimulation has been demonstrated (Lawlor and Alessi, 2001). In addition, PKC has been reported to play a crucial role in cell growth, division and cell differentiation (Clemens *et al.,* 1992; Iwamoto *et al.*, 1992). C-peptide has been reported to stimulate these kinases apart from Akt (for review see sections 1.7. and 1.8).

The precise role of C-peptide, or the lack thereof, in the modulation of these signalling pathways in diabetes and the pathogenesis of diabetic nephropathy remains uncertain. Thus, the aims of these experiments were to identify an appropriate cell type for study, to establish a repertoire of C-peptide signalling effects focusing especially on those pathways involved in cell growth and those potentially relevant to the development of diabetic nephropathy, and finally to act as a platform for future studies.

3. 2. RESULTS

3.2.1. Preliminary experiments

3.2.1.1 Preliminary experiments using primary cultures of HPTC

Most of the previously reported intracellular effects of C-peptide have been observed in cell models of rodent origin. However, my first choice was to use primary cultures of PTC derived from human kidney cells as a model to examine the intracellular effects of human C-peptide.

Effects of C-peptide on ERK and Akt phosphorylation in HPTC

These preliminary studies demonstrate that HPTC in primary culture responded to acute C-peptide exposure. Stimulation of HPTC with different concentrations of C-peptide (0.1-10nM) for 5 minutes resulted in concentration-dependent phosphorylation of ERK as detected by immunoblotting (**Fig 3.1**). Phosphorylation of ERK markedly increased 1.47 ± 0.12, fold over basal at C-peptide concentration as low as 0.1nM, with maximal stimulation of 2.3 ± 0.25, fold over basal at C-peptide concentration of 0.5nM. Loading controls total ERK antisera demonstrated equivalent protein loading between lanes.

Likewise, C-peptide induced concentration dependent activation of Akt, with substantial activation of Akt at a concentration of 0.1nM. Maximal phosphorylation of Akt about 3.3 ± 0.31, fold over basal was observed with 1nM C-peptide (**Fig 3.2**).

Thus, similar to its effects on ERK phosphorylation, the most marked effects of C-peptide on Akt phosphorylation were at concentrations close to physiological concentrations and progressively less with concentrations of C-peptide above 0.5nM and 1nM for ERK and Akt phosporylation respectively.

Blotting for β-actin as loading control demonstrated equivalent protein loading between lanes.

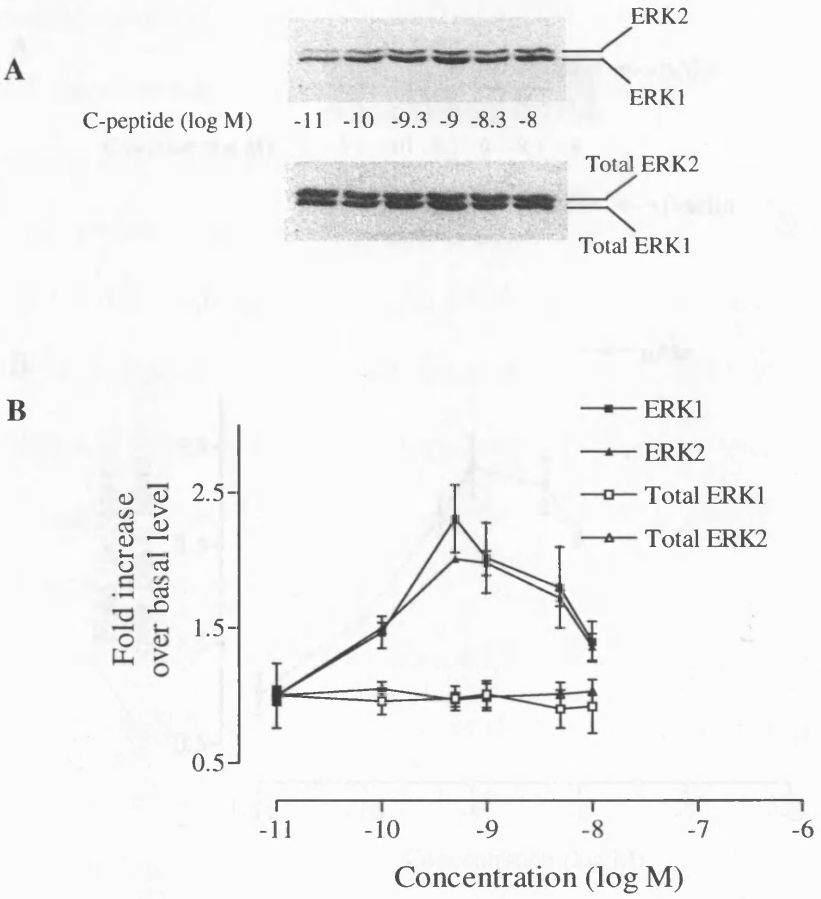


Fig 3.1. Concentration-dependent stimulation of ERK phosphorylation by C-peptide in HPTC. Cells were treated with indicated concentrations of C-peptide for the 5 minutes and total ERK or pERK detected by immunoblotting. **(A)** A representative blot is shown. **(B)** Phosphorylated bands were quantified and values were expressed graphically. Within the graph data are means ± SEM, n=3 positive experiments out of 18.

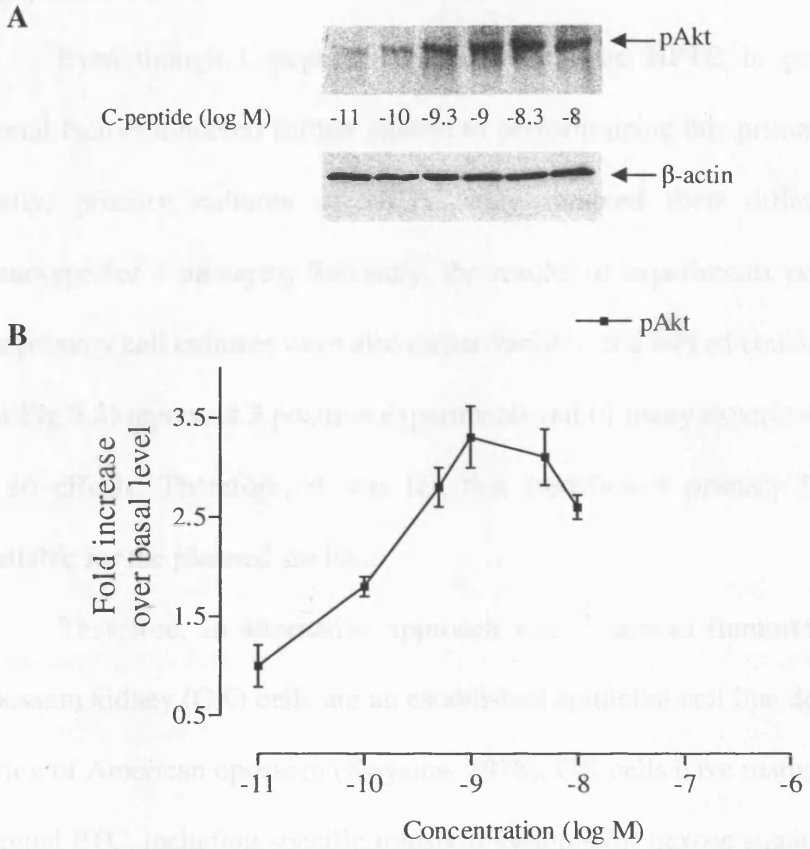


Fig 3.2. Concentration-dependent stimulation of Akt phosphorylation by C-peptide in HPTC. Cells were treated with the indicated concentrations of C-peptide for 5 minutes and pAkt detected by immunoblotting. **(A)** A representative blot is shown. **(B)** Phosphorylated bands were quantified and values were expressed graphically. Within the graph data are means ± SEM, n=3 positive experiments out of 10.

3.2.1.2. Opossum kidney cells as an alternative approach to examine effects of C-peptide on PTC

Even though C-peptide seemed to activate HPTC in primary culture, several factors hindered further studies to perform using this primary cell culture. Firstly, primary cultures of HPTC only retained their differentiated PTC phenotype for 3 passages. Secondly, the results of experiments performed using this primary cell cultures were also rather variable and lacked consistency (**Fig 3.1** and **Fig 3.2**) represent 3 positive experiments out of many experiments with slight or no effects. Therefore, it was felt that insufficient primary HPTC may be available for the planned studies.

Therefore, an alternative approach was to use an immortalised cell line. Opossum kidney (OK) cells are an established epithelial cell line derived from the cortex of American opossum (Koyama, 1978). OK cells have many characteristics of renal PTC, including specific transport systems for hexose sugars, amino acids, phosphate (Malmstron *et al.,* 1987), and Na^+-H^+ exchange (Moran *et al.,* 1988; Pollock *et al.,* 1986). In addition, OK cells express receptors for parathyroid hormone (Teitelbaum and Strewler, 1984), angiotension receptors (AT1) (Bandary *et al.,* 2005), α_2-receptor (Murphy and Bylund, 1988), serotonin (Murphy and Bylund, 1989) and dopamine (Guimaraes *et al.,* 1997). OK cells also have insulin receptors, and the binding, internalisation, and degradation of insulin by these cells has been studied (Yagil *et al.,* 1988a; Yagil *et al.,* 1988b; Yagil *et al.,* 1988C).

One key concern in the investigation of signalling effects of C-peptide in OK cells was that because the structure of C-peptide varies considerably between species this may make it difficult to anticipate binding and molecular effects of

human C-peptide in this cell line. However, C-peptide binding may depend on a single or few residues, conserved in mammals. For example, free Glu and C-terminal pentapeptide of human C-peptide (EGSLQ) are able to partially displace membrane-bound C-peptide (Pramanik *et al.*, 2001). Moreover, human C-peptide was reported to elicit effects in diabetic rat (Ido *et al.*, 1997). Likewise, a bioassay based on Ca^{2+} release showed cross-reactivity of both rat C-peptide and its C-terminal pentapeptide in human cells (Shafqat *et al.*, 2002). Therefore, further preliminary C-peptide studies were performed in OK cells.

Binding of human C-peptide to OK cell

Reassurance that C-peptide may signal in OK cells was sought by examining the potential for binding of fluorescent labelled C-peptide to OK cells. $5x10^3$ cells were seeded onto 25mm diameter non-coated glass coverslips and cultured for 24 hours then serum-starved overnight. Tetramethylrhodamine (Rh) lablled human C-peptide was added to serum-starved OK cells excited at λ569 nM and imaged by confocal microscopy. A comparison of phase image and fluorescent image with or without addition of 100nM Rh-C-peptide is shown in (**Fig 3.3**). Fluorescence was localised to the plasma membrane and no fluorescence was observed in cells excited at λ569 in absence of Rh-C-peptide. Photobleaching occurred very rapidly regardless of adjustment in excitation intensity and temperature. Thus, it was very difficult to pursue further studies to establish the kinetics and pharmacology of Rh-human C-peptide binding to OK cells. Nevertheless, this preliminary study gave reassurance that human C-peptide may bind and potentially trigger signalling pathways in OK cells.

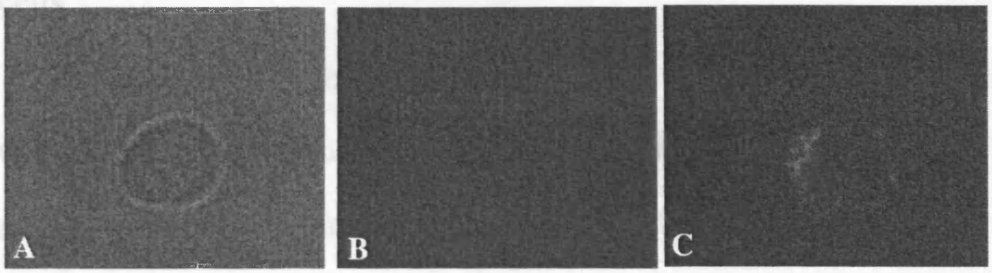


Fig 3.3. Binding of Rh-C-peptide to plasma membrane of OK cells. OK cells were cultured on non-coated cover-slips for 24 hours and imaged using confocal microscope. The phase image (image A) was compared with the same image excited at λ 569 nM in absence of Rh-C-peptide (image B) or following addition 100nM Rh-C-peptide (image C). Images are representative of 2 independent experiments.

3.2.2. Identification some of C-peptide signalling effects in OK cells

Having found that human C-peptide bound to OK cells, it was decided that OK cells would be a suitable PTC model to investigate the signalling effects of C-peptide. All subsequent experimental results depict observations in OK cells.

Effects of C-peptide on ERK activation

Western blotting was used to examine effects of C-peptide on ERK phosphorylation. Incubation of OK cells with C-peptide resulted in both a time- (**Fig 3.4**) and concentration-dependent (**Fig 3.5A**) increase in phosphorylated ERK. Phosphorylated ERK appeared rapidly following stimulation, displaying a peak and plateau. A maximal stimulation of 3.3 ± 0.1 fold of basal occurred at 3 minutes, which fell to a plateau between 10 and 30 minutes. Phosphorylation of ERK markedly increased 1.8 ± 0.1 fold of basal at C-peptide concentrations as low as 0.1nM, with maximal stimulation of 2.1 ± 0.1 fold of basal at 0.3nM. The phosphorylation of ERK-1 and -2 was progressively less with concentrations of C-peptide above 0.3nM. Loading controls using total ERK-1 antisera demonstrated equivalent protein loading between lanes. Scrambled C-peptide did not affect phosphorylation of either ERK-1 or –2 (**Fig 3.4B**).

To confirm ERK activation in OK cells by C-peptide, ERK activity was examined by immune complex *in vitro* kinase assay using a synthetic peptide corresponding to a portion of EGF receptor as substrate. **Fig 3.6** shows a concentration-dependent stimulation of ERK activity in response to C-peptide for 5 minutes, and displays a very similar concentration response to that observed in the phosphorylated ERK immunoblots, maximal ERK activity (135 ± 1%) relative

to control being observed with 0.5nM C-peptide and declining thereafter. SC-peptide had no significant effect on ERK activity (**Fig 3.6**).

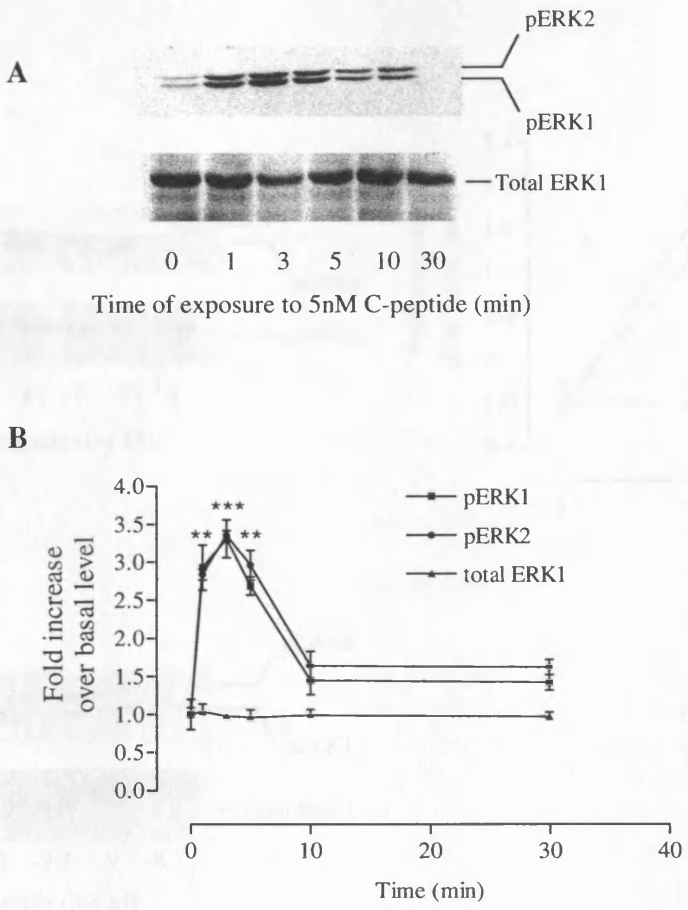


Fig 3.4. Time course-dependent stimulation of ERK phosphorylation by C-peptidein OK cells. Cells were treated with 5nM C-peptide for the indicated times and total ERK or pERK detected by immunoblotting. **(A)** A representative blot is shown. **(B)** Phosphorylated bands were quantified and values expressed graphically. **p<0.01, ***p<0.001, relative to 0 time. Within the graph data are means ± SEM, n=3 experiments.

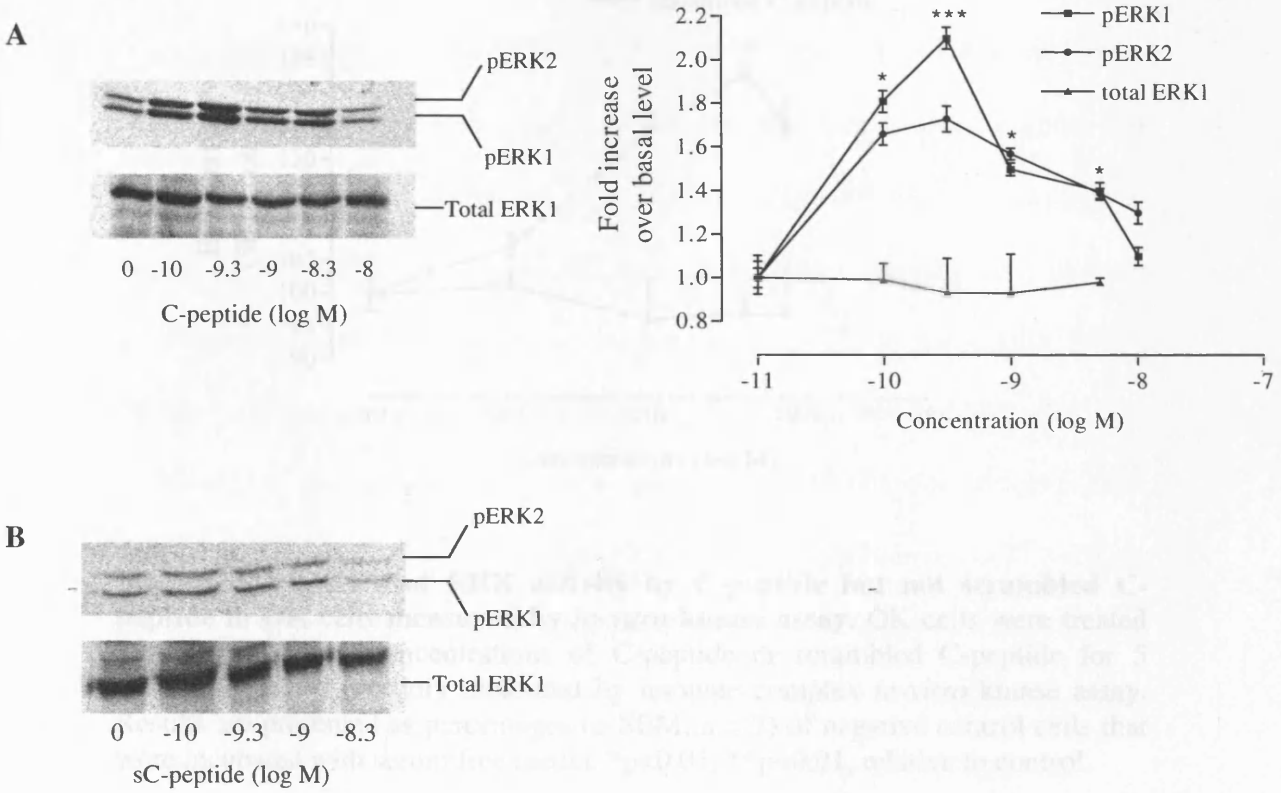


Fig 3.5. Concentration-dependent stimulation of ERK phosphorylation by C-peptide but not sC-peptide in OK cells. Cells were treated with the indicated concentrations of C-peptide for 5 minutes and total ERK or pERK detected by immunoblotting. **(A)** A representative blot is shown (left panel). Phosphorylated bands were quantified and values expressed graphically (right panel). *p<0.05,***p<0.001, relative to control time. **(C)** Effect of scrambled C-peptide on ERK phosphorylation in OK cells. Cells were treated with the indicated concentrations of scrambled C-peptide for 5 minutes and total ERK or pERK detected by immunoblotting. A representative blot is shown. Within the graphs data are means ± SEM, n=3 experiments.

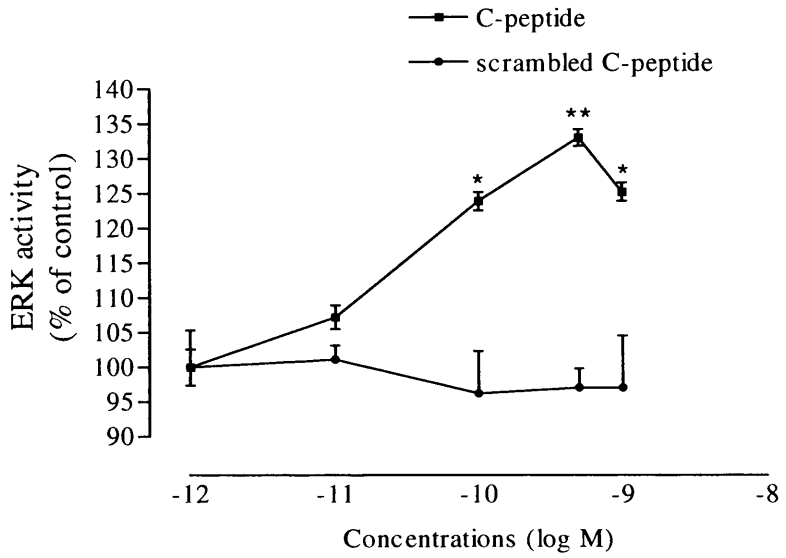


Fig 3.6. Stimulation of ERK activity by C-peptide but not scrambled C-peptide in OK cells measured by *in-vitro* kinase assay. OK cells were treated with the indicated concentrations of C-peptide or scrambled C-peptide for 5 minutes and ERK activity measured by immune complex *in-vitro* kinase assay. Results are presented as percentages (± SEM, n = 3) of negative control cells that were incubated with serum free media. *$p<0.05$, **$p<0.01$, relative to control.

Activation of ERK by C-peptide is PTX sensitive and depends on PKC but not PI 3-kinase

To determine the role of $G\alpha_{i/o}$ G-proteins in activation of ERK, cells were pre-incubated overnight with 100ng/ml PTX in serum-free media, an agent that ADP ribosylates and inhibits $G\alpha_{i/o}$ proteins. Pre-treatment of the cells with PTX entirely blocked the activation of ERK induced by 5nM C-peptide for 5 minutes as it was measured using immune complex *in vitro* kinase assay (**Fig 3.7A**). Similarly, pre-treatment with 10μM bisindolylmaleimide (Ro-31-8220), the PKC inhibitor, for 30 minutes also significantly attenuated C-peptide induced ERK activity (**Fig 3.7A**). Pre-treatment with 100nM wortmannin (wort), the PI 3-kinase inhibitor, for 30 minutes had no significant effects on C-peptide induced ERK activity (**Fig 3.7A**). PTX also abolished the ability of C-peptide to cause phosphorylation of ERK-1 and -2 as detected by immunoblotting (**Fig 3.7B**).

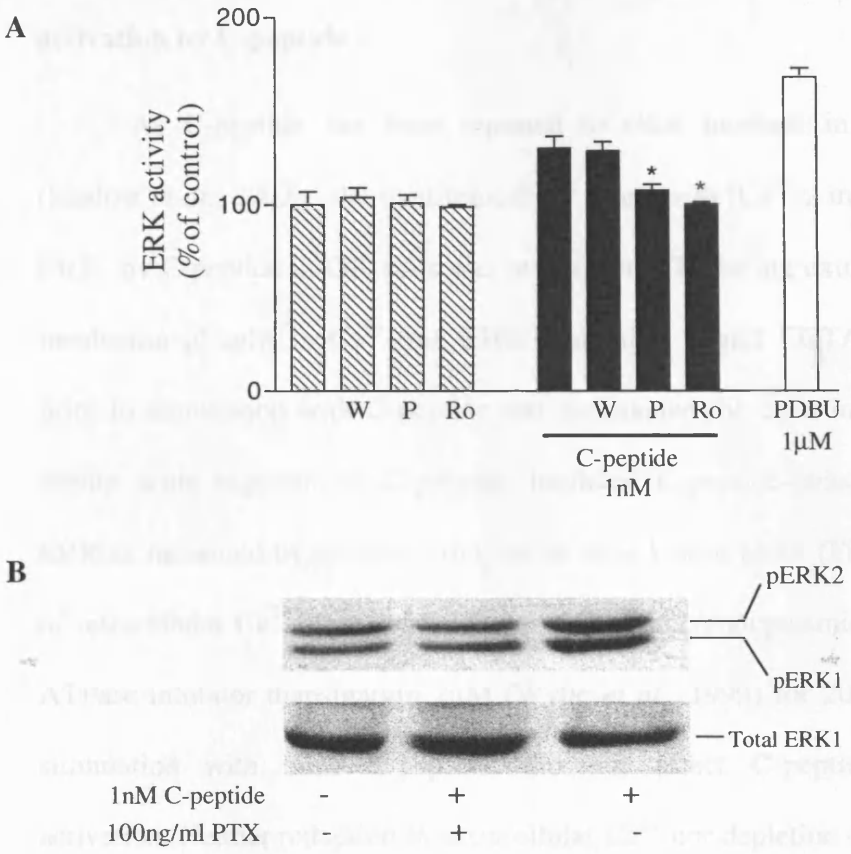


Fig 3.7. Effects of PTX (P), Ro-31-8220 (Ro) and wortmannin (W) on basal and C-peptide- induced ERK activity in OK cells. **(A)** OK cells were pre-treated with 100 ng/ml PTX for 18 hours, or Ro-31-8220 10μM for 30 minutes, or wortmannin 100nM for 30 minutes and were then incubated with 1nM C-peptide for 5 minutes. The effect of phorbol dibutyrate (PDBU) is shown as a positive control. ERK activity was measured by *in-vitro* kinase assay. Results are presented as percentages (± SEM, n=3 experiments) of control cells incubated with 1nM C-peptide alone. * $p<0.05$, relative C-peptide alone. **(B)** PTX inhibits C-peptide induced ERK phoshorylation detected by immunoblotting in OK cells. OK cells were pre-treated with 100ng/ml PTX for 18 hours and then incubated with 1nM C-peptide for 5 minutes. Blot representative of 3 independent experiments.

