Oxygen radical generation by lymphoblast NADPH oxidase in hypertension

Thesis submitted for the degree of

Doctor of Medicine

at the University of Leicester

by

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July 2006

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Acknowledgements

I would like to take the opportunity to thank the following

British Heart Foundation for funding the research project and my junior research fellowship.

Prof Leong Ng for his supervision of the project.

Dr Richard Wong for help with both performing and discussing experiments.

Pauline Quinn and Sonja Jennings for teaching myself many of the experimental techniques used.

And finally my family Kirsty, Isobel and Sophie Pettit for their patient support.

Publications resulting from this thesis:

1 A I Pettit *et al* (2002) Increased free radical production in hypertension due to increased expression of the NADPH oxidase subunit $p22^{phox}$ in lymphoblast cell lines **J Hypertension** 20:677-683

2 Andrew I Pettit, Richard KM Wong, Sonja C Jennings, Leong L Ng. Tyrosine kinase inhibition attenuates the respiratory burst of hypertensive and normotensive human lymphoblasts through blocking activation rather than priming pathway. To be published

Abbreviations

AA	Arachidonic Acid
ACE-	Angiotensin Converting Enzyme Inhibiter
AGII	Angiotensin II
AGE	Advanced glycation end products
BSA	Bovine Serum Album
cAMP	Cyclic adenosine monophosphate
CL	Chemiluminescence
CLA	Cypridina Luciferin Analogue
DAG	Diacyl Glycerol
DBP	Diastolic Blood Pressure
DETC	Diethyldithiocarbamate
DMSO	Dimethyl Sulfoxide
DPI	Diphenyleneiodonium chloride
FAD	Flavine-adenine dinucleotide
fMLP	n-formyl-methionyl-lenoyl-phenylalamine
GM CSF	Granulocyte Colony Stimulating Factor
GTPase	Guanine Triphosphate Hydrolase
HBSS	Hanks Buffered Salt Solution
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRP	Horse Radish Peroxidase
HRT	Hormone replacement therapy
HT	Hypertension

IHD	Ischaemic heart disease
IP_3	Inositol 1,4,5 Triphosphate
K_{ATP}	ATP dependent potassium channels
LDL	low density lipoprotein
MAPK	Mitogen activated protein kinase
MAFP	Methyl arachidonyl fluorophosphonate
NADPH oxidase	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NFκB	Nuclear Factor κ B
NO	Nitric Oxide
NOS	Nitric oxide synthase
N3 PUFA	n-3 long chain polyunsaturated fatty acid
NT	normotensive
oxLDL	oxidized low density lipoprotein
OZ	Opsonified Zymosan
PA	Phosphatidic Acid
PAF	Platelet activating factor
PI3K	phosphatidylinositol-3 kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKD	Protein Kinase D
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PAK	Protein activated kinase
PMN	polymorphoneutrophils

ROS	reactive oxygen species
Ser	Serine
SH_3	src homology 3 domain
SOD	Superoxide dismutase
SpA	Staphylococcal Protein A
TK	tyrosine kinase
TNFα	Tumour necrosis factor α
TPA	12-O-Tetradecanoylphorbol-13-Acetate
TPR	Tetratricopeptide motifs
Tyr	Tyrosine
Thr	Threonine
VSMC	Vascular Smooth Muscle cells

Contents

Abstract: Oxygen radical generation by lymphoblast NADPH oxidase in hypertension

Dr Andrew Ian Pettit

Objectives. The aim of this thesis was to investigate increased reactive oxygen species production originating from NADPH oxidase in lymphoblasts from hypertensive subjects. The subcomponents of NADPH oxidase and priming pathways of NADPH oxidase were subsequently investigated to elucidate the mechanism of increased reactive oxygen species production in hypertension.

Methods. Lymphocytes from hypertensive and matched normotensive subjects were immortalized. Luminol, isoluminol and Cypridina Luciferin Analogue chemiluminescence were used to assay reactive oxygen species production. NADPH oxidase subcomponents, general protein tyrosine phosphorylation and tyrosine/threonine phosphorylation of MAPK were assayed by western blots. The effect of inhibiting tyrosine kinase, p38 and p44/p42 MAPK, PI3K and $cPLA_2$ were studied on basal and stimulated reactive oxygen species production.

Results. Combined intra and extracellular stimulated reactive oxygen species production was increased in hypertensive cell lines. The ROS production was abolished by Diphenyleneiodonium chloride but not by rotenone, indicating that a non-mitochondrial flavoprotein, such as NADPH oxidase, was involved. Analysis of NADPH oxidase subcomponents revealed an increase in $p22^{phox}$ in lymphoblasts from hypertensive subjects, accounting for some of the increased reactive oxygen species production.

There was increased basal Tyr/Thr phosphorylation of p44/p42 MAPK in the hypertensive lymphoblasts, but the difference was lost on stimulation. The various kinase inhibitors had no effect on basal reactive oxygen species production. Tyrosine kinase inhibition produced up to 70% reduction in stimulated reactive oxygen species production in both cell lines. Inhibition of p44/p42 MAPK kinase, PI3K, and PLA_2 produced a small reduction in stimulated ROS production. There was no differential reduction in ROS production from the hypertensive group with these inhibitors, suggesting no role of these pathways in priming of NADPH oxidase.

Conclusions. We have shown there is increased reactive oxygen species production in lymphoblasts from hypertensive subjects probably originating from NADPH oxidase. Increased expression of $p22^{phox}$ in hypertension lymphoblasts accounts for approximately a third of the increased reactive oxygen species production. We could not demonstrate tyrosine kinase, p38 MAPK, p44/p42 MAPK, PI3K or PLA_2 priming of reactive oxygen species production.

Chapter 1: Introduction

Over the last decade there has been a growing body of evidence to suggest that there is an increased oxidative burden in hypertension. This oxidative stress could be involved in the development of hypertension and in some way account for the acceleration of atheroma seen in hypertension. Potentially there are numerous sources of oxidative stress in biological systems, but one possible cause for the excess in hypertension is NADPH oxidase. This enzyme is most commonly associated with neutrophils, where it serves to produce superoxide O_2^- and hydrogen peroxide H_2O_2, which are utilized as part of the host defence mechanism. The enzyme is also found in other white cell lines, microglial cells, fibroblasts, vascular smooth muscle cells, vascular endothelial cells, osteoclasts, glomerular mesangial cells and thyroid cell lines. ROS (reactive oxygen species) generated by any white cell line, endothelial cell or vascular smooth muscle cell could play a role in the development of hypertension because of their location within the blood vessel wall or close proximity to it. We propose to study lymphoblasts from normotensive (NT) and hypertensive (HT) subjects because the immortalized lines provide a readily available source of material for study. We propose to assess the ability of these cells to produce ROS, to confirm that it is derived from NADPH oxidase and then to study how the enzyme is up-regulated or primed in hypertensive lines.

In the introduction I aim to describe how ROS is linked with hypertension and atheroma. As numerous antioxidant studies have shown no reduction of cardiovascular end points, I have then discussed potential reasons why this might have occured and why oxidants are still important in cardiovascular disease. I will then discuss NADPH oxidase structure and assembly into an active enzyme followed by how NADPH oxidase ROS output can be increased. Finally the 2 aspectsof the experimental methods are discussed, lymphoblasts and chemiluminescence.

1.1:Background

Hypertension has long been associated with an increased mortality and morbidity. Population studies have revealed an excess of deaths in hypertensive groups, predominantly from stroke and cardiovascular disease (1). Treatment of hypertension reduces the mortality and morbidity from stroke in most major trials. Blood pressure control in the elderly (greater than 65 years) also reduces mortality and morbidity from cardiovascular disease (2-4), whilst treatment of younger people produces a non-significant reduction (5). This apparent difference is probably due to the older subjects being at higher risk and therefore receiving more benefit.

The trials described above suggest a link between hypertension and formation of atheroma and hence the myriad of studies into this synergism. ROS have generated much interest lately, as they may have roles in the development of hypertension, and initiation and progression of atheroma formation.

1.2:ROS and hypertension

Blood pressure is a product of cardiac output and peripheral resistance. Peripheral resistance in turn is a reflection of the balance between vasoconstrictors and vasodilators. A major vasodilator is NO (Nitric Oxide). ROS interact with NO to form peroxynitrite, which no longer acts as a vasodilator (6;7). ROS found in the adventitia also act as a barrier to NO because of the interaction with ROS. The decrease in biologically available NO could explain the impaired endothelial relaxation seen in hypertension (8) and consequently could increase peripheral resistance and blood pressure (9). There may also be reduced activity of NOS (Nitric oxide synthase) associated with increased ROS (10), but others have found that certain ROS induce increased transcription of NOS (11). Antioxidants can improve endothelial mediated relaxations (12), and reduce blood pressure in spontaneously hypertensive rats (9) and in human essential hypertension (13;14).

In certain circumstances ROS can vasodilate, particularly in the cerebral circulation. There are three ways this can be achieved: increased adenylate cyclase activity causing increased cAMP (Cyclic adenosine monophosphate) (15), opening of K_{ATP} (ATP dependent potassium channels), or opening of Ca activated channels (16). The differential effect of ROS on peripheral and cerebral circulation is thought to protect cerebral blood flow during adverse circumstances such as shock.

1.3:ROS: a potential role in atherogenesis

ROS may be pathologically significant at a number of points in the formation of atheroma and thrombosis:

1. Oxidation of low density Lipoprotein (ox LDL)
2. Interaction with NO to form peroxynitrite
3. Second messenger in mitogenesis of vascular smooth muscle cells
4. Endothelial cell apoptosis
5. Platelet aggregation
6. Over-expression of ICAM1 and VCAM1

It is well established that elevated LDL (low density lipoprotein) cholesterol levels are a risk factor for ischaemic heart disease. However it is becoming increasingly apparent that it is the oxLDL (oxidized low density lipoprotein) component that is most atherogenic (7;17-19), and hence a better indicator of risk (20). ROS produced by monocytes/macrophages, and possibly fibroblasts and smooth muscle cells, will contribute to the burden of oxLDL (21;22).

As discussed above, ROS interacts with NO removing a potent vasodilator and replacing it with peroxynitrite, a more noxious compound. At an intra-cellular signaling level ROS may act as a second messenger resulting in activation of p21 ras, NFκB (Nuclear Factor κ B), MAPK (Mitogen activated protein kinase), PI3K (phosphatidylinositol-3 kinase), PKC (Protein Kinase C), protein kinase B/Akt and protein tyrosine kinase (23-26). These signals may

account for the changes seen in cell growth from mitogenic signaling in smooth muscle cells (23), to apoptosis and delayed division in endothelial cells and in vascular smooth muscle cells (27). This means that ROS could account for both smooth muscle hyperplasia and the endothelial damage encountered in atheroma.

Superoxide destroys acetyl hydrolase, the enzyme responsible for the breakdown of PAF (Platelet activating factor); therefore as superoxide rises so does PAF (28). PAF has aggregatory effects on platelets as well as activating neutrophils and monocytes and reducing cardiac output.

1.4:The source of ROS

There are a number of enzyme sources of reactive oxygen species:

1. NADPH oxidase (White blood cells and Vascular Smooth Muscle cells (VSMC)).

2. Xanthine oxidase.

3. NADH oxidase of mitochodrial electron transport chain.

4. Microsomal cytochrome P450.

Although mitochondria are potentially the largest source of ROS in biological systems, much of the work in the cardiovascular field has concentrated on Xanthine oxidase and NADPH oxidase.

Numerous authors have studied ROS production from neutrophils in rat (29) and human subjects with hypertension (7;10;30-32), finding excess ROS. ROS production has also been located in vessel walls, either in endothelium of pig coronaries (33) or smooth muscle cells and adventitia cells of rabbit aortas(34;35). Increased vascular ROS production has also been demonstrated in AGII (Angiotensin II) induced hypertension (8) and renovascular models of hypertension (36) in rat models. The ROS production has been confirmed to be from NADPH oxidase by inhibitor studies (8;33;34;36) and the various components of NADPH oxidase have been located within the adventitia (35). The association of hypertension with oxidative stress may not be universal to all forms of hypertension, as the

group studying the effects of AGII (8) found no increase in ROS production in hypertension induced by infusions of norepinephrine. Lacy (37) found that ROS correlated better with a family history of hypertension, and other groups have not been able to repeat the findings of increased ROS production from neutrophils (38;39).

NADPH oxidase can be found within the various white cell lines and in VSMC and endothelial cells. Both the white cell lines and and the cells located within the cell wall seem to produce increased ROS in HT. It is not known if the mechanism of upregulation is the same in both groups of cells and which is of primary importance (40). Many believe that the increased production within the vessel wall is the supreme pathogen, as ROS production is located where the deleterious effect is taking place. Equally though the white cells are in intimate contact with the vessel wall and the relative amounts of ROS produced by white cells and the vessel wall sources are not known.

1.5:Antioxidants

As mentioned above antioxidants improve endothelial function and reduce blood pressure. However if the hypothesis is correct, antioxidants should also slow or prevent atherogenesis and result in reduced cardiovascular mortality. The evidence of such benefit is at best limited and probably at odds with the hypothesis.

The antioxidants can be subdivided into natural and pharmacological. The naturally occurring antioxidants are vitamin C, vitamin E, β carotene and

the flavonols. The flavonols have drawn a lot of attention particularly in the form of tea and wine. Pharmacological antioxidants are aspirin, probucol, HMG CoA reductase inhibitors (3-hydroxy-3-methylglutaryl coenzyme A) and hormone replacement therapy, as well as a number of commonly used antihypertensives.

1.5A:Vitamin Antioxidants

The theoretical arguments for vitamin antioxidants being valuable treatment options in HT are persuasive, but the clinical data are less clear-cut. Use of surrogate end points, such as the amount of oxLDL, shows favorable results (41;42). Vitamin E, as it is fat soluble, is incorporated into LDL particles making them resistant to oxidation. Vitamin C is water-soluble and it too makes LDL more resistant to oxidation. However in an animal atheroma model, although vitamin E reduced oxLDL, it did not halt the progression of atheroma (43).

The descriptive, case control and prospective studies, shown in table 1(adapted from table produced by Diaz et al (44)) show the beneficial effect of vitamins E and C in reducing cardiac events. Most of the studies are concerned with intake of the vitamins from food-stuffs rather than supplements.

The randomised control trials of vitamin antioxidant use have been less promising, with only 2 showing beneficial results. The ATBC trial was initially conceived to study if vitamin antioxidants reduce malignancies in Finnish male

smokers, but also found that α-tocopherol reduced cardiac events (45). The CHAOS study showed a reduction in non-fatal myocardial infarction but an apparent rise in fatal myocardial infarction with vitamin E use (46).

Study **Descriptive**	Population Studied	Dose (per day)	Findings
Verlangieri (47)	US	NA	Fruit and vegetable consumption was inversely proportional to cardiovascular mortality
Gey & Puska (48)	16 European regions	NA	Plasma α tocopherol was inversely proportional to cardiovascular mortality
EPIC study (49)	19436 patients in Norfolk	NA	Plasma ascorbic acid was inversely proportional to all cause mortality, cardiovascular mortality and ischaemic heart disease
ARIC study (50)	6318 females and 4989 males aged 45-64 years	NA	Dietary and supplementary intake of vitamins C, E and β carotene: weak inverse relationship to carotid intimal thickness (a marker of coronary atherosclerosis) especially in those >55 years

Table 1a, Summary of vitamin antioxidant trials

Case Control			
Riemersma et al (51)	110 patients with angina and 394 controls	NA	Low plasma α tocopherol levels in the angina group
Ramirez & Flowers (52)	101 patients with coronary artery disease on angiography and 49 controls	NA	Reduced leucocyte ascorbic acid levels in patients with coronary artery disease

Table 1b, Summary of vitamin antioxidant trials

Prospective studies			
Nurses' Health Study (53)	87245 female US nurses	NA	α tocopherol intake was inversely proportional to coronary artery disease
Health professionals follow up study (54)	39910 American male health professionals	NA	α tocopherol intake was inversely proportional to coronary artery disease.β carotene intake was inversely proportional to coronary artery disease in smokers and ex smokers
Physicians Health Study (55)	22071 American male physicians	NA	Portions of fruit and vegetable eaten a day were inversely proportional to IHD (Ischaemic heart disease) especial in over weight and smokers
Nurses' health study (56)	85118 women in the US	NA	Ascorbic acid intake was inversely proportional to coronary artery disease
Losonczy et al (57)	11178 elderly US citizens	Self reported Vitamin C&E supplementation	Reduced rates of coronary artery disease in those taking α tocopherol and vitamin C

Table 1c, Summary of vitamin antioxidant trials

Randomised Control Trials			
ATBC(58)	22269 Finnish male smokers	50mg Vitamin E 20mg β carotene	Small reduction for α tocopherol. β carotene had no effect on cardiac events.
CHAOS (46)	2002 British patients with angiographically proven coronary artery disease	Initially 800IU then 400IU of vitamin E	77% reduction in non fatal Myocardial infarction
GISSI study (59)	11324 patients post MI	Vitamin E 300mg, n 3 PUFA 1g	Vitamin E had no effect on death, MI or stroke. N-3-PUFA (n-3 long chain polyunsaturated fatty acid)reduced death, MI and stroke.
Primary prevention project (60)	4495 subjects with one or more cardiovascular risk factors.	Vitamin E 300mg	Vitamin E had no effect on cardiac events.
Hope study (61)	9294 patients over the age of 55 years at high risk of cardiovascular disease.	Vitamin E 400IU	Vitamin E had no effect on cardiovascular events.
Heart Protection Study (62)	20536 subjects at high risk of cardiovascular disease with normal or low cholesterol levels.	Vitamin E 300mg, Vitamin C 250mg, β carotene 20mg	Antioxidant cocktail had no effect on cardiovascular disease.

Table 1d, Summary of vitamin antioxidant trials

There are some pitfalls in the use of vitamins C and E that can confound the benefit they may have.

1.5B:Dose of Vitamin C or E

Vitamin C in too low (63) or too high a concentration (64) becomes a pro-oxidant and is associated with increased oxidative damage of DNA and oxidation of LDL. Barry Halliwell (65), in a review in the Lancet, posed some interesting hypotheses as to why vitamin E may also act as a pro-oxidant. In the later stages of atherogenesis, transition metals are found within atheromatous plaques (66). These metal ions in their reduced form catalyse the free radical damage. Although vitamin E is an antioxidant it will also maintain the transition metals in their reduced form, and therefore in the presence of atheroma, there is the potential for vitamin E to shift the balance towards oxidative damage. This may explain why there is some evidence to show that vitamin antioxidants are advantageous in primary prevention but not secondary prevention. The dosing of vitamin E in some studies has been criticised as it was thought to be a sub-therapeutic dose.

1.5C:Other Antioxidants

The antioxidant effects of other medications may have compromised the results of some antioxidant studies. Aspirin, carvedilol, probucol and HMG CoA reductase inhibitors are all known to be antioxidants or attenuate ROS formation. There are also theoretical grounds to suggest that ACE-(Angiotensin Converting Enzyme inhibitors) or Angiotensin II Receptor Blockers may reduce ROS formation in certain situations. The GISSI-

prevenzione trial (59) showed no effect of treatment with vitamin E. As this study was conducted in Italy the beneficial effect of a Mediteranean diet may have masked any benefit of vitamin E treatment.

1.5D:Inability to reverse established Atherosclerosis

Fatty streaks, the precursor of atheroma, can be found in infants, and established but clinically insignificant atheroma is present by early adult life. The animal models of atherogenesis have applied or induced an oxidant stress and simultaneously applied antioxidants. Positive results of intervention trials, in those older than 55 years, when atheroma is already established, are perhaps not to be expected. The positive results of the CHAOS study may represent reduction in re-stenosis in those undergoing angioplasty or stent insertion.

Special Note

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Food stuff	Comments	References
Tea	The antioxidant component is thought to be flavonoids, but tea also contains catechins, theaflavins and thearubigins that may also exert an antioxidant effect. There is no overall significant effect on ambulatory blood pressure. Increased flavonol consumption, assessed by dietary questionnaire, is associated with reduced relative risk for MI and death in most trials. The Caerphily study possibly showed an increased risk of heart disease in the highest quartile of tea drinkers	(45;67;68)
Red Wine	The flavinol content of wine is thought to produce the beneficial antioxidant effect. Red wine has been shown to have antioxidant potential for 4.5 hours post consumption, it also improves endothelial function, prevents LDL oxidization and NFκβ activation.	(69-71)
Drug	**Comment**	**References**
Aspirin (Mostly in vivo studies or based on aspirin infussions)	Aspirin increases plasma antioxidant capacity by scavenging hydroxyl radicals and reducing NADPH oxidase activity and in so doing is able to inhibit oxidation of LDL and improve endothelial function	(72-75)
HMG CoA reductase inhibitors (In vitro, numerous statins reduce ROS. In vivo studies show improved endothelial function, dose and statin used not specified)	The statins, as these are collectively known, are also able to improve endothelial function and reduce blood pressure in a manner that is separate from their cholesterol lowering action. The mechanism mediating improved endothelial function is thought to be due to attenuation of ROS formation by prevention of post-translational modification of rac2 , see section 1.7 for information on mode of action of rac 2.	(76-78)

ACE inhibitors	In vivo Both ACE- and ARBs (Enalapril 10mg od, Ramipril 10mg od and Losaratan 100mg od) can improve endothelial function, inhibit LDL oxidation and protect myocardium against reperfusion injury, all of which are attributed to antioxidant activity. Captopril has intrinsic antioxidant activity, and ACE- as a group are able to increase availability of super oxide dismutase, catalase, and glutathione peroxidase (in vitro studies of ACE- and ARBs). Angiotensin II causes an increased expression of $gp91^{phox}$ that is antagonized by ARBs (in vitro).	(79-83)
Calcium Channel Blockers (all in vitro studies)	The calcium channel blockers are able to inhibit lipid peroxidation (amlodipine >verapamil>diltiazem) and reduce ROS production by stimulated neutrophils (verapamil). There are two proposed mechanisms: the first a non-specified membrane effect, reducing lipid peroxidation, the second a reduced activation of NADPH oxidase by Protein Kinase C (PKC) as there is reduced intracellular Ca to activate PKC.	(84;85)
α Blockers	In vitro doxazosin does act as a free radical scavenger but in vivo the same group was unable to show any effect on ROS production or lipid peroxidation (doxazosin 1-8mg/day). Cabell *et al* have demonstrated that terazosin is able to increase myocardial SOD (Superoxide dismutase) in SHRs.	(81;86)

β Blockers	In vitro the β blocker carvedilol is not a free radical scavenger but is thought to sequester ferric ions, thus preventing Fenton type production of free radicals. In vitro it has been shown that propranolol is able to prevent production of ROS by stimulated neutrophils. The two possible reasons for the antioxidant activity of propranolol are that it has membrane stabilizing effects and that it increases free radical defences by increasing production of SOD and glutathione peroxidase. Maximally tolerated doses of carvedilol and bisoprolol had no antioxidant effect in heart failure patients	(87-90)
Hydralazine (In vitro work on SHR)	There is some work to suggest in certain models of hypertension that hydralazine increases SOD and glutathione peroxidase and reduces lipid peroxidation.	(81;91)

Table 2 The various antioxidant foodstuffs and drugs

1.6:Effect of age on ROS production

The evidence on the effect of age on ROS production is inconsistent. In animal studies some have found up to a 40% reduction in ROS production from the young to the aged of the species (92;93), whilst others have found the converse (94;95). Hamilton found an increase in ROS production from aortic rings of older rats, causing endothelial dysfunction. The mechanism seemed to be due to increased $p22^{phox}$ expression (94).

In humans the evidence is more in favour of reduced ROS production with age, the magnitude varying from as little as a 17% reduction in neutrophils (96) to a potential 70% reduction in stimulated ROS production from monocytes (92). The mechanism of reduced ROS production with age is thought not to be due to alterations in NADPH oxidase but in the pathway activating NADPH oxidsae. Lipschitz found reduced DAG (Diacyl Glycerol) and IP3 (Inositol 1,4,5 Triphosphate) in neutrophils from the elderly (96).

1.7:NADPH oxidase

Babior has reviewed the properties of this enzyme (97). NADPH oxidase catalyses the production of superoxide (equation 1) and indirectly hydrogen peroxide via the action of superoxide dismutase (equation2).

equation 1 $$NADPH + H^+ + 2O_2 \longrightarrow NADP^+ + 2H^+ + 2O_2^-$$

equation 2 $$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

The main physiological function of NADPH oxidase is in the respiratory burst of phagocytic cell lines, but it also contributes to carotid sinus oxygen sensing (98), to oxygen sensing in the lung allowing ventilation perfusion matching (99) and to the organification of iodine in the thyroid.

The enzyme is comprised of 3 cytosolic components; $p47^{phox}$, $p40^{phox}$, $p67^{phox}$, and 2 membrane bound components; $p22^{phox}$ and $gp91^{phox}$, collectively known as cytochrome b_{558}. Cytochrome b_{558} is a heterodimer composed of $p22^{phox}$, $gp91^{phox}$, one FAD (Flavine-adenine dinucleotide) and two hemes. It is thought that the FAD subunit acts as the electron donor/receptor rather than the heme subunit, as the kinetics of NADPH oxidase most closely match FAD, and the flavin antagonist DPI (Diphenyleneiodonium chloride) inhibits NADPH oxidase. Cytochrome b_{558} is the catalytic core of the enzyme but is dormant until activation, when the cytosolic components translocate to the membrane. Two GTPases (Guanine Triphosphate Hydrolase) are closely related with the function of the oxidase, rac2 and Rap1A. Rac2 is involved with signal transduction of neutrophils activated with fMLP (n-formyl-methionyl-lenoyl-phenylalamine) (100), and isoprenylated rac 2 facilitates translocation of the cytosolic components of NADPH oxidase.

Activation of NADPH oxidase can be achieved by phosphorylation of the subcomponents or directly by Arachidonic Acid (AA); the combination of both mechanisms has a synergistic effect (101). Common to both mechanisms of activation are conformational changes within the cytosolic phox subunits, which unmask Src Homology 3 domains (SH3) allowing the

following chain of events: translocation of cytosolic components, interaction with rac2/rap1a, association with Cytohrome b_{558} and catalytic activity.

1.8:Activation by Phosphorylation of Subcomponents

1.8A:$p47^{phox}$

Translocation of all the cytosolic phox subunits appears to be dependent upon $p47^{phox}$, as translocation of the other subunits does not occur in $p47^{phox}$ deficient cells. The $p47^{phox}$ subunit is also thought to promote tighter binding of the subcomponents. $p47^{phox}$ is extensively phosphorylated upon activation and has been most studied. The course of events leading to activation are thought to be partial $p47^{phox}$ phosphorylation, allowing translocation of the cytosolic subcomponents to the plasma membrane; subsequently there is further phosphorylation of $p47^{phox}$ resulting in catalytic activity.

There are 8 or 9 Serine (Ser) residues in the C terminus that are phosphorylated. The serine residues would appear to be phosphorylated in a specific order and each phosphorylation step has a different functional significance. The phosphorylation of serine 379 is the only amino acid that, when mutated alone, results in both loss of activity and translocation. The mutation of serines 303 and 304 to arginine results in reduced activity, but translocation is still able to occur. Serines 345 and 348 are phosphorylated by MAPK, but the mutation of these amino acids to arginine has no effect on oxidase activity. When serines 359 and 370 are exchanged for arginine, $p47^{phox}$ becomes resistant to phosphorylation on the other serines, resulting in loss of translocation and oxidase activity. These mutation studies together

suggest that the phosphorylation sequence resulting in activity is as follows: Phosphorylation of serine 359 and possibly 370 has a permissive function allowing the phophorylation of serine 379. Once serine 379 is phosphorylated translocation of the cytosolic components can occur. This in turn allows serines 303 and 304 to be phosphorylated allowing assembly of the active enzyme, probably by unmasking an N terminal $SH3_A$ domain of $p47^{phox}$, which binds to the cytoplasmic C terminal of $p22^{phox}$ (102;103). Protein Kinase A (PKA) and Protein Kinase C (PKC) can achieve activation of NADPH oxidase by this phosphorylation sequence. The action of PKA can be inhibited by cAMP agonist, whilst that of PKC cannot.

The action of arachidonic acid on $p47^{phox}$ is discussed later (section 1.9B).

1.8B:$p67^{phox}$

The phosphorylation of $p67^{phox}$, probably by p21 Activated Kinase (PAK) (104) on Thr233 (105), results in disruption of intramolecular SH3 domain polyproline interactions, unmasking a rac2 binding site. The $p67^{phox}$ /rac2 interaction occurs at the N terminus of $p67^{phox}$, which has 4 Tetratricopeptide repeat (TPR) motifs, which are thought to undergo an ionic interaction with rac2 (106). The activated complex translocates with $p47^{phox}$ to the plasma membrane, where amino acids 199-210 undergo an important interaction with cytochrome b_{558} (107), allowing or facilitating electron transfer from NADPH through FAD, and possibly heme to oxygen (108;109). Rac2 plays an important supporting role increasing the affinity of $p67^{phox}$ for $gp91^{phox}$ (110), and is required in the electron transfer (111).

The $p67^{phox}$ subunit is closely associated with $p40^{phox}$, but there are conflicting reports as to the nature and function of the interaction. On stimulation, $p40^{phox}$ and $p67^{phox}$ may associate and both translocate to the plasma membrane (112). Others have suggested that $p67^{phox}$ and $p40^{phox}$ form a complex in the resting state, and that on activation the rac2 binding causes dissociation of $p67^{phox}$ and $p40^{phox}$ (113).

1.8C:$p40^{phox}$

The role of this subunit is unclear and the published data on it is potentially conflicting. As mentioned above the $p40^{phox}$ interacts with $p67^{phox}$, but whether that is in the dormant or active state is unclear. Sathyamoorthy et al (114) found $p40^{phox}$ to down regulate NADPH oxidase activity, whilst others have found activation of NADPH oxidase by PKC results in phosphorylation of both $p47^{phox}$ and $p40^{phox}$ and enzyme activity (115).

1.8D:$p22^{phox}$

On activation, the cytosolic C tail of $p22^{phox}$ forms a high affinity bond with N terminal SH3 domain of $p47^{phox}$ (103), an interaction that is essential for enzyme activity. Activation of NADPH oxidase is also associated with $p22^{phox}$ phosphorylation, which is proportional to enzyme activity, but the function of which is unknown. Phosphorylation of $p22^{phox}$ has been shown to be by Protein Kinase C (PKC), Protein Kinase D (PKD) and by a phosphatidic acid activated protein kinase (116;117).

1.8E:gp91^{phox}

This subunit, with p22^{phox}, forms the membrane-spanning electron transport chain cytochrome b558, which is the catalytic core of NADPH oxidase. Unlike the other subunits, it is glycosylated on its N terminus at residues Asn131, Asn148, and Asn239 (118). The C terminus interacts with both p47^{phox} (119) and rac2 (120), and is thought to act as the reductase of NADPH. In addition it forms a protein channel allowing efflux of generated hydrogen ions (121). The hydrogen channel is located within the N terminal of gp91, and is voltage and Arachidonic Acid activated(122).

A simplified sheme of NADPH oxidase activation is represented in figure 1.

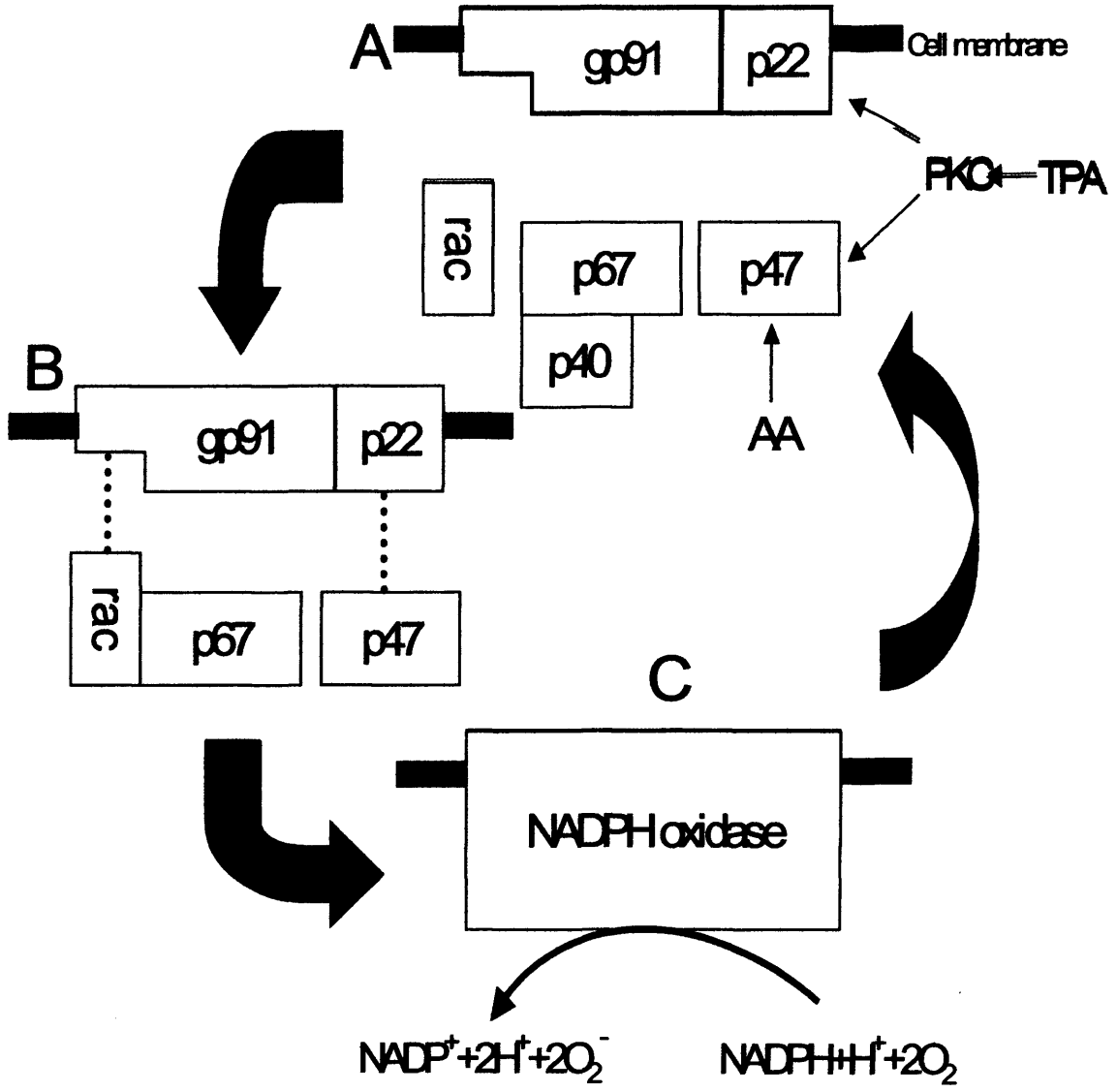

Figure 1. Activation of NADPH oxidase. In A gp91 and p22 are located in cellmembrane and p40, p47, p67 and rac lie dormant in cytosol. TPA (12-O-Tetradecanoylphorbol-13-Acetate) activates p47 by phosphorylation and AA by conformational changes in p47. Phosphorylation of p67 may result in dissociation of p40. In B association of rac and p67 and changes in p47 facilitates their translocation to the membrane components. In C, NADPH oxidase is now assembled and able to produce ROS. The NADPH oxidase is deactivated by type 1and 2A phosphatases.

1.9:Stimulation pathways

There are numerous agents capable of activating the respiratory burst of leucocytes; commonly used agents are 12-O-tetradecanoylphorbol-13-acetate (TPA), Arachidonic Acid (AA), Opsonified Zymosan (OZ), Staphylococcal Protein A (SpA), Calcium Ionophore (A23187) and n-formyl-methionyl-lencyl-phenylalamine (fMLP). Lymphoblasts do not posses fMLP receptors and we found that OZ, A23187 and SpA were unable to stimulate ROS production from lymphoblasts (unpublished observations).

1.9A:TPA

It has been well recognised that TPA stimulates protein kinase C (PKC) to activate white blood cells (123). There is no clear-cut work showing whether it is a specific isoform of PKC, or a general property of PKC, that activates NADPH oxidase. PKC is dependent on trace amounts of calcium for its function (124) and in vivo it is activated by DAG (125). Activation of PKC acts both directly, and via a cascade, to stimulate NADPH oxidase. The direct actions of PKC are to phosphorylate $p47^{phox}$ (126) and $p22^{phox}$ (116). The indirect pathway is serine phosphorylation of ERK (p42/44 MAPK) by PKC; the activated ERK then phosphorylates cytosolic phospholipase A_2 ($cPLA_2$) (127;128). The active PLA_2 produces AA, that in high concentrations stimulates NADPH oxidase as described in 1.9B, and in low concentrations has a permissive but essential role in the activation process (129;130). The process may be more complex, as PKC could be activating $cPLA_2$ directly and

via ERK (131). Both PKC and ERK phosphorylate $cPLA_2$ but at separate locations (132). The stimulation pathway is summarised in figures 1 and 2.

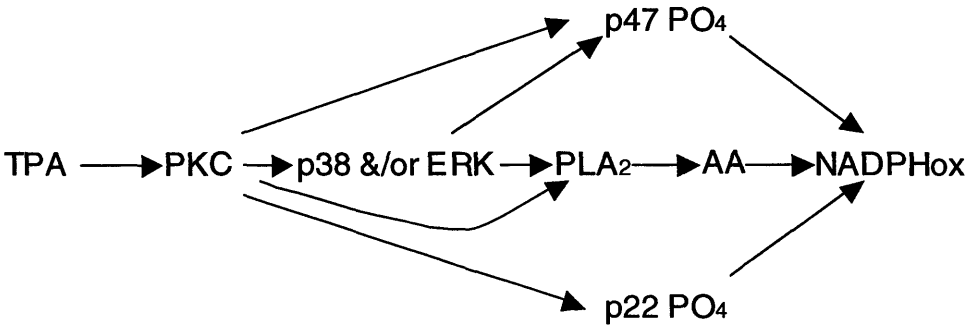

Figure 2, TPA activation pathway of NADPH oxidation.

The activation pathway is switched off by type1 and type2A protein phosphatases (133). Regulation of NADPH oxidase is also achieved by cAMP dependent Protein Kinase A (PKA) (134) at a messenger up stream of PKC.

1.9B:Arachidonic Acid

A number of groups (7;129;135;136) feel that AA is pivotal in the signal pathways which activate NADPH oxidase, with the formation of AA being more significant than phosphorylation of $p47^{phox}$. In vivo AA is formed by PLA_2. Inhibition of this enzyme inhibits ROS production but translocation of $p47^{phox}$ and $p67^{phox}$ still occurs, and phosphorylation of $p47^{phox}$ is unaffected.

AA exerts a number of direct and indirect effects on NADPH oxidase. The indirect effects have received less attention and are probably less

important, but are stimulation of p38 MAPK, ERK1/2, PKC (137;138) and calmodulin (139).

A simplified diagram of AA activating NADPH oxidase is shown in figure 1. The direct action of AA is on $p47^{phox}$ and $gp91^{phox}$. AA is able to translocate $p47^{phox}$ to the plasma membrane (101;140). AA-induced translocation acts synergistically with phosphorylation of $p47^{phox}$, and occurs by unmasking an SH3 domain in $p47^{phox}$, allowing an interaction with $p22^{phox}$. The action of AA on $gp91^{phox}$ appears to be 2 fold: it increases the subunits' affinity for O_2 (141) and activates the hydrogen channel in $gp91^{phox}$ (121;122;142). The actions of AA on $gp91^{phox}$ are independent of those on $p47^{phox}$.

1.10:Potential mechanisms of increased ROS production

1.10A:Abnormalities or over expression of $p22^{phox}$

Recent work suggests that $p22^{phox}$ may be important in the pathogenesis of atheroma, particularly when related to hypertension, either by increased expression of $p22^{phox}$, or genetic polymorphisms of $p22^{phox}$, which modulate NADPH oxidase function.

A post mortem study showed that coronary arteries containing atheroma expressed more $p22^{phox}$ than those without atheroma (143). It may be possible to explain the increased expression of $p22^{phox}$ by an increase of macrophage/foam cells in atheroma boosting the $p22^{phox}$ content; however a couple of rat models of hypertension have also shown increased $p22^{phox}$ RNA expression. Spontaneous hypertensive rats (SHR), by the age of 30 weeks, show increased $p22^{phox}$ RNA, increased ROS production and impaired

endothelial relaxation (144). Interestingly these changes were not present at 16 weeks of age and could be prevented by treatment with irbesartan, an angiotensin II receptor blocker. Rats infused with angiotensin II (AGII) also express more $p22^{phox}$ RNA (145). The rise in $p22^{phox}$ is abolished by losartan (an angiotensin II receptor blocker), hydralazine (a vasodilator) and superoxide dismutase. These results suggest that $p22^{phox}$ may well play a role in the perpetuation of hypertension but does not seem to be the initiating event. The data also suggest that superoxide itself may be a second messenger for increased production of $p22^{phox}$ RNA and therefore there is the potential for positive feedback to develop.

Two polymorphisms of $p22^{phox}$ have been potentially linked with atherogenesis, the most frequently studied being C242T. The mutation C242T of $p22^{phox}$ probably codes for a heme binding locus, and exchanges the histidine at residue 72 for tyrosine. There are therefore 3 combinations of the polymorphism: CC, CT and TT. The CT and TT polmorphism, in human saphenous vein samples, are associated with a 30% reduction in NADPH oxidase ROS production as compared to the CC polymorphism (146). It would therefore be expected that the CC polymorphism should be associated with hypertension and atherosclerosis. However the experimental results are inconsistent, with possible ethnic variation. Iwai found an expected association between CC polymorphism and HT (147) but Fricker et al could not demonstrate that the C242T polymorphism had any effect on endothelial function (148).

If we consider coronary atherosclerosis, 2 moderately sized trials seemed to suggest that TT and TC polymorphisms were more common in

ischaemic heart disease and were associated with a more rapid loss of luminal diameter (149;150). These findings were not in keeping with the hypothesis however, and have not been reproduced in a study of over 2000 subjects (151). In Oriental and Asian populations the studies have either shown no correlation (152), or they have shown an increased frequency of TC and TT polymorphisms in the control populations, suggesting that the CC polymorphism is associated with atherosclerosis, or the TC and TT polymorphisms protect against atherosclerosis (153). In caucasians with peripheral vascular disease no association with C242T polymorphisms has been demonstrated (154), whilst in Japanese patients with ischaemic cerebrovascular events there is an association with genotypes TT and TC (155).

The other mutation is A640G, which is thought to lie within a 3' terminal exon. The function of the exon is not clear and therefore how ROS production is modified is not known. The polymorphisms are GG, GA and AA. The presence of the GG polymorphism is associated with a small but significant relative risk of 1.27 of developing HT (156). There have only been 2 studies into A640G polymorphisms and atherogenesis. They are both of ischaemic heart disease, one suggesting a correlation between IHD in Caucasians and genotype AA (151), whist the other showed no correlation in a Japanese population (153). In conclusion the information on the A640G polymorphism is limited, the mechanism is not understood and the polymorphism associated with hypertension (GG) is not that associated with atheroscelrosis (AA).

1.10B:Expression of $p67^{phox}$ and $gp91^{phox}$

In vitro studies have shown that Angiotensin II can induce increased expression of $p67^{phox}$ in adventitial fibroblasts (157), and of $p67^{phox}$ and $gp91^{phox}$ in rabbit aortic arch homogenates (158). In both groups there was an increase in ROS production.

1.10C:Priming of NADPH oxidase

In addition to the activation/inactivation process described above, and increased expression or expression of more active components of NADPH oxidase, NADPH oxidase can undergo priming. Priming places NADPH oxidase in an increased state of readiness, so that upon stimulation the release of ROS is increased. Agents that have been shown to prime neutrophils in vitro are GM-CSF (159), TNFα (Tumour necrosis factor α) (160), PAF (161), LPS (162;163), substance P (160), Influenza A virus (164), Interferon-γ (165), Advanced Glycation end products (166) and IL 8 (167). In vitro, primed neutrophils have been described in sepsis, rheumatoid arthritis, after blunt trauma and in adult respiratory distress syndrome. Just as there are multiple agents able to prime white cells it is likely there are a number of receptors and pathways involved, depending on the priming agent and cell type. The majority of previous work on priming of cells has been conducted on fresh neutrophils, and occasionally monocyte models such as U937. Two good reviews of priming are by Hallett MB & Lloyds D, and Condliffe AM, Kitchen E & Chilvers ER (168;169). The work has focused on the process of priming in vitro, whereas the model we have used has been primed in vivo

and then studied in vitro. Figure 3 attempts to summarise the priming of NADPH oxidase.

1.10C(i):*G proteins*

There is evidence suggesting that lymphocytes and lymphoblasts from HT subjects have higher stimulated G protein activity (170;171). Heximer et al have shown that heterozygotes and homozygotes with an inactivating mutation in RGS2 GTPase activity, are more prone to developing hypertension (172). This all suggests that in the presence of other triggers, such as AGII and PAF, HT lymphoblasts have increased G protein activity. (171). We have not pursued this pathway as our lymphoblasts are divorced from their priming agent. Also, TPA and AA do not activate NADPH oxidase via G proteins, therefore G protein activity would be expected to exert no effect in the lymphoblast model stimulated with these agonists. Basal G protein activity would be worth investigating if we were able to detect differences in basal HT and NT ROS production. We searched for other NADPH oxidase agonists that stimulate through G proteins, but found no agents after investigating fMLP, LPS, OZ and Protein A.

1.10C(ii): *Tyrosine kinases (including p44/p42 MAPK and p38 MAPK)*

The tyrosine kinases are a group of 95 different enzymes that act as intracellular second messengers. The tyrosine kinases can be receptor linked or non-receptor mediated. There is a large body of evidence suggesting that agents which prime WBCs cause activation of tyrosine kinases, therefore increasing tyrosine phosphorylation. The tyrosine kinases phosphorylate

numerous proteins within the cells, most frequently a 40-43kDa protein, thought to be p44/p42 MAPK, and a unknown 72kDa protein (169). The identity and function of the 72kDa protein is unclear. p44/p42 MAPK are known to partially phosphorylate $p47^{phox}$, and this phosphorylation may be involved in the priming and activation of NADPH oxidase (173). There is evidence to show that GM-CSF priming of neutrophils results in tyrosine phosphorylation of p38 MAPK (174).

1.10C(iii): *PI3K*

In some priming models it has been found that PI3K has a central role in the priming pathway at a number of points (175). PI3K can be activated by tyrosine phosphorylation and by G proteins. PI3K in turn activates PAK kinase to phosphorylate and therefore prime $p47^{phox}$ (176). PI3K may also play a role in activating rac2, causing enhanced NADPH oxidase activity, by facilitating translocation of the cytosolic components of NADPH oxidase (177). The inhibitor of PI3K, wortmannin, has been used by a number of investigators, who have found that although PI3K activity was abolished, priming was still able to occur.

1.10C(iv): *Phospholipase C (including PKC and $_{i}Ca$)*

$PLC\beta_2$ (Phospholipase C), the prominent subtype in neutrophils, is activated via a G protein pathway (178), whilst other subtypes of PLC are activated by tyrosine phosphorylation. PLC produces both IP3 and DAG. IP3 mobilises intracellular calcium. This may activate rac2, which is a cofactor for PKC, PLD (Phospholipase D) and $cPLA_2$. Rac2 facilitates the translocation of

cytosolic components of NADPH oxidase. DAG is the naturally occurring agonist of PKC. Some have argued that the sub-stimulating concentrations of DAG may prime rather than stimulate NADPH oxidase.

The priming of NADPH oxidase by PLC is not confirmed for the following reasons: when the current most reliable assay for IP3 is used, no increase is found on priming of PMNs (polymorphoneutrophils) (179). Assays of iCa whilst PMNs are primed are interesting, because no investigators have been able to show a rise in iCa attributed to priming with TNFα (160;180). Variable results have been found for the other agents, for example GM CSF (Granulocyte Colony Stimulating Factor) priming of neutrophils is associated with increased iCa (181).

There is no doubt that DAG activation of PKC produces activation of NADPH oxidase, but the evidence that PKC is involved in priming of NADPH oxidase is weak. GM-CSF priming of neutrophils may be associated with a small increase in DAG (182), but Sullivan failed to demonstrate the same findings (183). There is no evidence that PKC is involved in LPS (162) or TNFα priming (184). Some authors have actually found that inhibition of PKC with staurosporine enhances priming (185).

1.10C(v):*Phospholipase D*

PLD catalyses the conversion of phosphatidyl Choline to phosphatidic Acid (PA). PA activates a kinase capable of phosphorylating $p47^{phox}$. PA is also converted to DAG by the action of phosphatidate phosphohydrolase, and therefore can prime or stimulate NADPH oxidase via PKC. Bourgoin (186) has demonstrated that in human neutrophils primed with GM-CSF there is

increased PLD activity, and the priming can be inhibited by pertussis toxin, inhibition of tyrosine kinase and calcium chelation.

1.10C(vi):*cPLA$_2$*

The product of cPLA$_2$ is AA. Higher concentrations activate NADPH oxidase whilst lower concentrations are thought to prime the enzyme. A number of authors have shown that agents that prime PMNs result in priming of cPLA$_2$, in that it is translocated to the plasma membrane where its substrate (187) is located, and it is phosphorylated by p44/p42 MAPK and therefore more active (188). Primed neutrophils have also been shown to produce more AA. Despite the priming of cPLA$_2$ some authors have failed to show that inhibition of cPLA2 in primed cells, with mepacrine or quinacrine, abolishes priming of NADPH oxidase (189;190).