Effects of removal of extracellular Ca^{2+} and depletion of $[Ca^{2+}]_i$ on ERK activation by C-peptide

As C-peptide has been reported to elicit increase in $[Ca^{2+}]_i$ in PTC (Shafqat *et al.,* 2002), the requirement for changes in $[Ca^{2+}]_i$ in the activation of ERK by C-peptide in OK cells was investigated. Reducing extracellular Ca^{2+} by incubation of cells in Ca^{2+}-free KHB containing 10mM EGTA for 20 minutes prior to stimulation with C-peptide and maintained the cells in the same media during acute exposure to C-peptide, inhibited C-peptide-induced activation of ERK as measured by immune complex *in vitro* kinase assay (**Fig 3.8**). Depletion of intracellular Ca^{2+} stores by addition of the sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin 2μM (Wylie *et al.,* 1999) for 20 minutes prior to stimulation with 5nM C-peptide, did not affect C-peptide-induced ERK activation. Neither reduction in extracellular Ca^{2+} nor depletion of $[Ca^{2+}]_i$ had any effect on basal ERK activation. These data suggest that ERK activation by C-peptide was dependent on extracellular Ca^{2+}.

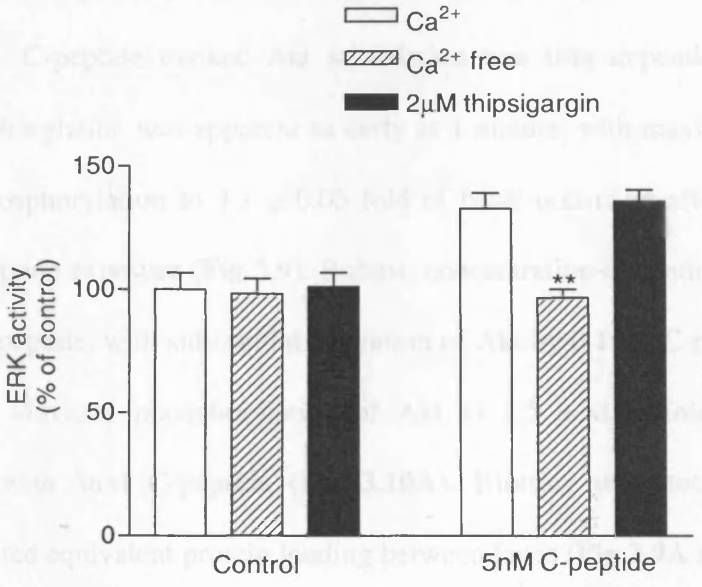


Fig 3.8. Effects of Ca^{2+} free and depletion of $[Ca^{2+}]_i$ on C-peptide-induced ERK activation in OK cells. Cells were either incubated in normal KHB (1.3mM Ca^{2+}), Ca^{2+}-free KHB supplemented with 10mM EGTA or normal KHB with 2μM thapsigargin for 20 minutes prior to stimulation with 1nM C-peptide for 5 minutes. ERK activity was measured by *in-vitro* kinase assay. Results are presented as percentages (± SEM, n=3 experiments) of negative control cells that were incubated with serum free media. **$p<0.01$, relative to C-peptide stimulated cells in presence of Ca^{2+}.

Effects of C-peptide on Akt phosphorylation

To investigate the effects of C-peptide on Akt activation in OK cells, Western blotting analyses with Akt-S473-phospho-specific antibodies were performed. C-peptide evoked Akt stimulation was time-dependent. Significant Akt phosphorylation was apparent as early as 1 minute, with maximal stimulation of Akt phosphorylation to 4.1 ± 0.05 fold of basal occurring after 5 minutes of 5nM C-peptide exposure (**Fig 3.9**). Robust, concentration-dependent activation of Akt by C-peptide, with substantial activation of Akt by 0.1nM C-peptide was also observed. Maximal phosphorylation of Akt to 2.5 ± 0.03 fold of basal was observed with 5nM C-peptide (**Fig 3.10A**). Blotting with total Akt antisera demonstrated equivalent protein loading between lanes (**Fig 3.9A and Fig 3.10A**). Activation of Akt was not stimulated by scrambled C-peptide (**Fig 3.10B**).

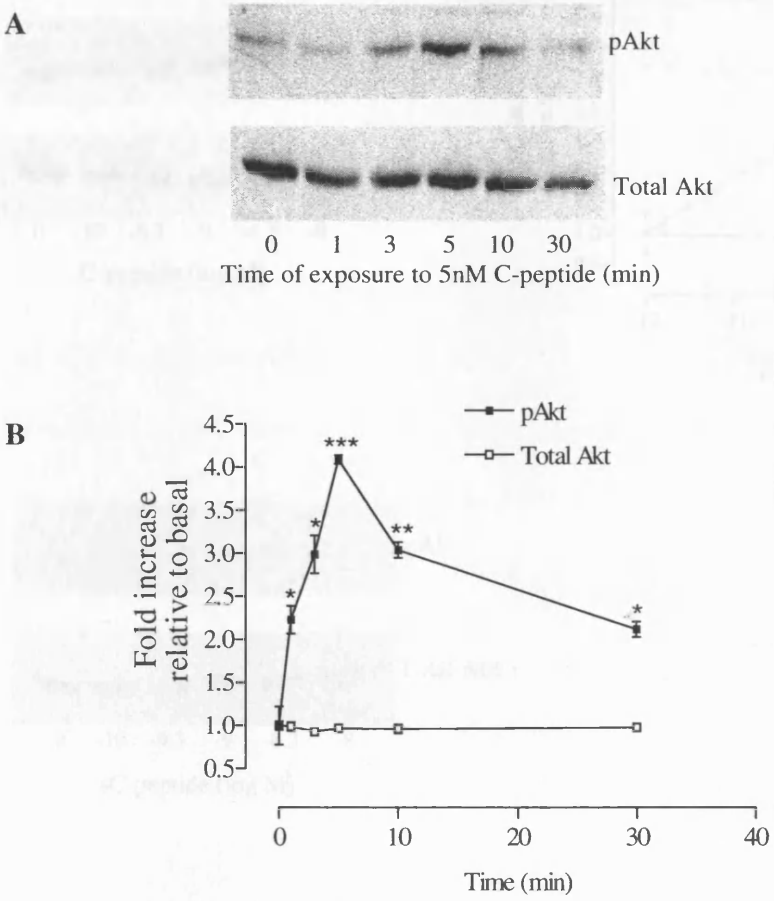


Fig 3.9. Time course-dependent stimulation of Akt phosphorylation in OK cells by C-peptide. Cells were treated with 5nM C-peptide for the indicated times and total Akt or pAkt detected by immunoblotting. **(A)** A representative blot is shown. **(B)** Phosphorylated bands were quantified and values expressed graphically.*p<0.05,**p<0.01,***p<0.001, relative to 0 time. Within the graph data are means ± SEM, n=3 experiments.

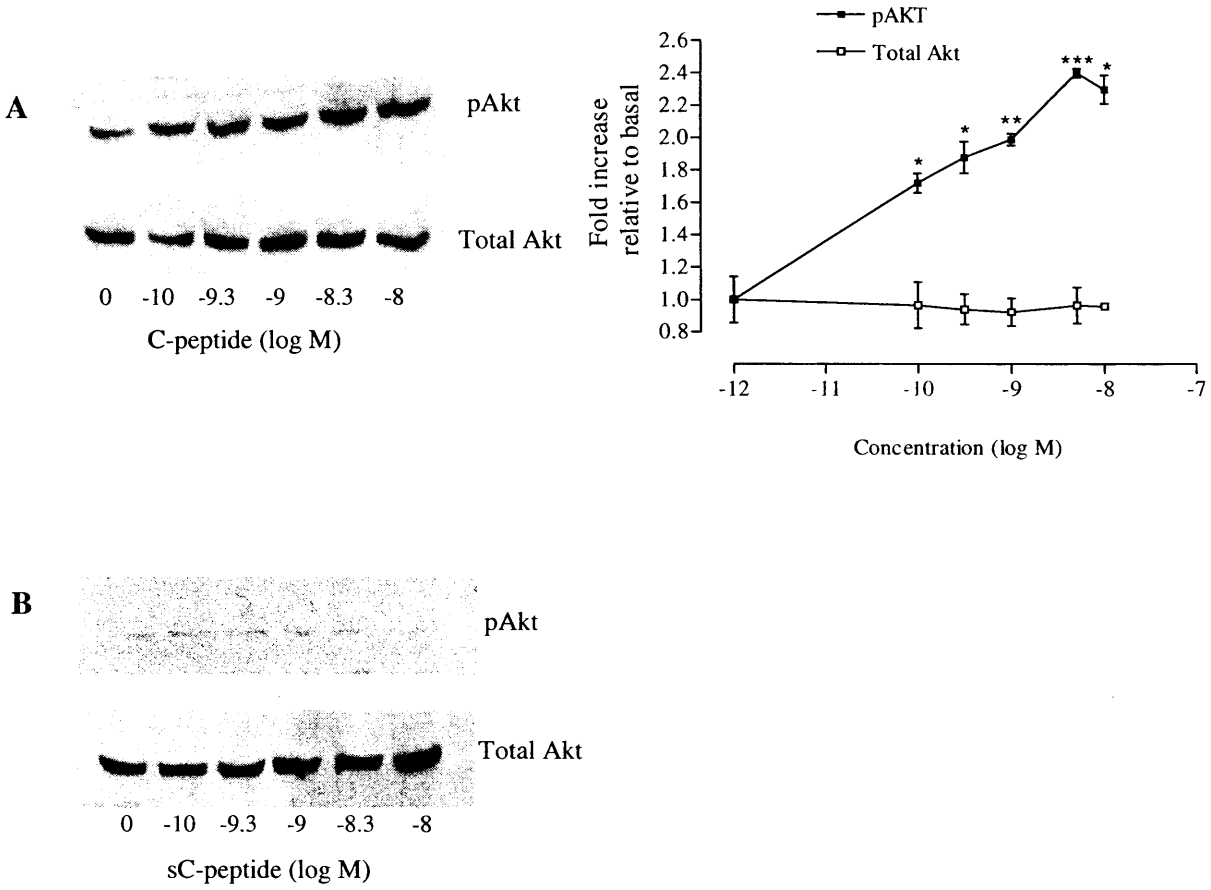


Fig 3.10. Concentration-dependent stimulation of Akt phosphorylation in OK cells by C-peptide but not sC-peptide. (A) Cells were treated with the indicated concentrations of C-peptide for 5min and total Akt or pAkt detected by immunoblotting. A representative blot is shown (left panel). Phosphorylated bands were quantified and values expressed graphically (right panel). *p<0.05,**p<0.01,***p<0.001, relative to control. **(B)** Effect of scrambled C-peptide on Akt phosphorylation in OK cells. Cells were treated with the indicated concentrations of scrambled C-peptide for 5min and total Akt or pAkt detected by immunoblotting. A representative blot is shown. Within the graph data are means ± SEM, n=3 experiments.

Effects of PTX and PI 3-kinase inhibitor on Activation of Akt by C-peptide

To study the role of $G\alpha_{i/o}$ G-proteins in activation of Akt, cells were pre-incubated overnight with 18ng/ml PTX in serum-free media. Pre-treatment of the cells with PTX entirely blocked the physphorylation of Akt induced by 5nM C-peptide for 5 minutes as detected by immunoblotting (**Fig 3.11**).

To determine whether phosphorylation of Akt by C-peptide was dependent on PI 3-Kinase activity, the effect of the PI 3-kinase inhibitor, wortmannin on Akt phosphorylation was examined. Pre-treatment OK cells with wortmannin 100nM for 30 minutes prior to exposure to 5nM C-peptide for 5 minutes completely abolished C-peptide-induced Akt phosphorylation as detected by immunoblotting (**Fig 3.12**).

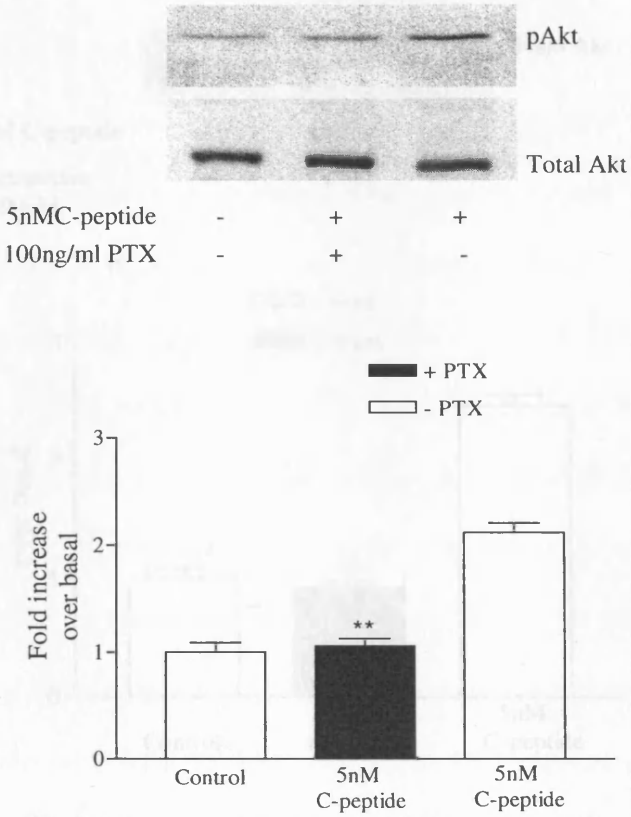


Fig 3.11. PTX inhibits C-peptide induced Akt/PKBα phosphorylation in OK cells. Cells were pre-treated with 100ng/ml PTX overnight and then incubated with 5nM C-peptide for 5 minutes and total Akt or pAkt detected by immunoblotting. A representative blot is shown (upper panel). Phosphorylated bands were quantified and values expressed graphically (lower panel). **p<0.01 relative to non-inhibitor C-peptide stimulated cells. Within the graph data are means ± SEM, n=3 experiments.

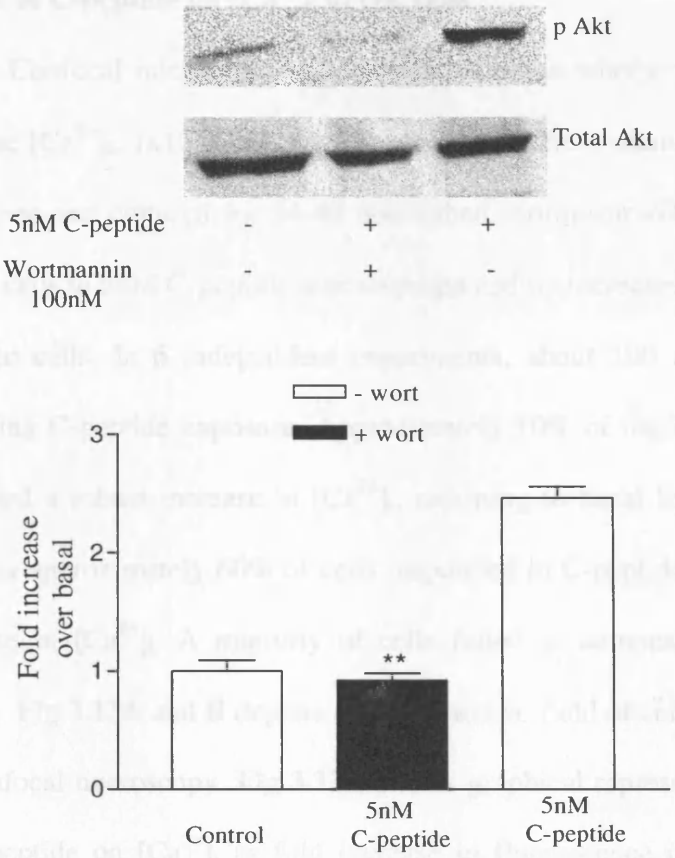


Fig 3.12. PI 3-kinase inhibitor inhibits C-peptide induced Akt/PKBα phosphorylation in OK cells. Cells were pre-treated with 100nM wortmannin for 30min and then incubated with 5nM C-peptide for 5 minutes and total Akt or pAkt detected by immunoblotting. A representative blot is shown (upper panel). Phosphorylated bands were quantified and values expressed graphically (lower panel). **p<0.01 relative to non-inhibitor C-peptide stimulated cells. Within the graph data are means ± SEM, n=3 experiments.

Effects of C-peptide on $[Ca^{2+}]_i$ in OK cells

Confocal microscope was used to examine whether C-peptide is able to increase $[Ca^{2+}]_i$. $1x10^4$ Cells were seeded onto 25mm diameter non-coated glass coverslips and cultured for 24-48 hours then serum-starved overnight. Exposure of OK cells to 5nM C-peptide was accompanied by increases in $[Ca^{2+}]_i$ that varied between cells. In 6 independent experiments, about 100 cells were examined, following C-peptide exposure. Approximately 10% of total OK cells population exhibited a robust increase in $[Ca^{2+}]_i$, returning to basal levels within 1 minute whereas approximately 60% of cells responded to C-peptide with a more modest increase in $[Ca^{2+}]_i$. A minority of cells failed to demonstrate any increase in $[Ca^{2+}]_i$. **Fig 3.13A** and **B** depicts a representative field of cells viewed in real time by confocal microscopy. **Fig 3.13C** shows graphical representation of the effects of C-peptide on $[Ca^{2+}]_i$ as fold increase in fluorescence intensity compared to basal levels. Cell 1(**Fig 3.13C**) exhibits a robust increase in fluorescence intensity to 3.3 fold of basal, returning to basal within 1 minute. Cell 2 (**Fig 3.13C**) shows more modest increase in fluorescence intensity about 1.1 of basal, while cell 3 (**Fig 3.13C**) failed to demonstrate any increase in $[Ca^{2+}]_i$, following C-peptide exposure.

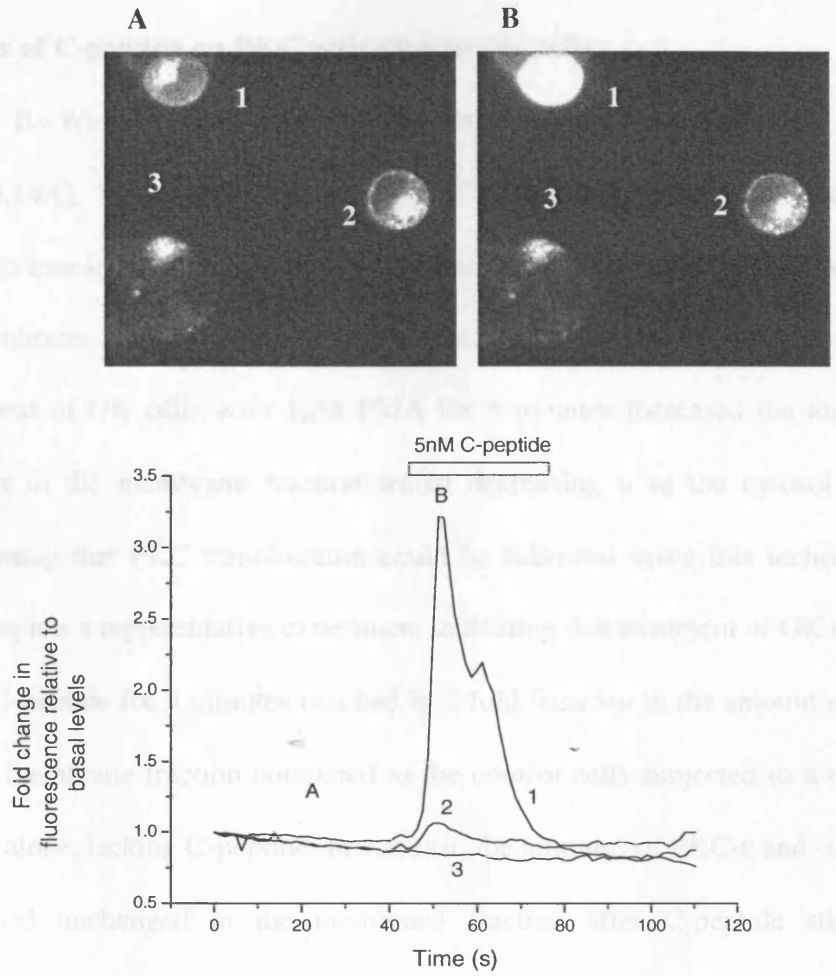


Fig 3.13. $[Ca^{2+}]_i$ responses to C-peptide in OK cells. Cells plated on coverslips and loaded with flu-3-AM were excited at 488nM and imaged using a confocal microscope under non-stimulated conditions **(A)** or following stimulation with 5nmol/l C-peptide in the perfusate **(B)**. **(C)** Changes in fluorescence in cell 1,2 and 3 in the field of view are represented graphically as fold increases in fluorescence relative to basal levels. Image A was obtained at 20 seconds and image B obtained at 55 seconds. The traces derived from cells 1, 2 and 3 are numbered respectively. Data are representative of 6 experiments.

Effects of C-peptide on PKC activation in OK cells

By Western blotting the PKC isoforms -α, -ε, -ι were detected in OK cells (**Fig 3.14A**). To examine the effects of C-peptide on PKC activity, C-peptide induced translocation of these PKC isoforms from a cell cytosolic compartment to a membrane compartment was analysed. Control experiments showed that treatment of OK cells with 1μM PMA for 5 minutes increased the amounts of PKC-α in the membrane fraction whilst decreasing it in the cytosol fraction, confirming that PKC translocation could be followed using this technique. **Fig 3.14** depicts a representative experiment indicating that treatment of OK cells with 5nM C-peptide for 5 minutes resulted in 2-fold increase in the amount of PKC-α in the membrane fraction compared to the control cells subjected to a change of media alone, lacking C-peptide. In contrast, the amounts of PKC-ε and -ι isoforms remained unchanged in the membrane fraction after C-peptide stimulation, although membrane translocation of PKC-ε was seen after PMA treatment (**Fig 3.14A**). Thus, C-peptide specifically translocates PKC-α in OK cells.

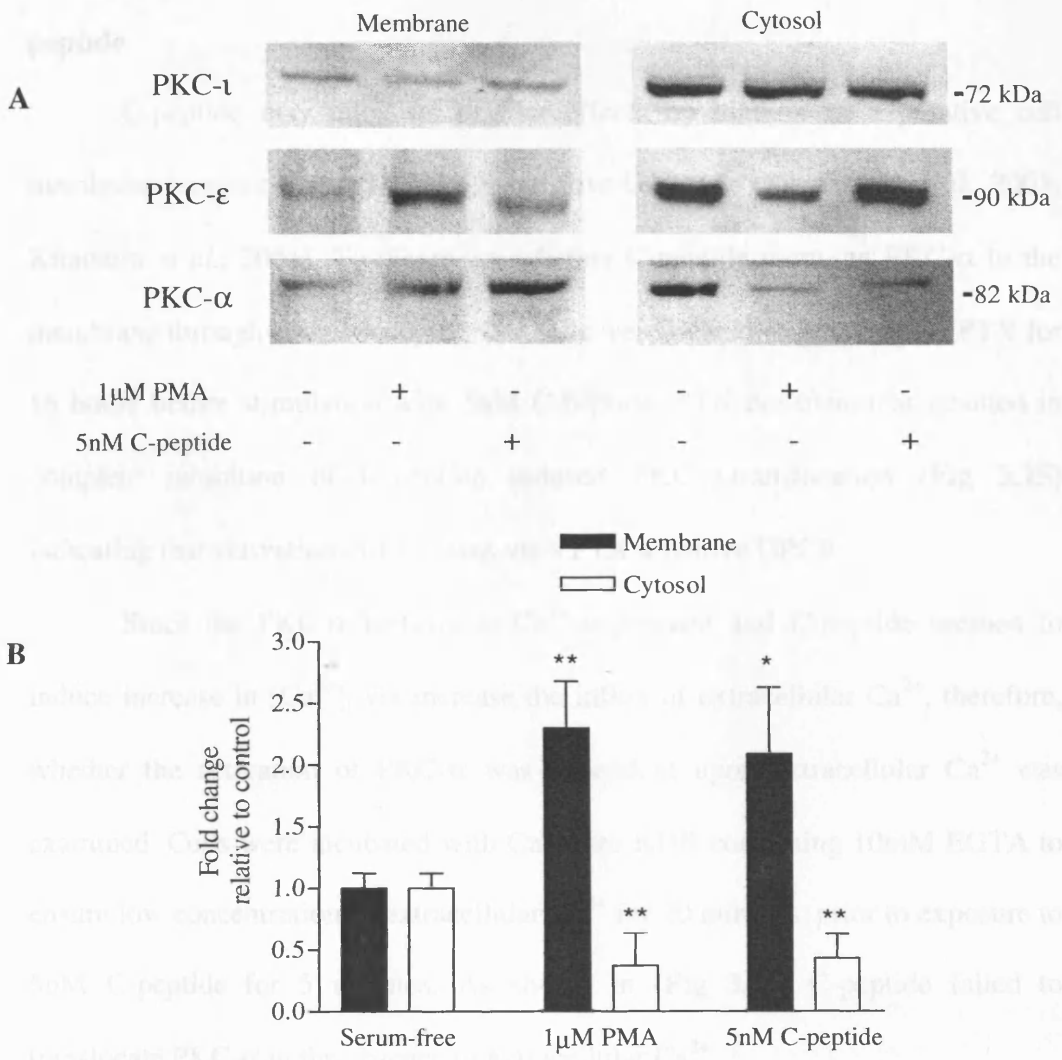


Fig 3.14. Effects of C-peptide on PKC-ι, ε and α isoforms translocation in OK cells. Serum starved cells were treated with either 5nM C-peptide or 1μM PMA for 5 minutes. Membrane and cytosol where prepared as described in methods chapter. **(A)** PKC isoforms were detected by Western blotting of membrane and cytosol fractions with isoform specific anti- PKC-ι, ε and α antibody. **(B)** The amounts of PKC-α in membrane and cytosolic fractions were quantified using Scion Image analysis software and results are expressed relative to those of untreated control cells. Data are means ±SEM, n=5. * $p<0.05$, ** $p< 0.01$, relative to control.