1.10C(vii): *Consequences of NADPH oxidase Priming*

The net effect of the above priming steps is presumed to be conformational changes in the sub units of NADPH oxidase, either as a result of partial phosphorylation (173) or the direct action of AA. The conformational changes may allow translocation to occur or improve the efficiency of NADPH oxidase.

1.10C(viii): *Depriming*

It is intuitive that increased phosphatase activity will oppose the action of tyrosine kinase, thus tending to counteract the priming effect. The role of PKC is controversial, in that it may be part of the priming or depriming pathway. GM-CSF may prime NADPH oxidase through PKC (182) but inhibition of PKC by staurosporine can increase ROS production (185).

Special Note

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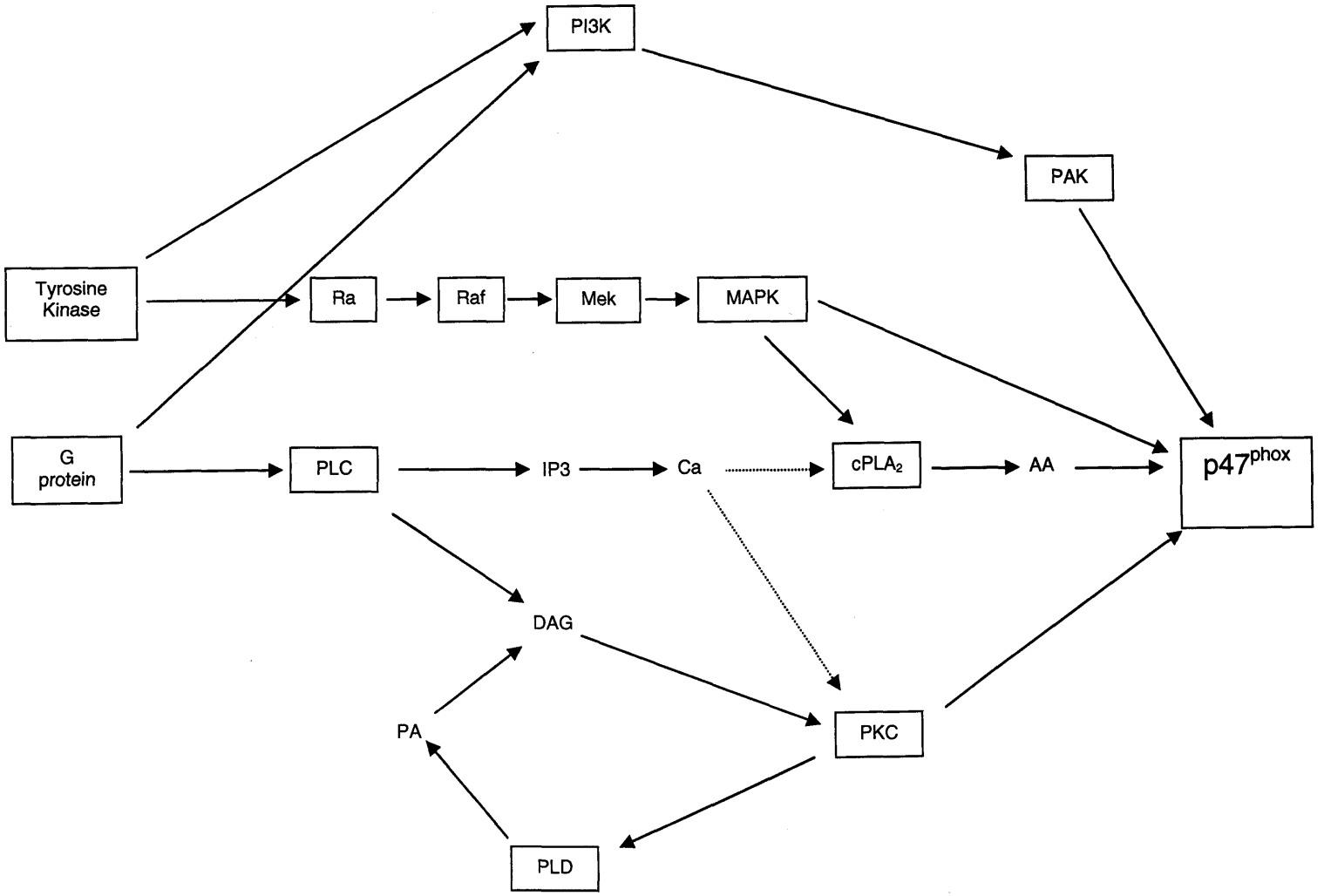


Figure 3, Potential priming pathways of NADPH oxidase. For explanation see body of text

1.10D:Reduced Oxidative defenses

As well as ingested antioxidants the enzymes SOD, catalase and glutathione peroxidase, which catalyse the break down of ROS, also act as antioxidant defences. It could be argued that depletion of these defences would result in increased ROS; however in animal models of hypertension the levels of these enzymes in aortic rings, kidney and cardiac muscle are the same as the normotensive controls or increased (191-193). The increase suggests an adaptive response to the oxidative burden of hypertension.

1.11:Lymphoblasts as a model for studying increased ROS production in HT

1.11C: Leucocytes and hypertension

Because of the intimate nature of the blood and blood vessels it is not unreasonable to assume that changes in leucocyte ROS production have an impact on the vasculature. As mentioned in section 1.4 there is also an NADPH oxidase located within endothelial and vascular smooth cells. It is matter of debate which NADPH oxidase is pathologically most significant (40).

T Lymphocytes and Monocyte/Macrophage cells are thought to be important as they enter the arterial walls and are implicated in the development of atheroma. Monocytes are also thought to be responsible for oxidation of LDL making it more atherogenic (194). Monocytes produce approximately a third of the ROS produced by neutrophils (195) therefore in quatitative terms neutrophils ROS production could be significant. We are using lymphoblasts to follow what is

happening with neutrophils ROS production, the reasons are outline bellow. There is no information on how closely neutrophil or lymphoblast NADPH oxidase mirrors vascular wall NADPH oxidsase.

1.11B: The disadvantage of Neutrophils in the study HT ROS production

Many other groups have used neutrophils to study the respiratory burst in hypertension and demonstrated increased ROS production; this does have the advantage of using fresh cells but has 3 problems:

- Numerous other conditions, not apparent at sampling, may prime or activate the neutrophils.
- The extraction of neutrophils can activate them.
- Patient medication and diet will alter the respiratory burst of fresh neutrophils. For a complete list of antioxidants see Tables 1 & 2.

1.11C: Lymphoblast

It is believed that using lymphoblast avoids the variables introduced by the sensitivity and susceptibility of the neutrophils to activating agents, such as infections and cigarette smoke, and antioxidants the subject was ingesting. This assumes that lymphoblast NADPH oxidase expresses its genotype rather than a reflection of factors influencing ROS production at time of sampling. Dr VM Lee and Prof LL Ng have activated lymphocytes with TPA and then immortalized them. They found that there was no difference between stimulated and unstimulated lymphoblast (unpublished data). We therefore believe that

lymphoblasts closely represent the lymphocyte genotype and avoid confounding environmental factors at the time of sampling.

Lymphoblasts are derived from B lymphocytes by transforming them into blast cells using Epstein Barr Virus in the presence of cyclosporin (196). Numerous groups have studied lymphoblasts as a tissue culture model of Chronic Granulomatous Disease. Chronic Granulomatous Disease is a heterogenious condition in which the NADPH oxidase respiratory burst is impaired and consequently the subject is prone to recurrent bacterial infections. Using lymphoblasts has the advantage that there is a ready supply of white cells without having to resort to repeated and large volume venesection. The lymphoblasts behave similarly to the neutrophils particularly with regard to the respiratory burst, which although a 100 fold less, correlates well with the neutrophil respiratory burst. The reduced free radical production is attributed to lower levels of subunits $p22^{phox}$ and $gp91^{phox}$ (197).

Lymphoblasts have also been used in the study of hypertension by ourselves and Siffert (171;198). It is felt that lymphoblasts retain many of the features of the native cell lines(40;198;199) but Siffert's group are alone in comparing the hypertensive phenotype in native and transformed cells (199), particularly focusing on the properties of the sodium hydrogen exchanger. As the majority of the lymphoblasts we used were formed previously we were unable to compare lymphoblast and nutrophil respiratory burst.

It is felt that more immature cell lines, such as HL60 and EBV transformed lymphocytes, have increased potential for kinase signalling and activity. With

respect to EBV transformed lymphocytes, a number of authors have found some protein tyrosine kinases are up-regulated, or that latent EBV protein is associated with increased tyrosine kinases signalling potential (200;201). As the experiments are controlled, any potential for false positive results should be removed. There is also concern that lymphoblast phenotype may change after increasing passage number.

1.11D:Summary of information on lymphoblast NADPH oxidase

Advantages

1. Lymphoblasts provide a large and replenishable source of material.
2. Lymphoblasts are removed from confounding dietary, medicinal and serum factors.
3. Stimulating factors at time of transformation do not seem to persist post transformation.
4. Lymphoblasts probably reflect genotype.
5. Lymphoblast NADPH oxidase has been used as a model of neutrophil NADPH oxidase in the study of chronic granulomatous disease.
6. Lymphoblasts have been used to study changes in leucocytes due to HT.

Disadvantages

1. Lymphoblasts may not be an ideal model for study of priming (see section 3.1E).
2. Lymphoblast phenotype may alter with increasing passage number.

3. Lymphocyte immortalization process increases the potential for kinase signaling but should be controlled for.
4. Lymphoblasts have reduced expression of receptors, limiting number of agonists available.
5. Lymphoblasts have diminished $p22^{phox}$and $gp91^{phox}$, thus producing only 5% the ROS of neutrophils.

1.12:Background to Chemiluminescence

There are a number of methods available to assay ROS production in biological systems: cytochrome c reduction, spin trapping and chemiluminescence. Amplified chemiluminescence using lucigenin, CLA (Cypridina Luciferin Analogue), luminol and isoluminol are simple techniques, at least methodologically, which provide sensitive real time data on ROS production. There are a few pitfalls with each of the luminescent chemicals that I will consider in turn.

1.12A:Lucigenin (Bis-*N*-methylacridinium)

Lucigenin is the most commonly used agent for amplified chemiluminescence of neutrophil superoxide production. It is known that lymphoblasts produce 5% the amount of superoxide compared to neutrophils, this probably is due to less $p22^{phox}$ & $gp91^{phox}$ (202). In our preliminary

experiments we found that lucigenin was not sufficiently sensitive to detect superoxide production from lymphoblasts.

1.12B:CLA (Cypridina Luciferin Analog)

This luminescent probe has not been utilised much but it has been found to be sensitive, specific and able to measure superoxide production in biological systems (203;204). The chemical structure is depicted in figure 4 and the photochemical reaction is shown in figure 5. Skatchkov et al found that CLA was more sensitive than lucigenin (204). Early results with CLA showed that the luminescence of stimulated macrophages could be almost abolished with SOD (205) suggesting that CLA is specific for superoxide free radicals.

We, like some other authors (204), have obtained a high background signal that was inhibited by SOD. Their group felt that the high background signal was due to traces of transition metals in the buffers used. Their group found that on addition of Diethyldithiocarbamate (DETC) the high background was abolished. We found that background levels were consistent on a day to day basis and within specific cell lines. The high background did not impair CLA's sensitivity and we found it consistently detected differences in stimulated production of superoxide between NT and HT cell lines; consequently we did not use DETC.

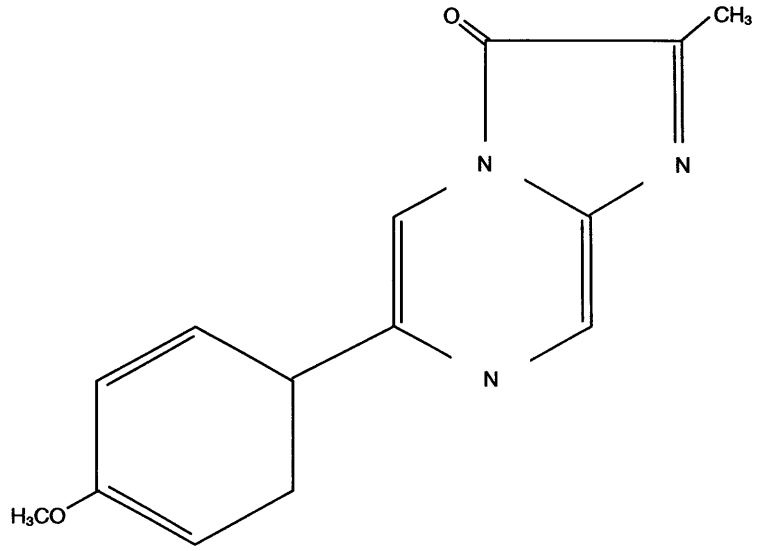


Figure 4, Chemical structure of Cypridina Luciferin Analogue (CLA).

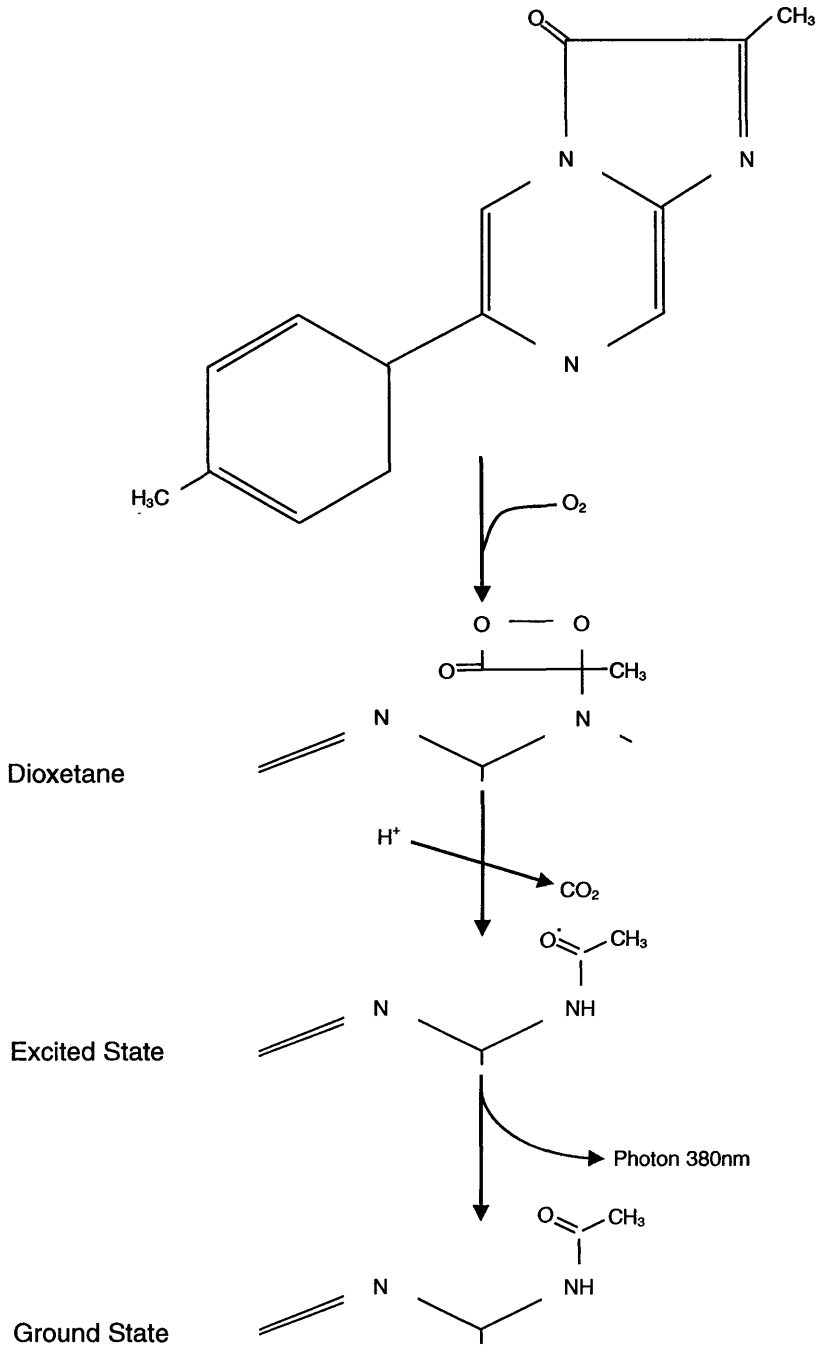


Figure 6, Simplified diagram depicting the photochemical reaction of Cypridina Luciferin Analogue (CLA).

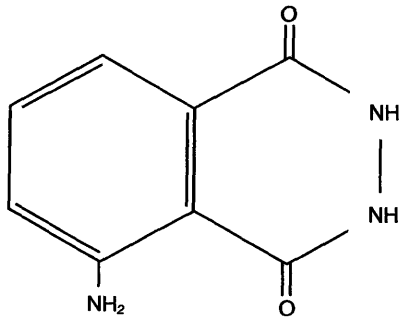
1.12C:Luminol and Isoluminol

The structure of luminol is depicted in figure 6a (206). It is a membrane permeant probe and therefore measures intra and extra-cellular ROS production. Luminol generates luminescence to a variety of free radicals (superoxide, hydrogen peroxide and hydroxyl radicals). Luminol needs to be reduced prior to becoming active, thus explaining why it is unable to detect superoxide production in patients with MPO deficiency, who should have increased superoxide production (207). It is for this reason that HRP (Horse Radish Peroxidase) is added to luminol and isoluminol solutions so that it is in excess.

As with the other luminescent probes luminol has some potential drawbacks. Luminol was able to inhibit the priming of neutrophils by aggregated IgG (208). The authors felt that luminol, a lipid soluble molecule, was disrupting the pathway signalling the priming. When isoluminol was substituted for luminol, priming was demonstrated again. This probably occurs as the change in position of the amine group (figure 6b) makes isoluminol lipid insoluble, and therefore unable to enter the cell and disrupt the intracellular pathway signalling priming.

Isoluminol is very similar to luminol in structure, function and practise. The difference is that it is solely extracellular; therefore as mentioned above it does not inhibit priming, but is only able to assay extracellular ROS.

A



B

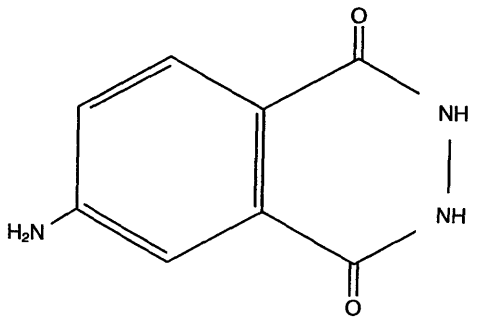


Figure 7, Structure of Luminol (A) and Isoluminol (B).

1.12D:General considerations

When using luminescence to measure oxidative burden in biological systems, the complexity of interactions involved needs to be borne in mind. The luminescent probes are not measuring the product of one enzyme, but potentially a number of enzymes contributing to the oxidative burden. Cells are not only a source of ROS but also have a number of scavengers (SOD, catalase and glutathione peroxidase) to mop up free radicals. ROS may also interact with NO and antioxidants vitamins or medication, neither of which are an issue in cultured lymphoblasts, but become problematical in studying neutrophils and endothelial cells (206). The luminescent probes, particularly Luminol, are pH and temperature dependent (209).

As well as considering the complexity of interactions of the luminescent probes and cells, the composition of the solutions used may be significant. As has been previously mentioned trace elements (copper, iron, cobalt and amino acids) may act as oxidants (206), possibly explaining the high background seen with CLA (204).

1.12E:Summary of chemiluminescence

1. Lucigenin and CLA are thought to be superoxide specific.
2. Isoluminol detects extracellular ROS.
3. CLA used instead of lucigenin as it is more sensitive.
4. Luminol inhibits the process of priming.
5. Luminol in particular is pH and temperature sensitive.
6. The probes are measuring the outcome of a complex interaction of radical producers and scavengers.

1.13:Brief Aims of Project

1. To confirm excess free radical production in 12 hypertensive (HT) subjects compared to 12 normotensive (NT) controls.
    a. 3 chemiluminescent probes (Luminol, Isoluminol and CLA) were used to check reproducibility of ROS production and to see if ROS production was predominantly intra or extracellular.
    b. Basal ROS production was assayed.
    c. AA and TPA will also be used to stimulate ROS production because it is possible that various triggers could inappropriately activate neutrophil or macrophage ROS production in hypertension. AA is thought to directly activate NADPH oxidase and TPA acts indirectly, therefore these 2 agonists may help to show if NADPH oxidase or the activation pathway is upregulated in hypertension.
    d. Tiron and catalase were used to see which ROS are produced and to check the specificity of the CL probes.
    e. DPI and Rotenone were used to confirm that a non-mitochondrial flavoprotein, presumably NADPH oxidase, was producing the ROS.
2. To compare NADPH oxidase subunit expression in NT and HT subjects.
    a. Using western blot analysis of p22phox, p47phox, gp91phox and rac2 to see if there was differential expression in the HT compared to the NT group.
3. To analyse differences in HT and NT cell line protein tyrosine phosphorylation.

a. Using western blot analysis of general tyrosine phosphorylation. This gave the advantage of highlighting possible areas of interest
b. Using western blot analysis to study potential proteins of interest, highlighted in 3a. We studied p44/42 MAPK phosphorylation.
c. The western blot analysis of general tyrosine phosphorylation and p44/42 MAPK phosphorylation was performed in basal and activated states, to see if priming of tyrosine phosphorylation is present in the basal or activated cells.

4. To examine possible reversal of the priming of HT lymhoblasts, using inhibitors of Tyrosine Kinase (Genistein, Herbimycin A & Tyrophostin), p44/p42 MAPK (PD98059), p38 MAPK (SB203580), PI3K (Wortmannin) and $cPLA_2$ (MAFP & Mepacrine). As these enzymes have been implicated in activation and priming of NADPH oxidase, we would expect a proportionally larger attenuation of ROS production if priming was causing increased ROS production. Aims 3 to 4 are an attempt to see if lymphoblast NADPH oxidase is primed to produce more ROS.

Chapter 2: Materials, Methods and Results

2.1A:Chemicals

A23187, AA, Bovine Serum Albumin (BSA), DPI, Foetal Calf Serum, Genistein, Herbimycin A, Histopaque 1077, Horseradish Peroxidase type II (HRP), Luminol, L-α-Lysophosphatidic Acid (LPA), N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP), RPMI 1640, Sodium Orthovanadate, 12-O-Tetradecanoylphorbol-13-Acetate (TPA), Tissue Culture Media 199, Tyrphostin A25, Wortmannin and Zymosan were all purchased from Sigma (Dorset, UK). Isoluminol was purchased from Fluka, 2-methyl-6-phenyl-3, 7dihydro-imidazo[1,2-a]pyrazin-3-one (CLA) from Tokyo Kasei Kogyo Co. Rac 2 polyclonal antibody was supplied by Santa Cruz Biotechnology Inc (California, USA), the polyclonal rabbit phospho p44/p42 MAP kinase antibody was purchased from Cell Signal and the monoclonal mouse phospho-tyrosine antibody was supplied by Transduction Laboratories (USA). Anti-rabbit IgG conjugated to horseradish peroxidase was supplied by Amersham Life Science (Little Chalfont, UK). Calbiochem (Dormstadt, Germany) supplied the PD98059, SB 203580 and mepacrine (also known as quinacrine). Methyl arachidonyl fluorophosphonate (MAFP) was purchased from Biomol (Plymouth Meeting, Philadelphia, USA).

2.1B:Tissue Culture

Lymphoblast cultures had previously been established (198). Briefly, subjects were unrelated, non-diabetic whites from Boston, Massachusetts or Leicester, UK. Systolic and diastolic (fifth Korotkoff sound) blood pressure was the average of two results taken 5 minutes apart with the patient seated. Samples from non-diabetic whites were taken as both diabetes (210) and African race (211) can be associated with increased ROS formation. Hypertension was defined as a diastolic greater than 90mmHg or a systolic of 140mmHg or more (WHO definition of hypertension). Secondary hypertension was excluded on the basis of clinical examination and biochemical evaluation. A family history of hypertension was established if either of the parents had been diagnosed as being HT and/or were on antihypertensive therapy. This information was obtained either directly from the parent or from the study subject. We recognize that the information on family history of HT may be inaccurate because of recall bias. Other than HT the subjects were otherwise well.

Fasting blood samples were taken for immortalisation of the lymphocytes after gaining informed consent from the study subjects. The peripheral lymphocytes were suspended in Iscove's modified Dulbecco's growth medium and were transformed using Epstein Barr Virus, the process being facilitated by cyclosporin A. The immortalised cells were harvested by centrifugation (800rpm for 7 minutes) suspended in complete growth media and stored in liquid nitrogen.

The Samples from Boston Massecheusetts were transported on dry ice and then cultured in growth medium in Leicester.