Effects of PTX and removal of extracellular Ca^{2+} on PKC-α activation by C-peptide

C-peptide may elicit its cellular effects by binding to a putative cell membrane receptor coupled to a PTX-sensitive G-protein (Tismaratos *et al.,* 2003; Kitamura *et al.,* 2001). To determine whether C-peptide recruited PKC-α to the membrane through such a receptor, OK cells were treated with 100ng/ml PTX for 18 hours before stimulation with 5nM C-peptide. PTX pre-treatment resulted in complete inhibition of C-peptide induced PKC-α translocation (**Fig 3.15**) indicating that activation of PKC was via a PTX sensitive GPCR.

Since the PKC-α isoform is Ca^{2+} dependent and C-peptide seemed to induce increase in $[Ca^{2+}]_i$ via increase the influx of extracellular Ca^{2+}, therefore, whether the activation of PKC-α was dependent upon extracellular Ca^{2+} was examined. Cells were incubated with Ca^{2+} free KHB containing 10mM EGTA to ensure low concentration of extracellular Ca^{2+} for 20 minutes, prior to exposure to 5nM C-peptide for 5 minutes. As shown in (**Fig 3.15**) C-peptide failed to translocate PKC-α in the absence of extracellular Ca^{2+}.

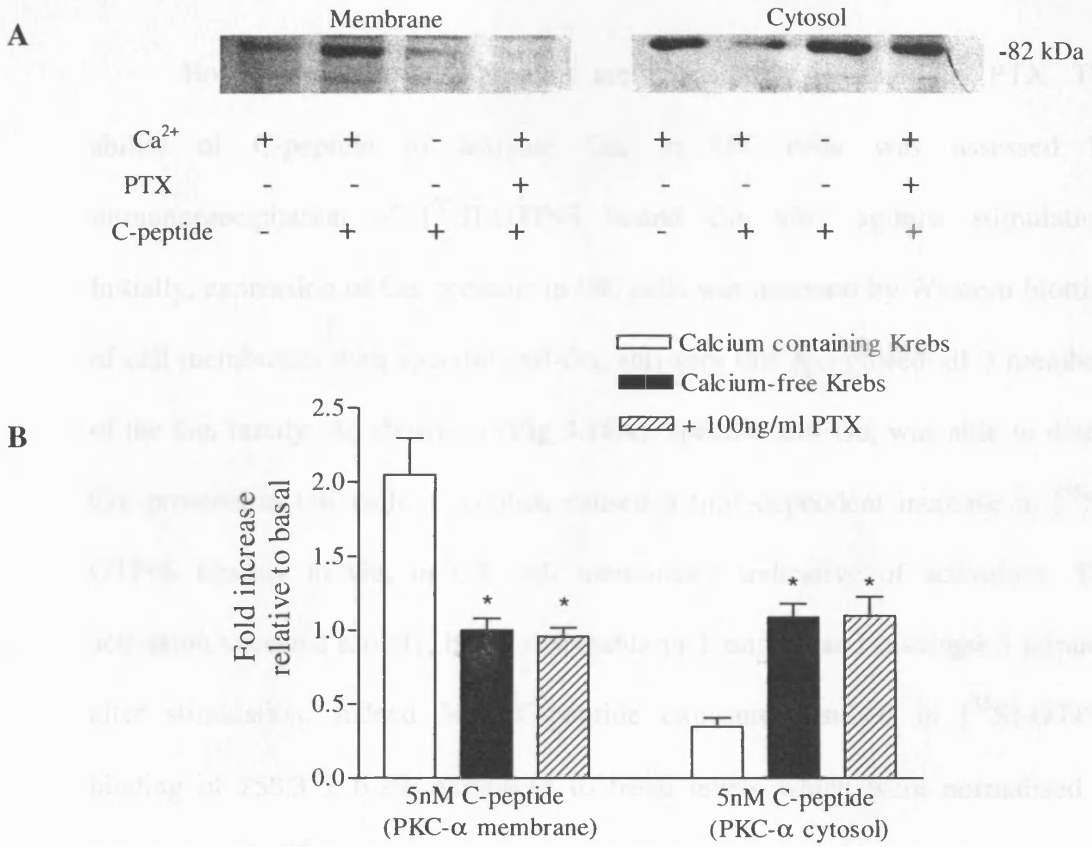


Fig 3.15. PKC-α translocation is inhibited by PTX and in Ca^{2+} free medium. OK cells were pre-treated with 100ng/ml PTX for 18 hours or incubated with Ca^{2+} free KHB prior to stimulation with 5nM C-peptide for 5 minutes. **(A)** Membrane and cytosol were prepared and PKC-α was detected by Western blotting. A representative immunblot is depicted. **(B)** The amount of PKC-α detected in immunoblots was quantified using Scion Image analysis software. The results are expressed relative to those of untreated control cells that have been normalized to a value of 1. Data are means ± SEM, n=4. $^{*}p<0.05$ relative to control (C-peptide stimulated cells in Ca^{2+} containing KHB).

Activation of PTX sensitive GPCR by C-peptide

Both $G\alpha_i$ and $G\alpha_o$ G-proteins are functionally inhibited by PTX. The ability of C-peptide to activate $G\alpha_i$ in OK cells was assessed by immunoprecipitation of [^{35}S]-GTPγS bound $G\alpha_i$ after agonist stimulation. Initially, expression of $G\alpha_i$ proteins in OK cells was assessed by Western blotting of cell membranes with specific anti-$G\alpha_i$ anti-sera that recognised all 3 members of the $G\alpha_i$ family. As shown in (**Fig 3.16A**), specific anti-$G\alpha_i$ was able to detect $G\alpha_i$ proteins in OK cells. C-peptide caused a time-dependent increase in [^{35}S]-GTPγS binding to $G\alpha_i$ in OK cell membranes indicative of activation. The activation occurred rapidly, being detectable in 1 minute and maximal 3 minutes after stimulation. Indeed 5nM C-peptide exposure resulted in [^{35}S]-GTPγS binding of 258.3 ± 6.2% compared to basal levels which were normalised to 100%. Basal [^{35}S] counts were ~ 350-500 cpm, and non-specific binding accounted for ≤ 20% of total counts. In contrast, sC-peptide did not activate [^{35}S]-GTPγS binding to any significant level (**Fig 3.16B**).

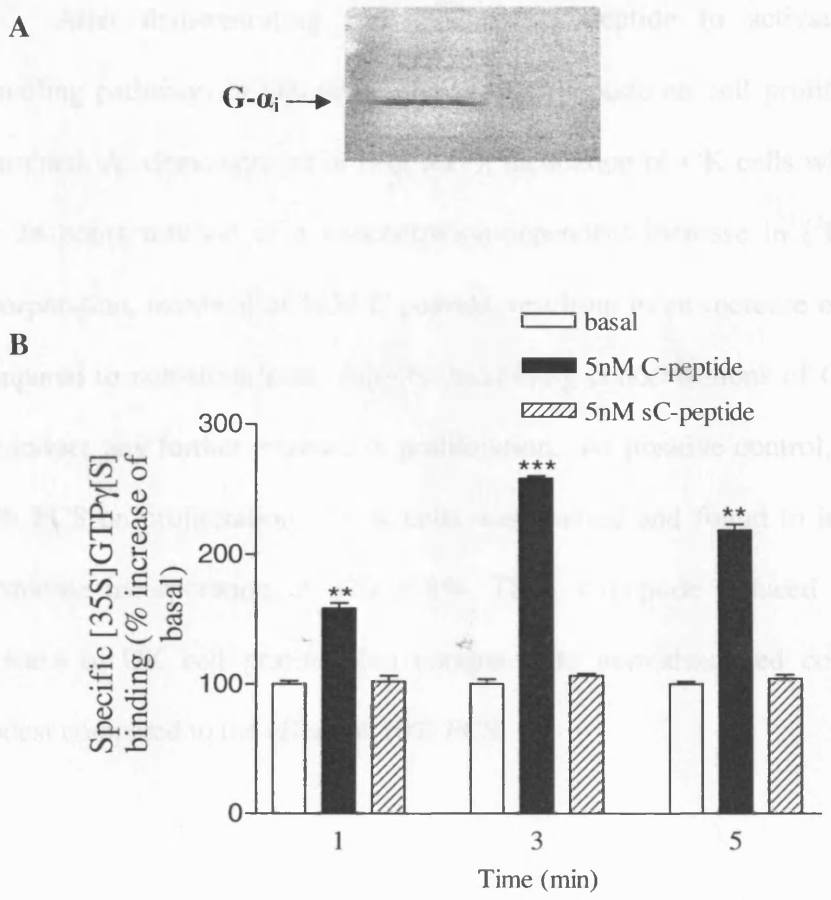


Fig 3.16. C-peptide activates $G\alpha_i$. **(A)** Expression of $G\alpha_i$ in OK cell membranes. Cell membranes (30μg protein) were solubilized, proteins separated and transferred to nitrocellulose for immunoblotting. **(B)** Time-course C-peptide-stimulated [^{35}S]-GTPγS binding to $G\alpha_i$ in OK cell membranes. Cell membranes prepared from OK cells were incubated in the absence or presence of 5nM C-peptide or 5nM scrambled C-peptide for the time indicated at 37°C. Results are expressed as means ± SEM, n=3 experiments. ** $p< 0.01$, ***$p<0.001$ relative to basal.

Effects of C-peptide on proliferation of OK cells

After demonstrating the ability of C-peptide to activate mitogenic signalling pathways in OK cells, effects of C-peptide on cell proliferation were examined. As demonstrated in (**Fig 3.17**), incubation of OK cells with C-peptide for 24 hours resulted in a concentration-dependent increase in [^{3}H]-thymidine incorporation, maximal at 5nM C-peptide, resulting in an increase of 130 ±1.5% compared to non-stimulated controls. Increasing concentrations of C-peptide did not induce any further increase in proliferation. As positive control, the effect of 10% FCS on proliferation of OK cells was studied and found to increase [^{3}H]-thymidine incorporation of 370 ± 8%. Thus, C-peptide induced a significant increase in OK cell proliferation compared to non-stimulated controls, albeit modest compared to the effect of 10% FCS.

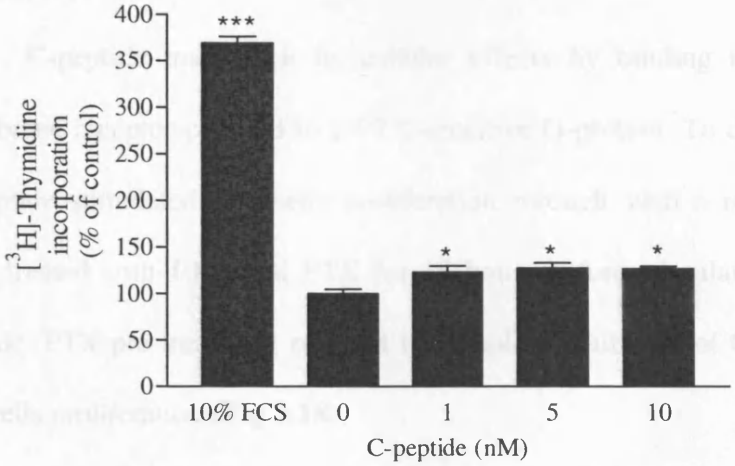


Fig 3.17. C-peptide stimulates proliferation of OK cells. Serum-starved OK cells were stimulated with various concentrations of C-peptide in serum-free media for 24 hours or 10% FCS as positive control. Proliferation was measured by [^{3}H] thymidine incorporation and counts accumulated in non-stimulated cells were arbitrarily assigned a value of 100%. Results are expressed as percentage of control (non-stimulated cells). Data are means ± SEM, n=4. *$p<0.05$, ***$p< 0.001$ relative to control (non-stimulated cells).

Effects of PTX and PI 3-kinase inhibitor on C-peptide-induced Proliferation of OK cells

C-peptide may elicit its cellular effects by binding to a putative cell membrane receptor coupled to a PTX-sensitive G-protein. To determine whether C-peptide stimulated OK cells proliferation through such a receptor, OK cells were treated with 100ng/ml PTX for 18 hours before stimulation with 5nM C-peptide. PTX pre-treatment resulted in complete inhibition of C-peptide induced OK cells proliferation (**Fig 3.18**).

To determine whether stimulation of OK cells proliferation by C-peptide was dependent on PI3-K activity, effect of the PI-3 kinase inhibitor, wortmannin was examined. Pre-treatment OK cells with wortmannin 100nM for 30 minutes prior to exposure to 5nM C-peptide for 24 hours significantly attenuated C-peptide-induced OK cells proliferation (**Fig 3.18**).

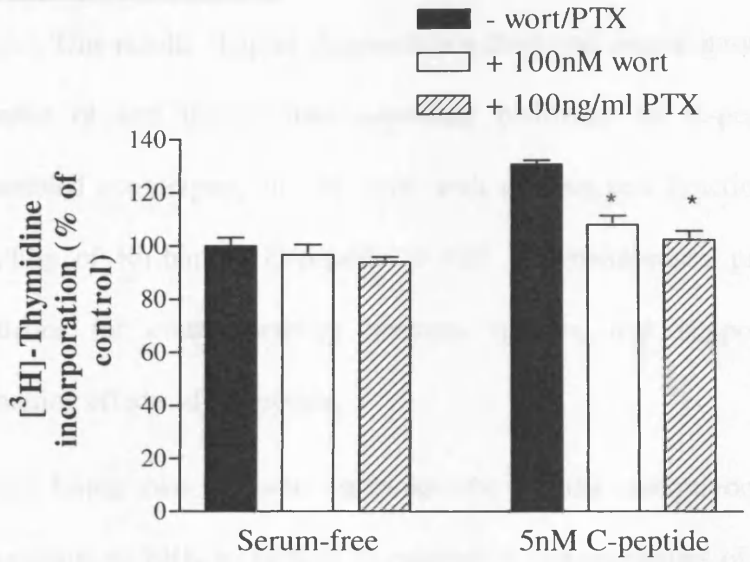


Fig 3.16. PI 3-kinase inhibitor and PTX inhibit C-peptide-induced proliferation of OK cells. OK cells were pre-treated with either 100nM wortmannin for 30 minutes, or 100ng/ml PTX for 18 hours prior to stimulation with the indicated concentration of C-peptide. Cell proliferation was measured by [^{3}H] thymidine incorporation. Results are expressed as percentage of control (non-stimulated cells). Data are means ± SEM, n=4. *$p<0.05$, relative to non-inhibitors C-peptide-stimulated cells.

3.3. DISCUSSION

This results chapter demonstrates clear and unambiguous stimulation of a number of key intracellular signalling pathways by C-peptide, but not its scrambled counterpart, in OK cells with distinct cell functional consequences. Binding of Rh-human C-peptide to OK cell membranes provided reassuring evidence for cross-reactivity between species, and supported the detected signalling effects of C-peptide.

Using two different methods the results unequivocally demonstrated stimulation of ERK by typical physiological concentrations of C-peptide (Dreyer *et al.,* 1986; Horwitz *et al.*, 1975; Faber *et al.,* 1978) in the mid-picomolar to low-nanomolar range. Furthermore phosphorylation of Akt induced by C-peptide is indicative of PI 3-kinase activation. Although both of these phenomena demonstrate similar rapid kinetics of activation, C-peptide is a more potent activator of ERK than PI 3-kinase. Also the relationship between ERK stimulation and C-peptide concentration is described by a bell-shaped curve, whereas a sigmoidal relationship is observed between C-peptide concentration and Akt stimulation. This may be a consequence of the rapid desensitisation of the C-peptide receptor (s), or may be due to the activation of another cell receptor, masking the low concentrations effect of C-peptide. However, there is little evidence that C-peptide signals via more than one receptor, and the explanation for these differences is unclear.

The above data are consistent with recent studies in Swiss 3T3 fibroblasts, showed that phosphorylation of ERK was stimulated by C-peptide with conserved glutamic acid residues at position 3, 11 and 27 and by the presence of helix-promoting residues in the N-terminal segment (Henriksson *et al.,* 2005). Zhong *et*

al., (2005) reported that phosphorylation of ERK and JNK but not p38K was observed in human renal tubular cells upon treatment with homologous C-peptide or its C-terminal pentapeptide segment.

Consistent with the observations of others (Ohtomo *et al.,* 1998; Shafqat *et al.,* 2002), this study demonstrated that C-peptide evoked increases in $[Ca^{2+}]_i$. In OK cells only a minority of cells demonstrated a robust rise in $[Ca^{2+}]_i$, the majority showing more modest increases, and ~10% failing to show any response. Whilst such heterogeneity of response may reflect heterogeneity in the cell culture population, no such variability was seen in the earlier studies of lysophosphatidic acid induced Ca^{2+} transients in this cell type (Dixon *et al.*, 1999). However, in the latter study $[Ca^{2+}]_i$ was assessed in large number of cells in suspension. Under these conditions it is more difficult to observe such variability, compared to confocal studies where only few cells are directly monitored. Moreover, in the same study, the magnitude of $[Ca^{2+}]_i$ increase was relatively modest compared with the $[Ca^{2+}]_i$ responses observed in other cell types after lysophosphatidic acid (Moolenaar., 1997). That may reflect variability of responses in OK cells where although some cells respond with a large $[Ca^{2+}]_i$ transient, the signal may be diluted due to a proportion of cells showing no response. In practical terms however this variability in observed Ca^{2+} responses limited any further attempt to dissect the mechanisms underlying C-peptide induced changes in $[Ca^{2+}]_i$.

Observation of Ca^{2+} dependent membrane translocation of PKC-α however supports the activation of Ca^{2+} signalling by C-peptide in OK cells, and provides support for the concept that Ca^{2+} dependent pathways play an important role in C-peptide mediated actions (Ohtomo *et al.,* 1996; Shafqat *et al.,* 2002). Robust PKC-α translocation was also observed in response to C-peptide at

physiologically relevant low nanomolar concentrations. Abolition of this phenomenon in Ca^{2+}-free medium and by PTX indicates that it is mediated via a GPCR, and dependent on entry of Ca^{2+} into the cell rather than release from intracellular stores. The PKC isoforms -ε and -ι were detected by Western blotting but did not translocate. Efendiev *et al* (1999) indicated that OK cells express all the PKC isoforms except γ. In the current study other PKC isoforms could not be detected in OK cells, although detailed evaluation of PKC isoforms was not intended. These differences most likely relate to problems of antibody specificity, and consequent failure to detect opossum PKC isoforms. This finding is consistent with recent studies showing that C-peptide induced PKC-α translocation to the membrane fraction in rat medullary thick ascending limb tubular cells (Tsimaratos *et al.,* 2003) and activated PKC-δ and ε in human renal tubular cells (Zhong *et al.,* 2005).

A plasma membrane receptor for C-peptide has not yet been identified, and non-receptor-mediated membrane interactions have been suggested to explain C-peptide effects (Ido *et al.,* 1997). However, as in previous studies C-peptide actions were abolished by PTX pre-treatments (Maestroni *et al.,* 2005; Zhong *et al.,* 2005; Walcher *et al.,* 2004; Marx *et al.,* 2004; Shafqat *et al.,* 2002; Kitamura *et al.,* 2001; Ohtomo *et al.,* 1996). This PTX inhibition of C-peptide signalling in OK cells implicated a G-protein coupled receptor, coupled to either $G_{\alpha i}$ or $G_{\alpha o}$, in the transduction of these events. Using [^{35}S]-GTPγS binding the current experiments now for the first time unequivocally demonstrate specifically that $G\alpha_i$ proteins are activated by C-peptide binding to a GPCR. Earlier work (Brunskill *et al.,* 1996) showed appreciable amounts of G_i proteins especially G_{i-3} are expressed in OK cells, while G_o proteins are not detected in kidney cortex by

immunoblotting (Murakami *et al.,* 1989). Such observations are in agreement with studies demonstrating PTX sensitive C-peptide binding to kidney cell membranes (Rigler *et al.,* 1999), and provide strong support for the existence of a specific C-peptide receptor. Furthermore, the C-peptide concentrations stimulating signalling in the current studies correlate well with the affinity of the previously described binding sites (Rigler *et al.,* 1999).

C-peptide induced ERK activity via $G\alpha_{i/o}$ coupling receptor suggests possible $G\beta\gamma$ involvement. A common intermediate step is via PI 3-kinase acting upstream of Ras (Crespo *et al.,* 2001). However, C-peptide induced ERK activity was not affected by wortmannin, suggesting that PI 3-kinase is not involved in this stimulation. Attenuation of ERK activity by Ro-31-8820 pre-treatment suggests that C-peptide induced ERK activity is PKC-dependent in OK cells as proposed by Kitamura *et al* (2001) in Swiss 3T3 fibroblasts. Abolition of ERK activation in Ca^{2+} free medium is in agreement with a recent study demonstrated that the L-Type Ca^{2+} channels blockers verapamil and nifedipine abolish the effect of C-peptide on phosphorylation (Zhong *et al.,* 2005)

Stimulation of Akt signalling pathway is not necessarily contradictory to the proposed G-protein involvement. Stimulation of Gi/Go-protein-coupled receptors may result in activation of PI 3-kinase-γ via its association with the dissociated $\beta\gamma$ subunits of the G-protein complex (Stoyanov *et al.,* 1995; Toker and Cantley, 1997). Particularly, PI 3-kinase-γ is highly expressed in kidney PTC (Bernstein *et al.,* 1998). Zhong *et al.,* (2005), have also demonstrated similar inhibition of Akt phosphorylation by PTX pre-treatment in human renal tubular cells.

Following the observation that C-peptide stimulated kinases with well-described roles in the regulation of growth, proliferation and cell survival (Hagemann and Blank, 2001; Gutkind, 2000; Marte and Downward, 2001; Lawlor and Alessi, 2001; Clemens *et al.,* 1992; Iwamoto *et al.*, 1992), whether C-peptide is a functional mitogen in OK cells was examined. Accordingly, physiological concentrations of C-peptide were found to stimulate OK cell proliferation in a manner that was dependent on PI 3-kinase activity and sensitive to PTX. Previous studies reported that similar concentrations of C-peptide were mitogenic for human neuroblastoma cells (Li *et al.,* 2003), with higher concentrations stimulating MEK dependent proliferation of fibroblasts (Hehenberger *et al.,* 1997). This latter finding is compatible with the present results and suggests that ERK is involved in C-peptide induced cell proliferation. Interpretation of concentration dependency data in OK cells is difficult however because PTC are active in peptide catabolism. Thus, after the obligate exposure of OK cells to C-peptide for 24 hours in [^{3}H]-thymidine incorporation studies, C-peptide concentrations in the culture medium may be considerably less than those applied at the start of the experiment. It is however tempting to suggest that the absence of this mitogenic effect in diabetes may contribute to the hypertrophic renal growth abnormalities seen in nephropathy.

Salutary effects of C-peptide observed in patients with diabetes are not paralleled by similar observations in normal individuals, and C-peptide binding data suggest that under normal physiological conditions binding sites may be fully occupied. Thus, reduced C-peptide signalling may only become important in diabetic individuals when levels fall or become undetectable. In this situation it is interesting to speculate that balanced interactions between insulin, C-peptide and

glucose signalling pathways may be disturbed leading to the development of diabetic complications.

Some preliminary evidence of cross-talk between insulin and C-peptide has been found. Using L6 rat myoblasts, inhibition of protein tyrosine phosphatase by C-peptide with associated increased phosphorylation of the insulin receptor and insulin receptor substrate-1 has been demonstrated (Li *et al.,* 2001). These findings in myoblasts were subsequently extended to show insulinomimetic C-peptide effects manifest as induced phosphorylation of glycogen synthase kinase, ERK, and p90Rsk (Grunberger *et al.,* 2001). No effect of C-peptide on phosphorylation of Akt was seen in that study, although C-peptide did increase phosphotyrosine associated PI 3-kinase activity as measured by immune complex *in-vitro* kinase assay. Some synergy was observed between C-peptide and insulin, but only at sub-maximal concentrations.

Taken together, the available data indicates important GPCR mediated effects of C-peptide at the cellular level which translate into vital effects on insulin action and glucose homeostasis when measured at the whole animal level. The cellular effects of C-peptide are not apparent in all cells investigated but are clearly present in the OK PTC.

In conclusion, C-peptide signal transduction may involve activation of a PTX sensitive membrane receptor probably $G\alpha_i$ which induced prompt increase in $[Ca^{2+}]_i$ concentration and subsequently activation of PKC and ERK. A parallel activation of Akt/PKB conceivably via PI 3-kinase by the dissociated $\beta\gamma$ subunits of G-protein is also observed. Activation of ERK and PI 3-kinse could regulate transcription factors and downstream kinases responsible for chromatin remodelling, gene expression and cell proliferation. The identification of the

molecular mechanism of C-peptide action will bring new understanding of the physiological role of C-peptide and providing new impetus for the investigation of C-peptide as a therapeutic agent in diabetes mellitus.

CHAPTER 4
ACTIVATION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-γ BY C-PEPTIDE IN OK CELLS

4.1. INTRODUCTION

The signalling effects of C-peptide documented in (chapter3) supported the notion that C-peptide may possess multiple and significant biological functions. In addition, C-peptide has been shown to improve glucose utilization both *in-vivo* and *in-vitro*. Specifically in STZ-induced diabetic rats, C-peptide stimulates whole body glucose utilization (Wu *et al.,* 1996) and stimulates glucose transport in healthy human skeletal muscle (Zierath *et al.,* 1991; Zierath *et al.,* 1996). In BB/Wor rat model of Type 1 diabetes chronic C-peptide infusion prevents both acute and chronic metabolic, functional and structural changes (Sima *et al.,* 2001; Ido *et al.,* 1997). Also, enhancement by C-peptide of insulin-stimulated insulin receptor autophosphorylation and TKA (Grunberger *et al.,* 2001) Suggests commonality between insulin and C-peptide signalling pathways.

Furthermore, C-peptide has shown to increase gene transcription, recent work by Kitamura *et al.,* (2003), has shown that C-peptide enhanced NO production by increasing eNOS protein via ERK-dependent up-regulation of eNOS gene transcription. Moreover, C-peptide also enhanced DNA-CREB/ATF1 interactions via the p38K pathway in mouse lung capillary endothelial cell (Kitamura *et al.,* 2002).

The peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand activated transcription factors. Three PPAR isoforms, PPARα, PPARβ (also known as δ) and PPARγ have been identified, and all isoforms are present in the kidneys of humans and other species

(Kliewer *et al.,* 1994; Yang *et al.,* 1999; Asano *et al.,* 2000). PPARs regulate gene expression by binding as heterodimers with one of three retinoid X receptor proteins to *cis*-acting peroxisome proliferator response elements (PPRE) within the promoter regions of target genes (Kliewer *et al.,* 1992). Each PPAR isoform exerts distinct actions on cellular function and exhibits different specificity in ligand-binding properties (Kersten *et al.,* 2000; Kliewer and Willson, 1998; Basu-Modak *et al.,* 1999).

Glitazones or thiazolidinediones (TZDs) are synthetic high-affinity PPARγ ligands that act as insulin sensitizing agents, and are currently used for the treatment of Type 2 diabetes mellitus, (Nolan *et al.,* 1994). They improve lipid profiles together with insulin sensitivity and glucose tolerance in patients with Type 2 diabetes, and thus control hyperglycemia (Iwata *et al.,* 2001; Rangwala and Lazar, 2004). These metabolic effects of TZDs are mediated via PPARγ regulated transcription of genes involved in glucose and lipid homeostasis. Similarly, insulin also promotes altered expression of important genes regulating glucose and fatty acids metabolism (O`Brien and Granner, 1996; Girard *et al.,* 1994; Rieusset *et al.,* 1999). Several lines of evidence indicate convergence between insulin and PPARγ signalling pathways. Firstly the TZD, pioglitazone, augments insulin-stimulated tyrosine phosphorylation of IR/IRS-1 (Iwata *et al.,* 2001), secondly insulin also participates in the control of PPARγ expression (Rieusset *et al.,* 1999; Vidal-Puig *et al.,* 1997), and finally insulin mediates ligand independent activation of PPARγ in a phosphorylation-dependent manner (Werman *et al.,* 1997).

Given this evidence of cross-talk between signalling pathways, thus, it was postulated that C-peptide, insulin signalling and PPARγ functions could be

linked. In order to extend the results of the C-peptide signalling studies, the effects of C-peptide and insulin on PPARγ transcriptional activity were investigated in OK PTC.

4.2. RESULTS

Expression of PPARγ in OK cells

Western blotting was used to determine PPAR isoform expression in OK cells using whole cell lysates. Previous PPRE-luciferase reporter studies in OK cells (Chana *et al.*, 2004) indicated transcriptional activation only by PPARγ or PPARβ ligands. In agreement with this observation both PPARγ and PPARβ, but not PPARα proteins were detected in OK cells by Western blotting in the current study using specific anti-sera against PPARγ, PPARβ and PPARα (**Fig 4.1**).

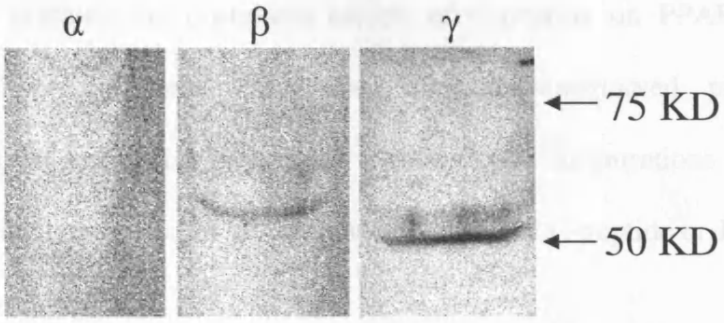


Fig 4.1. Expression of PPAR subtypes in OK cells. PPAR subtypes were detected by immunoblotting of cell lysates. Blots are representative of 3 experiments.

Effects of C-peptide and insulin on PPARγ activation

To examine the postulated effects of C-peptide on PPARγ activity, a luciferease reporter gene assay was used. Serum-starved pPPRE-TK-luc transfected OK cells were stimulated with various concentrations of C-peptide, insulin, or ciglitazone for 24 hours or with either 5nM C-peptide or 100nM insulin for various time points.

Both agents induced a late increase in luciferase expression in pPPRE-TK-luc transfected OK cells that was significant after 6 and 4 hours after exposure to 5nM C-peptide or 100nM insulin respectively (**Fig 4.2**). Maximal stimulation of 169.6 ± 2.1 and 235.1 ± 6.4% of control occurred 18 hours after exposure to C-peptide or insulin respectively (**Fig 4.2**).

A concentration dependent increase in luciferase expression in pPPRE-TK-luc transfected OK cells was observed after stimulation with the TZD ciglitazone (max 207 ± 2.8% of control, EC_{50} 80nM), C-peptide (max. 170 ± 6.5% of control, EC_{50} 4nM) and insulin (max. 250 ± 4.5% of control, EC_{50} 10nM) (**Fig 4.3**).

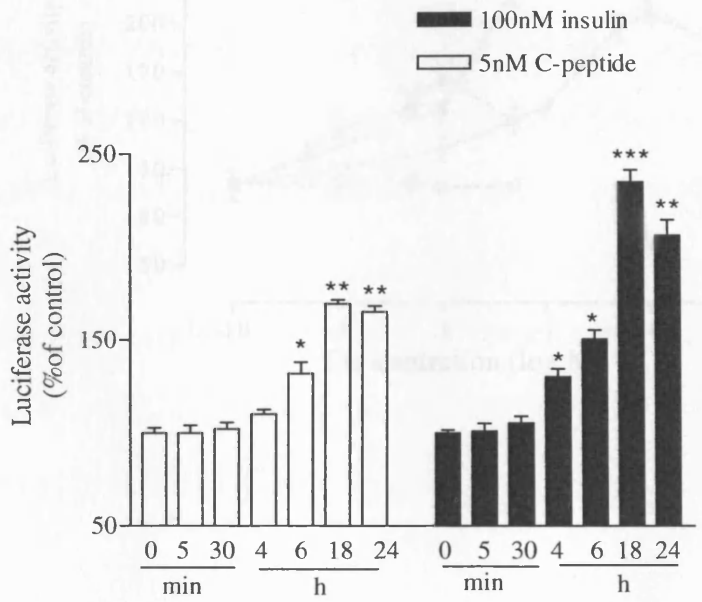


Fig 4.2. Time-dependent activation of PPARγ in OK cells. OK cells transiently transfected with pPPRE-TK-luc and pSV-βgal were treated for different period (5min-24h) with the indicated concentrations of C-peptide or insulin. Cells were lysed, and luciferase activity in lysates was determined and normalized for transfection efficiency using β-gal activity. Normalized luciferase activity is expressed as percentage compared with control in non-stimulated cells. Results are expressed as means ± SEM, n=3 experiments. * $p < 0.05$, ** $p< 0.01$, ***$p<0.001$ relative to 0 time).

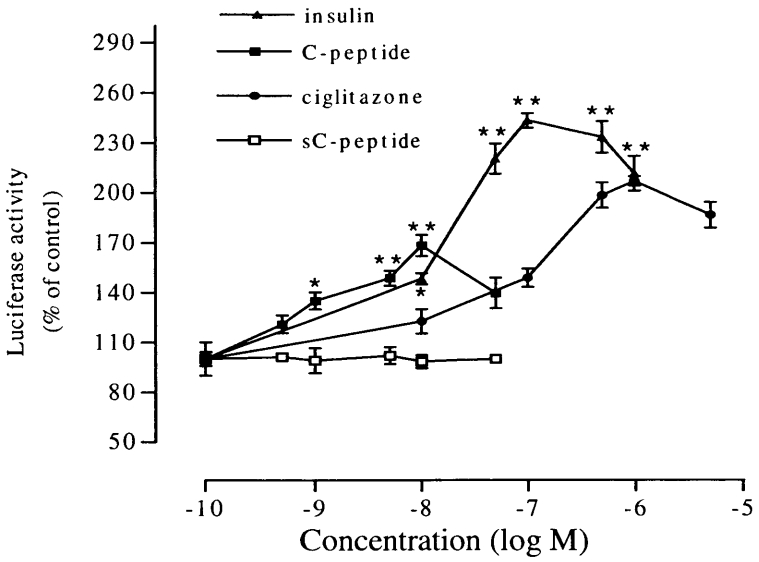


Fig 4.3. Concentration-dependent activation of PPARγ in OK cells. OK cells transiently transfected with pPPRE-TK-luc and pSV-βgal were treated for 24h with the indicated concentrations of C-peptide, insulin or ciglitazone. Cells were lysed, and luciferase activity in lysates was determined and normalized for transfection efficiency using β-gal activity. Normalized luciferase activity is expressed as percentage compared with control in non-stimulated cells. Results are expressed as means ± SEM, n=3-4 experiments. * $p < 0.05$, ** $p < 0.01$, relative to control.

C-peptide- and insuin-stimulated of PPRE activity is mediated by PPARγ

To examine whether effects of C-peptide or insulin were mediated via activation of PPARγ, cells were transfected with pPPRE-TK-luc ± pCMX-PPAR-γ (**Fig 4.4A**). Luciferase activity was measured after stimulation with 5nM C-peptide or 100nM insulin for 24 hours. Over-expression of PPARγ enhanced both basal PPRE activity and that induced by 5nM C-peptide, 100nM insulin or 5μM ciglitazone by 11 ± 2.0 , 20 ± 1.0, and 14 ± 1.0 fold respectively above that observed in non-stimulated, wild type pPPRE-TK-luc transfected OK cells. Increased levels of PPARγ protein in pCMX-PPAR-γ transfected cells were confirmed by Western blotting (**Fig 4.4B**).

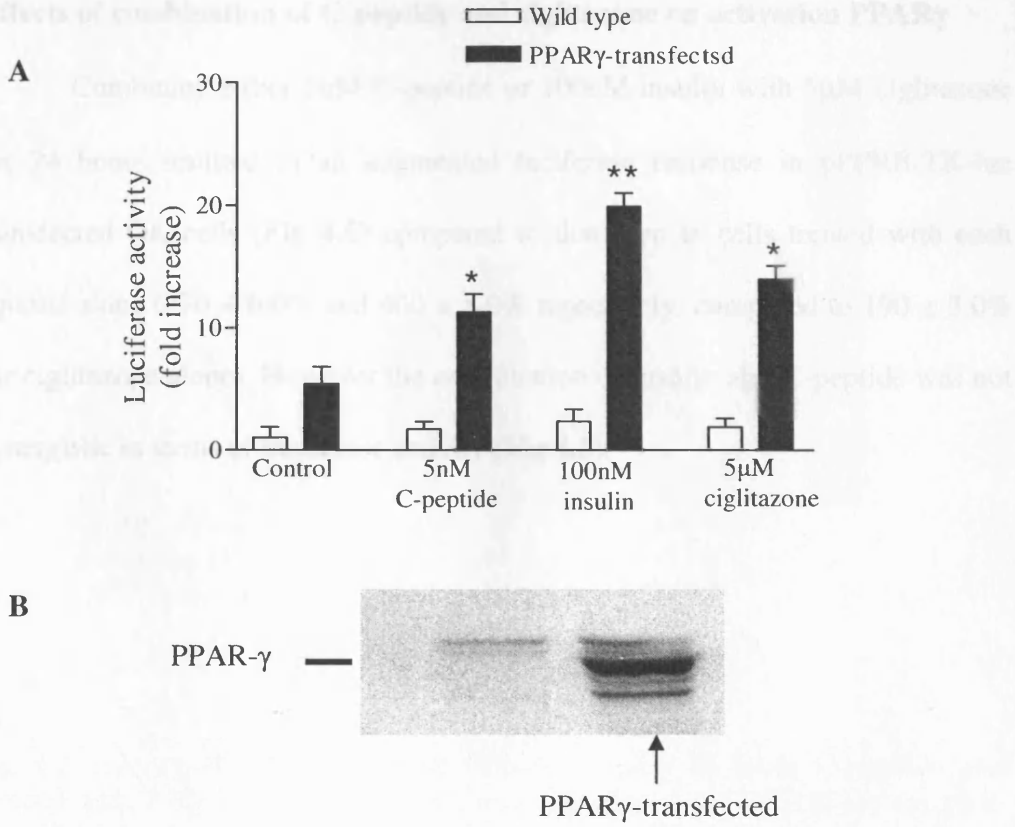


Fig 4.4. C-peptide and insulin stimulated PPAR activity are mediated through PPARγ. **(A)** OK cells were transiently transfected with pPPRE-TK-luc and pSV-βgal co-transfected with pCMX-PPARγ or empty vector. Transient transfected cells were treated with the indicated concentrations of C-peptide, insulin or ciglitazone. Cells were lysed, and luciferase activity was determined and normalized for transfection efficiency using β-gal activity. Normalized luciferase activity is expressed as a percentage compared to that measured in non-stimulated pPPRE-TK-luc and pSV-βgal transfected wild type cells. Results are expressed as means ± SEM, n=3 experiments. **(B)** Overexpression of PPARγ in transfected cells. Whole cell lysates of wild type and pCMX-PPARγ-transfected cells were immunoblotted using monoclonal anti-PPARγ. This blot is representative of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ relative to control.

Effects of combination of C-peptide and ciglitazone on activation PPARγ

Combining either 5nM C-peptide or 100nM insulin with 5μM ciglitazone for 24 hours resulted in an augmented luciferase response in pPPRE-TK-luc transfected OK cells (**Fig 4.5**) compared to that seen in cells treated with each agonist alone (270 ± 6.0% and 400 ± 8.0% repectively, compared to 190 ± 3.0% for ciglitazone alone). However the combination of insulin and C-peptide was not synergistic in terms of luciferase activity (**Fig 4.5**).

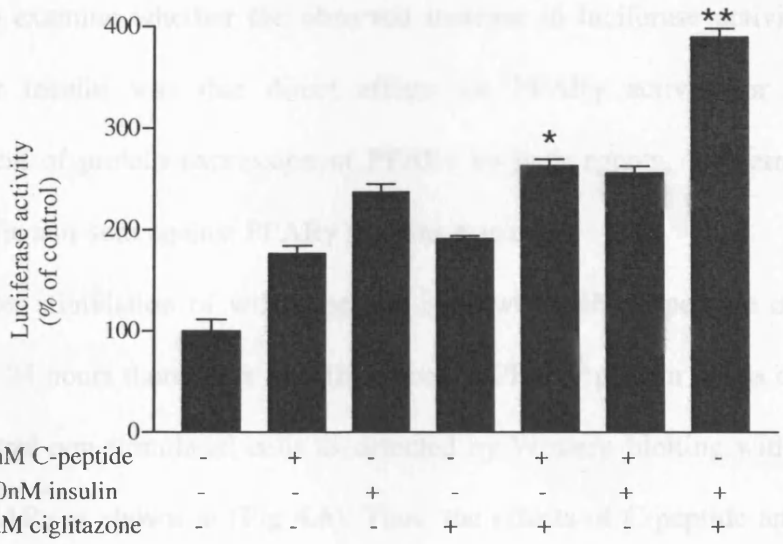


Fig 4.5. Synergistic stimulation of PPARγ activity by both C-peptide and insulin with TZD. Cells were transient transfected with pPPRE-TK-luc and pSV-βgal. Transiently transfected OK were treated for 24 hours with the indicated concentrations of C-peptide, insulin, ciglitazone or combination. Cells were lysed, and luciferase activity in lysates was determined and normalized for transfection efficiency using β-gal activity. Normalized luciferase activity is expressed as percentage compared to control in non-stimulated cells. Results are expressed as means ± SEM, n=3 experiments. * $p < 0.05$, ** $p < 0.01$ relative to ciglitazone alone.

Effects of C-peptide and insulin on protein expression levels of PPARγ

To examine whether the observed increase in luciferase activity by C-peptide or insulin was due direct effects on PPARγ activity or reflected enhancement of protein expression of PPARγ by both agents, Western blotting with specific anti-sera against PPARγ proteins was used.

After stimulation of wild type OK cells with 5nM C-peptide or 100nM insulin for 24 hours there were no differences in PPARγ protein levels compared to the control non-stimulated cells as detected by Western blotting with antisera against PPARγ as shown in (**Fig 4.6**). Thus, the effects of C-peptide and insulin on PPARγ mediated activation of PPRE were not related to changes in expression of PPARγ itself.

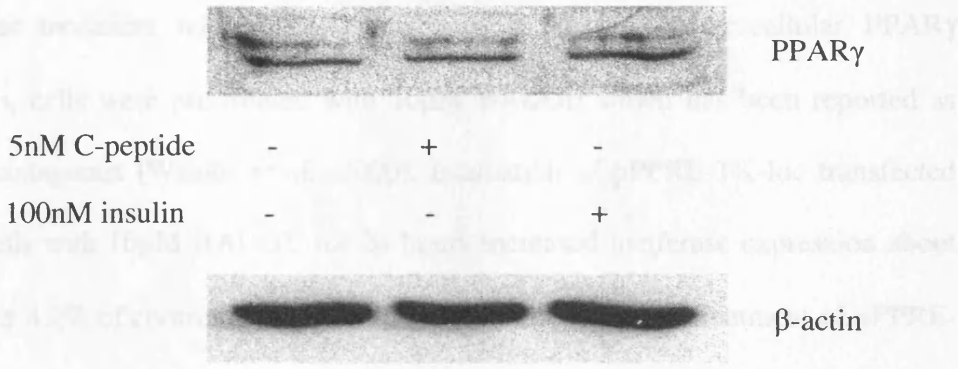


Fig 4.6. Effects of C-peptide and insulin on protein levels of PPARγ in OK cells. Cells were treated with indicated concentrations of C-peptide or insulin for 24h and PPARγ detected by immunoblotting. Membranes were stripped and re-probed for β-actin as loading control. This is a representative blot of 4 independent experiments.

Effects of PPARγ antagonist on C-peptide- and insulin-induced activation of PPARγ

To determine whether C-peptide or insulin *per se* were PPARγ ligands, or whether treatment with these agents caused release of intracellular PPARγ ligands, cells were pre-treated with 10μM BADGE which has been reported as weak antagonist (Wright *et al.,* 2000). Incubation of pPPRE-TK-luc transfected OK cells with 10μM BADGE for 24 hours increased luciferase expression about 139.7 ± 4.2% of control non-stimulated cells. Moreover, pre-treatment of pPPRE-TK-luc transfected OK cells with BADGE for 1 hour and combining it with either 5nM C-peptide or 100nM insulin for further 24 hours resulted in an augmented luciferase response (**Fig 4.7**) compared to that seen in cells treated with each agonist alone (189.3 ± 6.1% and 298.7 ± 2.7% of control respectively, compared to 154.4 ± 4.0% and 201.3 ± 3.0% of control for C-peptide and insulin alone respectively). Pre-treating the cells with BADGE had no effect on 5μM ciglitazone induced activation of PPRE. Thus, these data suggest that BADGE may act as a ligand PPARγ agonist in this cell line.

Alternatively, GW9662 a selective irreversible PPARγ antagonist was used. Pre-treatment of pPPRE-TK-luc transfected OK cells for 30 minutes before application of 100nM insulin, 5nM C-peptide or 5μM ciglitazone. GW9662 pre-treatment significantly inhibited the effects of ciglitazone on PPRE activity to about 60% of that observed compared to non-GW9662 pre-treated cells. However, neither C-peptide nor insulin-induced PPRE activity was affected by GW9662 treatment as shown in (**Fig 4.8**). Therefore, C-peptide and insulin activate PPARγ in a manner different from direct binding and that does not include enhanced release or increase synthesis of endogenous PPARγ ligands.

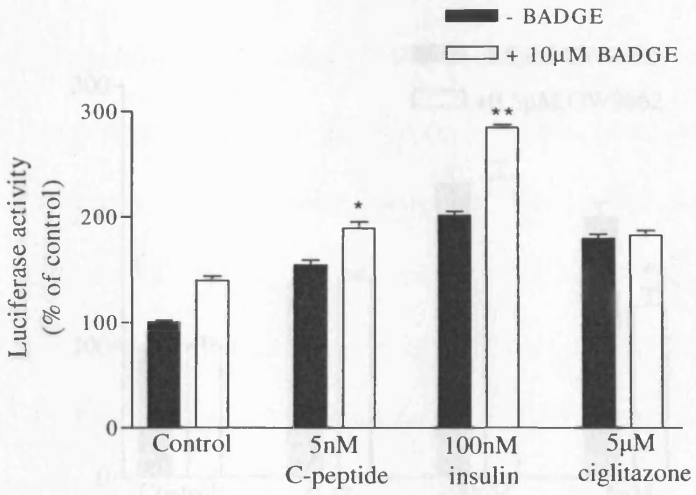


Fig 4.7. PPARγ antagonist BADGE acts as an agonist in OK cells and augments C-peptide and insulin-induced PPARγ activity. Cells were transient transfected with pPPRE-TK-luc and pSV-βgal to normalize transfection efficiency. Transiently transfected cells were treated with 10μM BADGE for 30 minutes before stimulation and during treatment for 24 hours with the indicated concentrations of C-peptide, insulin and ciglitazone. Cells were lysed, and luciferase activity in lysates was determined. Normalized luciferase activity is expressed as percentage compared with control in non-stimulated, non BADGE treated cells. Results are expressed as means ± SEM, n=3 experiments. * $p< 0.05$, ** $p< 0.01$ relative to non-BADGE treated cells.

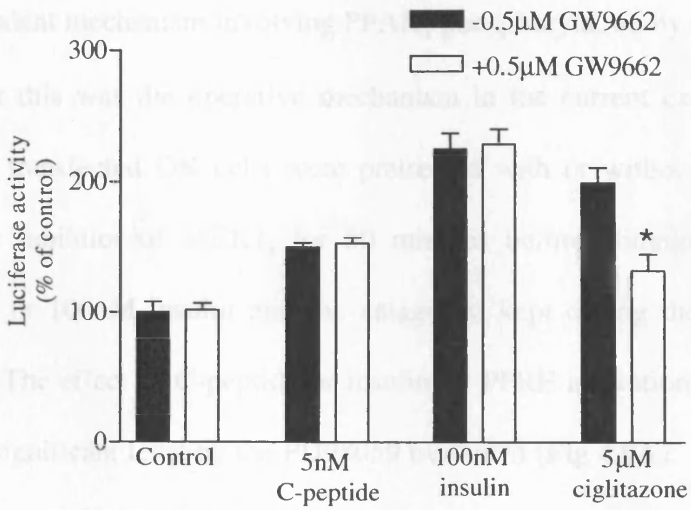


Fig 4.8. PPARγ antagonist GW9662 has no effect on C-peptide or insulin-induced PPARγ activity. Cells were transient transfected with pPPRE-TK-luc and pSV-βgal to normalize transfection efficiency. Transiently transfected cells were treated with GW9662 for 30 minutes before stimulation and during treatment for 24 hours with the indicated concentrations of C-peptide, insulin and ciglitazone. Cells were lysed, and luciferase activity in lysates was determined. Normalized luciferase activity is expressed as percentage compared with control in non-stimulated, non GW9662 treated cells. Results are expressed as means ± SEM, n=3 experiments. * $p < 0.05$ relative to ciglitazone alone.