The lymphoblasts were recovered from storage and maintained in RPMI 1640 growth medium containing glutamine, Penicillin and streptomycin and 10% fetal calf serum at a cell density of less than 10^6 cells per ml. The cells were incubated at 37°C in an atmosphere of 4% CO_2 and 96% O_2. A study group of 12 HT patients and 12 age and sex matched NT subjects were chosen at random. Cells were studied between passages 20-30.

Aliquots were taken on the day of experiment and cells were recovered by centrifugation (1400rpm for 10 minutes). The cells were spun through Histopaque 1077 at 1400rpm for 10 minutes to isolate the viable cells and washed in TC199 before being suspended in 1-5mls of TC199. The cells were stored at 37°C prior to the experiments. The cell number was estimated using a Beckman Coulter Counter.

2.1C:Lymphoblast Agonists

A 50mM solution of AA was made up in distilled H_2O, 20µl aliquots were freeze dried then stored in liquid nitrogen. A stock solution of fMLP was made up in DMSO (Dimethyl Sulfoxide) and stored at –70°C. Lysophosphatidic acid stock solution was made up in distilled H_2O to a concentration of 1mM and stored at -20°C. Opsonified zymosan was formed by sterilizing zymosan by boiling it in

Hanks Buffered Salt Solution (HBSS) for 20 minutes. The zymosan was washed with further HBSS and then incubated with 2mls of fresh human serum at 37°C for 30 minutes. The opsonified zymosan (OZ) was washed in further HBSS and then reconstituted in HBSS at a concentration of 10mg/ml and stored at –70°C. Pervanadate was formed from 25mM sodium vanadate and 25mM H_2O_2, excess H_2O_2 was degraded using catalase. TPA stock solution was made up in DMSO to a concentration of 2mM and stored at –20°C.

2.1D:Chemiluminescence

ROS estimation was based on the technique described by Liu (212). The detection of ROS was enhanced by using CLA (203;205), luminol and isoluminol (208) CL (Chemiluminescence), and recorded using an EG & G Berthold microplate luminometer LB96V. The frequently used CL probe lucigenin was also used but was found to be insufficiently sensitive to detect ROS production from lymphoblasts using the LB96V luminometer. All experiments were performed at 37°C. The luminescent probes were made up on the day in a balanced salt solution containing NaCl 140mM, HEPES 15mM, KCl 5mM, glucose 5mM, $CaCl_2.2H_2O$ 1.8mM and $MgSO_4.7H_2O$ 0.8mM. The final concentrations were luminol 5µM, isoluminol 50µM and CLA 1µM. The solutions of luminol and isoluminol contained 6 units HRP/ ml as luminol and isoluminol need to be reduced before they are able to detect ROS (Section 1.12C). The experiments were performed in triplicate, with 10^5 cells per well.

A number of agonists were assessed prior to use of AA and TPA on all the cell lines. The agonists A23187 (concentration 5nM to 50μM), fMLP (concentration 100nM) and LPA (concentration 100nM) had no detectable effect on ROS production as assessed by CLA, luminol and isoluminol CL. OZ (concentration 31.25μg/ml to 1mg/ml) produced very inconsistent results and was therefore not used as an agonist. Very high background CL counts were encountered with pervanadate (concentration 1μM). The high background counts will have been due to the presence of residual H_2O_2 from the manufacture of the pervanadate. The high background count still persisted despite attempting to remove the excess H_2O_2 with catalse.

The lymphoblasts were stimulated with both 500nM TPA and 50μM AA. The dose of TPA used was as previously used by Dr. VM Lee, retrospectively dose response curves were performed in 5 NT and 5 HT cell lines for both AA and TPA and it was found that the dose used was supra maximal (figure 7). The dose response curves for AA were performed prior to stimulation experiments and are shown in figure 7. A dose of 50μM AA was chosen due to concern that AA can act as a detergent and damage the cell membrane at high concentrations and therefore produce nonspecific unwanted effects.

The peak ROS production was used for AA stimulated ROS production, as there was a rapid rise in ROS generation followed by a rapid decay in ROS formation. The area under the curve was used to calculate TPA stimulated ROS

production as there was often a slow steady rise in ROS production, the formula is shown in Equation 3 and to standardize the value it was divided by the total assay time.

Equation 3 $$AUC = \tfrac{1}{2} \sum_{i=0}^{n-1} (t_{i+1}-t_i)(y_i+y_{i+1})$$

The 3 methods of CL correlated well with each. For AA stimulated ROS production r=0.79 for the correlation of Luminol with CLA, r= 0.96 for the Luminol with Isoluminol and r=0.74 for CLA with Isoluminol, all $P<0.001$. For TPA stimulated ROS production r=0.75 for the correlation of Luminol with CLA, r= 0.63 for the Luminol with Isoluminol and r=0.55 for CLA with Isoluminol, all $P< 0.01$.

The coefficiant of variation (CV) of ROS production from individual cell lines with each probe ranged from 23% with Luminol, 47% with CLA and 69% with Isoluminol. The poor CVs with CLA probably result from the high background and with Isoluminol because of the overall reduced extracellular ROS production.

One concern we had was that the different cell lines would be in different phases of their lifecycle and that this might influence the ROS production results. We therefore quiesced 6 NT and 6 HT cell lines by incubating aliquots of the chosen cell lines over night in quiescing media (RPMI with 10µg/ml insulin, 5µg/ml of transferring and 0.1% BSA). On the day of the experiments the cell lines were processed as described above and luminol CL was performed. The NT cell lines mean ROS production of non-quiesced cells was 773.3 (358.7) RLU

and 553.3(282.5) RLU for the quiesced cells. The HT cell lines ROS production for the non-quiesced cells was 703.3 (138.8) RLU and for the quiesced cell lines 565.0 (214.8) RLU. As there was no differential effect of quiescing these 12 cell lines it did not become a routine protocol.

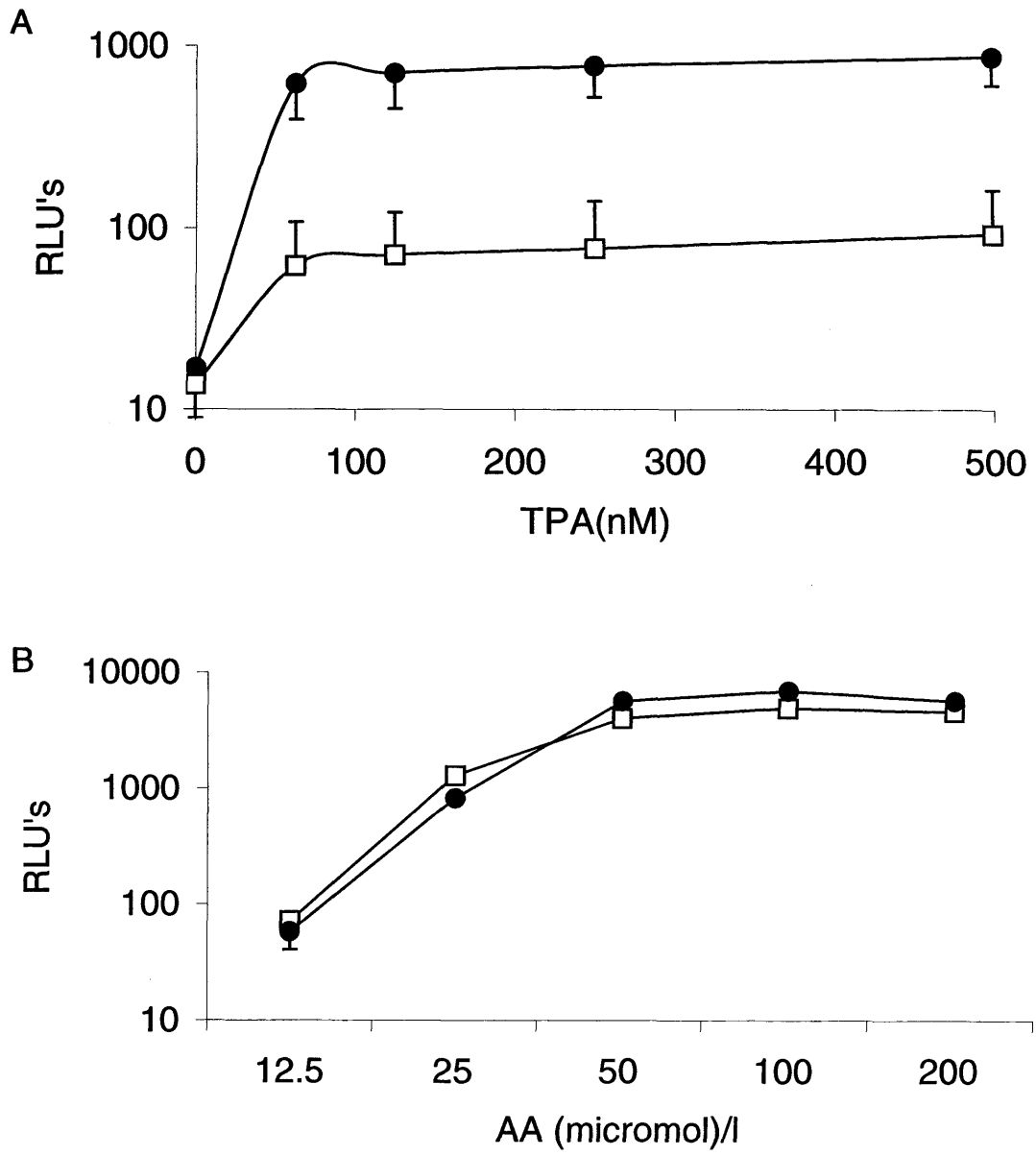


Figure 7, Representative TPA and AA dose response curves. Luminol enhanced chemiluminescance. The points represent peak ROS production for each stimulant dose and are the mean (SEM) of three recordings for an individual cell line. • represents HT cell line, □ represents NT cell line.

2.1E:Inhibitors

For the CL experiments with inhibitors, the concentrations and minimum incubation times are shown in table 1, the times and concentrations were based on predominantly neutrophil data and are referenced in the table.

Inhibitor	Target	Concentration	Incubation	Reference
Genistein	Tyrosine Kinase	100μM	20min	(185;213;214)
Tyrphostin A25	Receptor linked Tyrosine Kinase	100μM	20min	(215;216)
Herbimycin A	Src type Tyrosine Kinase	10μM	20min	(217)
PD98059	MEK (p44/p42 MAPK)	50μM	10min	(217;218)
SB203580	p38 MAPK	30μM	60min	(219)
Wortmannin	PI3K	100nM	15min	(220)
Mepacrine	PLA_2	100μM	30min	(189)
MAFP	$cPLA_2$	250nM	10min	(221)

Table 2. Inhibitor concentrations and incubation times.

2.1F:Antibodies

Polyclonal antibodies to $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and $gp91^{phox}$ were developed within the department using the following techniques. p22-Glutathione-S-Tranferase fusion protein was produced in E Coli transfected with the plasmid (obtained from Prof D Roos, the University of Amsterdam). The fusion protein was purified on a Glutathione Sepharose slurry column and eluted with free 10mM glutathione buffered by 50mM Tris to pH8.0. $p47^{phox}$ and $p67^{phox}$ were produced in SF9 insect cells infected with recombinant baculovirus expressing $p47^{phox}$ and $p67^{phox}$ (a gift from Dr David Lambeth, Emory University) and purified by ion exchange chromatography as described by Leto et al (222). The $gp91^{phox}$ was a thioredoxin-fusion protein obtained from Dr. David Lambeth (Emory University). Anti sera to the recombinant phox components were raised in rabbits by monthly subcutaneous injections of 500μg of the various proteins. After collection of the serum the antibodies were purified by protein A columns and eluted with 100mM Glycine, pH2.5. The specificity of the $p67^{phox}$ antibody was determined by performing 2 Western blots resolving the same protein extracts on each gel, one probed with the $p67^{phox}$ antibody alone (5μg/ml) and the other probed with a combination of the antibody (5μg/ml) and the antigen (10μg/ml) (*figure 8*). The band of interest was taken as the band whose intensity was decreased in the antigen blocked blot. The $p47^{phox}$ antibody produced a single band (*figure 9*).

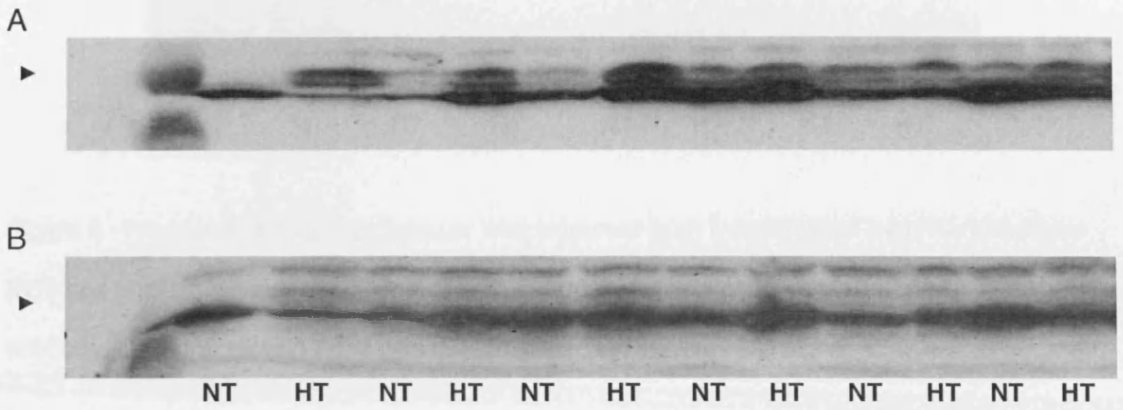


Figure 8. Western blot of 5μg protein extracted from lymphoblasts derived from Normotensive (NT) and Hypertensive (HT) subjects In figure A The Western blot was probed by 5μg/ml of $p67^{phox}$ antigen and figure B was probed by $p67^{phox}$ antigen and 10 μg/ml of antibody. Along the left hand lane of blots A and B Life science molecular weight markers have been resolved the 66kDa marker is shown (►). Figures A and B represent separate western blots performed simultaneously and the wells contain the same lymphoblast extract both containing 50μg of protein extract.

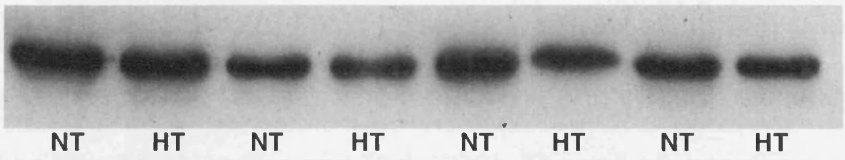


Figure 9. Western blot 1µg of protein per well extracted from lymphoblasts from Normotensive (NT) and Hypertensive (HT) subjects. The Western blot was probed with 1µg/ml of $p47^{phox}$ antibody. Only one protein band was demonstrated on the Western blot at 47 kDa.

To determine which was the specific band for $p22^{phox}$ and $gp91^{phox}$ a western blot was performed of whole cell protein extract along with cytosolic fraction and a membrane fraction. The membrane and cytosol fractions were separated as described by Abo and Pick (223). The cell suspensions were sonicated 3 times for 10 seconds, the homogenate was spun at 300g and the post nuclear fraction was retained. The post nuclear fraction was separated into cytosol (supernatant) and membranes (pellet) by centrifugation at 50 000g for 1 hour. The $p22^{phox}$ and $gp91^{phox}$ bands were enriched in the membrane fractions but absent or diminished in the cytosolic fractions (figures 10 & 11).

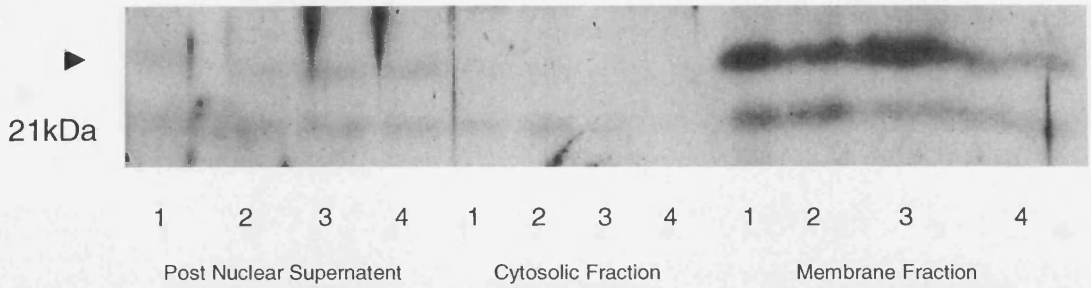
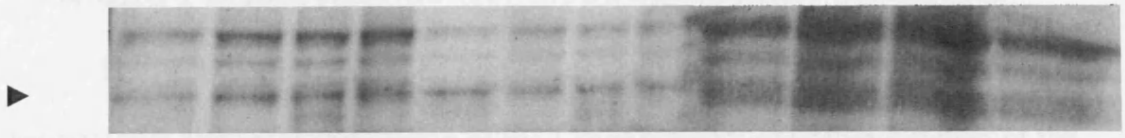


Figure 10. Western blot of protein extracted from U937 (Monocyte) cell line, probed for $p22^{phox}$. The U937 cell lines were fractionated by sonication and differential centrifugation. The ►marks the band that is deficient in the cytosol fraction and enhanced within the membrane fraction.



1 2 3 4 1 2 3 4 1 2 3 4

Post Nuclear Supernatent Cytosolic Fraction Membrane Fraction

Figure 11. Western blot of protein extracted from U937 (Monocyte) cell line, probed for $gp91^{phox}$. The U937 cell lines were fractionated by sonication and differential centrifugation. The ► marks the band that is deficient in the cytosol fraction and enhanced within the membrane fraction.

2.1G:SDS-Polyacrylamide Gel Electrophoresis

The technique was based on that described by Laemmli (224). For experiments requiring stimulation of the lymphoblasts, prior to extraction of the protein, a 1ml aliquot of cells in TC199 would be incubated with 500nM TPA, whilst the paired control was incubated in 1ml TC199. All control and stimulated cells were incubated at 37°C for 5 minutes. 50-500µg protein extracts from the lymphoblast cell lines were resolved on 7.5-15% SDS polyacrylamide gels and the resolved proteins were western blotted onto nitrocellulose.

For the assay of the phox and rac subunits the resolved proteins were probed with the polyclonal phox antibodies in PBS tween with 5% Marvel, for 1 hour at room temperature. The concentration of the antibodies was as follows:- $p22^{phox}$ 10µg/ml with 5% horse serum, $p47^{phox}$ 1µg/ml, $p67^{phox}$ 5µg/ml, $gp91^{phox}$ 10µg/ml and rac2 0.2µg/ml. The primary antibody was labeled with anti rabbit IgG conjugated to horseradish peroxidase diluted 1 in 1500 in PBS tween and 5% marvel, for one hour at room temperature.

For assays of phosphorylated MAPK and tyrosine kinase the resolved proteins were western blotted onto nitrocellulose, and probed with the primary antibodies in PBS tween with 3% BSA, for 1 hour at room temperature. Both the phospho-p44/p42 MAP kinase and the phospho-tyrosine antibodies were diluted 1:1000. The phospho-p44/p42 MAP kinase was labeled with anti rabbit IgG conjugated to horseradish peroxidase, diluted 1 in 1500 in PBS tween and 3%

BSA, for one hour at room temperature. The phospho-tyrosine antibody was detected using anti-mouse IgG conjugated to horseradish peroxidase, diluted 1:2500 in PBS tween and 3% BSA, for one hour at room temperature.

The protein bands were detected using enhanced chemiluminescence kits from Amersham, and band intensities analyzed on a BioRad Densitometer.

2.1H:Statistics

Each cell line underwent CL on 3 occasions on separate days for all conditions. Chemiluminescent values are presented as means of the log relative light units (logRLU) and SEMs unless otherwise stated. For the experiments with the inhibitors the percentage reductions in ROS production were calculated from untransformed RLU/min results. The Western blot results are presented as arbitrary densitometry units, and represent the mean and SEM of the standardised results from at least 3 Blots. Two tailed p values <0.05 were considered significant. The two-sample t test, Mann Whitey test and Spearman Correlation Coefficient were calculated using Minitab (Minitab Inc., PA). For the variation in ROS production found, the experiments have greater than 90% power to detect a 50% difference, at $p<0.05$. The Power of the study was estimated based on preliminary data of TPA stimulated ROS production assayed by luminol enhanced CL (NT ROS production 569.4 RLU, HT ROS production 1250.5 RLU, giving a difference of 681.0 RLU and a standard deviation of 487.0 RLU).

Chapter 2.2: Results

2.2A:ROS Production

2.2A(i):Study Subject Characteristics

The NT and HT groups were well matched for age and gender (Table 3). The systolic blood pressure (SBP) was 115(4) mmHg in NT subjects and 149(2) mmHg in the HT group ($P<0.01$); diastolic blood pressure (DBP) was 77(2) mmHg in the NT group and 91(2) in the HT subjects ($P<0.01$). Other than HT the subjects had no other medical complaints. We have limited other data on the NT and HT groups. The HT group has slightly increased creatinine levels but this is still within the normal range. Statistically there are no other significant differences. There is a tendency for the HT group to have a slightly increased fasting glucose and triglyceride. There is no data on smoking status or dietary history of subjects.

	NT	HT	
AGE	51.8(4.1)	59.5(3.8)	
SBP	115(4)	149(5)	P<0.001
DBP	77(2)	91(2)	P<0.001
n	12	12	
MALES	5	5	
Family history of HT	0	6	
Antihypertensive medication	0	3	
Creatinine (µmoles/L)	69.3(5.8)	94.71(8.12)	P=0.03
Fasting Plasma Glucose (mmol/L)	3.89(0.37)	4.62(0.43)	P=0.23
Cholesterol (mmol/L)	4.81(0.31)	5.68(0.43)	P=0.14
High Density Lipoprotein (mmol/L)	0.97(0.13)	1.00(0.07)	P=0.84
Triglycerides (mmol/L)	1.53(0.43)	2.82(0.57)	P=0.11

Table 3. Baseline characteristics of the study population.

2.2A(ii):Baseline CL

Aims of Baseline CL.

To assay baseline ROS production in the NT and HT cell lines. Three CL probes were used as there are potential disadvantages of each of the luminescent probes. The differences between the probes are: CLA is thought to be specific for superoxide production, Luminol and Isoluminol are less specific, Isoluminol is thought to measure extracellular ROS and Luminol under certain conditions has inhibited priming.

Results of Baseline CL.

Basal levels of ROS production with CLA (NT 178(12) vs. HT 182.5(16.1) P=0.83), luminol (NT 14.5(1.4) vs. HT 12.9(2.6) P=0.60) or isoluminol (NT 21.1(3.9) vs. HT 34.4(9.5) P=0.21) revealed no significant difference between NT and HT cell lines, results expressed as the mean RLU± SEM of 12 NT and 12 HT cell lines.

Discussion of Baseline CL.

The 3 CL probes were unable to detect a difference between the basal NT and HT cell lines. As lymphoblast ROS production is 5% of the equivalent neutrophil ROS production it is possible that the assays are insufficiently sensitive to detect a true difference. We therfore studied stimulated ROS production.

2.2A(iii):AA and TPA Stimulated ROS Production

Aims of AA and TPA Stimulated ROS production

In vivo white cells release ROS as a response to environmental triggers classically infections, therefore it was perhaps to be expected that basal ROS production was low and not significantly dfferent in NT and HT cell lines. We therefore studied stimulated ROS production in the NT and HT cell lines. We chose two agonists that stimulate ROS production, TPA activates NADPH oxidase through PKC (123) and AA activates NADPH oxidase directly. Using 2 agonists allows us to demonstrate the reproducibility of the ROS production and as AA acts directly and TPA acts via a stimulation pathway If there is increased ROS production just with TPA it will suggest that the increased ROS production is due to upregulation or priming of the activation pathway and not NADPH oxidase. Characteristic traces of ROS production are included to illustrate the nature of the raw data.

Results: Characteristic CL Traces

Typical luminol, Isoluminol and CLA enhanced CL traces are shown for both AA stimulation (figures 12A, 13A and 14A respectively) and TPA stimulation (figures 12B, 13B and 14B) with the same NT and HT cell line in A and B for each figure. For stimulation with 50μM AA, the cell lines produced a rapid peak of ROS production between 45 and 90 seconds, and then declined to baseline. The peaks for luminol enhanced CL were NT 679.3 (31.6) RLU and HT 2718 (65.6)

RLU (figure 12A), for Isoluminol were NT 919.3(86.7) RLU and HT 1438.7(17.6) RLU (figure 13A) and with CLA were NT 836.3(11.6) RLU and HT 2113.3(8.8) RLU (figure 14A). The maximum rate of ROS production with TPA (500nM) reached a plateau much later, between 5 and 40 minutes. The area under the curve for these particular cell lines were NT 777.5 RLU and HT 2084.8 RLU with luminol enhanced CL (figure 12B), NT 913.4 RLU and HT 1371.6 RLU with Isoluminol (figure 13B) and NT 556.4 RLU and HT 1709.2 RLU when assayed by CLA enhanced CL (figure 14B).

Discussion of Characteristic CL Traces

AA caused a rapid rise in ROS production as might be expected from an agent activating NADPH oxidase directly. ROS production rapidly returned to normal post stimulation. Peak ROS production was used to calculate mean ROS production.

TPA caused a slow rise in ROS production peaking after more than 5 minutes, sometimes taking more than 30 minutes to reach a peak in action. Due to the slow rise in action and the variability in peak of action, area under the curve was used to more accurately assess total ROS production. Before presenting the data on the HT and NT group's ROS production, the following 2 sections attempt to identify the source and type of ROS produced.

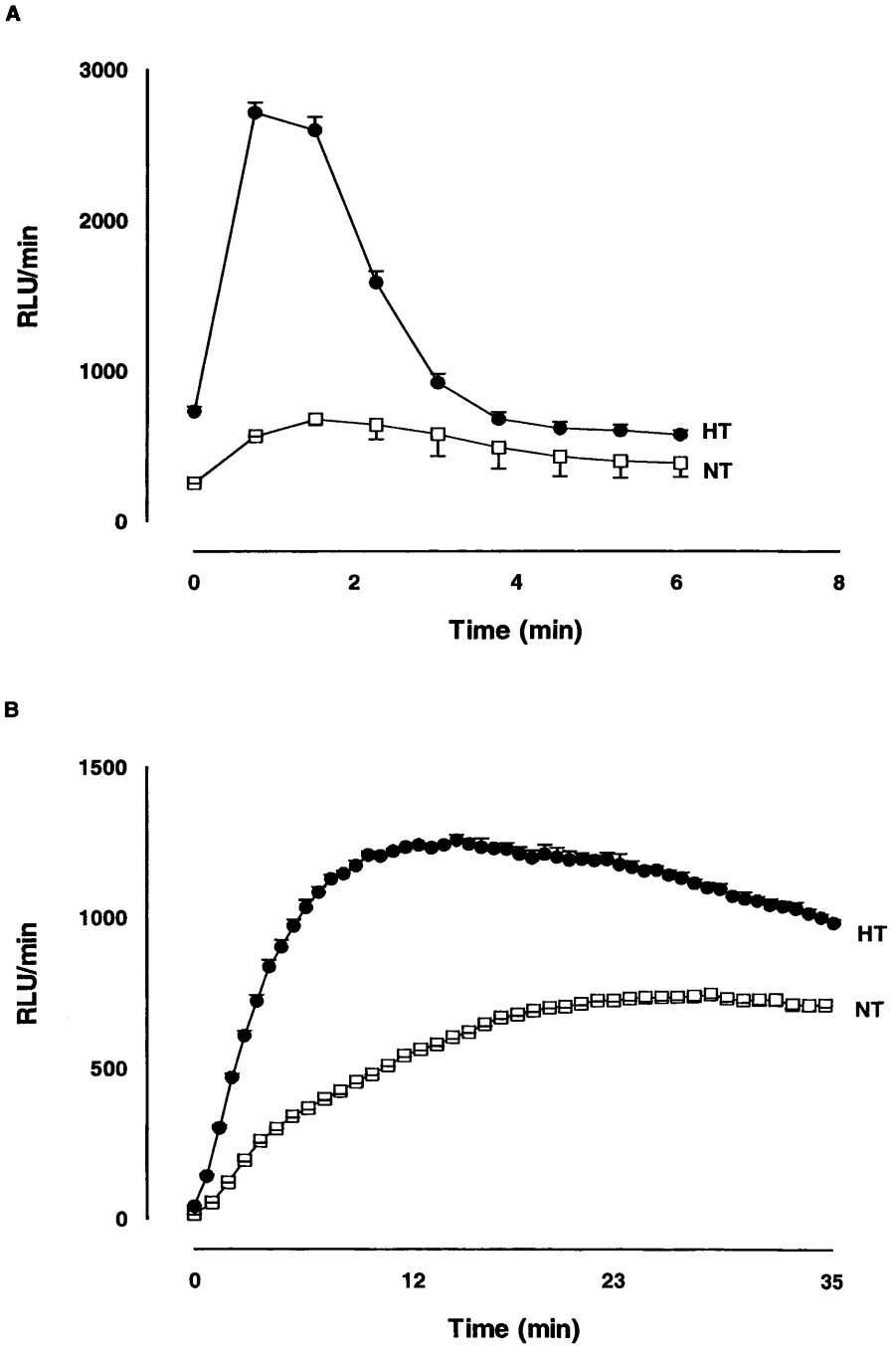


Figure 12, Luminol enhanced CL of AA (50μM) stimulated (A) and TPA (500nM) stimulated (B) cell lines. The results are from a single HT and NT cell line, the same cell lines in both A and B. The results for each cell line represent the mean of three separate aliquots containing 10^5 cells recorded simultaneously.

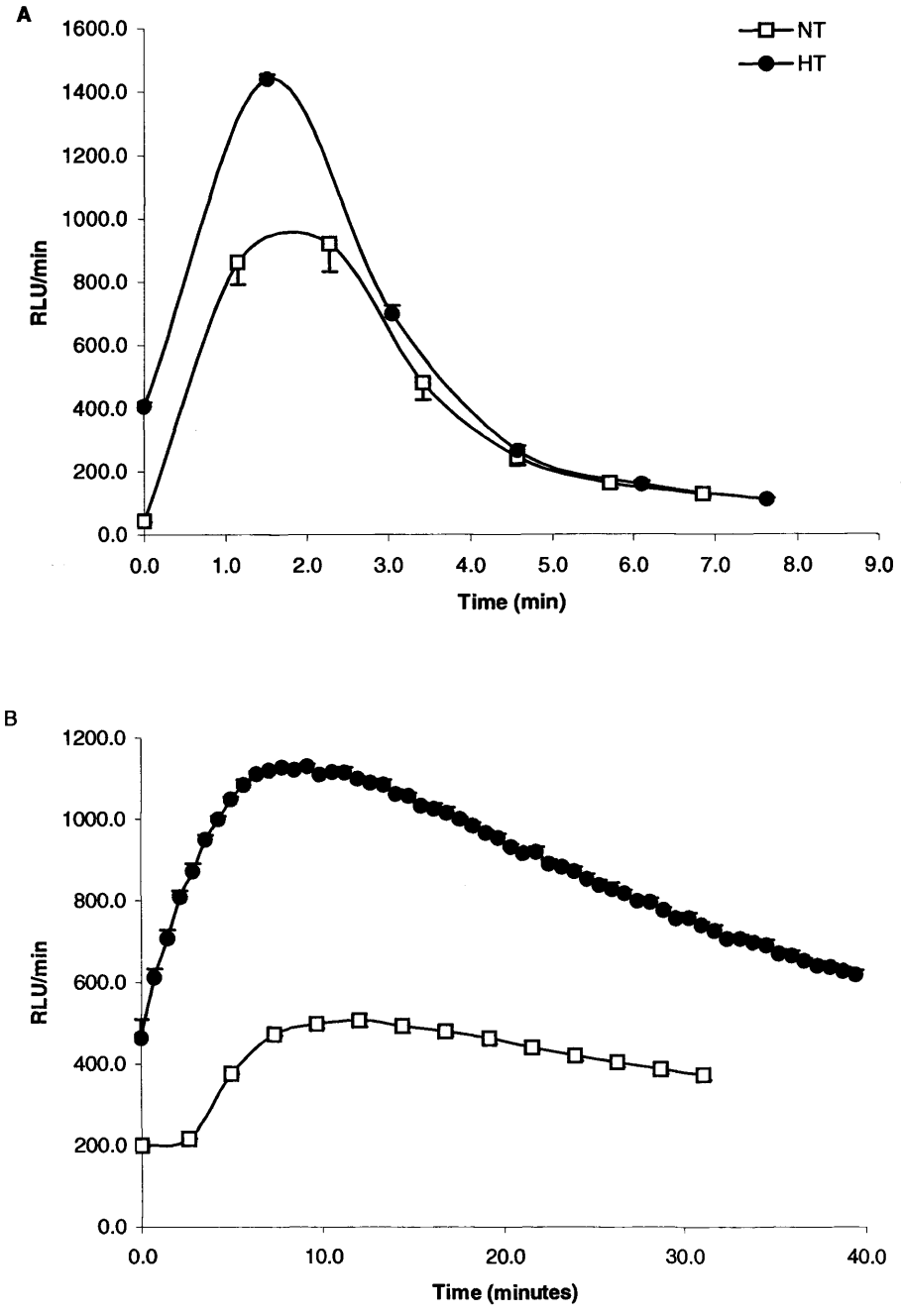


Figure 13. Isoluminol enhanced CL of AA (50μM) stimulated (A) and TPA (500nM) stimulated (B) cell lines. The results are from a single HT and NT cell line, the same cell lines in both A and B. The results for each cell line represent the mean of three separate aliquots containing 10^5 cells recorded simultaneously.

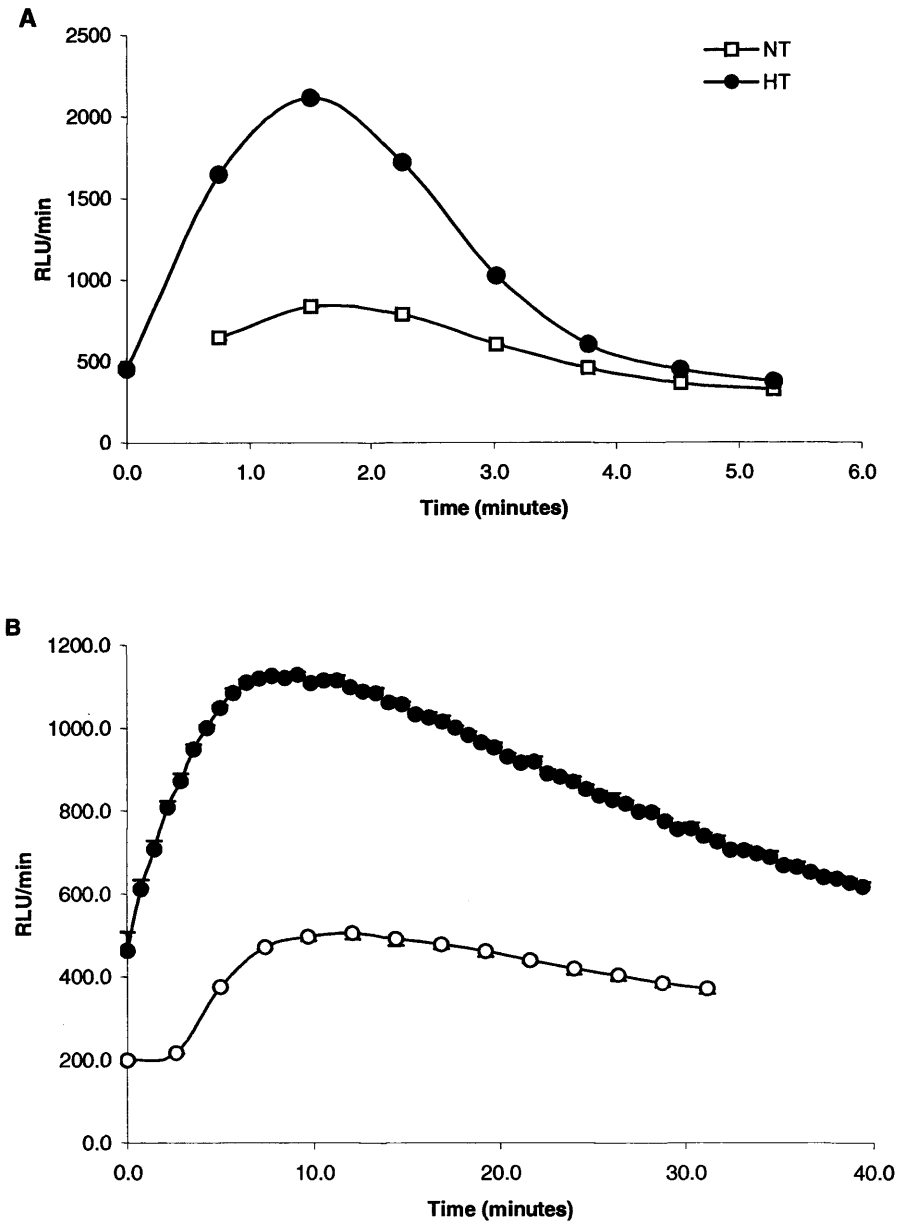


Figure 14. CLA enhanced CL of AA (50μM) stimulated (A) and TPA (500nM) stimulated (B) cell lines. The results are from a single HT and NT cell line, the same cell lines in both A and B. The results for each cell line represent the mean of three separate aliquots containing 10^5 cells recorded simultaneously.

2.2A(iv):Enzyme Inhibition of ROS Production

Aim of Enzyme Inhibition of ROS Production

The project was conceived with the assumption that there would be increased ROS production from HT lymphoblasts and that it would be due to priming or alterations in NADPH oxidase. Mitochondrial NADH oxidase and xanthine oxidase may also be a source of ROS. DPI is an inhibitor of flavoproteins such as NADPH oxidase and mitochondrial NADH oxidase. Rotenone inhibits the mitochondrial respiratory chain thus reducing mitochondrial ROS production. Inhibition of ROS production with DPI but not rotenone would suggest that NADPH oxidase is the source of ROS production.

Results of Enzyme Inhibition of ROS Production

The AA or TPA stimulated ROS production was abolished with 20μM DPI (*figure 15A*). 10μM rotenone had no effect on CLA or luminol enhanced CL, with either AA or TPA stimulated cells (*Figure 15B*). Rotenone reduced TPA stimulated ROS production measured with isoluminol, by a mean of 31(5)% but had no effect on AA stimulated ROS production.

Discussion of Enzyme Inhibition of ROS Production

DPI abolished ROS production whilst rotenone had no effect on ROS production, as detected by luminol and CLA CL. DPI inhibits flavoproteins and has been shown to inhibit ROS production from un-stimulated macrophage mitochondria (225). As the ROS production was inhibited by DPI but not

rotenone this shows that a non-mitochondrial flavoprotein is responsible for ROS production. In the context of the agonists used this strongly suggests that NADPH oxidase produces the increased ROS seen in HT. The 30% reduction in ROS production with TPA stimulated ROS production assayed with isoluminol, may represent inhibition of an energy requiring process, allowing TPA generated ROS to cross the cell membrane. Such a difference would not be apparent with the other CL probes as they are able to detect intra and extra cellular ROS.

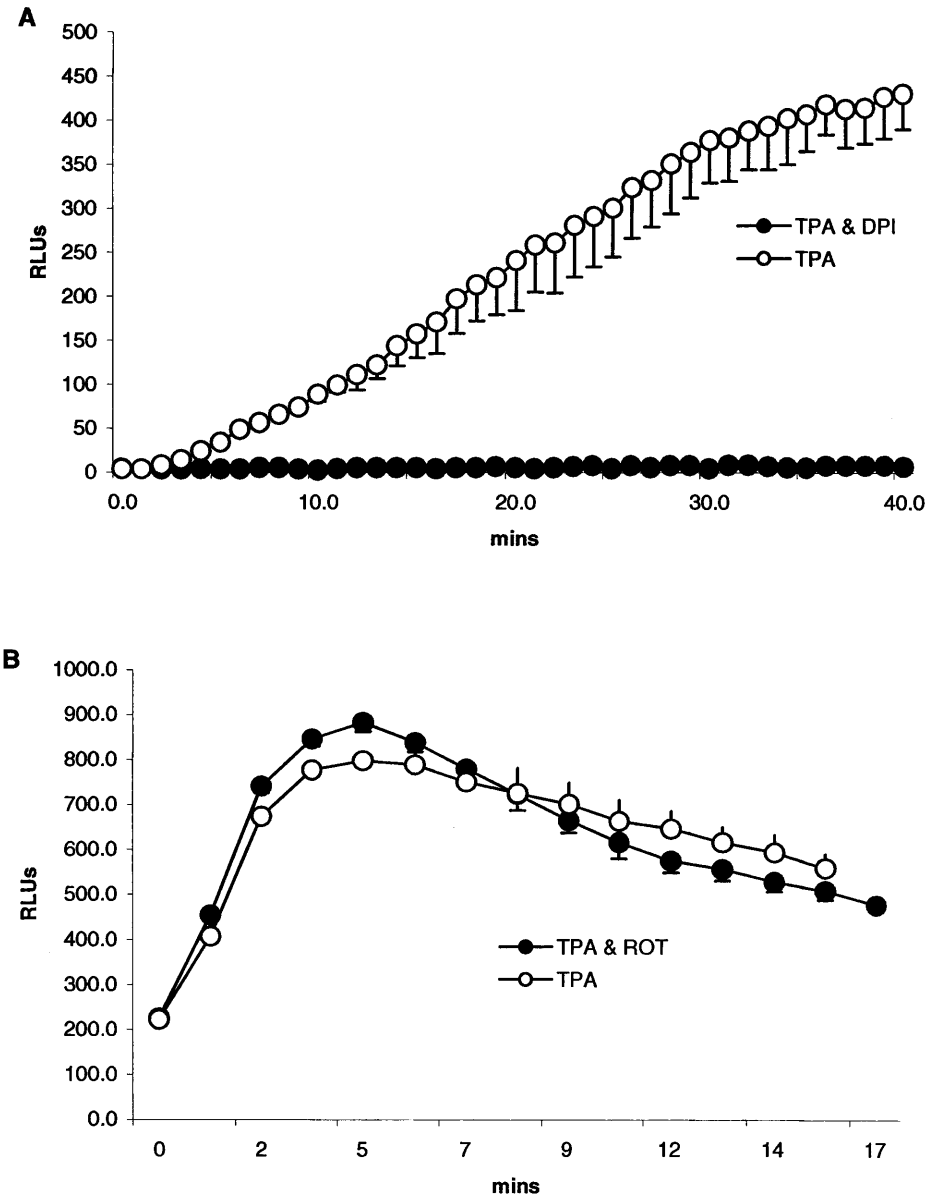


Figure 15. Luminol enhanced CL of TPA (500nM) stimulated different HT cell lines in A and B. The results for each cell line represent the mean (SEM) of three separate aliquots containing 10^5 cells recorded simultaneously. In A 20 μM DPI was used and an aliguot of cells in B were incubated with 50μM Rot for 20 minutes prior to stimulation with TPA.

2.2A(v):Super Oxide and Hydrogen Peroxide Chelators

Aims of Super Oxide and Hydrogen Peroxide Chelators

To assess if the stimulated ROS production is in the form of superoxide, hydrogen peroxide or a mixture we added chelators of superoxide and hydrogen peroxide. Tiron is a synthetic superoxide dismutase and catalase breaks down hydrogen peroxide. The chelators of ROS are also a check on the specificity of luminol, Isoluminol and CLA.

Results of Super Oxide and Hydrogen Peroxide Chelators

Tiron (10mM) abolished AA and TPA stimulated ROS production as detected by CLA, luminol and isoluminol (example trace shown in figure 16A). Catalase (300U/ml), the chelator of hydrogen peroxide, reduced but did not abolish detected ROS production (figure 16B). Catalase reduced stimulated ROS production of 2 NT and 2 HT cell lines; detected by CLA enhanced CL to 61.5(7.5)% for AA stimulation and to 55.5(8.7)% for TPA stimulation; for isoluminol enhanced CL to 53.9(5.4)% for AA stimulation and 62.1(3.1)% for TPA stimulation; and with luminol enhanced CL to 64.6(3.9)% for AA stimulation and 66.0(6.0)% for TPA stimulation.

Discussion of Super Oxide and Hydrogen Peroxide Chelators

The tiron abolished stimulated ROS production measured by CLA, luminol and isoluminol. This is in keeping with previous evidence (226-229). Catalase

reduces all stimulated ROS production by approximately 40%. This suggests that 40% of the superoxide produced by NADPH oxidase is converted to hydrogen peroxide by superoxide dismutase. The 40% reduction in CLA assayed ROS production is not in keeping with previous results (203), this suggests in this system CLA is detecting both superoxide and hydrogen peroxide.

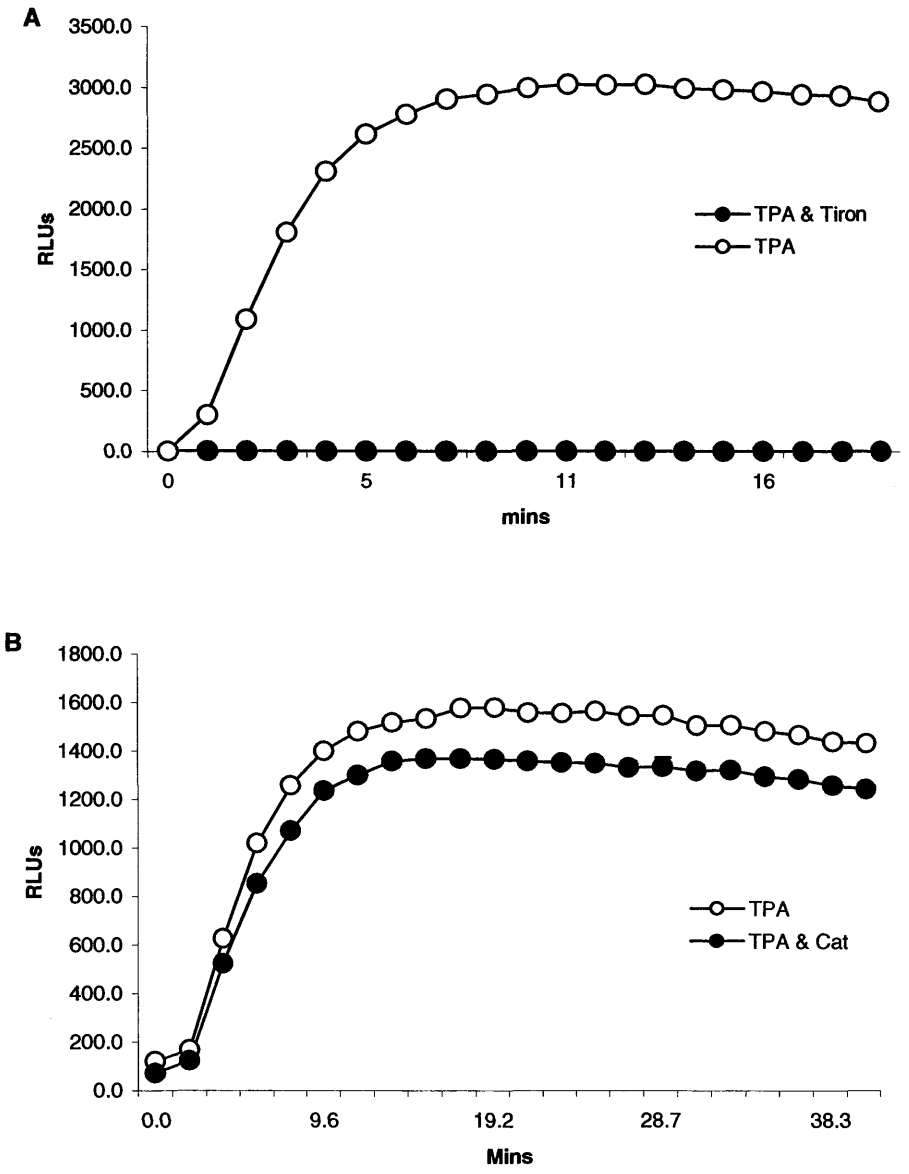


Figure 16, Luminol enhanced CL of TPA (500nM) stimulated different HT cell lines in A and B. The results for each cell line represent the mean (SEM) of three separate aliquots containing 10^5 cells recorded simultaneously. In A 10 mM Tiron was used and in B 300U/ml of catalase was used.

2.2A(vi):NT and HT ROS production

Aims of NT and HT ROS production

As there was no detectable increase in basal ROS production from HT lymphoblasts we then studied stimulated ROS production. In vivo white cells release ROS in response to stimuli such as infections. It is possible that chronic low-grade infections or inflammation could be a trigger for white cell ROS release in HT and atherosclerosis. We found that the lymphoblast agonists TPA and AA both produced reliable ROS production. TPA activates NADPH oxidase indirectly through PKC whilst AA acts directly on NADPH oxidase. We were keen to use a number of agonists; this allowed us to study direct and indirect activation of NADPH oxidase. Three chemiluminescent probes were used as they each measure different reactive oxygen species and isoluminol only measures extracellular ROS. Luminol may also inhibit priming therefore we did not want to use Luminol alone.

Results of NT and HT ROS production

As the stimulated CL results were not normally distributed as determined by a Anderson-Darling Probability plots, we used a log transformation to normalise the data and all results are expressed as mean (SEM) log RLUs, the normality of the log transformed data was confirmed by repeating the probability plot of the logged data. We found that stimulation of the cell lines with 50μM AA resulted in a significantly higher ROS production in HT, compared to NT cell lines (figure 17A) for CLA enhanced CL (NT 2.99(0.06) vs. HT 3.30(0.06) $P<0.01$), luminol enhanced CL (NT 2.60(0.18) vs. HT 3.26(0.12) $P<0.01$) and with

isoluminol enhanced CL (NT 2.64(0.14) vs. HT 3.04(0.12) P<0.05). When stimulating with 500nM TPA, we were able to detect a significantly increased ROS production in HT cell lines, compared to NT cell lines (figure 17B) with CLA (NT 2.59(0.05) vs. HT 3.14(0.03) P<0.001), luminol (NT 2.51(0.17) vs. HT 3.13(0.08) P<0.003) but not with isoluminol (NT 2.56(0.15) vs. HT 2.81(0.13) P=0.22).