Effects of ERK inhibitor on C-peptide-mediated activation of PPARγ

Insulin has been previously shown to activate PPARγ via a ligand independent mechanism involving PPARγ phosphorylation by ERK. To determine whether this was the operative mechanism in the current experiments, pPPRE-TK-luc transfected OK cells were pretreated with or without 5μM PD98059, a specific inhibitor of MEK1, for 30 minutes before stimulation with 5nM C-peptide or 100nM insulin and the antagonist kept during the entire stimulation period. The effect of C-peptide or insulin on PPRE activation was not attenuated to any significant level by the PD98059 treatment (**Fig 4.9A**). To confirm that the activation of PPARγ by C-peptide or insulin are not mediated via activation of MEK1, pPPRE-TK-luc transfected OK cells were co-transfected with pMEK1-CA (active MEK1). Basal unstimulated PPRE driven luciferase expression was increased by 270 ± 10% and no further significant stimulation by agonists was observed (**Fig 4.9B**). Furthermore, Co-transfection with pMEK1-KD (a mutant negative MEK1) markedly inhibited basal transactivation to about 55 ± 8.0%, but the effects of C-peptide or insulin were not attenuated compared to new basal (**Fig 4.9B**).

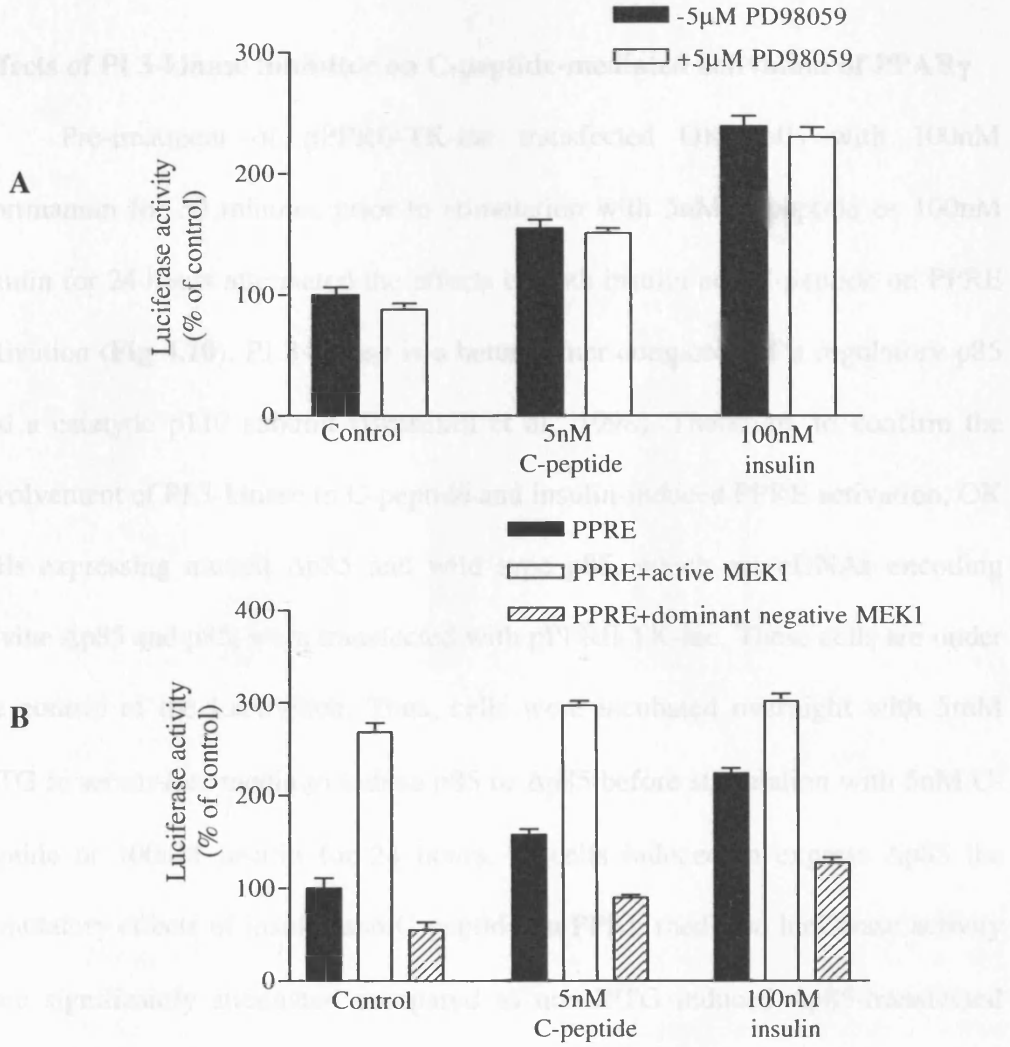


Fig 4.9. MAP kinase is not required for C-peptide or insulin-induced PPARγ activity. Cells were transient transfected with pPPRE-TK-luc and pSV-βgal to normalize for transfection efficiency. **(A)** Transiently transfected cells were treated with 5μM PD98059 for 30 minutes prior to stimulation and during treatment for 24 hours with the indicated concentration of C-peptide and insulin. **(B)** Cells were co-transfected with constitutive active MEK1, dominant negative MEK1, or empty vector. Transiently transfected cells were treated for 24 hours with indicated concentrations of C-peptide or insulin. Cells were lysed, and luciferase activity in lysates was determined. Normalized luciferase activity is expressed as percentage compared with control in non-stimulated cells. Results are expressed as means ± SEM, n=3 experiments.

Effects of PI 3-kinase inhibitor on C-peptide-mediated activation of PPARγ

Pre-treatment of pPPRE-TK-luc transfected OK cells with 100nM wortmannin for 30 minutes prior to stimulation with 5nM C-peptide or 100nM insulin for 24 hours attenuated the effects of both insulin and C-peptide on PPRE activation (**Fig 4.10**). PI 3-kinase is a heterodimer composed of a regulatory p85 and a catalytic p110 subunit (Brunskill et al., 1998). Therefore, to confirm the involvement of PI 3-kinase in C-peptide and insulin-induced PPRE activation, OK cells expressing mutant Δp85 and wild type p85, which are cDNAs encoding bovine Δp85 and p85, were transfected with pPPRE-TK-luc. These cells are under the control of the LacSwitch. Thus, cells were incubated overnight with 5mM IPTG in serum-free media to induce p85 or Δp85 before stimulation with 5nM C-peptide or 100nM insulin for 24 hours. In cells induced to express Δp85 the stimulatory effects of insulin and C-peptide on PPRE mediated luciferase activity were significantly attenuated compared to non-IPTG induced Δp85-transfected controls (**Fig 4.11A**). Induced expression of wild type p85 had no effect on C-peptide or insulin-induced activation of PPARγ (**Fig 4.11B**). Successful induction of recombinant p85/Δp85 was confirmed by Western blotting with antisera against PI3-kinase p85-α (**Fig 4.11C**).

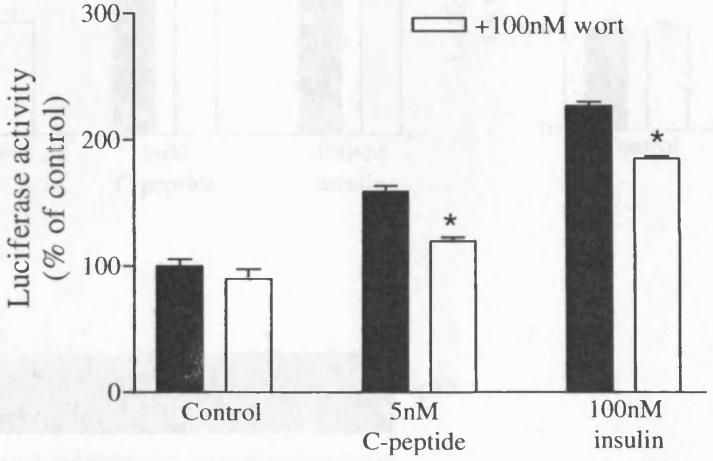


Fig 4.10. Effects of PI 3-kinase inhibitor on C-peptide- or insulin-induced PPRE activation. Cells were transiently transfected with pPPRE-TK-luc and pSV-βgal to normalize for transfection efficiency. Transient transfected wild type cells were treated with or without 100nM wortmannin for 30 minutes before stimulation for 24 hours with indicated concentrations of C-peptide or insulin. Results are expressed as means ± SEM, n= 3-4 experiments. *$p< 0.05$ relative to non-wortmannin treated.

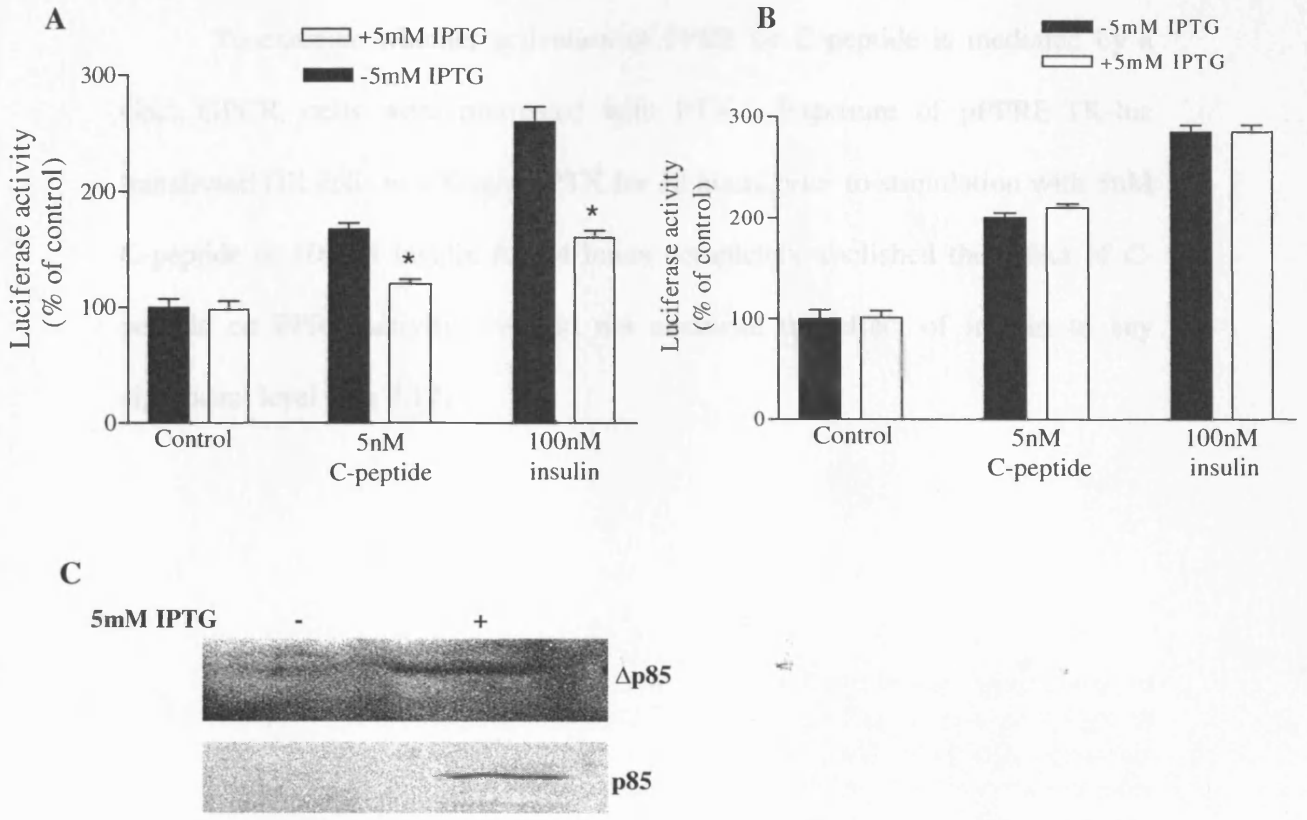


Fig 4.11. Effects of induction of expression of mutant Δp85 or wild p85 transfected OK cells on C-peptide- or insulin-induced PPRE activation. (A) pPPRE-TK-luc and pSV-βgal transient transfected Δp85-transfected OK cells were treated with or without 5mM IPTG overnight prior to stimulation for a further 24 hours with indicated concentrations of C-peptide or insulin. **(B)** pPPRE-TK-luc and pSV-βgal transient transfected wild type p85-transfected OK cells were treated with or without 5mM IPTG overnight prior to stimulation for a further 24 hours with indicated concentrations of C-peptide or insulin. Cells were lysed, and luciferase activity in lysates was determined. Normalized luciferase activity is expressed as percentage compared with control in non-stimulated. Results are expressed as means ± SEM, n=3-4 experiments. *p< 0.05 relative to non-IPTG treated cells. D) Induction of Δp85 and p85 expression by IPTG in OK cells. Δp85-transfected OK cells and p85-transfected OK cells were treated with 5mM IPTG for 24 hour and lysates were immunoblotted with anti-PI3-kinase p85α. Blots are representative of 3 independent experiments.

Effects of PTX on C-peptide induced PPRE activation

To examine whether activation of PPRE by C-peptide is mediated by a $G\alpha_i/_o$ GPCR, cells were pretreated with PTX. Exposure of pPPRE-TK-luc transfected OK cells to 100ng/ml PTX for 18 hours prior to stimulation with 5nM C-peptide or 100nM insulin for 24 hours completely abolished the effect of C-peptide on PPRE activity, but did not attenuate the effect of insulin to any significant level (**Fig 4.12**).

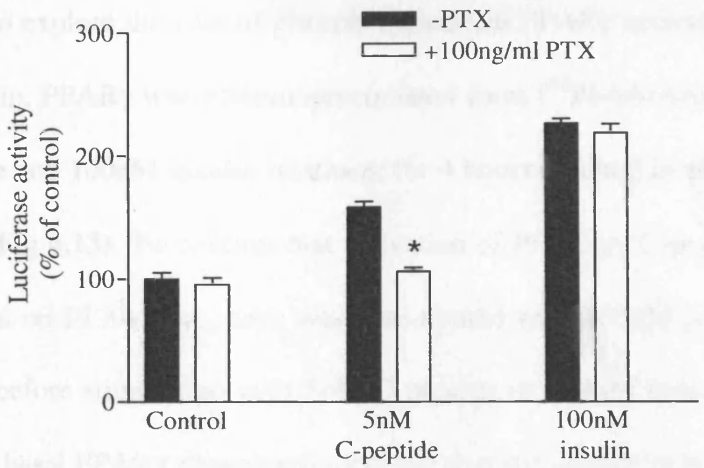


Fig 4.12. PTX inhibits C-peptide but not insulin-induced activation of PPARγ. Cells were transiently transfected with pPPRE-TK-luc and pSV-βgal to normalize for transfection efficiency. A) Transiently transfected wild type OK cells were treated with or without 100 ng/ml PTX for 18 h prior to stimulation for 24 h with indicated concentrations of C-peptide or insulin. Cells were lysed, and luciferase activity in lysates was determined. Normalized luciferase activity is expressed as percentage compared with control in non-stimulated cells. Results are expressed as means ± SEM, n=3 experiments. * $p < 0.05$ relative to non-PTX treated, C-peptide stimulated cells.

Effects of C-peptide and insulin on PPARγ phosphorylation

To explore the role of phosphorylation in PPARγ activation by C-peptide and insulin, PPARγ was immunoprecipitated form [^{32}P]-labelled cells. Both 5nM C-peptide and 100nM insulin treatment for 4 hours resulted in phosphorylation of PPARγ (**Fig 4.13**). To confirm that activation of PPRE by C-peptide or insulin is dependent on PI 3-kinase, cells were pre-treated with 100nM wortmannin for 30 minutes before stimulation with 5nM C-peptide or 100nM insulin. There was no effect on basal PPARγ phosphorylation, but that stimulated by both insulin and C-peptide was abolished.

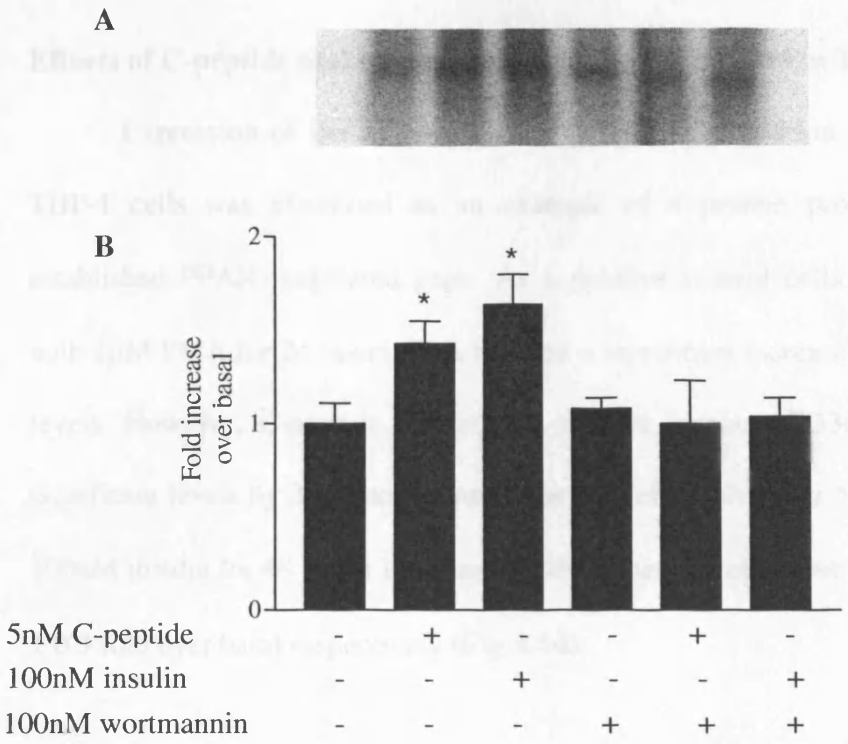


Fig 4.13. Phosphorylation of PPARγ in response to C-peptide and insulin. Wild type OK cells were labelled with [^{32}P]-orthophosphate, treated with indicated concentrations of C-peptide and insulin with or without wortmannin pre-treatment and then immunoprecipitated with anti-PPARγ. Immunoprecipitates were collected on Protein A sepharose and then subjected to PAGE followed by autoradiography. **(A)** The labelled band represents phosphorylated PPARγ. **(B)** The combined results of densitometric analysis from three identical experiments. *$p < 0.05$ relative to non-wortmannin-inhibited control condition.

Effects of C-peptide and insulin on protein levels of CD36 in THP-1 cell

Expression of the monocyte/macrophage differentiation marker CD36 in THP-1 cells was examined as an example of a protein product of a well-established PPARγ regulated gene. As a positive control cells were stimulated with 1μM PMA for 24 hours. This induced a significant increase in CD36 protein levels. However, C-peptide and insulin did not increase CD36 protein to any significant levels by 24 hours. Stimulation the cells with either 5nM C-peptide or 100nM insulin for 48 hours increased CD36 protein levels about 2.0 ± 0.2 and 2.2 ± 0.3 fold over basal respectively (**Fig 4.14**).

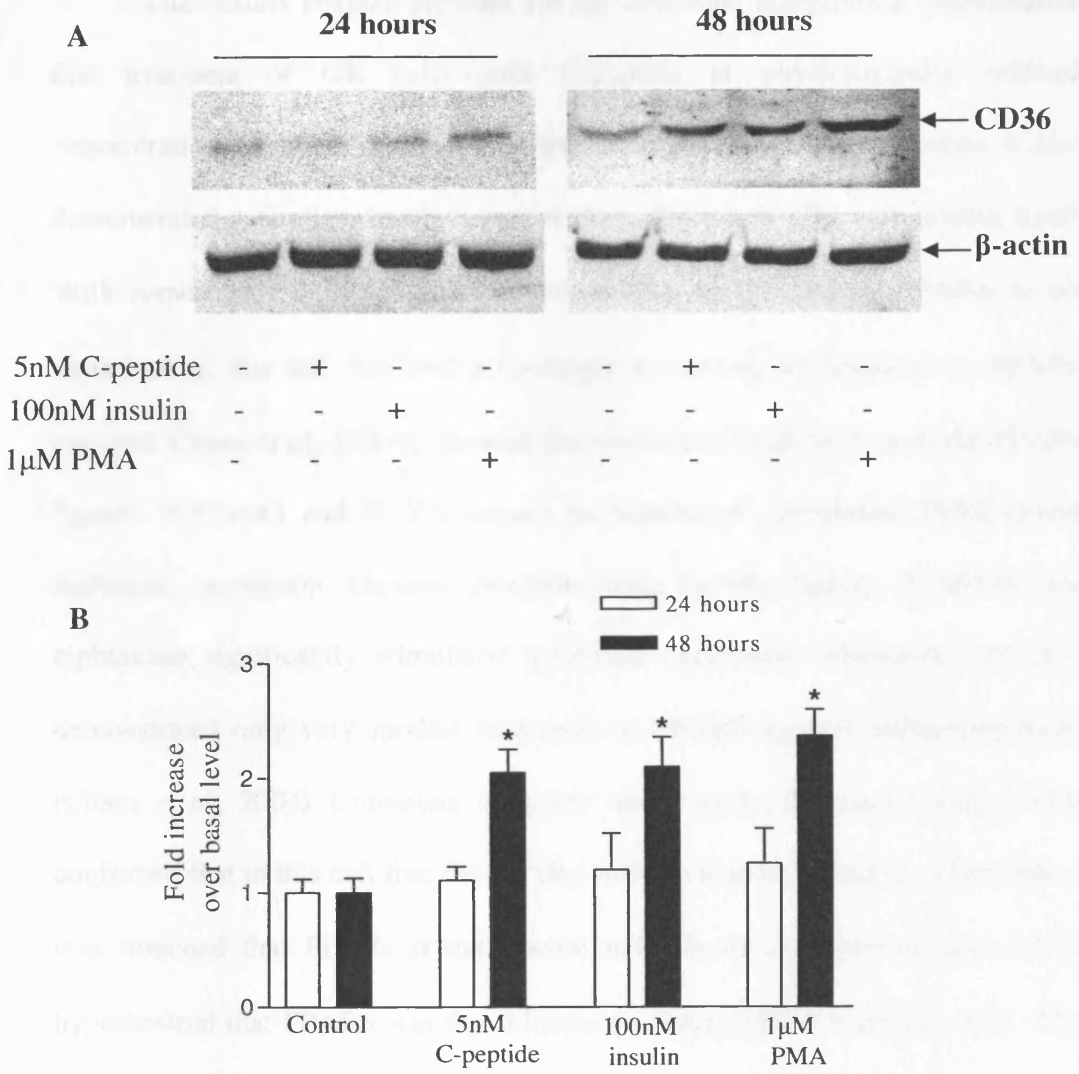


Fig 4. 14. C-peptide and insulin increase CD36 protein levels in THP-1 cells. Cells were treated with indicated concentrations of C-peptide, insulin or PMA in serum free media for the indicated times. CD36 protein was detected by immunoblotting. **(A)** A representative blot is shown. **(B)** Bands were quantified by densitometry and values expressed graphically as fold increase over the basal. Results are expressed as means ± SEM, n=3. *p<0.01 relative to control at the same time point.

4.3. DISCUSSION

This results chapter provides for the first time unequivocal confirmation that treatment of OK cells with C-peptide at physiologically relevant concentrations resulted in significant transactivation of PPRE. Likewise it also demonstrated a similar, but less potent dose dependent effect of insulin itself. With respect to the PPAR isoform responsible for this effect, PPARα is not expressed in this cell line and accordingly it showed no response to PPARα agonists. Chana *et al.,* (2004), showed that treatment of OK cells with the PPARα ligands WY14643 and ETYA caused no significant stimulation PPRE-driven luciferase expression whereas treatment with PPARγ ligands 15-dPGJ$_2$ and ciglitazone significantly stimulated luciferase expression. Moreover, OK cells demonstrated only very modest responses to PPARβ agonist carbaprostacyclin (Chana *et al.,* 2004). Consistent with this earlier study, the current experiments confirmed that in this cell line the PPARγ isoform is most abundant. Therefore, it was reasoned that PPARs α and β were unlikely to be involved, and it was hypothesized that PPARγ was the subtype mediating PPRE transactivation. This hypothesis was suggested by demonstrating augmentation of C-peptide and insulin evoked PPRE activity in cells overexpressing recombinant PPARγ.

Several studies suggest that insulin might participate in the control of PPAR-γ expression (Vidal-Puig *et al.,* 1997; Vidal-Puig *et al.,* 1996). In rodents, PPARγ$_1$ and γ$_2$ expression is decreased by fasting and by insulin deficiency in adipose tissue (Vidal-Puig *et al.,* 1996), and treatment of diabetic mice with insulin leads to a partial restoration of PPARγ mRNA levels in epididymal fat cells (Vidal-Puig *et al.,* 1996). In cultured human adipocytes insulin, in the presence of dexamethasone, has been shown to upregulate PPARγ$_1$ and γ$_2$ (Vidal-

Puig *et al.,* 1998). Furthermore, in human adipocytes insulin treatment leads to increased expression of both PPARγ mRNA and protein both *in vivo* and in mature isolated cells (Rieusset *et al.,* 1999). However, enhanced expression of PPARγ protein was not induced by insulin or C-peptide in OK cells in the current study, and thus changes in PPARγ expression levels can not explain the demonstrated increase in PPRE transactivation by insulin and C-peptide in wild type OK cells.

The TZD ciglitazone is a prototypic PPARγ ligand that also transactivates PPRE in OK cells as shown in this chapter and previously (Chana *et al.,* 2004). Combining ciglitazone with C-peptide or insulin resulted in synergistic PPARγ-mediated transactivation of PPRE suggesting distinct means of PPARγ activation. Conversely, combining C-peptide with insulin did not increase the effect of insulin alone, suggesting that the same signalling elements may be used by both agents.