Discussion of NT and HT ROS production

AA stimulated ROS production was always increased in the HT compared to the NT cell lines when estimated by all the chemiluminescent probes. TPA stimulated ROS production was significantly greater in the HT group when assayed by CLA and luminol. We were unable to detect a significant difference in TPA stimulated ROS production between HT and NT cell lines using isoluminol. If the difference between the TPA stimulated ROS production in the NT compared to HT groups is less than 50% the study would be under powered to detect a genuine difference. Isoluminol is only able to assay extracellular ROS whereas CLA and luminol can assay both intra and extracellular ROS production therefore it is possible that although TPA results in increased ROS production but not release.

For increased stimulated ROS production in HT to be significant clinically, there needs to be a stimulus for ROS generation. In hypertension, AGII can activate neutrophils to form ROS (8), providing a potential trigger for ROS generation. Established atheroma is now thought to be a lowgrade inflammatory

process, so it is possible that a factor present in the inflammatory milieu could activate NADPH oxidase. For the increased ROS production to have a clinical impact the stimulus must also result in a significant extracellular release of ROS.

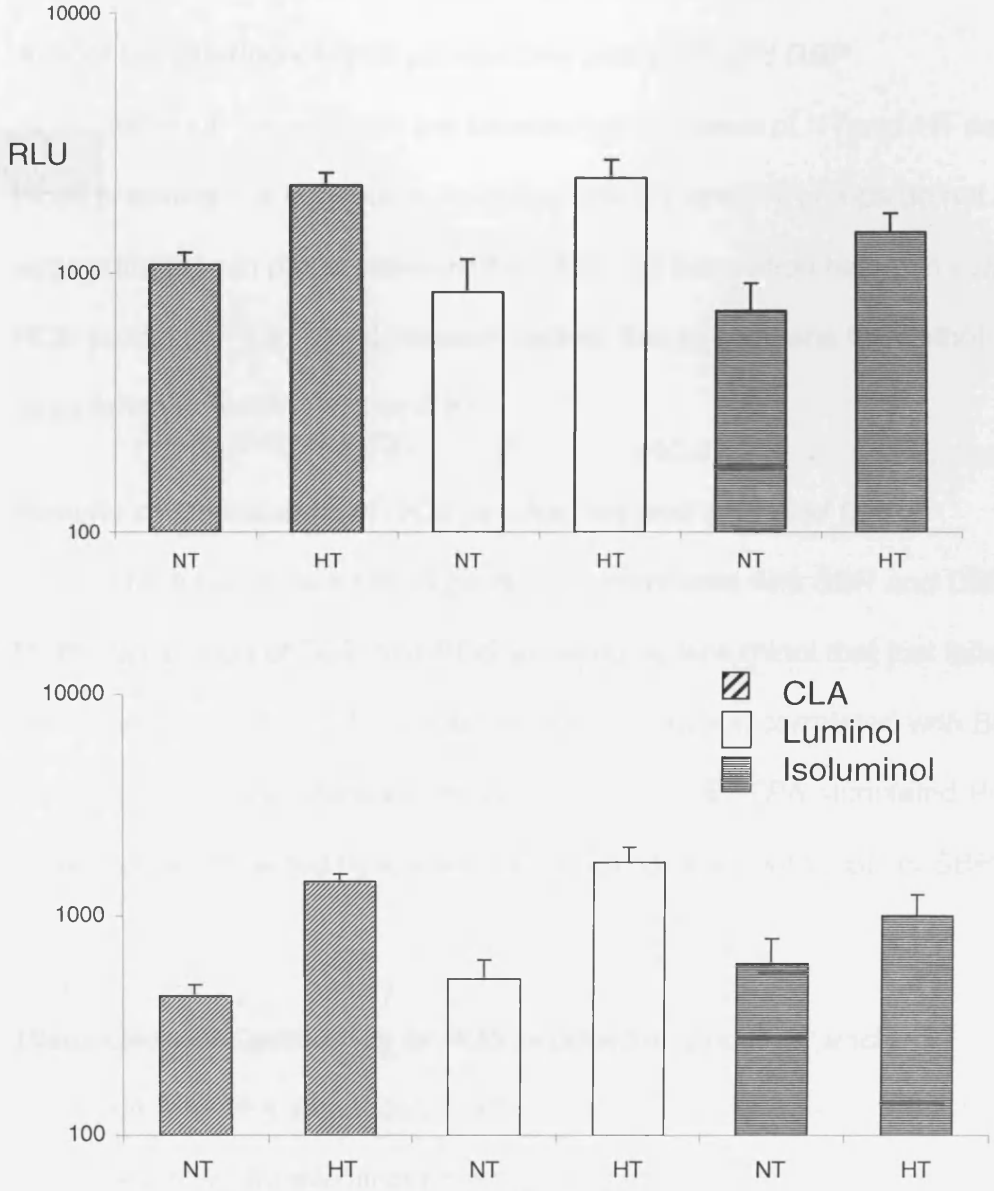


Figure 17, Peak Enhanced CL with CLA, luminol and isoluminol, stimulated with 50µM AA in (A) and area under the curve for 500nM TPA in (B). The results represent the mean of 12 NT and 12 HT cell lines. The result for the individual cell lines also represented the mean of the 3 separate recordings on different days.

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2.2A(vii):Correlation of ROS production and SBP and DBP

Aim of Correlation of ROS production and SBP and DBP

Although the subjects are selected on the basis of NT and HT categories, blood pressure is a continuous variable. The NT and HT groups do not have a large difference in blood pressure therefore if a correlation between stimulated ROS production and blood pressure exists, this strengthens the pathological association between ROS and HT.

Results of Correlation of ROS production and SBP and DBP

The AA stimulated ROS production correlated with SBP and DBP except for the correlation of DBP and ROS assayed by Isoluminol that just fails to be significant (Table 4). TPA Stimulated ROS production correlated with BP when detected by CLA and luminol enhanced CI (Table 5). TPA stimulated ROS production as detected by isoluminol did not correlate with DBP or SBP (Table 5).

Discussion of Correlation of ROS production and SBP and DBP

AA and TPA stimulated ROS production significantly correlates with DBP and SBP except for isluminol enhanced CI that can only detect a correlation for SBP and DBP with AA stimulated ROS production. The inability for Isoluminol to detect a correlation with BP and TPA stimulated ROS production is to be expected as it gives reduced separation between the NT and HT groups probably because it only detects extracellular ROS production.

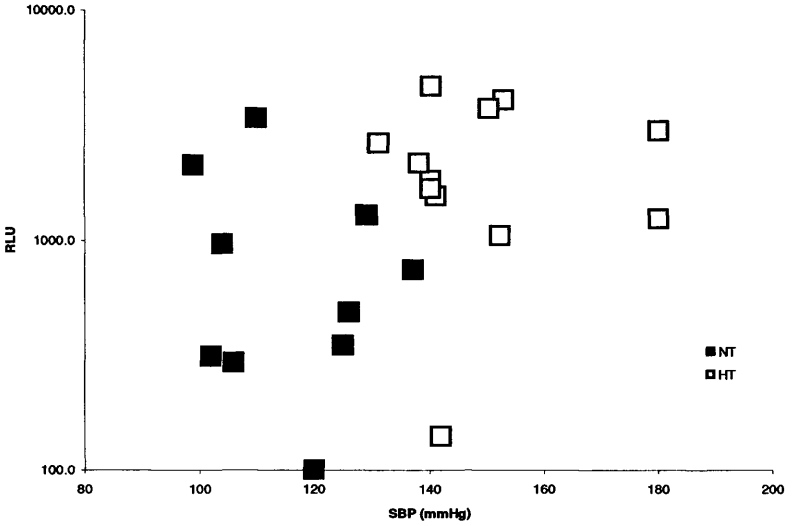
	DBP		SBP	
CLA	R=0.44	P=0.045	R=0.58	P=0.002
Luminol	R=0.66	P=0.002	R=0.59	P=0.014
Isoluminol	R=0.46	P=0.039	R=0.43	P=0.042

Table 4. Spearman's correlation of peak 50μM AA stimulated CLA, luminol and isoluminol enhanced CL, measured as log RLU, with SBP and DBP.

	DBP		SBP	
CLA	R=0.57	P=0.002	R=0.75	P=0.001
Luminol	R=0.55	P=0.005	R=0.50	P=0.014
Isoluminol	R=0.20	P=0.34	R=0.26	P=0.22

Table 5. Spearman's correlation of area under the curve 500nM TPA stimulated CLA, luminol and isoluminol enhanced CL, measured as log RLU, with SBP and DBP.

A



B

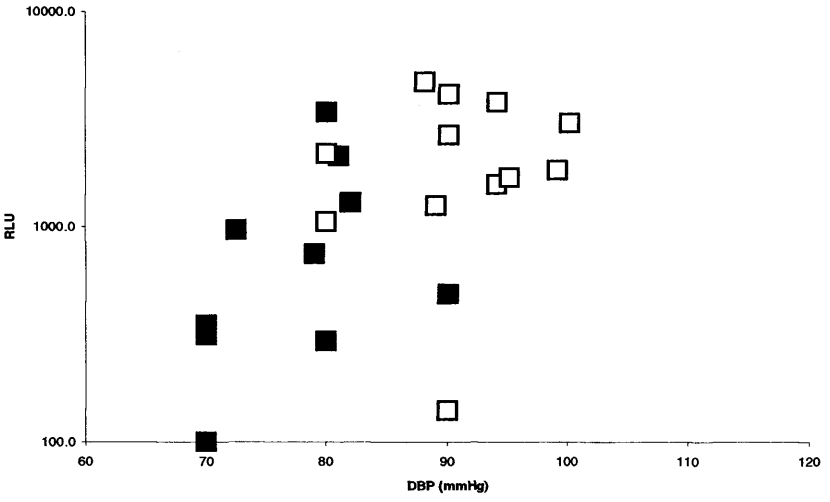


Figure 18. Luminol enhanced CL of 50µM AA stimulated lymphoblasts (12 NT closed box and 12 HT open box) plotted vs. SBP (A) and DBP (B). Each point represents the mean of 3 values recorded on separate days. For SBP r=0.59 (P=0.014) and for DBP r=0.66 (P=0.002).

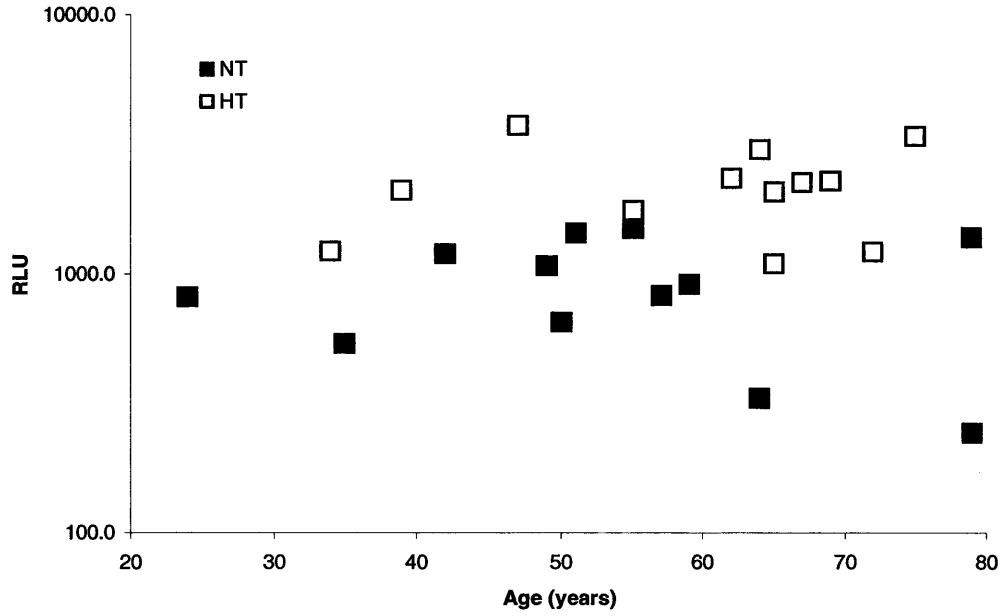


Figure 19. AUC of CLA enhanced CL of TPA stimulated lymphoblast (12 NT and 12 HT) plotted vs. age. Each point represents mean of at least 3 readings on separate days. For correlation of ROS with age r=0.34, P=0.1.

	TPA	AA
Luminol	r=-0.05	r=0.10
Isoluminol	r=0.22	r=0.01
CLA	r=0.34	r=0.35

Table 6. Spearman's correlation of peak 500nM TPA and 50μM AA stimulated CLA, luminol and isoluminol enhanced CL, measured as log RLU, with age. None of the values are significant.

2.2A(viii):Correlation of ROS Production with Age

Aim of Correlation of ROS Production with Age

As ROS production may decline with age (96) it was decided to analyse the effect of age of lymphocyte donor on lymphoblast ROS production, primarily to reassure ourselves that age was not confounding our experimental findings. We were aware this was a retrospective analysis of the data and the study was not powered to make firm conclusion about the association of age with ROS.

Results of Correlation of ROS Production with Age

Table 6 shows the correlation coefficients for both AA and TPA stimulated ROS production measured with luminol, isoluminol and CLA. The correlations are week (ranging from r=-0.09 to 0.14) and none reached statistical significance. Figure 19 shows CLA enhanced CI of TPA stimulated ROS production vs. age, the NT values are represented by closed boxes and the HT values by open boxes.

Discssion of Correlation of ROS Production with Age

The HT population was 7.7 years older than the NT group. The possible reduction in ROS production with age seen by Lipschitz (96) would off set any difference seen between the HT and NT groups. Our results found less of a correlation of ROS production with age. I believe this allows us to say that the age difference is not the cause of the increase in ROS production between the HT and NT groups but we could not draw any robust conclusions on effect of age on ROS production.

2.2A(ix):Subgroup Analysis of ROS Production and Family History of HT

Aims of Subgroup Analysis of ROS Production and Family History of HT

Lacy et al (37) found that family history (FH) of hypertension was more strongly related to ROS production than HT alone, therefore in an attempt to look for confounding factors we have broken the HT group into HT and HT FH.

Results and Discussion of Subgroup Analysis of ROS Production and Family History of HT

Although this is retrospective analysis on a small subgroup, we have found that both HT and HT FH groups produce more ROS than NT cell lines. There is no significant difference between the HT and HT FH cell lines (table 7), in fact the HT group rather than the HT FH group tended towards producing more ROS (P=0.07) when assayed by luminol. These results would suggest that family history of HT is probably not confounding the observed difference but we cannot comment on the difference in ROS production in those with and without a FH of HT.

We believe our data illustrates an increase in stimulated ROS production from HT lymphoblasts. The results are in keeping with increased ROS production from neutrophils of HT subjects (7;10;29-32). The lymphoblast ROS production correlates well with blood pressure. We believe the source of ROS is likely to be NADPH oxides as the ROS production is abolished by DPI but not consistently by rotenone. The ROS is probably a combination of superoxide and 30-40%

hydrogen peroxide. Although other factors, such as age and family history of hypertension, may also affect ROS production, we believe these variables do not account for the difference we have demonstrated.

In the remaining results section we have attempted to investigate the mechanism of increased ROS production in HT. We have investigated the NADPH oxidase subcomponents and priming of NADPH oxidase.

	NT	HT	HT FH
n	12	6	6
CLA TPA	431.9(49.8)	1375.0(165.3)	1482.5(134.2)
CLA AA	1082(121)	1867(447)	2473(223)
Luminol TPA	508.4(113.7)	2255.9(456.4)	1207.0(238.2)
Luminol AA	847(291)	2154(648)	2509(495)

Table 7. NT, HT and HT FH subgroup analysis of ROS. Enhanced CL values are expressed as RLU (SEM), AA stimulated values represent peak ROS production and TPA stimulated values are the AUC. There is no significant difference between the HT and HT FH cell lines for all 4 variables as assessed by Mann Whitney test, although the ROS production assayed by luminol tends toward the HT group generating more ROS (P=0.07).

2.2B:NADPH oxidase Subcomponent Analysis

2.2B(i):Expression of NADPH oxidase subunits in lymphoblasts from NT and HT subjects

Aims of measuring NADPH oxidase subcomponents

As we had demonstrated increased ROS production from HT lymphoblasts produced by a non-mitochodrial flavo protein, probably NADPH oxidase, we went onto to analyse NADPH oxidase subcomponents. Some authors have found increased expression of RNA for subcomponent $p22^{phox}$ in HT (145) and others have demonstrated increased expression of $p22^{phox}$, $p67^{phox}$ and $gp91^{phox}$ in some HT models . Using western blot analysis we assayed the NADPH oxidase components $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $gp91^{phox}$ and the G protein rac2 in the NT and HT cell lines.

Results of measuring NADPH oxidase subcomponents

We found that $p22^{phox}$ was over-expressed in HT versus the NT cell lines. A representative Western Blot is shown in figure 20. Using standardised results from 4 separate Western Blots, in arbitrary densitometry units, we found the median of the 12 NT $p22^{phox}$ levels to be 0.9, and that of the 12 HT cell lines to be 1.4. The 95% confidence interval for the difference between the $p22^{phox}$ content of the NT and HT lines was 0.15-1.06 (P=0.01 as calculated by Mann Whitney test).

We could detect no significant difference between the cell lines in $p47^{phox}$ (NT 1.0(0.06) vs. HT 1.0(0.11) P=0.97) (figure 21), $p67^{phox}$ (NT 1.03(0.33) vs. HT

1.14(0.28) P=0.41) (figure 22), gp91^{phox} (median NT 1.04 vs. median HT 1.14 (95% CI for difference –0.49 to 0.13) P=0.39) (figure 23) and rac2 (NT 1.13(0.15) vs. HT 0.88(0.12) P=0.21) (figure 24). The results for all the phox subunits and rac2 are the average of 3 standardised Western Blots.

Discussion of measuring NADPH oxidase subcomponents

The increased expression of p22^{phox} is in keeping with previous findings (143-145) and provides a cause for the increased ROS production in HT lymphoblasts. Lymphoblasts are known to contain reduced levels of the membrane bound subunits (p22^{phox} and gp91^{phox}). It may be possible that we were unable to detect significant but small differences in gp91^{phox} because of the low levels expressed in lymphoblasts.

Figure 20. A representative Western blot for $p22^{phox}$. 100µg of the protein extract was loaded in each lane and was resolved on 15% gel. The $p22^{phox}$ antibody was used at a concentration of 10µg/ml. Densitometric analysis revealed less $p22^{phox}$ in the NT cell lines (median 0.9) compared to the HT cell lines (median1.4, 95% CI for difference 0.15-1.06 P=0.01). HT, hypertensive. NT normotensive.

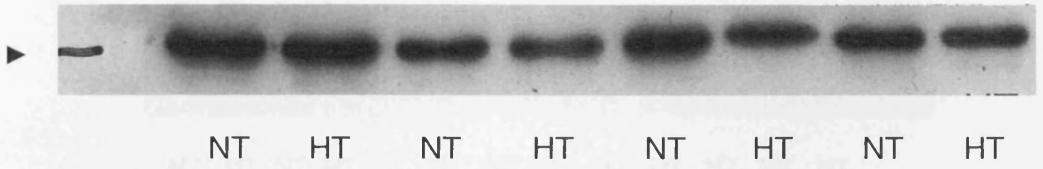
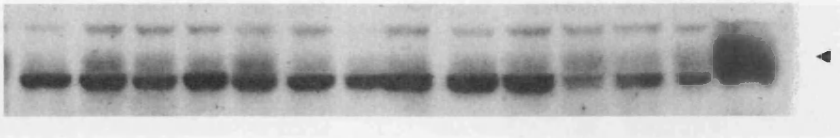


Figure 21. A representative Western blot for $p47^{phox}$. 50µg of the protein extract was loaded in each lane and was resolved on 7.5% gel. The $p47^{phox}$ antibody was used at a concentration of 1µg/ml. The marked line (►) corresponds with the 45kDa marker. Densitometric analysis revealed no significant difference between NT and HT expression of $p47^{phox}$ (NT 1.0(0.06) vs. HT 1.0(0.11) P=0.97). HT, hypertensive. NT normotensive.



NT HT NT HT NT HT NT HT NT HT NT HT NT

Figure 22. A representative Western blot for $p67^{phox}$. 100µg of the protein extract was loaded in each lane and was resolved on 7.5% gel. The $p67^{phox}$ antibody was used at a concentration of 5µg/ml. The 66kDa marker is shown in the right well (◄). Densitometric analysis revealed no significant difference between NT and HT expression of $p67^{phox}$ (NT 1.03(0.33) vs. HT 1.14(0.28) P=0.41). HT, hypertensive. NT normotensive.

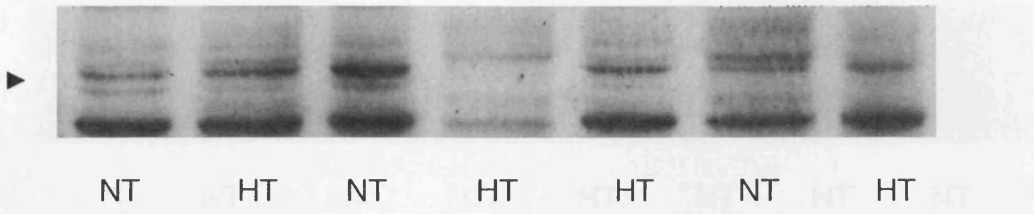


Figure 23. A representative Western blot for $gp91^{phox}$. 100µg of the protein extract was loaded in each lane and was resolved on 7.5% gel. The $gp91^{phox}$ antibody was used at a concentration of 10µg/ml. ► marks the band of interest. Densitometric analysis revealed no significant difference between NT and HT expression of $gp91^{phox}$ (median NT 1.04 vs. median HT 1.14 (95% CI for difference –0.49 to 0.13) P=0.39). HT hypertensive. NT normotensive.

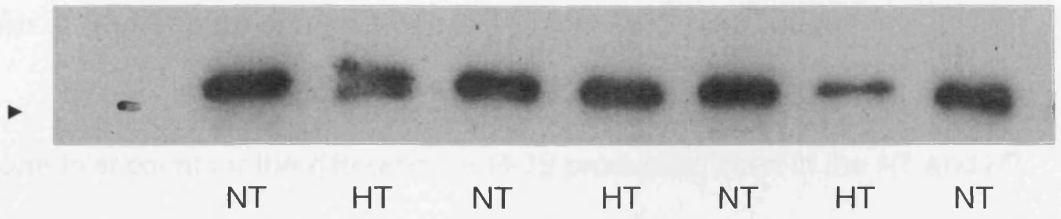


Figure 24. A representative Western blot for rac2. 100µg of the protein extract was loaded in each lane and was resolved on 15% gel. Rac2 antibody was used at a concentration of 0.2µg/ml. The hand drawn line corresponded with 20.1kDa marker. Densitometric analysis revealed no significant difference between NT and HT expression of rac2 (NT 1.13(0.15) vs. HT 0.88(0.12) P=0.21). HT, hypertensive. NT normotensive.

2.2B(ii):Regression analysis of $p22^{phox}$ and ROS production

Aim of Regression analysis of $p22^{phox}$ and ROS production

We felt it was important to know if the variation in $p22^{phox}$ was sufficient alone to account for the difference in ROS production seen in the NT and HT groups. We used a forward and backward stepwise regression analysis, analysing categorical and contnuous variables (DBP, SBP, family history of hypertension or $p22^{phox}$) to see which predict stimulated ROS production (AA stimulated, assayed by luminol CL). We selected AA stimulated ROS production as assayed by luminol chemiluminescence as there was the largest differential between the NT and HT groups using this stimulant and luminescent probe. For individual variables we selected a cut off of P=0.15 for inclusion or exclusion into the model.

The regression analysis produced a model of stimulated ROS production (AA stimulated, luminol CL) = -5841 + (94.5 x DBP) – (351 x $p22^{phox}$). The regression matrix is shown in table 8, with both DBP and $p22^{phox}$ accounting for about 28% of the variance in AA stimulated ROS production.

Discussion of Regression analysis of $p22^{phox}$ and ROS production

As DBP and $p22^{phox}$ each only account for about a third of the variance other factors are likely to be involved in the increased ROS seen in HT.

	Step 1 (DBP)	Step 2 ($p22^{phox}$)
Constant	-5036	-5841
DBP	79	94.5
T	2.84	3.27
P	0.009	0.004
$p22^{phox}$		-351
T		-1.51
P		0.146
R^2	26.87	34.04
Adjusted R^2	23.55	27.76

Table 8. Matrix of multiple forward and backward regression of variables SBP, DBP, family history of HT and $p22^{phox}$ to predict stimulated ROS production (AA stimulated, luminol CL). For individual variables an inclusion/exclusion cut off of P=0.15 was used for inclusion in the multiple variable model.

2.2C:Tyrosine Phosphorylation

2.2C(i):Basal Tyrosine Phosphorylation

Aims of Assaying Basal Tyrosine Phosphorylation

Although we could detect no difference in the basal ROS production in HT and NT subjects we still felt it was important to investigate basal priming. Priming could be upregulating NADPH oxidase without altering basal ROS production. Hallet and Lloyds (169) believe tyrosine phosphorylation is a key event in leucocyte priming. Priming is another way of upregulating NADPH oxidase ROS production. To make an initial screen of potential causes of increased ROS production we assayed basal tyrosine phosphorylation in the NT and HT cell lines using western blot analysis, probing with a non specific phosphotyrosine antibody. By using a non-specific phosphotyrosine antibody we were able to look at differences in multiple tyrosine phosporylated proteins before performing a more focused analysis.

Results of Assaying Basal Tyrosine Phosphorylation

Basal protein tyrosine phosphorylation was studied in all 12 NT and 12 HT cell lines, at least 6 bands were detected at approximately 110, 60, 44, 42, 38, 28kDs. The band at 42kD revealed an increase in tyrosine phosphorylation (figure 25) of the HT cell lines (NT 0.97(0.12) vs. HT 1.49(0.18) P=0.024)), all other bands showed no difference in tyrosine phosphorylation between HT and NT cell lines (110kDa NT 1.72(0.37) vs. HT 1.69(0.34) P=0.62, 60kDa NT

1.06(0.17) vs. HT 1.00(0.21) P=0.31, 38kDa NT 3.19(0.21) vs. HT 3.59(0.44) P=0.37).

Discussion of Assaying Basal Tyrosine Phosphorylation

The apparent difference in tyrosine phosphorylation of a 42kDa protein suggests a potential difference in a 42kD protein probably basal p44/42 MAPK between basal NT and HT lymphoblasts.

2.2C(ii):Basal phosphorylation of MAPK

Aims of Basal phosphorylation of MAPK

To see if this difference at 42kD represented differential tyrosine phosphorylation of MAPK in unstimulated lymphoblasts, the experiment was repeated with phospho tyrosine/threonine MAPK antibody (figure 26).

Results of Basal phosphorylation of MAPK

We were able to detect increased tyrosine/threonine phosphorylation of HT subjects, in both p42 MAPK (NT 1.02(0.12) vs. HT 1.49(0.17) (P=0.036)) and p44 MAPK (values were not normally distributed and were therefore analysed by the Mann Whitney test; NT median 0.98 vs. HT median 1.71 (95% CI for difference 0.24-1.22) (P=0.007)). The lymphoblasts of NT and HT subjects had the same MAPK content (230) therefore these results indicate increased tyrosine phosporylated and therefore active MAPK in basal HT lymphoblasts.

Discussion of Basal phosphorylation of MAPK

The results suggest a difference in Basal tyrosine phosphorylation of p44/42 MAPK. As we were unable to detect a difference in basal ROS production between NT and HT lymphoblast, assuming the ROS assay is sufficiently sensitive to detect a difference (section 2.2A(ii)), this difference is not producing a differential in basal ROS. As activation and priming of NADPH oxidase ROS production are distinct but intertwined processes the lack of difference in basal ROS does not exclude priming. Likewise the increased basal tyrosine phosphorylation of p44/42 MAPK does not confirm priming of NADPH oxidase.

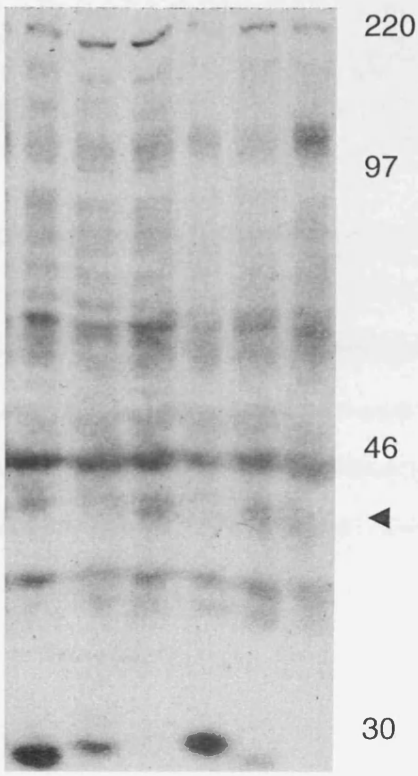


Figure 25, Basal protein tyrosine phosphorylation of NT and HT lymphoblasts. The western blot was probed with phospho-tyrosine primary antibody (1 in 1000). There was 500µg of protein per lane, resolved on 15% SDS gels. The ◄ indicates the band with increased basal tyrosine phosphorylation in HT vs. NT cell lines, NT 0.97 (0.12) vs. HT 1.49 (0.18) (P=0.024). Protein assayed in arbitrary densitometric units. NT, normotensive. HT, hypertensive.

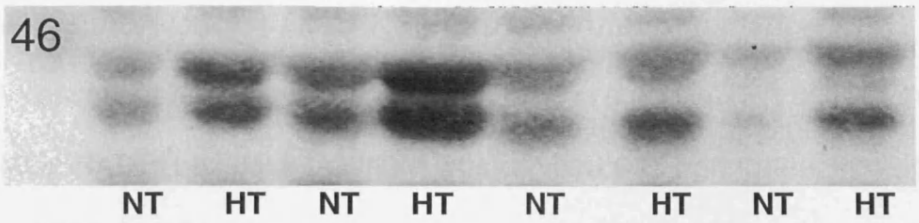


Figure 26. Basal NT and HT lymphoblast protein tyrosine phosphorylation. The western blot was probed with a threonine/tyrosine dual phosphorylated p44/p42 MAPK (1 in 1000). There was 500µg of protein per lane, resolved on 15% SDS gels. p42 MAPK phosphorylation NT 1.02 (0.12) vs. HT 1.49 (0.17) (P=0.036) and p44 MAPK phosphorylation median NT 0.98 vs. HT 1.71 (95% CI for difference 0.24-1.22) (P=0.01 calculated using Mann Whitney test as data are not normally distributed). Protein assayed in arbitrary densitometric units. NT, normotensive. HT, hypertensive.

2.2C(iii):Phosphorylation of MAPK Before and After Stimulation

Aim of Phosphorylation of MAPK Before and After Stimulation

To ascertain if tyrosine phosphorylation has a role in TPA activation of lymphoblast NADPH oxidase, a single western blot was performed, in which pairings of 500nM TPA stimulated and unstimulated extracts from the same 6NT and 6HT cell lines were resolved. This experiment would also enable us to compare p44/42 MAPK tyrosine phosphorylation after activation between the NT and HT groups to see if a differential still exsts.

Result of Phosphorylation of MAPK Before and After Stimulation

The resolved proteins were probed with threonine and tyrosine dual phosphorylated MAPK (figure 27). There was increased phosphorylation of both p44/p42 MAPK in TPA stimulated fractions (p42 phospho MAPK NT unstimulated 0.80(0.08) vs. NT stimulated 3.44(0.33) (P=0.001) and HT unstimulated 0.83(0.14) vs. HT stimulated 3.30(0.35) (P=0.003); p44 phospho MAPK NT unstimulated 0.38(0.10) vs. NT stimulated 2.37(0.22) (P=0.001) and HT unstimulated 0.36(0.10) vs. HT stimulated 2.23(0.30) (P=0.002)). We were unable to detect any difference in TPA stimulated tyrosine/threonine phosphorylation of either p42 MAPK (NT 1.00(0.1) vs. HT 0.97(0.08) P=0.81)) or p44 MAPK (NT 0.95(0.10) vs. HT 0.89(0.12) P=0.73)) between the NT and HT cell lines (results of 3 normalised western blots). The protein extracts were also probed with the phospho tyrosine antibody. No other protein bands revealed any differences in tyrosine phosphorylation between NT and HT cell lines upon TPA

stimulation. There was no significant difference between stimulated NT and stimulated HT cell lines for phospho MAPK content.

Discussion of Phosphorylation of MAPK Before and After Stimulation

There is increased phosphorylated tyrosine / threonine p44/42 MAPK after activation with TPA. Suggesting a tyrosine kinase is involved in activation of NADPH oxdase and will be confirmed with inhibitor studies (section 2.2D(i)). We were unable to demonstrate increased tyrosine phosphorylation of p44/42MAPK in the HT compared to the NT group after activation with TPA. If p44/42 MAPK is priming NADPH oxidase through tyrosine phosphorylation the priming is occurring independently of the activation process. The increase in basal phosphorylation of p44/p42 MAPK in HT may indicate priming of the lymphoblasts. To confirm if tyrosine kinase / p44/p42 MAPK were priming NADPH oxidase, we inhibited tyrosine kinase and p44/p42 MAPK, and then stimulated NADPH oxidase to study the effect on ROS output.

HT HT NT NT HT HT NT NT

\+ - + - + - + - TPA

Figure 27. Paired basal and 500nM TPA stimulated protein tyrosine phosphorylation of 6 NT and 6 HT lymphoblasts. Basal and stimulated phospho-p44/p42 MAPK was assayed with threonine/tyrosine dual phosphorylated p44/p42 MAPK (1 in 1000). For p44 MAPK basal NT 0.38(0.1) vs. stimulated NT 2.37(0.22) (P=0.001) and basal HT 0.36(0.1) vs. stimulated HT 2.23(0.30)(P=0.002). For p42 MAPK basal NT 0.8(0.08) vs. stimulated NT 2.37(0.22) (P=0.001) and for basal HT 0.80(0.14) vs. stimulated HT 3.30 (0.35) (p=0.003). NT, normotensive. HT, hypertensive. TPA, 12-O-tetradecanoylphorbol-13-acetate.

2.2D:Inhibition of tyrosine kinase, MAPK, p38 MAPK, PI3K and cPLA2

Aim of all inhibitor experiments

The inhibitor studies have a number of aims. The primary goal was to see if Tyrosine kinase, p44/42 MAPK, p38 MAPK, PI3K and $cPLA_2$ have a role in the priming of NADPH oxidase. Although we would be unlikely to find a difference in basal ROS production, as ROS production is so low, the effects of the inhibitors on basal ROS production was studied. Then TPA stimulated ROS production was studied with all the inhibitiors, we chose TPA stimulation, as its action is upstream of NADPH oxidase. The inhibitors could have a number of effects; firstly we were looking to see if the inhibitors have an effect and the degree of the attenuation of ROS production. If the inhibitors have an effect on stimulated ROS production this could be due to inhibition of activation, inhibition of priming or inhibition of both. If the inhibitors were having an effect on priming we would expect an increased percetage reduction in inhibited HT ROS production as compared with the inhibited NT group.

2.2D(i):Tyrosine Kinase Inhibition

Results of Tyrosine Kinase Inhibition

Basal ROS production was not different in NT and HT cell lines, after stimulation of the lymphoblasts with 500nM TPA and 50μM AA there was increased ROS production in the HT cell lines compared with NT cell lines (section 2.2A(vi)). The tyrosine kinase inhibitors produced no detectable

reduction in baseline luminol CL. A characteristic luminol enhanced CL trace is shown in figure 28, after stimulation with 500nM TPA. The traces demonstrate genistein inihibition of one NT and one HT cell line recorded simultaneously, each point represents the mean of 3 separate wells. The NT cell line stimulated with 500nM TPA produced a maximum rate of ROS production of 1080.7(9.6) RLU/min, which was reduced to 187.7(1.2) RLU/min by 100μM genistein. The HT cell line produced a maximum rate of ROS production of 1595.0(11.4) when stimulated with 500nM TPA, genistein reduced the peak to 286.3(5.2).

TPA stimulated luminol enhanced CL was performed on all 12 HT and 12 NT lymphoblast cell lines with 3 tyrosine kinase inhibitors, genistein a non-specific tyrosine kinase inhibitor, tyrphostin A25 an inhibitor specific for receptor linked tyrosine kinases and herbimycin A, which is specific for src type tyrosine kinases. The genistein reduced NT cell line ROS production to 30.6 (9.6)% of NT control ROS production (P=0.002) and HT cell line ROS production to 29.4 (8.5)% of HT control ROS production (P=0.004) (figure 29). There was no significant difference in the percentage reduction in ROS production in the NT and HT groups.

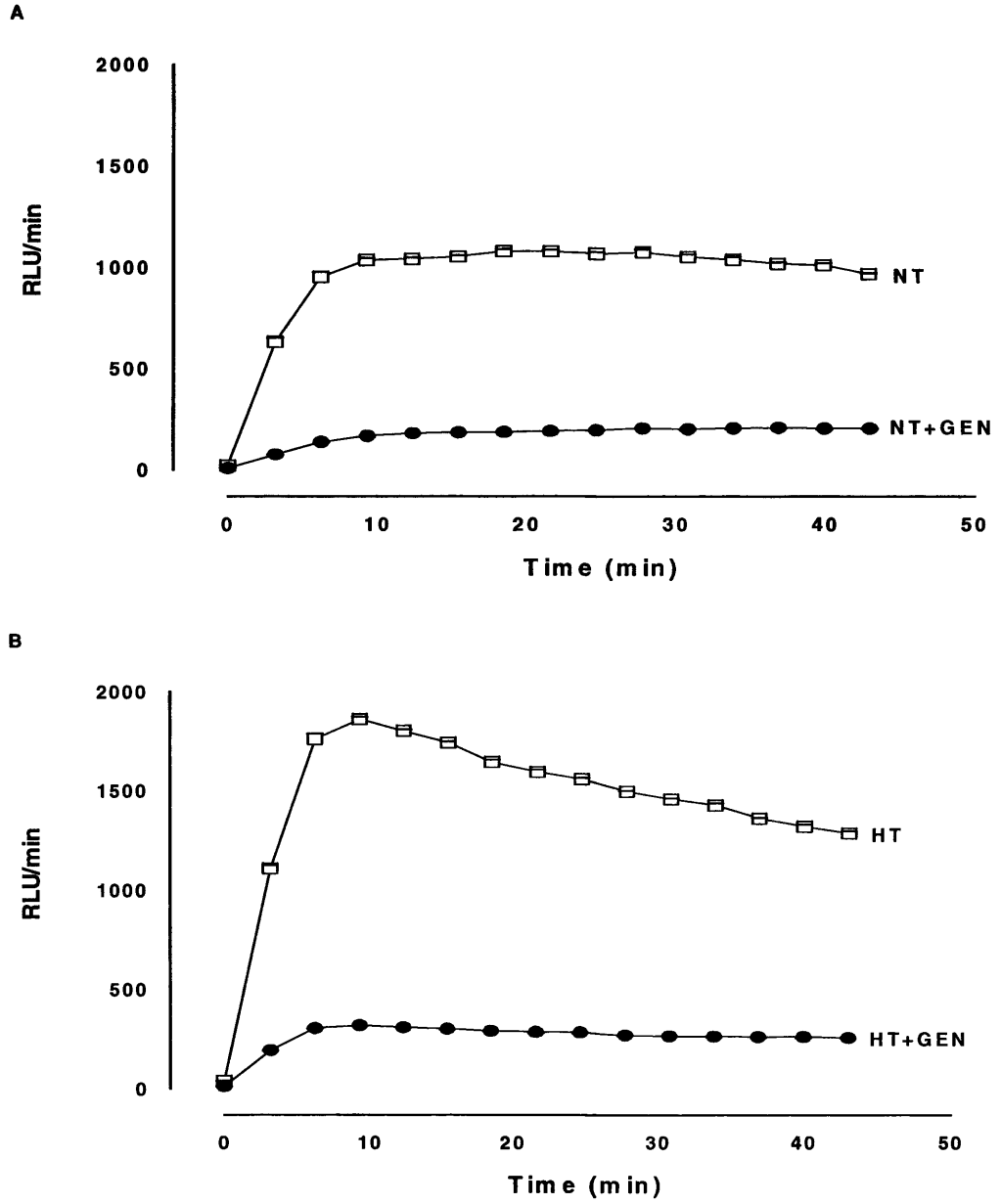


Figure 28, Representative luminol enhanced chemiluminescence of 500nM TPA stimulated NT cell line (A) and HT cell line (B), both inhibited with 100μM genistein. Each trace is the mean (SEM) of 3 wells recorded simultaneously. Each well contained 10^5 cells. Peak reactive oxygen species production for NT control 1080.7(9.6) RLU/min vs. NT genistein 187.7(1.2) RLU/min and for the HT control 1595.0(11.4) RLU/min vs. HT genistein 286.3 (5.2). NT, normotensive. HT, hypertensive. RLU, relative light units. GEN, genistein.

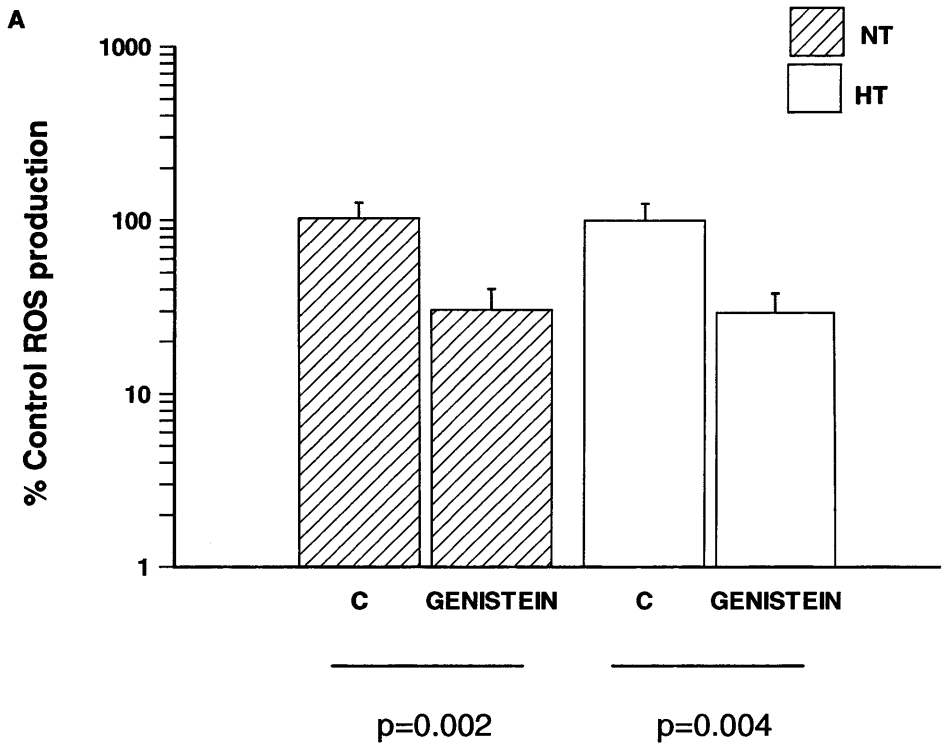


Figure 29. Mean TPA (500nM) stimulated ROS production after tyrosine kinase inhibition with 100μM genistein (a non specific inhibitor), of 12 NT and 12 HT cell lines. Results are expressed as a percentage of the uninhibited ROS production. Genistein reduced NT ROS production to 30.6 (9.6)% and HT ROS production to 29.4 (8.5)%. Results were both significant but there was no difference between percentage reductions in NT Genistein and HT Genistein. NT, normotensive. HT, hypertensive. C, control or uninhibited ROS production. ROS, reactive oxygen species.

Tyrphostin A25 (figure 30) significantly reduced ROS production in both NT (37.0 (18.4)% of NT control ROS production ($P<0.001$)) and HT cell lines (26.6 (7.1)% of HT control ROS production ($P<0.001$)). There was no significant difference in the percentage reduction in ROS production in the NT and HT groups.

Herbimycin A (figure 31) significantly reduced ROS production in both NT (51.6 (16.3)% of NT control ROS production ($P<0.001$)) and HT cell lines (70.4 (20.7)% of HT control ROS production ($P=0.007$)). There was no significant difference in the percentage reduction in ROS production in the NT and HT groups.

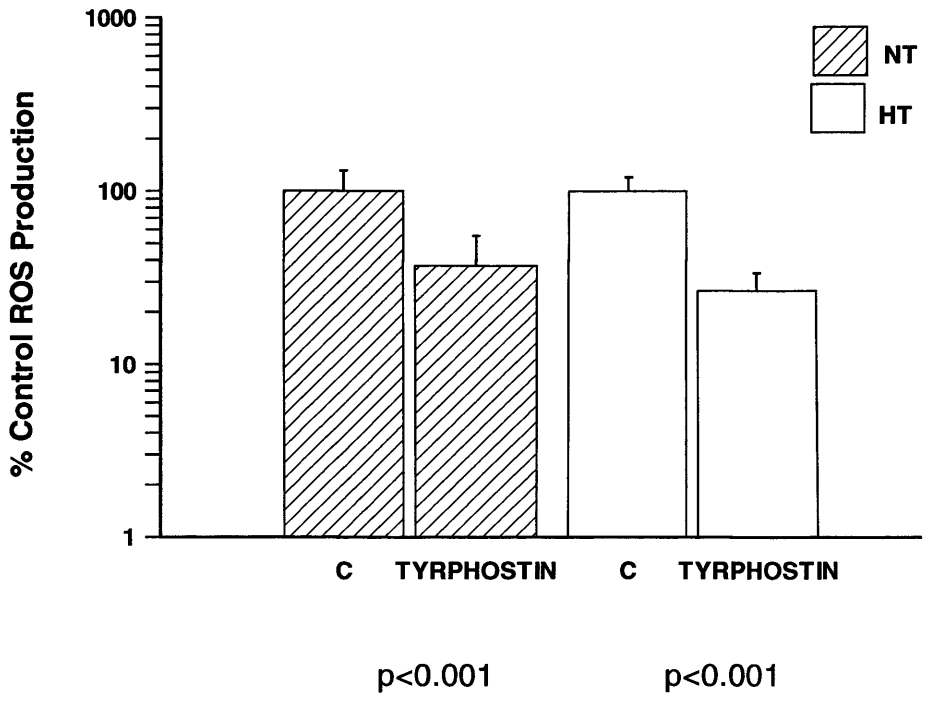


Figure 30. Mean TPA (500nM) stimulated ROS production after tyrosine kinase inhibition with 100μM tyrphostin A25 (receptor linked tyrosine kinase inhibitor) of 12 NT and 12 HT cell lines. Results are expressed as a percentage of the uninhibited ROS production. Tyrophostin reduced NT ROS production to 37.0 (18.4)% and HT ROS production to 26.6 (7.1)%. Results were significant (P<0.001) but there was no difference between the percentage tyrophostin inhibition of NT or HT ROS production. Normotensive shaded bars, Hypertensive represented by open bars. C, control or uninhibited ROS production. ROS, reactive oxygen species.

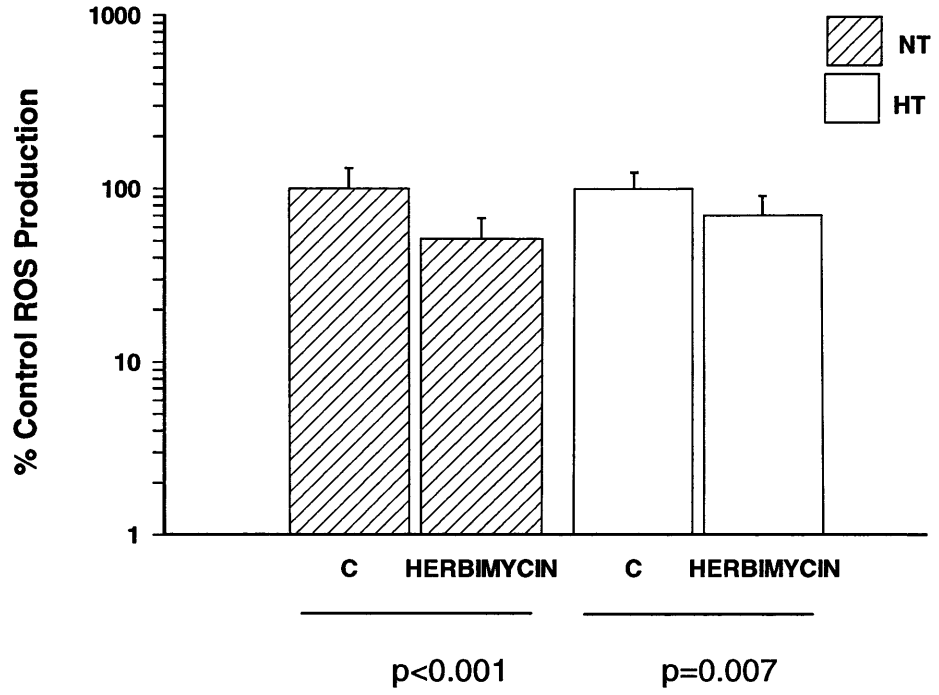


Figure 31. Mean TPA (500nM) stimulated ROS production after tyrosine kinase inhibition with 10μM herbimycin A (a src type tyrosine kinase inhibitor) of 12 NT and 12 HT cell lines. Results are expressed as a percentage of the uninhibited ROS production. Herbimycin A reduced NT ROS production to 51.6 (16.3)% and HT ROS production to 70.4 (20.7)%. All results are significant P<0.01) but there was no difference between the percentage herbimycin inhibition of NT or HT ROS production. Normotensive represented by shaded bars and Hypertensive by open bars. C, control or uninhibited ROS production. ROS, reactive oxygen species.