Even though it seemed unlikely that either insulin or C-peptide themselves could be ligands for PPARγ it remained possible that an endogenous intracellular PPARγ ligand may be produced as a result of their respective signalling capabilities. Therefore, PPARγ antagonists were used to examine this question. BADGE has been reported as a weak antagonist of PPARγ (Wright *et al.,* 2000). Although BADGE inhibits adipocyte differentiation in cell culture, its use as an antagonist to elucidate the importance of PPARγ in biological processes may be limited by low binding affinity and potency (Wright *et al.,* 2000). In OK cells BADGE appeared to act as PPARγ agonist rather than antagonist and increase PPRE luciferase expression. Indeed Combining BADGE with C-peptide or insulin resulted in synergistic PPARγ-mediated transactivation of PPRE suggesting

distinct means of PPARγ activation induced by these agents. Conversely, combining ciglitazone and BADGE did not increase the effect of ciglitazone alone, suggesting that the both agents may compete to the same binding site. This type of complex behaviour has been reported for some of PPARγ ligands such as BADGE and LG100641. These compounds act as antagonists in some assays or cell lines, but agonists in others (Bishop-Bailey *et al.,* 2000; Mukherjee *et al.,* 2000).

By contrast, GW9662 binds to the ligand-binding domain of PPARγ resulting in covalent modification of Cys^{285}, thus conferring upon GW9662 properties of a full PPARγ antagonist due to irreversible loss of ligand binding (Leesnitzer *et al.,* 2002). Interestingly in the current study GW9662 was able to inhibit ciglitazone, but not C-peptide- or insulin-mediated PPRE transactivation. Therefore unlike the situation with the prototypic TZD PPARγ ligands, activation of PPARγ by insulin and C-peptide appears to be independent of ligand binding.

Overall these results are consistent with a model whereby insulin and C-peptide stimulate PPARγ mediated PPRE activation via signalling cascades downstream of their cell surface receptors. Indeed, for C-peptide at least, pertussis toxin sensitivity of PPRE activation is coherent with the evolving concept of C-peptide mediated signalling via a $G\alpha_i$ or $G\alpha_o$ coupled receptor (Maestroni *et al.,* 2005; Zhong *et al.,* 2005; Walcher *et al.,* 2004; Marx *et al.,* 2004; Shafqat *et al.,* 2002; Kitamura *et al.,* 2001; Ohtomo *et al.,* 1996; Rigler *et al.,* 1999).

To understand the possible mechanism (s) for this finding, it is necessary to review recent observations on the regulation of PPARγ activity by phosphorylation. This may be linked with positive or negative regulation of ligand-independent transcriptional activity. Shalev *et al.,* (1996) demonstrated that

PPARγ is a phosphoprotein and that phosphorylation is capable of enhancing its transcriptional activation potency, although mapping of responsible sites was not carried out. Zhang *et al* (1996) showed that insulin and TZD combined synergistically to enhance the ability of full-length PPARγ to stimulate aP2 gene expression and speculated that this might be mediated by the ERK dependent phosphorylation that they also demonstrated. However, several groups of workers have demonstrated that ERK mediated phosphorylation of serine 82 of PPARγ1, and serine 112 of PPARγ2 was accompanied by a reduction in transcriptional activity (Adams *et al.,* 1997; Camp, and Tafuri, 1997). In contrast Lazennec and co-workers (2000) demonstrated that activators of protein kinase A evoked ligand-independent activation and phosphorylation of PPARγ.

Despite the previous reports that ERK is a key regulator of ligand independent PPARγ activity, the results of this chapter provided several lines of evidence to suggest that the ERK pathway was not involved in C-peptide or insulin mediated alterations in PPARγ activity in OK cells. Firstly, although a robust ERK activation by C-peptide in OK cells was specified previously (section 3.2.2), the stimulatory effects of C-peptide and insulin on PPRE transactivation were not affected by a chemical ERK inhibitor. Secondly, whilst overexpression of a dominant negative MEK1 significantly depressed basal transactivation of PPARγ activity, the effects of C-peptide and insulin on PPRE transactivation were preserved. In OK cells, rather than inhibiting PPARγ transcriptional activity as demonstrated by others (Adams *et al.,* 1997; Camp and Tafuri, 2000), expression of constitutively active MEK1 leads to maximally enhanced basal activation of endogenous PPARγ activity such that ligand-dependent and independent activation can no longer be detected. Indeed, the mechanism of C-peptide or

insulin and ERK-stimulated PPARγ activation is not clear. Zhang *et al.,* (1996), reported that PPARγ containing Ala instead of Ser^{112} (site of ERK action) was also activated by TZD and/or insulin in a manner similar to that observed with wild-type. In addition, insulin was capable of activating PPARα and PPARδ, both of which lack the consensus ERK physphorylation site at Ser^{112} (Juge-Aubry *et al.,* 1999). Furthermore, PPARα and PPARδ transactivation was blocked by dominanant negative MEK1 (Juge-Aubry *et al.,* 1999). These results suggest that the ERK pathway regulates PPARs via an indirect mechanism that may be independent of direct phosphorylation. These findings are in agreement with those described in this chapter whereby ERK may regulate the basal PPARγ activity in OK cells but was not involved in that induced by C-peptide or insulin.

In view of the earlier observations (section 3.2.2) that C-peptide activated PI 3-kinase in OK cells, a capacity also shared by insulin (Brunskill *et al.,* 1998), the reliance of ligand independent PPARγ activation by these agents on activity of PI 3-kinase was studied. Wortmannin induced attenuation of PPRE transactivation induced by both C-peptide and insulin strongly implicates PI 3-kinase in this effect, and the concordant results of the experiments using Δp85 confirmed the involvement of Type 1A PI 3-kinase. Furthermore, ligand independent activation of PPARγ by C-peptide and insulin was clearly associated with PI 3-kinase dependent phosphorylation of PPARγ, although the precise kinase mediating the phosphorylation was not established by these studies.

These observations raise a major question as to whether the PPRE activation induced by C-peptide and insulin observed using luciferase reporter constructs is mirrored by transcriptional activation of established PPARγ regulated genes in native cells. Data regarding PPARγ function in proximal

tubular cells is beginning to accrue, Arici *et al.*,(2003) have shown that albumin-bound fatty acids were able to activate PPARγ in human proximal tubular cells and the consequences of this activation included apoptotic cell death. Moreover, several authors suggest a potential role for PPAR agonists in the treatment of renal disease. Recent study in STZ-induced diabetes showed that TZD was not only able to prevent diabetic glomerular hyperfiltration and albumin excretion, but also to reduce mRNA expression of extracellular matrix proteins and TGF-β1 (Routh *et al.,* 2002). However, little information is available regarding specific PPARγ regulated genes in proximal tubular cells and in kidney in general.

A variety of PPARγ regulated genes have been identified in macrophages and it has been reported that expression of PPARγ is observed in atherosclerotic legions co-localised with macrophages (Marx *et al.,* 1998; Ricote *et al.,* 1998) and that PPARγ ligand, troglitazone, significantly inhibits the development of atherosclerotic foam cell lesions (Li *et al.,* 2000; Collins *et al.,* 2001). Co-localisation of C-peptide with macrophages in atherosclerotic lesions described in diabetic patients suggests a potential role for C-peptide in regulation of function in this cell type (Max *et al.,* 2004). The type B scavenger receptor CD36 is expressed during differentiation of monocytes to macrophages and its expression is regulated by PPARγ (Nicholson, 2004; Chawla *et al.,* 2001; Nagy *et al.,* 1998). PPARγ modulates CD36 gene expression through direct interaction with the proximal promoter via a specific response element (Nicholson, 2004). The primary functions of CD36 relate to clearance of apoptotic cells arising during development, normal homeostasis, and inflammation (Savill *et al.,* 1992; Bird *et al.,* 1999). CD36 is also important in protection against insulin resistance and is essential for the actions of insulin sensitising actions of TZDs (Hevener *et*

al., 2001; Qi *et al.,* 2002; Seda *et al.,* 2003). Tontonoz *et al.,* (1998), reported that PPARγ ligands up-regulated expression of CD36, and promoted uptake of modified LDL via CD36 in THP-1 cells, suggesting a novel role of PPAγ in gene regulation, differentiation and lipid metabolism in macrophages. Their studies showed that treatment of THP-1 cells with troglitazone alone resulted in increase in CD36 mRNA, and the effect of combination of troglitazone and phorbol ester was much greater than that of troglitazone alone. By treating the human monocyte cell line THP-1 with insulin and C-peptide, the current studies provide proof of principle that C-peptide has the ability to induce expression of PPARγ regulated genes. Similar effects could not be confirmed in OK cells due to cross reactivity of multiple proteins with CD36 specific anti-sera (data not shown).

In summary this results chapter demonstrates that C-peptide and insulin evoked, phosphorylation associated ligand independent activation of PPARγ with the requirement for PI 3-kinase activity in OK cells. These effects measured using reporter constructs were mirrored by C-peptide and insulin stimulated expression of PPARγ regulated genes in a monocyte cell line. The findings may indicate a potentially major new role for C-peptide in the regulation of cell biology, insulin sensitivity, glucose homeostasis and PPARγ function.

CHAPTER 5
C-PEPTIDE PROTECT AGAINST TNF-α-MEDIATED APOPTOSIS OF OKCELLS VIA ACTIVATION OF NFκB

5.1. INTRODUCTION

Evolving evidence suggests that C-peptide might have a protective role in diabetic nephropathy and ameliorates diabetic renal complications (Sjoquist *et al.,* 1998, Samnegard *et al.,* 2001, Huang *et al.,* 2002: Samnegard *et al.,* 2004: Samnegard *et al.,* 2005). The mechanism(s) underlying these beneficial effects of C-peptide are incompletely understood. However, C-peptide has been found to stimulate numerous intracellular signalling pathways in PTC such as MAPK, PI 3-kinase/Akt, PKC as documented in this thesis (section 3.2.2), resulting in increases in $[Ca^{2+}]_i$ (Shafqat *et al.,* 2002). C-peptide has also been shown to stimulate eNOS in endothelial cells (Kitamura *et al.,* 2003), and Na^+, K^+ ATPase activity in both glomerular and tubular cells (Zhong *et al.,* 2004).

Tumor necrosis factor-α (TNF-α) is a pleiotropic 157 amino acid peptide cytokine, capable of eliciting a wide spectrum of cellular responses including differentiation, proliferation, inflammation, and cell death (Wajant *et al.,* 2003) via interaction with two members of the TNF receptor family, TNF-R1 and TNF-R2. Predominantly produced by monocytes/macrophages but also by T and B-lymphocytes and glomerular mesangial cells (Baud *et al.,* 1989; Hruby and Lowry, 1991). TNF-α binding to TNF-R1 may simultaneously trigger apoptotic pathways by recruitment of death effector adaptor molecules with subsequent activation of caspase cascades, and anti-apoptotic pathways by a pathway involving TRAF2 and NFκB (for review see Wajant *et al.,* 2001). Integration of these events determines the eventual cellular response to TNF-α stimulation. In

particular NFκB stimulates transcription of anti-apoptotic factors that modulate the caspase cascade, and thus NFκB activity acts as a checkpoint in a cell's decision to survive or apoptose in response to a given stimulus.

TNF-α is a key player in the pathogenesis of diverse renal diseases. For example in IgA nephropathy elevated intrarenal TNF-α production and increased urinary concentrations of TNF-α have been demonstrated (Wu *et al.,* 1996). In rodent studies of diabetic nephropathy amplified renal expression of TNF-α mRNA is observed (Nakamura *et al.,* 1993). Circulating, urinary and renal interstitial TNF-α levels were increased after induction of diabetes with STZ, preceding the rise in urinary albumin excretion (Kalantarinia *et al.,* 2003). Similarly increased serum levels of TNF-α have been found in patients with Type 2 diabetes and nephropathy (Moriwaki *et al.,* 2003).

Apoptosis is an active mode of cell death contributing to the late structural abnormalities seen in diabetic nephropathy, including tubular atrophy and tubulointerstitial fibrosis (Ortiz *et al.,* 1997a). Kumar *et al* (2004), found a significant increase of apoptotic cells in the tubulointerstitium, epithelial and endothelial but not glomeruli of diabetic mice. At present, however, there is little information on the factors that promote or regulate apoptosis in kidney. The balance between factors that contribute to survival, growth or lethality often impacts on the chance of cell to death (Louis *et al.,* 1993; Raff *et al.,* 1992). In fact, there is evidence that a relative deficit of survival factors contributes to renal failure. Multifunctional cytokines such as IGF-I and EGF have survival factor activity for tubular epithelium (Hise *et al.,* 1995). The local expression of cytokines with survival factor activity is decreased in renal failure (Hise *et al.,* 1995; Verstrepen *et al.,* 1993). Other multifunctional cytokines such as TNF-α

also promote apoptosis and induce acute tubular necrosis (Ortiz *et al.,* 1997b; Gresser *et al.,* 1987), while anti-TNF-α anti-bodies protect against renal failure in model system (Shulman *et al.,* 1993). Therefore, TNF-α in the kidney in diabetes may contribute to apoptosis. Furthermore, the absence of C-peptide and insulin in patients with Type 1 diabetes may render the kidney more susceptible to apoptosis induced by lethal cytokines such as TNF-α. This hypothesis is strengthened by the previous findings (section 3.2.2) showing that C-peptide is a functional mitogen in OK cells. Therefore, it could be hypothesised that C-peptide may be able to antagonise cytotoxic effects of lethal cytokines such as TNF-α on PTC.

The effect of TNF-α on OK cell viability was examined. Whether TNF-α cytotoxicity may be modulated by administration of C-peptide or insulin was assessed. The mechanisms by which C-peptide or insulin mediated cytoprotective effects in the face of TNF-α-induced cytotoxicity were also addressed.

5.2. RESULTS

Effects of C-peptide and insulin on TNF-α-induced cytotoxicity in PTC

The first step was to investigate the susceptibility of PTC to TNF-α induced cytotoxicity and to examine whether C-peptide or insulin may have cytoprotective effects against TNF-α-toxicity. Therefore, OK cells were pre-incubated with or without 5nM C-peptide or 100nM insulin in serum-free medium for 24 hours before stimulation with different concentrations of TNF-α (50-300ng/ml) for 24 hours in presence or absence of insulin or C-peptide and Cell viability was assessed by MTT assay.

Incubation of OK cells with increasing concentrations of TNF-α resulted in declining viability, becoming significant at the higher concentration of 300ng/ml TNF-α, when cell viability was reduced to about 60.8 ± 2.7% of control (**Fig 5.1**). Both C-peptide and insulin treatment were able to abolish TNF-α toxicity.

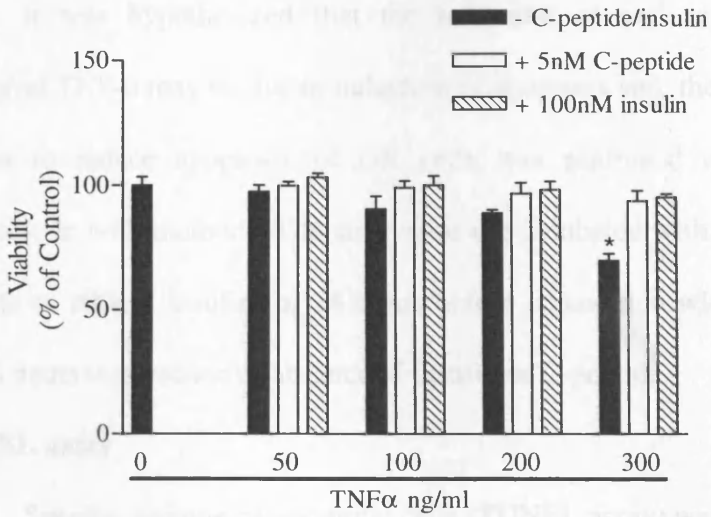


Fig 5.1. C-peptide and insulin protect against TNF-α-induced cytotoxicity. MTT assay of cells pre-incubated with 5nM C-peptide, 100nM insulin or medium alone before exposure to TNF-α. Results are expressed as means ± SEM, n=3 experiments. *p< 0.05 relative to control.

Effects of C-peptide and insulin on TNF-α-induced apoptosis of PTC

It was hypothesized that the reduction of cell viability induced by 300ng/ml TNF-α may be due to induction of apoptosis and, therefore the ability of TNF-α to induce apoptosis of OK cells was examined using two different methods. In both methods, OK cells were pre-incubated with or without 5nM C-peptide or 100nM insulin for 24 hours before stimulation with 300ng/ml TNF-α for 24 hours in presence or absence of insulin or C-peptide.

TUNEL assay

Specific staining of apoptotic cells (TUNEL assay) was directly visualized with a light microscopy (original magnification X 400) and hematoxylin was used as counterstain. For quantification of apoptotic cells, 50 cells from 6 random microscopic fields were counted and the results are depicted in (**Fig 5.2A** and **2B**). Serum starvation was accompanied by low-level background apoptosis of 3.0 ± 0.5% of cells. This was significantly exacerbated by 300ng/ml TNF-α when 12.0 ± 1.1% of cells were observed to be apoptotic. The TNF-α-induced apoptosis was completely attenuated by 5nM C-peptide and/or 100nM insulin when 2.3 ± 0.7% and 2.0 ± 0.5% of cells respectively were found to be apoptotic.

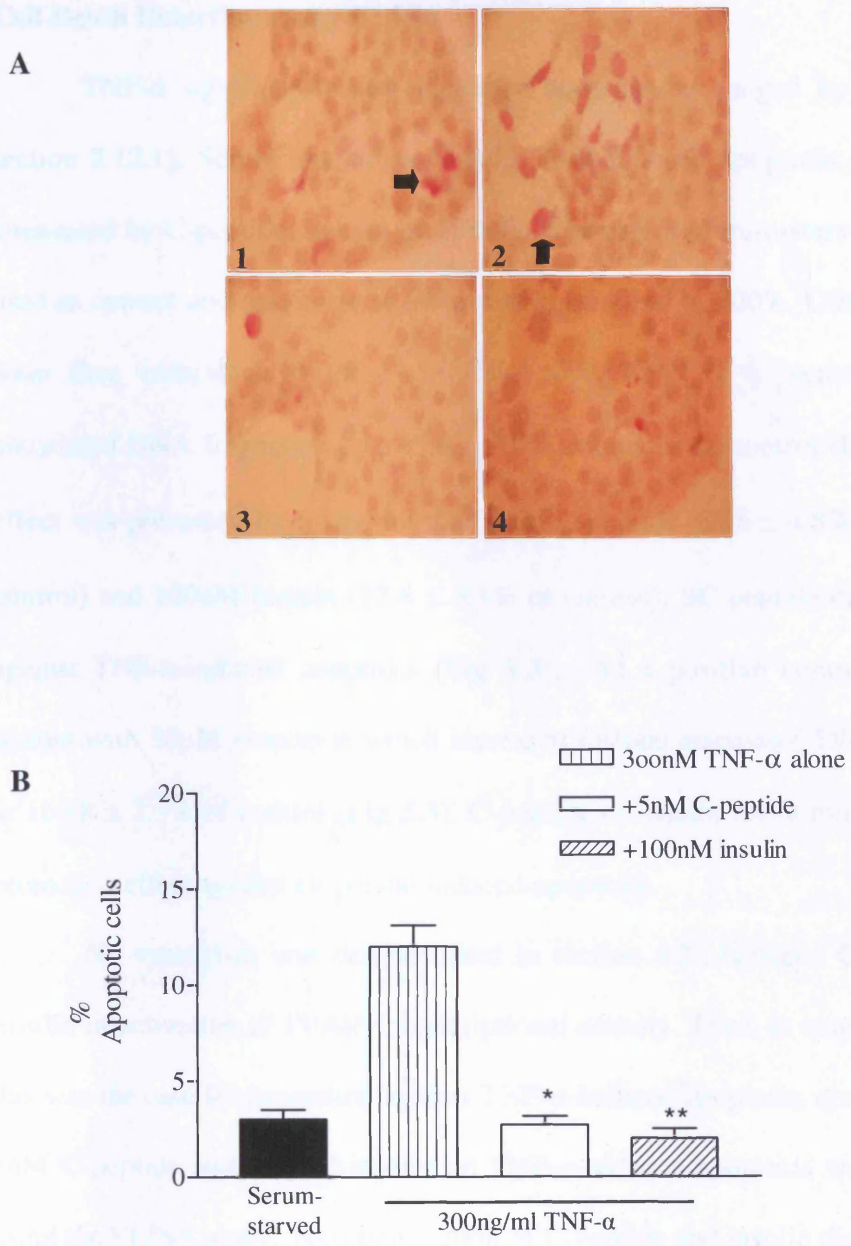


Fig 5.2. C-peptide and insulin protect against TNF-α-induced apoptosis (TUNEL assay). (A) Some apoptotic cells are identified by arrows. Panels: 1=serum-starved control, 2=300ng/ml TNF-α treatment, 3=C-peptide pre-treatment prior to 300ng/ml TNF-α, 4=insulin pre-treatment prior to 300ng/ml TNF-α. **(B)** Quantification of TUNEL stained apoptotic cells was represented graphically. Results are mean of percentage of apoptotic cells ± SEM, n=3 experiments.*$p < 0.05$ and **$p < 0.01$ relative to TNF-α-treated cells.

Cell Death Detection assay ELISA

TNF-α significantly also increased apoptosis as judged by ELISA (see section 2.12.1). Serum starvation of cells itself induced apoptosis and this was attenuated by C-peptide, insulin or 10% FCS treatment. Serum-starved cells were used as control and apoptosis in these was normalized to 100%. Other conditions were then compared to these controls. 300ng/ml TNF-α increased histone-associated DNA fragments by 145.8 ± 5.9% compared to control (**Fig 5.3**). This effect was prevented by treatment with 5nM C-peptide (81.6 ± 4.8% compared to control) and 100nM insulin (77.4 ± 3.1% of control). SC-peptide did not protect against TNF-α-induced apoptosis (**Fig 5.3**). As a positive control cells were treated with 50μM etoposide which increased histone associated DNA fragments to 160.8 ± 2.5% of control (**Fig 5.3**). C-peptide or insulin had a minor protective protection effect against etoposide-induced apoptosis.

No synergism was demonstrated in section 4.2., between C-peptide and insulin in activation of PPARγ transcriptional activity. Thus, to examine whether this was the case for protection against TNF-α-induced apoptosis, combinations of 5nM C-peptide and 100nM insulin on TNF-α-induced apoptosis were examined using the ELISA assay. The combination of C-peptide and insulin did not increase the protection against TNF-α-induced apoptosis to any significant level over that induced by either C-peptide or insulin (**Fig 5.4**).

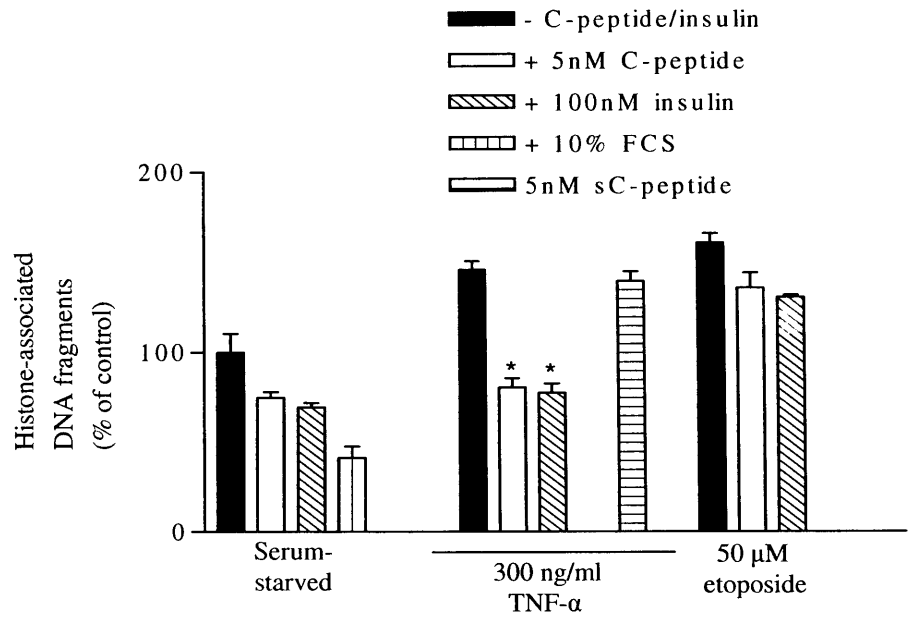


Fig 5.3. C-peptide and insulin protect against TNF-α-induced apoptosis (ELISA assay). Apoptosis was assessed by measuring cytoplasmic histone associated DNA fragments using ELISA technique. Cells were pre-incubated without or with 5nM C-peptide or 100nM insulin for 24 hours before exposure to TNF-α or etoposide. Results are expressed as means ± SEM, n=3 experiments.* $p< 0.05$, relative to TNF-α stimulated cells.

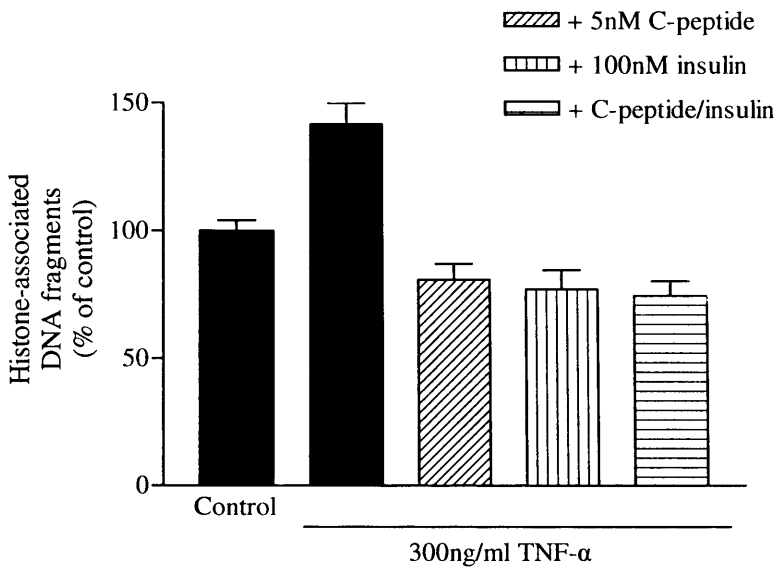


Fig 5.4. C-peptide does not augment the protective effect of insulin against TNF-α-induced apoptosis of OK cells. Cells were pre-incubated with or without 5nM C-peptide or 100nM insulin or combination for 24 hours before exposure to TNF-α. Apoptosis was assessed by measuring cytoplasmic histone associated DNA fragments using ELISA technique. Results are expressed as means ± SEM, n=3 experiments.

Effects of NFκB inhibitor MG-132 on the protective effects of C-peptide and insulin against TNF-α-induced apoptosis

It was reasoned that activation of NFκB might be responsible for the cytoprotective effects of C-peptide and insulin. To address this question the proteasome inhibitor MG-132 was used to block NFκB activity. This agent blocks the degradation of IκB thus preventing the translocation of NFκB to the nucleus (Palombella *et al.,* 1998). As shown in (**Fig 5.5**), treatment of serum starved cells with MG-132 induced no significant increase in histone-associated DNA fragments. Pre-treatment of cells for 1 hour with 5μM MG-132 completely abolished the ability of C-peptide and insulin to antagonise TNF-α induced apoptosis. Compared to 80.8 ± 3.6% apoptotic cells seen in TNF-α/C-peptide treated cultures MG-132 pre-treatment led to the appearance of 159.2 ± 4.2% apoptotic cells. Likewise pre-treatment with MG-132 resulted in an increase in apoptotic cells from 78.6 ± 4.1% to 154.9 ± 1.8% in TNF-α/insulin stimulated cultures.

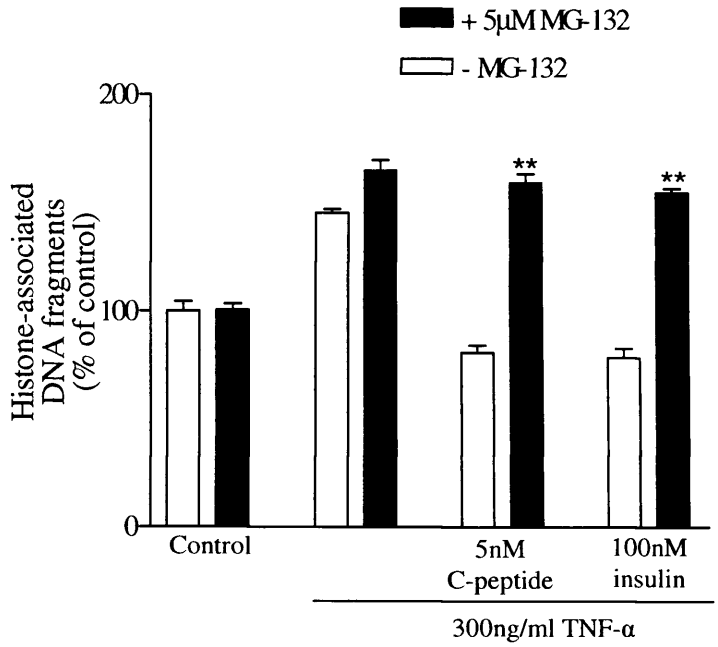


Fig 5.5. NFκB inhibitor blocks C-peptide and insulin protection against TNF-α-induced apoptosis. Cells were pre-incubated with or without MG-132 1 hour before incubation with or without C-peptide or insulin for 24 hours. Cells were then treated with TNF-α for 24 hours. Apoptosis was assessed by measuring cytoplasmic histone associated DNA fragments using ELISA technique as described in methods section. Results are expressed as means ± SEM, n=3 experiments.** $p < 0.01$, relative to non-MG-132 stimulated cells.

Effects of C-peptide and insulin on cytosolic IκB-α levels

To examine activation of NFκB, the levels of IκB-α protein were determined by Western blotting of OK cell cytosol fractions 24 hours after treatment of the cells with 5nM C-peptide, 100nM insulin and, as a positive control, 1μM PMA. 30μg of cytosol fractions were separated by PAGE and Iκ-B-α detected by Western blotting. As shown in (**Fig 5.6A**), after 24 hours levels of IκB-α protein were less in cells stimulated with C-peptide, insulin and PMA. Densitometry of immunoblots revealed that levels of IκB-α protein were decreased to 0.7 ± 0.07, 0.55 ± 0.06, and 0.5 ± 0.01 fold relative to basal by C-peptide, insulin and PMA respectively (**Fig 5.6B**). To confirm the equivalent loading of protein, both were stripped and re-probed for β-actin.

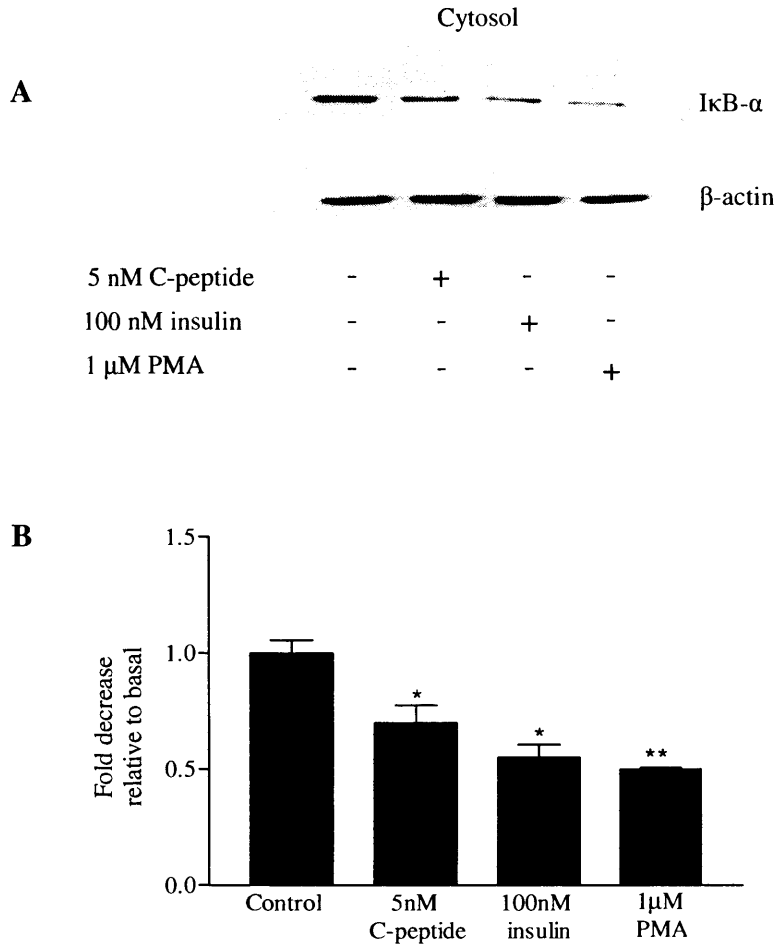


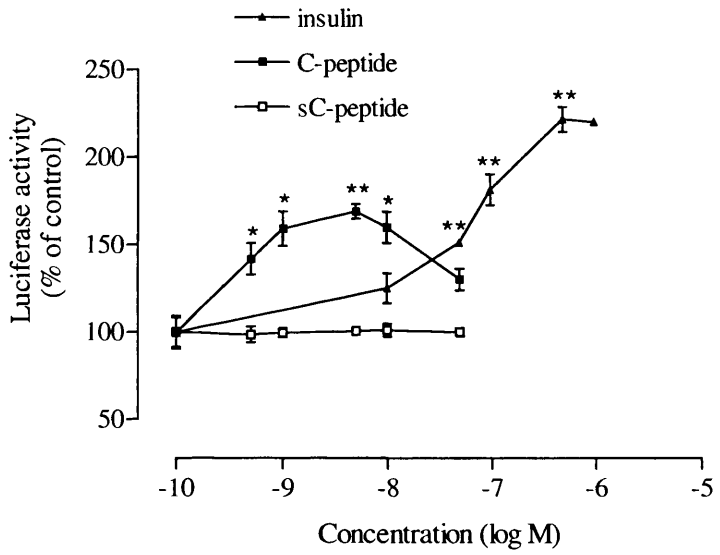
Fig 5.6. C-peptide and insulin enhance the cytosolic degradation of Iκ-B-α. **(A)** Cells were treated with 5 nM C-peptide, 100nM insulin or 1μM PMA for 24 hours. Iκ-B-α was detected by Western blotting of cytosol fraction with specific anti- Iκ-B-α.. **(B)** Density of immunoblots was quantified values were expressed graphically. Within the graph data are means ± SEM, n=4 experiments. *$p<0.05$, ** $p< 0.01$ relative to basal.

Effects of C-peptide and insulin on NFκB transcriptional activity

To establish unequivocally that C-peptide and insulin were able to induce NFκB activation, OK cells were transiently transfected with pNFκB-luc and pSVβgal to normalize for transfection efficiency. Cells were stimulated with various concentrations of C-peptide, insulin and as a positive control cells were stimulated with 1μM PMA for 24 hours. All three agents induced a concentration-dependent activation of NFκB transcriptional activity (**Fig 5.7**). C-peptide, at a concentration of 5nM, maximally stimulated NFκB to 169.2 ± 3.6% compared to control (**Fig 5.7A**). Similarly insulin, at a concentration of 100nM, maximally stimulated NFκB to 221.5 ± 5.2% compared to control (**Fig 5.7A**). As expected PMA was a potent stimulator of NFκB, at a concentration of 10μM, induced maximal activation of 1999.3 ± 15.9% compared to control (**Fig 5.7B**). Increasing concentrations of sC-peptide did not increase NFκB transcriptional activity to any significant levels.

The effect of combining 5nM C-peptide and 100nM insulin on NFκB activation was also examined, but did not increase NFκB transcriptional activity significantly over that observed with 100nM insulin (**Fig 5.8**).

A



B

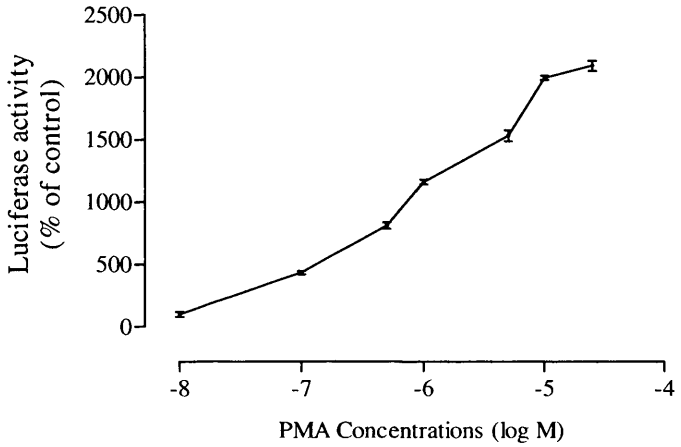


Fig 5.7. C-peptide and insulin stimulate NFκB transcriptional activity in OK cells. Cells transiently transfected with pNFκB-Luc and pSVβgal were treated with the indicated concentrations of **(A)** C-peptide, scrambled C-peptide, insulin or **(B)** PMA. Cells were lysed and luciferase activity in lysates was determined and normalized for transfection efficiency using β-gal activity. Normalised luciferase activity is expressed as percentage compared with control in non-stimulated cells. Results are expressed as means ± SEM, n=4 experiments.*$p<0.05$, **$p<0.01$, relative to control.

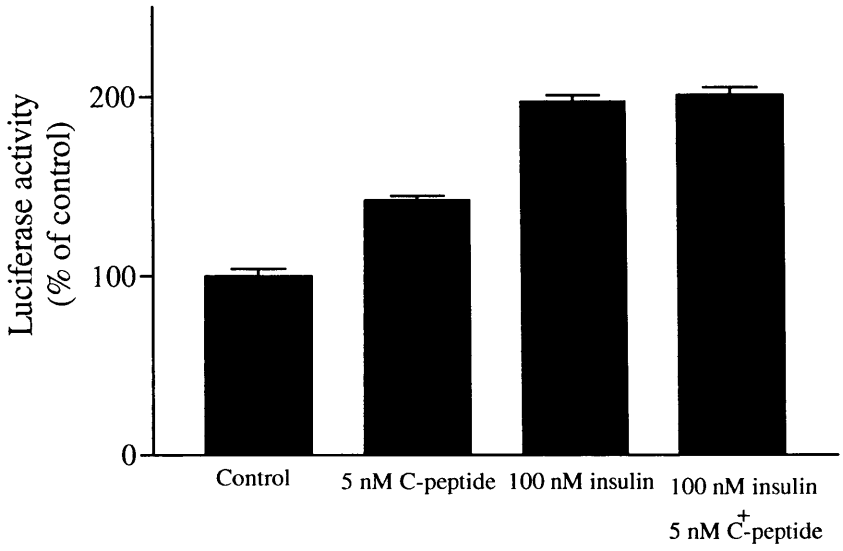


Fig 5.8. C-peptide does not increase effects of insulin on NFκB activity in OK cells. Cells were stimulated with indicated concentrations of C-peptide, insulin or combination. Cells were lysed and luciferase activity in lysates was determined and normalized for transfection efficiency using β-gal activity. Normalised luciferase activity is expressed as percentage compared with control in non-stimulated cells. Results are expressed as means ± SEM, n=4 experiments.

Effects of MG-132 on C-peptide- and insulin-induced activation of NFκB

Since MG-132 blocks protection effects of C-peptide and insulin against TNF-α-induced apoptosis as shown in this results section, it was crucial to examine whether MG-132 is also able to inhibit activation of NFκB by C-peptide and insulin.

OK cells were transiently transfected with pNFκB-luc and pSVβgal then serum-starved cells and pre-treated with 5μM MG-132 for 1 hour prior to stimulation with 5nM C-peptide or 100nM insulin for 24 hours. MG-132 pre-treatment significantly inhibited basal trans-activation of NFκB to 34.5 ± 10.7% of control and completely abolished the effects of C-peptide and insulin on NFκB transcriptional activity (**Fig 5.9**).

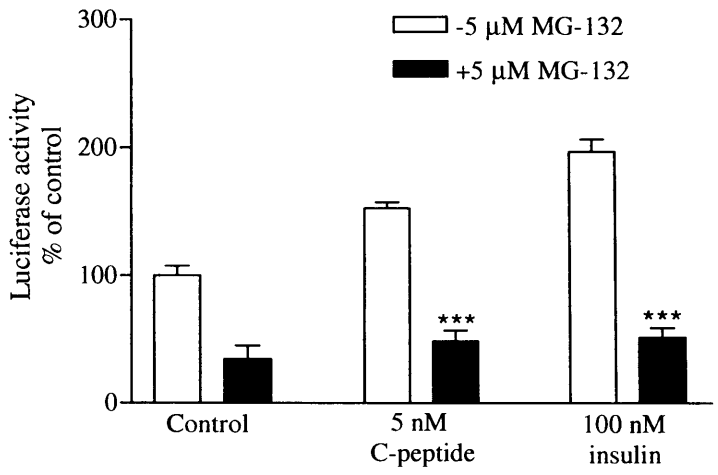


Fig 5.9. NFκB inhibitor abolishes C-peptide and insulin-induced activation of NFκB. Transiently transfected OK cells were pre-treated with indicated concentration of MG-132 for 1 hour prior to stimulation with indicated concentrations of C-peptide or insulin for 24 hours. Cells were lysed, and luciferase activity in lysates was determined. Normalized luciferase activity is expressed as percentage compared with control in non-stimulated cells. Results are expressed as means ± SEM, n=3 experiments. *** $p < 0.001$ relative to non-GM-132-treated cells.

Effects of PTX on C-peptide- and insulin-induced NFκB Activation

To examine whether C-peptide activated NFκB via a putative GPCR, OK cells were transiently transfected with pNFκB-luc and pSVβgal and then pre-treated with 100ng/ml PTX for 18 hours prior to stimulation with 5nM C-peptide or 100nM insulin for 24 hours.

PTX pre-treatment blocked stimulation of NFκB activity by C-peptide in the luciferase reporter assay (**Fig 5.10**). Activation of NFκB by insulin was unaffected by PTX.

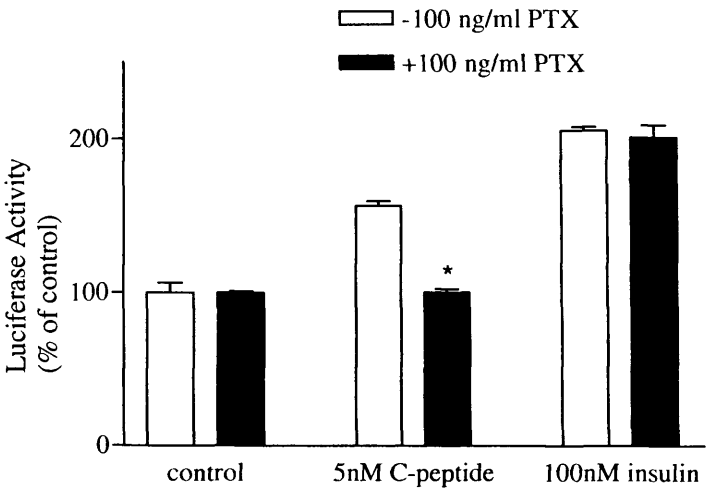


Fig 5.10. Activation of NFκB by C-peptide is PTX sensitive. Cells were transiently transfected with p-NFκB-Luc and pSV-βgal to normalize for transfection efficiency. Transiently transfected OK cells were treated with 100 ng/ml PTX for 18 hours prior to stimulation for 24 h with indicated concentrations of C-peptide or insulin. Cells were lysed, and luciferase activity in lysates was determined. Normalized luciferase activity is expressed as percentage compared with control in non-stimulated cells. Results are expressed as means ± SEM, n=3 experiments. * $p < 0.05$ relative to non PTX treated, C-peptide stimulated cells.

Effects of ERK inhibitor and PI 3-kinase inhibitor on C-peptide- and insulin-induced NFκB activition

Mechanisms by which C-peptide mediated activation of NFκB were examined according to the previously reported signalling effects of C-peptide in OK cells documented in section 3.2. To examine whether C-peptide activate NFκB via activation of ERK or PI3-kinase, OK cells were transiently transfected with pNFκB-luc and pSVβgal to normalize transfection efficiency. Pre-treatment of cells with 10μM PD98059, a MEK-1 inhibitor, for 30 minutes before stimulation with 5nM C-peptide or 100nM insulin failed to attenuate the effects of C-peptide or insulin on NFκB activity. Conversely pre-treatment the cells with 100nM wortmannin, a PI 3-kinase inhibitor, for 30 minutes significantly attenuated the effects of C-peptide and insulin on NFκB activity (**Fig 5.11**).

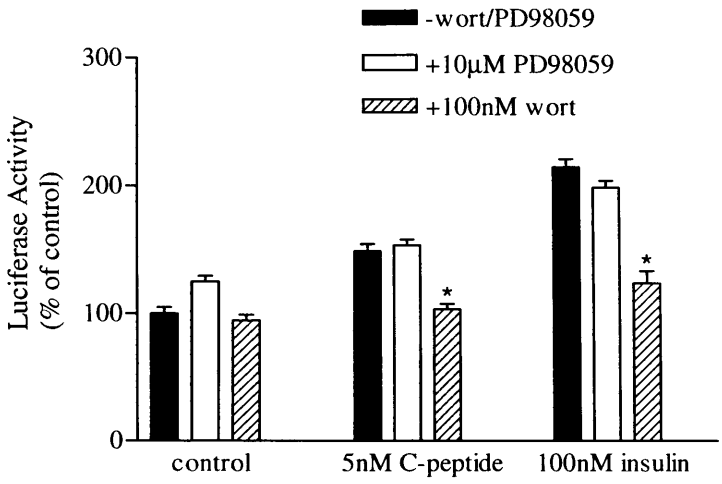


Fig 5.11. PI 3-kinase involvement in activation of NFκB by C-peptide and insulin. Cells were transiently transfected with p-NFκB-Luc and pSV-βgal to normalize for transfection efficiency. Transiently transfected OK cells were pre-reated with indicated concentration of wortmannin or PD98059 for 30 minutes prior to stimulation with indicated concentrations of C-peptide or insulin for 24 hours. Cells were lysed, and luciferase activity in lysates was determined. Normalized luciferase activity is expressed as percentage compared with control in non-stimulated cells. Results are expressed as means ± SEM, n=3 experiments. * $p< 0.05$ relative to non-wortmannin treated, C-peptide or insulin stimulated cells.

Effects of C-peptide and insulin on TRAF2 protein expression

To examine whether NFκB survival genes are increased in OK cells as a consequence of NFκB activation by C-peptide or insulin, TRAF2 protein was chosen for study as a previously described NFκB-dependent survival gene (Wang *et al.,* 1998).

Incubation of the cells with 5nM C-peptide or 100nM insulin for 24 hours increased the expression of TRAF2 proteins as detected by Western blotting of cytosolic fractions (30μg protein/sample) (**Fig 5.12A**). Gels then were stripped and re-probed for β-actin to ensure equal protein loading. Density of immunoblots was quantified and showed that C-peptide and insulin increased TRAF2 proteins 1.7 ± 0.03 and 1.8 ± 0.05 fold relative to basal for C-peptide and insulin respectively, (**Fig 5.12B**).

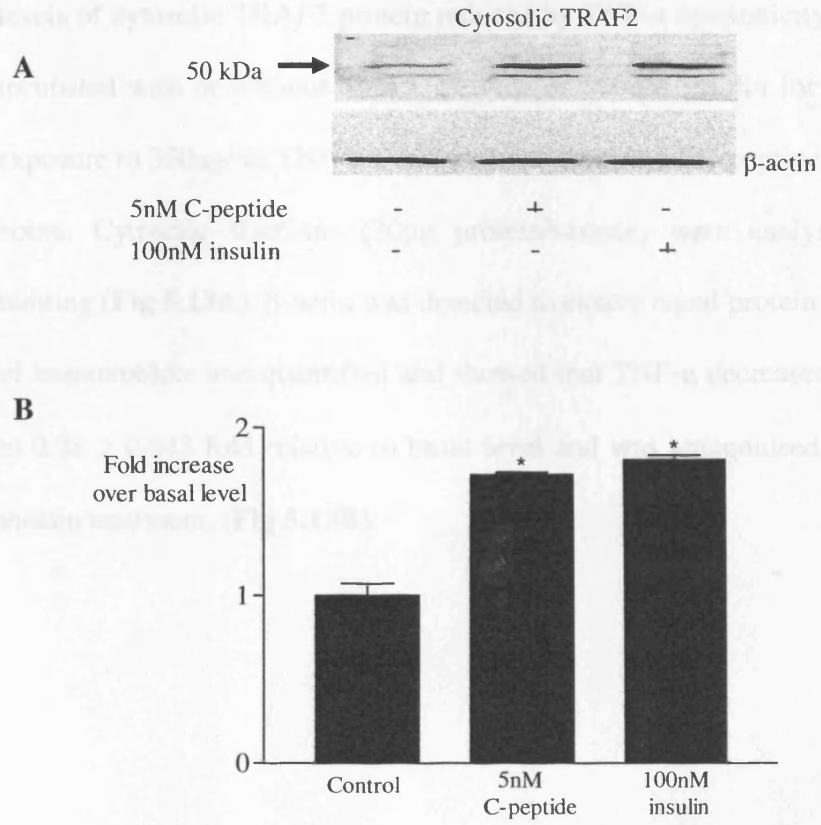


Fig 5.12. C-peptide and insulin increase the expression of NFκB-dependent survival gene, (TRAF2) protein. (A) Cells were stimulated with indicated concentrations of C-peptide or insulin for 24 hours. Cytosolic fractions were separated and immunoblotted with anti-TRAF2. A representative blot is shown. **(B)** Bands were quantified and values were expressed graphically. Results are expressed as means ± SEM, n=5 experiments. * $p< 0.01$, relative to non-stimulated cells.

Effects of C-peptide and insulin on TNF-α-induced reduction of TRAF2

To examine whether C-peptide or insulin may able to restore the basal levels of cytosolic TRAF2 protein reduced by TNF-α cytotoxicity, cells were pre-incubated with or without 5nM C-peptide or 100nM insulin for 24 hours before exposure to 300ng/ml TNF-α in presence or absence of C-peptide or insulin for 24 hours. Cytosolic fractions (30μg protein/sample) were analysed by Western blotting (**Fig 5.13A**). β-actin was detected to ensure equal protein loading. Density of immunoblots was quantified and showed that TNF-α decreased TRAF2 protein to 0.28 ± 0.043 fold relative to basal level and was antagonised by C-peptide or insulin treatment, (**Fig 5.13B**).

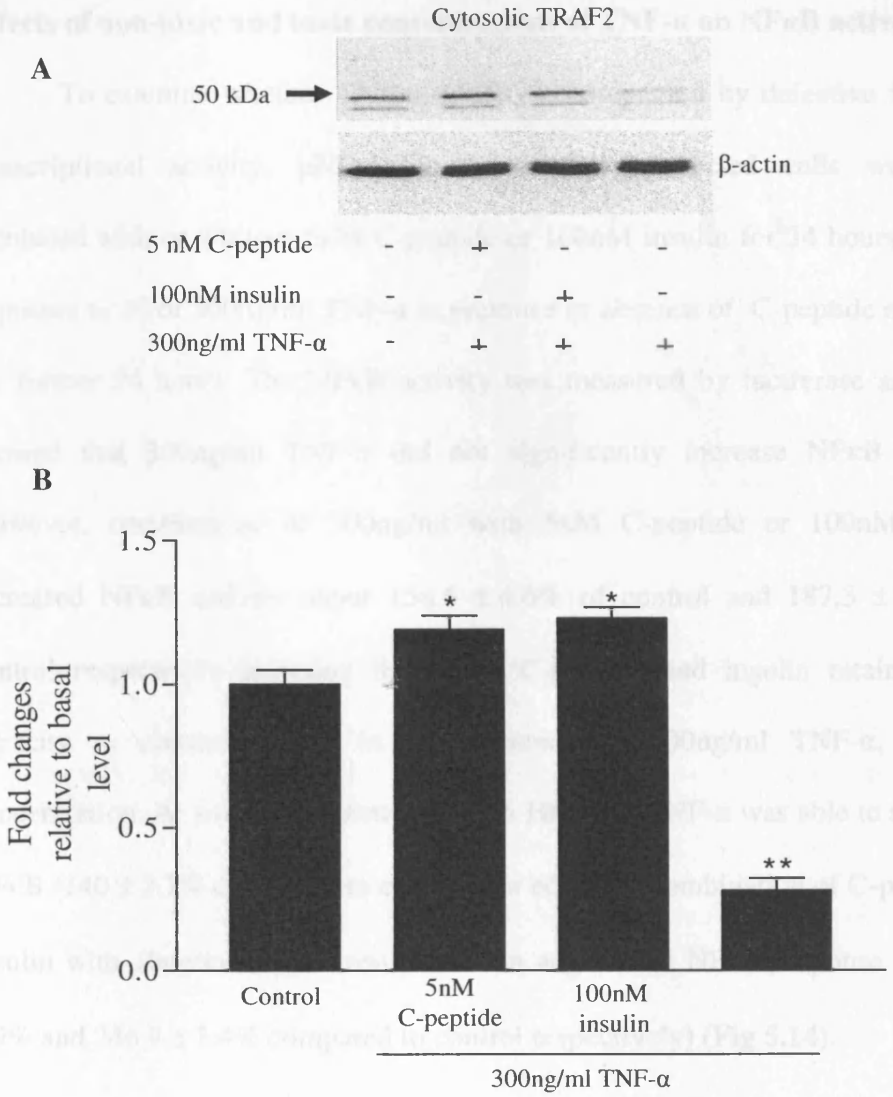


Fig 5.13. C-peptide and insulin antagonise TNF-α-induced reduction of TRAF2 protein. Serum-starved cells were pre-incubated with or without indicated concentrations of C-peptide or insulin for 24 h before stimulation with 300ng/ml TNF-α in-combination with or without C-peptide or insulin. Cytosolic fractions were separated as described in methods section. 30 μg per sample was subjected to SDS-PAGE electrophoresis and protein transferred to nitrocellulose membranes for immunoblotting with 1:1000 dilution of anti-TRAF2. A representative blot is shown. **(B)** Bands were quantified and values were expressed graphically. Results are expressed as means ± SEM, n=4.*$p < 0.05$ ** $p < 0.01$, relative to TNF-α stimulated cells.

Effects of non-toxic and toxic concentrations of TNF-α on NFκB activation

To examine whether TNF-α toxicity accompanied by defective in NFκB transcriptional activity, pNFκB-luc transiently transfected cells were pre-incubated with or without 5nM C-peptide or 100nM insulin for 24 hours prior to exposure to 10 or 300ng/ml TNF-α in presence or absence of C-peptide or insulin for further 24 hours. The NFκB activity was measured by luciferase assay and showed that 300ng/ml TNF-α did not significantly increase NFκB activity. However, combination of 300ng/ml with 5nM C-peptide or 100nM insulin increased NFκB activity about 158.6 ± 4.6% of control and 187.3 ± 2.1% of control respectively indicting that, both C-peptide and insulin retained their capacity to activate NFκB in the presence of 300ng/ml TNF-α, a toxic concentration. At a non toxic concentration 10ng/ml, TNF-α was able to stimulate NFκB (140 ± 3.2% compared to control). In addition, combination of C-peptide or insulin with 10ng/ml TNF-α resulted in an augmented NFκB response (198.7 ± 3.9% and 246.9 ± 3.4% compared to control respectively) (**Fig 5.14**).

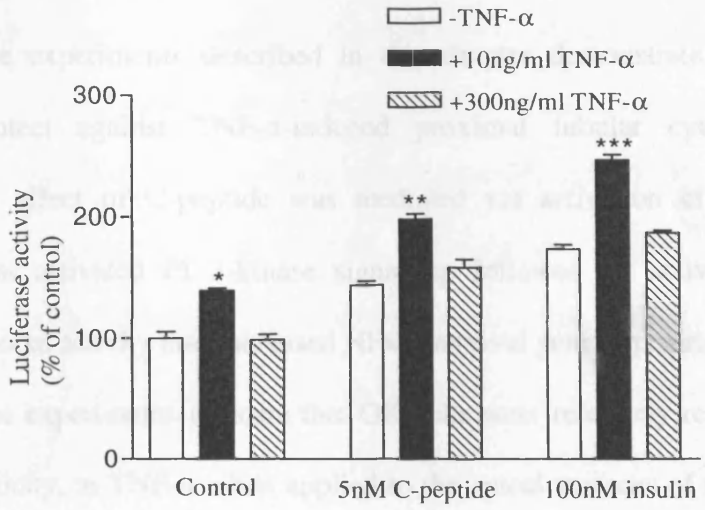


Fig 5.14. TNF-α at a non-toxic concentration activates NFκB. Cells were transiently transfected with p-NFκB-Luc and pSV-βgal to normalize for transfection efficiency. Transiently transfected OK cells were pre-incubated with or without indicated concentrations of C-peptide or insulin for 24 hours prior to stimulation with or without 10ng/ml TNF-α or 300ng/ml TNF-α . Cells were lysed, and luciferase activity in lysates was determined. Normalized luciferase activity is expressed as percentage compared to control in non-stimulated cells. Results are expressed as means ± SEM, n=3 experiments. * $p< 0.05$, **$p<0.01$ and ***$p<0.001$ relative to non-C-peptide or insulin treated, TNF-α-stimulated cells.

5.3. DISCUSSION

The experiments described in this chapter demonstrate that C-peptide could protect against TNF-α-induced proximal tubular cytotoxicity. This functional effect of C-peptide was mediated via activation of PTX sensitive GPCR that activated PI 3-kinase signalling followed by activation of NFκB transcriptional activity and increased NFκB survival gene expression.

The experiments indicate that OK cells were relatively resistant, in terms of cytotoxicity, to TNF-α when applied to the apical surfaces of nearly confluent monolayers. This resistance could result from species differences between the sequence of the human TNF-α used in these studies and that of the corresponding opossum cytokine, thus leading to reduced potency in this system. However, prevailing TNF-α levels in the kidney may be elevated in diabetes, although the precise proximal tubular concentration is unknown. Data from animal models of diabetes and from clinical studies in patients with diabetes has positioned TNF-α as a key mediator of nephropathy because: urinary and renal interstitial levels are elevated in diabetic rats prior to the development of albuminuria (Kalantarinia *et al.,* 2003); circulating TNF-α levels are elevated in patients with diabetic nephropathy (Lechleitner *et al.,* 2000; Foss *et al.,* 1992); and TNF-α is an independent predictor of urinary albumin excretion in individuals with diabetes (Lechleitner *et al.,* 2000). Moreover, TNF-α is known to be cytotoxic to kidney cells (Ortiz *et al.,* 1997b), and to directly compromise glomerular permeability leading to proteinuria and thus potentially to secondary proteinuric nephropathy that occurs as a result of increased protein delivery into the proximal tubule from any cause (Brunskill, 2004). In diabetic nephropathy TNF-α is an attractive candidate pro-apoptotic mediator. However, its effects on cell survival and death

are complex in that the sensitivity of cells to TNF-α induced cell death varies with the microenvironment. Therefore the absence of survival factors, and/or the presence of other lethal factors may promote apoptosis induced by TNF-α. Therefore, in type 1 diabetes, deficiency of insulin and/or C-peptide may play a role in the pathogenesis of diabetic nephropathy by rendering PTC more susceptible to the deleterious effects of cytokines such as TNF-α.

Kidney cell death by apoptosis has been widely documented in the course of renal injury, and proximal tubular cell loss through apoptosis contributes significantly to the tubular atrophy invariably observed in progressive renal failure (Ortiz *et al.,* 2001). C-peptide at physiologically relevant concentrations protected against apoptosis induced by both TNF-α and serum removal in OK cells, a property shared by insulin. This protective effect was to some extent specific for TNF-α as neither C-peptide nor insulin significantly inhibited cell death induced by the anti-tumour agent etoposide. Overall these findings are consistent with the earlier observations (section 3.2.2) that OK cells proliferated in the presence of C-peptide, and suggest that in kidney PTC both peptides released from pancreatic β-cells should be regarded as growth and survival factors.

Other workers have previously established that activation of NFκB may result in protection against TNF-α induced cell killing (Heyninck and Beyaert, 2001; Beg *et al.,* 1993; Yang *et al.,* 1996; Antwerp *et al.,* 1996). Therefore, in the absence of NFκB activity, cellular susceptibility to TNF-induced apoptosis may be increased, and enforced activation of NFκB may protect cells against apoptosis (Antwerp *et al.,* 1996). Mice deficient in NFκB died in utero from massive hepatocyte cell death (Beg *et al.,* 1995) and this phenotype rescued by concurrent deficiency of TNF or TNFR1 (Alcamo *et al.,* 2001; Dio *et al.,* 1999). In addition,

fibroblasts derived from these embryos demonstrated significant sensitivity to TNF-mediated apoptosis when culture *in vitro* (Beg and Baltimore, 1996). Therefore, it was postulated that C-peptide and insulin may inhibit TNF-α mediated apoptosis via stimulation of NFκB activity. The results of current studies support this hypothesis for the following reasons: i) both insulin and C-peptide activate NFκB in OK cells, ii) the NFκB inhibitor MG-132 abolished the anti-apoptotic effects of insulin and C-peptide, and iii) TNF-α failed to activate NFκB at concentrations required for the induction of apoptosis. Inhibition of NFκB with MG-132 only very slightly augmented apoptosis in OK cells treated with TNF-α alone, suggesting that basal cellular activity of NFκB is insufficient to control TNF-α induced cell injury.

Insulin possesses anti-apoptotic properties (Li *et al.,* 2003) dependent on activation of NFκB in mammalian cells (Bertrand *et al.,* 1998; Bertrand *et al.,* 1999). Until now however an NFκB stimulatory, anti-apoptotic role of C-peptide has never been demonstrated. Data from Li *et al* (2003) showed that insulin protected SH-SY5Y cells against glucose-induced apoptosis in association with nuclear translocation and presumed activation of NFκB. Although in their studies addition of C-peptide appeared to enhance the effects of insulin, Li *et al* (2003) were unable to demonstrate NFκB stimulatory, anti-apoptotic properties role of C-peptide by itself. These authors concluded that C-peptide effects on neuronal signalling were mediated through enhancement of insulin signalling.

No additive effects of C-peptide with insulin were observed in the current study, and it is clear that the cellular effects of C-peptide are a result of bioactivity of C-peptide and signalling in its own right. It was previously demonstrated activation of PI 3-kinase and ERK by C-peptide in OK cells (section 3.2.2) and

the current results now presented evidence that NFκB activation by these two agents, being sensitive to wortmannin but not PD98059, is dependent on PI 3-kinase activity but not that of ERK. Consistent with numerous reports of a crucial cell survival role for PI 3-kinase (Dudek *et al.,* 1997; Ozes *et al.,* 1999; Romashkova *et al.,* 1999; Shimamura *et al.,* 2003; Khwaja, 1999), the current data suggest that insulin and C-peptide both initiated NFκB dependent cell survival pathways downstream of PI 3-kinase activation.

Clear differences exist between the modes of action of insulin and C-peptide. Firstly, their NFκB activation dose response curves are quite different. Insulin displays a typical sigmoidal dose response curve of NFκB stimulation but is considerably less potent than C-peptide. However, low insulin potency is often observed *in vitro* where much higher than physiological insulin concentrations are generally required to elicit signalling effects (Abraham *et al.,* 1990; Bandary *et al.,* 2005). On the other hand the "bell shaped" activation curve seen when C-peptide activated NFκB was completely different to that of insulin, but identical to that seen for activation of other signalling pathways by C-peptide in this thesis (section 3.2.2 and 4.2) and others (Kitamura *et al.,* 2001; Zhong *et al.,* 2005). Secondly, PTX completely blocked NFκB activity evoked by C-peptide but had no effect on that evoked by insulin. Therefore this finding, together similar observations by others (Maestroni *et al.,* 2005; Zhong *et al.,* 2005; Walcher *et al.,* 2004; Marx *et al.,* 2004; Shafqat *et al.,* 2002; Kitamura *et al.,* 2001; Ohtomo *et al.,* 1996), indicates that C-peptide signalled its effects via a $G\alpha_{i/o}$ coupled GPCR in contrast to the receptor tyrosine kinase signalling of insulin.

Although degradation of IκB-α and activation of the NFκB-luciferase reporter suggested strongly that C-peptide and insulin had the capability to

regulate gene transcription in OK cells it was necessarily to document definitively at the protein level that NFκB regulated genes were altered under these conditions. TRAF2 was chosen to investigate for many reasons; it is a key effector mediating TNF-α responses (Wang *et al.,* 1998), and a critical regulator of TNF-α signalling to NFκB activation (Devin *et al.,* 2001; Woronicz *et al.*, 1997). TRAF2 gene transcription is NFκB regulated and may be increased by insulin (Bertrand *et al.,* 1999). In resting OK cells TRAF2 protein was increased in response to C-peptide or insulin in association with NFκB activation. This is the first evidence that C-peptide stimulates the expression of an adaptor protein recruited by activated TNF receptors. On the other hand, TNF-α treatment was followed by a reduction in TRAF2 protein, in agreement with previous observations of ubiquitination and degradation of TRAF2 in response to TNF-α (Habelhah *et al.,* 2004). However both insulin and C-peptide were able to counteract the reduction of TRAF2 resulting from higher concentration TNF-α treatment. These findings therefore suggest that at higher concentrations in OK cells TNF-α caused degradation of TRAF2 thus directing cells towards apoptosis. By activating NFκB however, both insulin and C-peptide enable cells to maintain healthy levels of TRAF2 thereby inhibiting pro-apoptotic signalling by TNF-α. Interestingly in the current studies in OK cells TNF-α at 10ng/ml was non-toxic and activated NFκB, a finding consistent with the report of Papakonstani and Stournaras, (2004) whose described survival factor like effects of 10ng/ml TNF-α in OK cells.

In conclusion, this work provides evidence for the ability of C-peptide, acting via a GPCR, to protect against TNF-α-induced apoptosis in kidney PTC. Altered levels of C-peptide may play a role in renal PTC damage in diabetic nephropathy.

CHAPTER 6

SUMMARY AND CONCLUDING DISCUSSION

The initial aim of this study was to determine whether C-peptide possessed the capacity to signal in PTC after studies in recent years had shown conclusively that C-peptide has biological effects. However, specific molecular mechanism(s) responsible for these observations remained unclear.

OK cells, an established immortalised epithelial cell line derived from the cortex of American opossum (Koyama 1978), was utilised as a PTC model to examine the intracellular effects of C-peptide and functional consequences. Binding of Rh-labelled human C-peptide to OK cells as detected by confocal microscopy, proving that human C-peptide cross-reacted with OK cells and strengthing the notion that C-peptide binding may depend on a single, or a few residues (Pramanik *et al.,* 2001).

As result of this work, multi-signalling effects of C-peptide have been identified in PTC. C-peptide transiently increased $[Ca^{2+}]_i$ followed by Ca^{2+} dependent membrane translocation of PKC-α, providing support for the concept that Ca^{2+} dependent pathways play an important role in C-peptide mediated actions (Ohtomo *et al.,* 1996; Shafqat *et al.,* 2002). Likewise, the data unambiguously demonstrated stimulation of ERK activation and that was PKC-dependent. Furthermore, phosphorylation of Akt induced by C-peptide is indicative of PI 3-kinase activation. Other investigators have examined signalling effects of C-peptide in human tubular cells (Zhong *et al.,* 2005). The results of this work, published contemporaneously with the experiments documented in this thesis, provide support for the results in this thesis.

This work provides evidence that C-peptide mediated its actions through PTX sensitive GPCR by 2 ways. Firstly, inhibition of C-peptide effects in OK cells by PTX implicates a GPCR, coupled to either $G\alpha_i$ or $G\alpha_o$, in the transduction of these events. Secondly, by using [^{35}S]-GTPγS binding this study has now for the first time unequivocally demonstrated specifically that $G\alpha_i$ proteins are activated by C-peptide binding to a GPCR.

Tubular atrophy is an important feature of diabetic nephropathy and is closely associated with the loss of renal function (Glibert and Cooper, 1999; Risdon *et al.,* 1968). The mechanisms by which diabetes causes renal loss are not well known. There is emerging evidence that tubular atrophy may be caused by apoptosis involving the death receptors (Schelling *et al.,* 1998; Schelling and Cleveland, 1999). Having demonstrated that C-peptide stimulated kinases with well-described roles in the regulation of growth, proliferation and cell survival (Hagemann and Blank, 2001; Iwamoto *et al.,* 1992; Marte and Downward, 2001) and that C-peptide was a functional mitogen, the physiological function of C-peptide in PTC can now be envisaged. Thus, in PTC C-peptide could be regarded as a growth and survival factor. This concept was supported by finding that C-peptide at physiologically relevant concentrations protected against apoptosis induced by TNF-α, a cytotoxic cytokine thought to play a role in development and progress of diabetic nephropathy (Mesah-Brown *et al.,* 2005; Furuta *et al.,* 1993; Helmy *et al.,* 2003). Furthermore, activation of NFκB by C-peptide and subsequently increased expression of TRAF2, the product of an NFκB-dependent survival gene (Wang *et al.,* 1998), supported a role for C-peptide in cell survival.

PPARγ has key roles in the regulation of adipogenesis, inflammation, lipid and glucose metabolism (Guan and Breyer., 2001; Fajas *et al.,* 2001; Desvergen

and Wahli., 1999; Willson *et al.,* 2001). This work provides for the first time unequivocal confirmation that C-peptide at physiologically relevant concentrations increased PPARγ activity in a ligand-independent manner and that C-peptide augmented effects of PPARγ ligands TZD. These findings suggest new important physiological roles for C-peptide in PTC and other sites that may include glucose homeostasis, adipocyte differentiation, monocyte differentiation, modulation of inflammation and modulation of insulin sensitivity. The work provides proof that C-peptide induced expression of CD36, a PPARγ regulated gene increased during differentiation of monocytes to macrophages (Nicholson, 2004; Chawla *et al.,* 2001; Nagy *et al.,* 1998). Thus, C-peptide may have a potential role in modulation of monocyte differentiation. This is supported by co-localisation of C-peptide with macrophages in atherosclerotic lesions described in diabetic patients (Max *et al.,* 2004). Further studies may essential to investigate and characterise the exact roles of ligand-independent activation of PPARγ by C-peptide in kidney.

In the current studies C-peptide-induced signalling effects by typical physiological concentrations (Dreyer *et al.,* 1986; Horwitz *et al.*, 1975; Faber *et al.,* 1978) in the mid-picomolar to low-nanomolar range and had no signalling effects in the supra-physiological concentrations. These findings suggest that C-peptide may have biological effects in Type 1 diabetes consistence with studies indicate that C-peptide has no measurable effects in healthy and patients with Type 2 diabetes (Wojcikowski *et al.,* 1983; Hoogwerf *et al.,* 1986; Johansson *et al.,* 1992b; Sima *et al.,* 2001). However, being demonstrated that C-peptide-induced PPARγ activation may suggest a role for C-peptide in Type 2 diabetes which is needed further studies.

This study provides evidence for the ability of C-peptide to stimulate intracellular signalling effects and evoke functional effects in kidney PTC. However, the exact nature of the C-peptide bioactivity, its membrane interactions and the resultant intracellular responses remain to be defined. Effort is now required to clone and characterise the C-peptide receptor. Moreover, future work should be directed towards further studies of the interaction between C-peptide and insulin signalling and the effects of changes in prevailing glucose concentrations that will enhance understanding of glucose metabolism, and pathogenesis of diabetic complications.

Despite being ignored for many years it is now clear that C-peptide possesses important biological properties and may potentially protect against diabetic complications. The gradual accretion of data in the field of C-peptide function over recent years argues strongly for the urgent consideration of clinical trials of C-peptide in diabetic nephropathy.

APPENDIX

General buffers and solutions contents

Kinase buffer

20mM HEPES, PH 7.2, 20mM β-glycerophosphate, 10mM $MgCl_2$, 50μM ATP, 1mM DTT and 50μM Na_3vo_4

Krebs-Hepes buffer

10mM HEPES, 4.2mM $NaHCO_3$, 11.7mM D-glucose, 1.18mM $MgSO_4.7H_2O$, 1.18mM KH_2PO_4, 4.69mM KCl, 118mM NaCl, 1.29mM CaCl2.$2H_2O$, pH 7.4

Laemmli's buffer

60mM Tris, PH 6.8, 10% glycerol, 2% SDS, 100mM DTT and 0.01% bromophenol blue

Lysis buffers

Lysis buffer1

20mM Tris-HCl, PH 8.0, 0.5% Nonidet P-40, 250mM NaCl, 3mM EDTA, 3mM EGTA, 1mM PMSF, 2mM Na_3vo_4 and 1mM DTT

Lysis buffer2

20mM Tris-HCl, 5mM EGTA, 2mM EDTA, 1mM DTT, 0.5mM phenylmethyl-sulphonyl fluoride (PMSF), 10μM iodoacetamide, pH 7.4

Lysis buffer3

10mM HEPES, 10mM EDTA, pH 7.4

Lysis buffer4

500mM HEPES, 2% Triton N101, 1mM $CaCl_2$, 1mM $MgCl_2$, pH 7.8

Lysis buffer5

1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 200μM Na_3vo_4

Solubilisation buffer

Solubilisation buffer1

9.2mM Tris, 0.92mM EGTA, 150mM NaCl, 0.1%(v/v) SDS, 1% (v/v) ethylphenylpolyethylene glycol (Nonidet P-40), 0.5% (w/v) deoxycholate, 0.5mM PMSF, 10μM benzamidine hydrochloride and 5μM iodoacetamide, pH 7.4

Solubilisation buffer2

100mM Tris, 200mM NaCl, 1mM EDTA, 1.25% Igepal, 0.2% SDS, pH 7.4

10% SDS PAGE running gels

(4ml dH2O, 3.3ml 30% acrylamide mix, 2.5ml 1.5M Tris (PH 8.8), 0.1ml 10% SDS (w/v), 0.1ml 10% ammonium persulfate (w/v) and 4μl TEMED in a total volume of 10ml) and stacking gels (3.4 ml dH2O, 0.83 ml 30% acrylamide mix, 0.63ml 1.0M Tris (PH 6.8) 0.05ml 10% SDS (w/v), 0.05ml 10% ammonium persulfate (w/v), and 4μl TEMED in a total volume of 5ml

12% SDS PAGE running gels

3.3ml dH2O, 4ml 30% acrylamide mix, 2.5ml 1.5 M Tris (PH 8.8), 0.1ml 10% SDS (w/v), 0.1ml 10% ammoniumpersulfate (w/v) and 4μl TEMED in a total volume of 10ml

Stacking gels

3.4 ml dH2O, 0.83 ml 30% acrylamide mix, 0.63ml 1.0M Tris (PH 6.8) 0.05ml 10% SDS (w/v), 0.05ml 10% ammonium persulfate (w/v), and 4μl TEMED in a total volume of 5ml

Running buffer

25mM Tris-base, 250mM glycine, 0.1% SDS (w/v)

Transfer buffer

39mM glycine, 48mM Tris base, 0.037% SDS (w/v) and 20% methanol (v/v)

TTBS

20mM Tris-base, 100mM NaCl and 0.05%Tween 20, pH 7.5

Stripping buffer

100M 2-Mercaptoethanol, 2% SDS, and 62.5mM Tris-HCl, pH 6.7

Lifting buffer

10mM HEPES, 0.2% EDTA, 0.9% NaCl, pH 7.4

Freezing buffer for cell membranes

10mM HEPES, 0.1mM EDTA

Assay buffer for [^{35}S]-(GTPγS) binding assay

10mM HEPES, 100mM NaCl, 10mM MgCl, pH 7.4

TE buffer

10mM Tris-base, 1mM EDTA, pH 7.4

Bradford's reagent

0.0035% (w/v) Coomassie brilliant blue, 3.5% (v/v) ethanol and 7.5% (v/v) phosphoric acid

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