2.2D(ii):Inhibition of p44/p42 MAPK, p38 MAPK, PI3K and $cPLA_2$,

Results of Inhibition of MAPK

To see if the differential MAPK phosphorylation in basal NT and HT cell lines (section 2.2C(ii)) was linked to increased NADPH oxidase activity and therefore ROS production, we used 50μM PD 98059, a specific inhibitor of MEK kinase (directly upstream of MAPK), to inhibit p44/p42 MAPK. Luminol enhanced CL was performed on all 12 NT and 12 HT cell lines with and without PD 98059. No difference in unstimulated CL could be detected, however after stimulation with 500nM TPA there was a small but significant reduction in NT ROS production (76.4 (19.5)% of NT control ROS production (P=0.012)) and in HT ROS production (92.4 (21.0)% of HT control ROS production (P=0.004)) (figure 32). The PD98059 percentage reduction in stimulated ROS production between NT and HT groups is not significantly different.

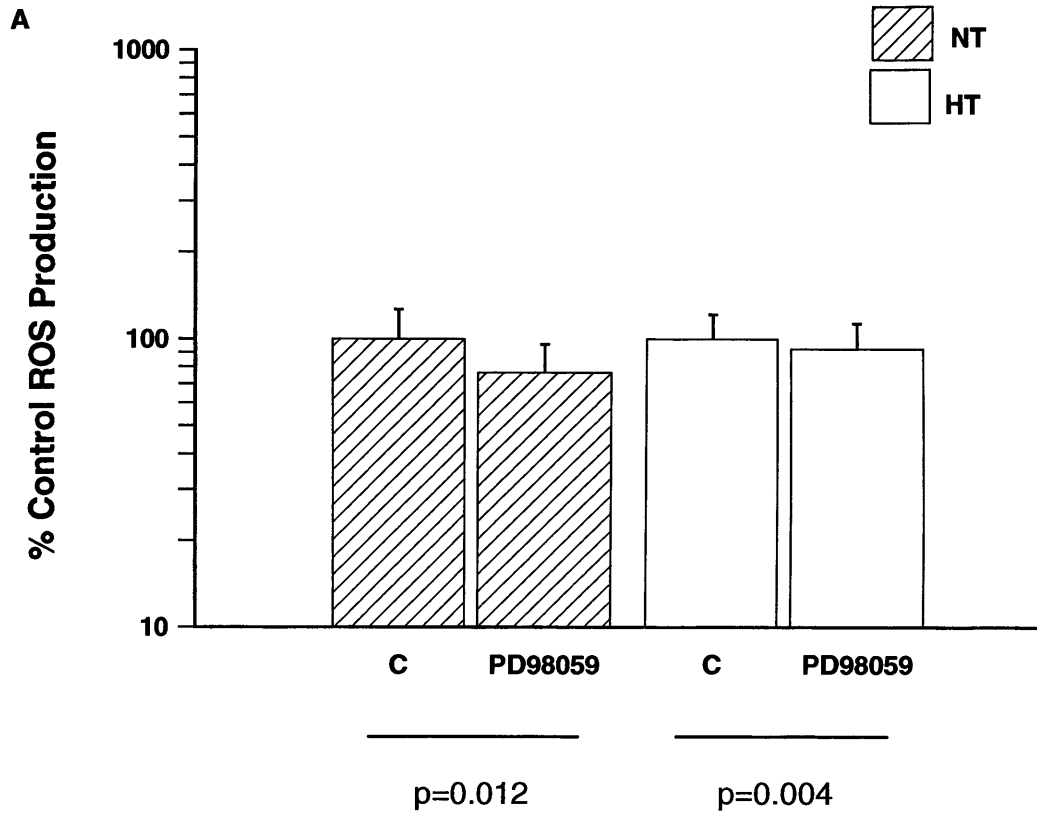


Figure 32. Inhibition of TPA stimulated ROS production by PD98059 (p44/p42 MAPK inhibitor). Resutls represent the % reduction of ROS production as assayed by luminol enhanced chemiluminescence. PD98059 reduced NT ROS production to 76.4(19.5)% (P=0.012) and HT ROS production to 92.4(21.0)% (P=0.004). NT, normotensive. HT, hypertensive. C, control or non inhibited ROS production. ROS, reactive oxygen species.

Results of Inhibition of PI3K, p38MAPK and cPLA$_2$

To further examine other messengers of the priming and or stimulation pathway we performed 500nM TPA stimulated, luminol enhanced CL, in the presence or absence of the following inhibitors: wortmannin (PI3K inhibitor), SB203580 (p38 MAPK), mepacrine (non specific PLA$_2$ inhibitor) and MAFP (specific cPLA$_2$ inhibitor). None of the inhibitors had any detectable effect on basal CL of NT or HT cell lines. Inhibition of NT and HT cell lines with SB203580 produced no significant reduction in TPA stimulated CL of NT or HT cell lines (figure 33).

The other inhibitors all produced a small reduction in TPA stimulated, luminol enhanced CL. Wortmannin reduced NT CL (91.9 (23.0)% of NT control ROS production (P=0.055)) and HT CL (90.8 (24.1)% of HT control ROS production (P=0.017)) (figure 34). There was no significant difference in the percentage ROS reduction by wortmannin in the NT and HT cell lines.

Mepacrine reduced NT CL to 69.8 (22.4)% of NT control ROS production (P=0.001)) and reduced HT CL to 62.0 (17.2)% of HT control ROS production (P=0.001)) (figure 35). There was no significant difference in the percentage reduction of ROS production by mepacrine in the NT and HT groups. MAFP reduced NT CL to 81.8 (29.2)% of NT control ROS production (P=0.001)) and HT CL to 91.7 (19.4)% of HT control ROS production (P=0.087)) (figure 36). The MAFP inhibition of ROS production was not significantly different in the NT and HT groups.

Discussion of Inhibitor studies

SB 203580 had no effect on TPA stimulated ROS production suggesting that p38 MAPK has no effect on either stimulation or priming of NADPH oxidase. The other inhibitors all reduced ROS production by varying degrees. The tyrosine kinases were the most potent, reducing stimulated ROS production between 50 and 70%. Inhibition of p44/42 MAPK, PI3K and PLA_2 all produced between 10-30% reduction in stimulated ROS production. There was no differential reduction in ROS production in the HT group therefore all the inhibitors seem to attenuate activation rather than priming.

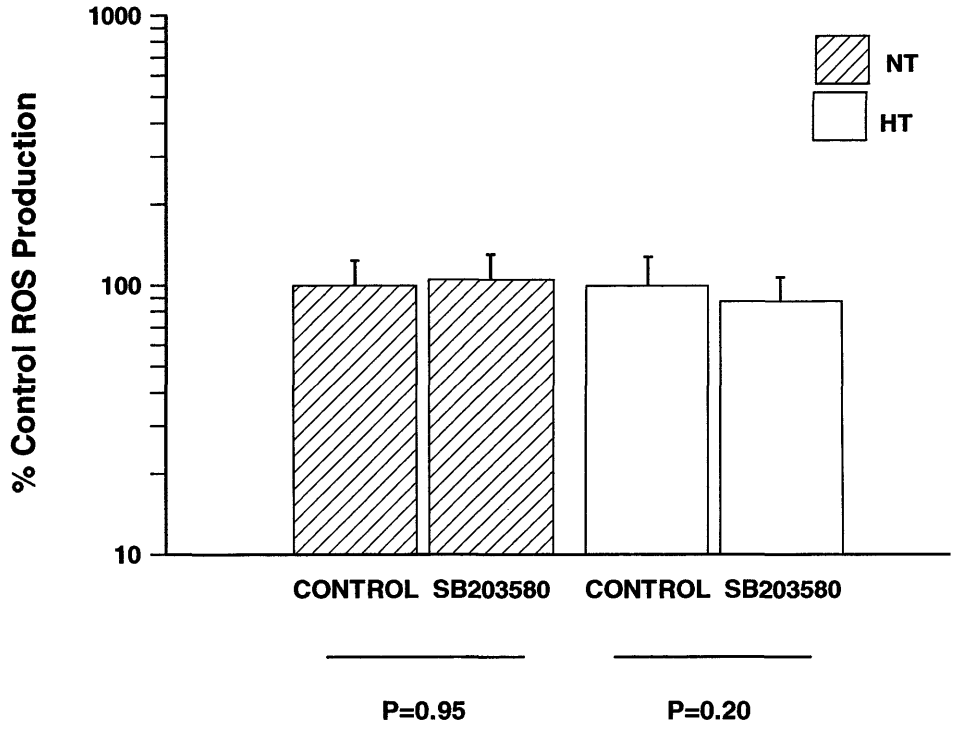


Figure 33. Effect of inhibiting p38 MAPK on TPA stimulated ROS production. p38 MAPK was inhibited with 30μM SB203580, results represent mean (SEM) of 8 NT and 8 HT cell lines. There was no significant reduction in ROS production on inhibition of p38. The ROS production is expressed as the percentage of control or uninhibited ROS production. Normotensive represented by shaded bars and Hypertensive by open bars. ROS, reactive oxygen species.

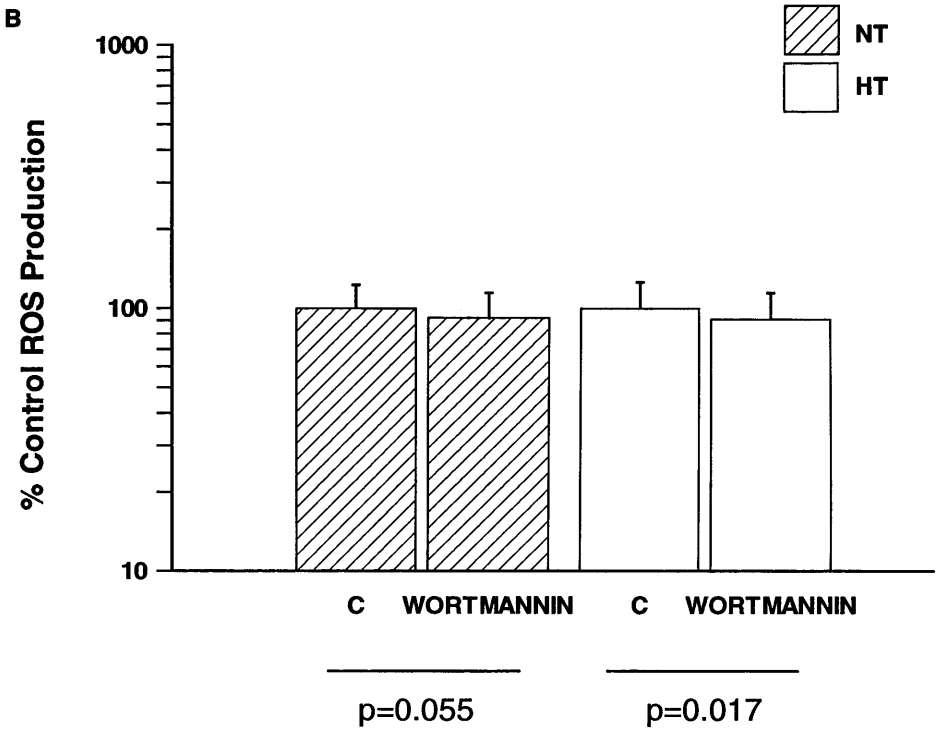


Figure 34. Effect of inhibiting PI3K on TPA stimulated ROS production. The inhibition of PI3K (wortmannin, 100nM) represent the mean (SEM) of 12 NT and 12 HT cell lines. The ROS production is expressed as the percentage of control or uninhibited ROS production. Wortmannin reduced NT ROS production to 91.9(23.0)% (P=0.055) and HT ROS production to 90.8(24.1)% (P=0.017). NT, normotensive. HT, hypertensive. ROS, reactive oxygen species.

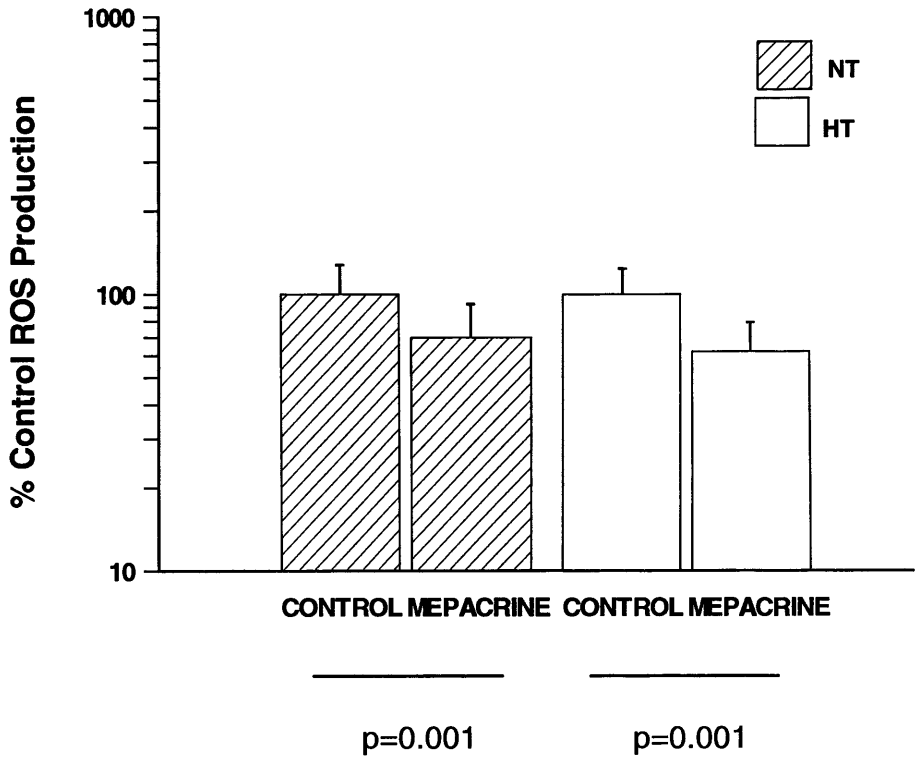


Figure 35. Effect of inhibiting PLA_2 with mepacrine on TPA stimulated ROS production. The inhibition of PLA_2 (mepacrine, 100μM) represent the mean (SEM) of 12 NT and 12 HT cell lines. The ROS production is expressed as the percentage of control or uninhibited ROS production. Mepacrine reduced NT ROS production to 69.8(22.4)% (P=0.001) and HT ROS production 62.0(17.2)% (P=0.001). Normotensive represented by shaded bars and Hypertensive by the open bars. ROS, reactive oxygen species.

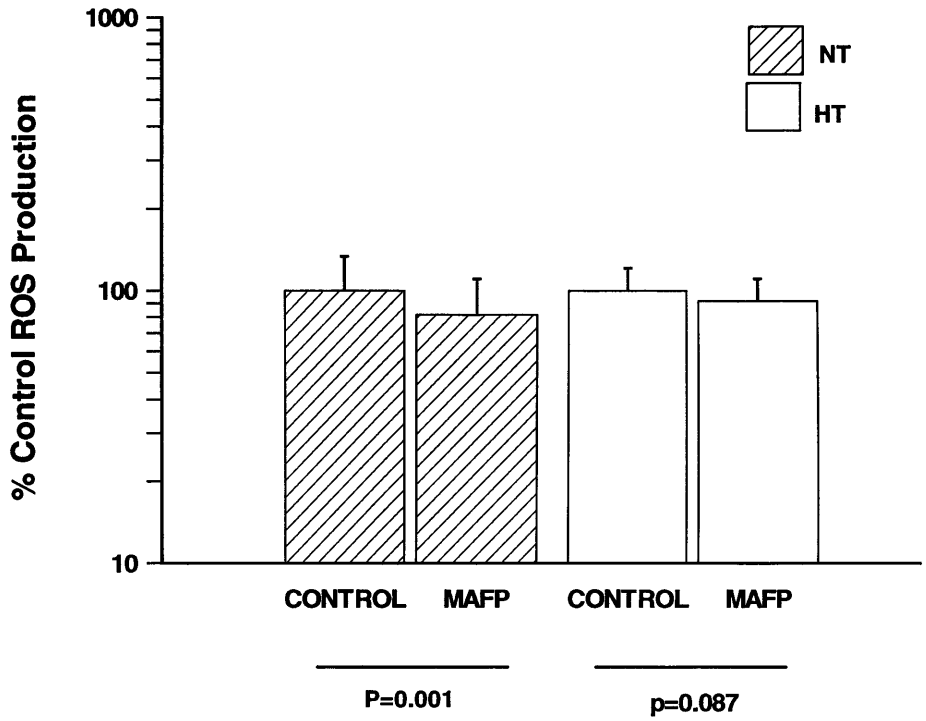


Figure 36. Effect of inhibiting PLA_2 with MAFP on TPA stimulated ROS production. The inhibition of PLA_2 (100μM and MAFP, 250nM) represent the mean (SEM) of 12 NT and 12 HT cell lines. The ROS production is expressed as the percentage of control or uninhibited ROS production. MAFP reduced NT ROS production to 81.8(29.2)% (P=0.001) and HT ROS production to 91.7(19.4)% (P=0.087). Normotensive represented by shaded bars and hypertensive by the open bars. ROS, reactive oxygen species. MAFP, Methyl arachidonyl fluorophosphonate.

Chapter 3: Discussion

3.1:Discussion of methods

3.1A:Lymphoblasts as a model for the study of hypertension

3.1A(i):Lymphoblast for the investigation of increased ROS production

Previous work with neutrophils was inconclusive, in that some groups have found an increase in ROS production in HT compared to NT subjects (31;32), whilst others have been unable to reproduce these results (38;39). We believe that neutrophil ROS production can be altered by the extraction process, and by dietary or medical antioxidants explaining the conflicting results. In choosing to use transformed cells these confounding factors are removed, but the ROS production is approximately 20-100 times less than neutrophils (197;202). TPA applied to lymphocytes had no lasting effect post immortalization (unpublished data Dr VM Lee and Prof LL Ng) supporting the hypothesis that lymphoblasts are free to express their genotype with fewer confounders than neutrophils. As discussed in section 1.10C, NADPH oxidase can be up regulated through a process of priming which is a separate from activation. It is currently not know if priming persists post transformation. I had assumed the priming would persist post transformation, hence we chose lymphoblasts to study priming, but this needs further investigation. I have proposed an experiment in section 3.5.

Immortalised human lymphoblasts are considered a good model for the study of the role of NHE (sodium Hydrogen exchanger) in hypertension

(198;199), the NHE correlating well with native and cultured cells. Lymphoblasts are also considred a good model for studying NADPH oxidase in chronic granulomatous disease (197). However experience in the study of hypertension priming of NADPH oxidase in lymphoblasts is limited. As our lymphoblasts were previously established it was not possible to compare neutrophil and lymphoblast ROS production from the same subjects but our results are not at variance with the previously published data on ROS production in HT.

Atheroma is now considered a low-grade inflammatory process with monocytes and lymphocytes accumulating within atheroma in the vessel wall. Even in the absence of atheroma the blood and vessel walls are in close proximity, so increases in ROS production from all white cells are likely to increase the vascular oxidative burden. Monocytes are also thought to be responsible for oxidation of LDL. An area of increasing interest is the increased ROS production by a NADPH oxidase within endothelial and vascular smooth muscle cells. It is likely that ROS production from within the vessel wall itself is the most significant in the generation of HT, as the ROS is being formed where NO is produced. The ROS interacts with NO reducing endothelial relaxation and therefore increasing vascular tone and blood pressure. The byproduct is also the noxious peroxynitrite. As far as I am aware there are no comparative data for ROS production from the endothelium and white cell lines in hypertension.

3.1A(ii):Lymphoblasts as a model to study mechanism of increased ROS production

Lymphoblasts have been used to study NADPH oxidase in chronic granulomatous disease allowing subcomponent analysis and assembly of the oxidase to be studied. The disadvantages were that ROS production is 20 to 100 times less than neutrophils probably due to decreased levels of $gp91^{phox}$ and $p22^{phox}$. Lymphoblasts have previously been used in the sudy of the mechanism of increased sodium hydrogen exchanger activity in hypetension, and it has been found that the lymphoblasts maintain their native sodium hydrogen exchanger activity.

Utilising lymphoblasts to study the priming process is more problematical. Most neutrophil priming studies involve in vitro incubation of unprimed cells with a potential primer and examine the effects of inhibitors on the priming process. In lymphoblasts the inhibitor studies will only produce a positive result if the process of priming results in changes in the lymphocytes which persists after removal from the priming agent, and that are maintained in immortalized cells. Most priming agents are considered to have an indefinite effect, but PAF (231) and AGE (Advanced glycation end products) rapidly prime and deprime (166). This needs to be explored further by priming lymphocytes and then transforming them to blasts, a comparison between primed and unprimed blast cells could then be performed.

Transformation of lymphocytes by EBV may also increase the potential for kinase signaling (200;201), this should be of an equal magnitude in both NT and

HT groups. However it is possible that this increased kinase signaling could amplify differences in the NT and HT groups. This would seem unlikely as the results we found are in keeping with previously published results (Chapter 1.4).

The primer agonist pairing is also important in human neutrophil priming. Bajaj et al (232) found that TNFα primed fMLP stimulated ROS production, whilst having no effect on phorbol myristate acetate (PMA, similar to TPA) stimulated ROS production; Utsumi et al (214) found that TNFα and rG-CSF priming of the PMA or AA responses were less than the fMLP stimulated response. Lymphoblasts are activated by fewer agonists than neutrophils (unpublished data, see section 2.1D); we found consistent stimulation only with TPA and AA. It is possible therefore that we were unable to reveal upregulation of a portion of the NADPH oxidase cascade upstream of PKC, e.g involving G-protein signalling.

3.1B:Demographics of the lymphoblast cell lines

The groups of patients supplying the NT and HT lymphoblasts are well matched for sex. The NT group is on average 7.7 years younger than the HT group (P=0.24). There is some data to suggest that ROS production from neutrophils declines with age (96). The reduction in stimulated ROS production from the young to the elderly is less than 20% (96). In our small cohort we could find no evidence for a reduction in ROS production with age (see table 6) but the study was not powered to investigate this effect (see methods). We feel that the confounding effect of age on lymphoblast ROS production, if ROS declines with

age, should actually attenuate any differences between the groups rather than accentuate any observed difference. We have therefore not attempted to match the groups any better with respect to age.

The groups do differ with respect to family history of hypertension, 50% of people with HT had a family history of HT whilst none in the NT group had a family history of hypertension. The absence of a family history in the NT group is probably as would be expected. The study was not powered to investigate the effect of family history on ROS production, and would require at least a doubling in the number of lymphoblast cell lines in each of the HT family history and HT no family history groups. Lacy et al studied only a slightly bigger group of subjects and found a differential between HT and HT FH groups (37). I therefore include the FH subgroup analysis to try and demonstrate that FH was not confounding the increased ROS production seen in HT.

Blood pressure is a continuous variable with an arbitrary cut off defining HT and NT. The NT group had good average blood pressure readings of around 115(4)/77(2) mmHg. The HT group's average blood pressure was 149(5)/91(2) mmHg which although high, in clinical terms is not dramatically so. Three of the study patients were on anti-hypertensive medication at the time of the study. The effect of the antihypetensive agents on the lymphoblasts is not known; we had assumed as the lymphoblasts are no longer exposed to the antihypertensive agent the lymphoblasts would revert to their genotype but this may not necessarily be true. If the antihypertensives still exert an effect on ROS production in the lymphoblasts, all the evidence suggests that most

antihypertensives would attenuate any effect seen in ROS production (see introduction). The comparatively small increase in blood pressure of the HT group may mean that a larger effect in ROS would have been seen if a more hypertensive group were selected.

Renal failure (233) diabetes (234) and hypertriglyceridaemia (235) all increase superoxide production. The potential small differences in fasting glucose and creatinine, both of which are well within the normal range, and triglycerides, are probably of insufficient magnitude to be significant confounding factors.

The lymphoblasts were predominantly collected many years previously for another study. Some of the demographic data was incomplete, in particular smoking status. Smoking is known to increase endothelial ROS production (236). It is not known if the increased neutrophil ROS production is also seen in immortalized cells from smokers. Dietary information on the patients was not taken and therefore it is not known if patients consumed large quatities of antioxidants. The customary view of antioxidants is that ingested antioxidants increase the break down of ROS without altering the production of ROS. This may not be true as ROS may also be an intracellular signal, creating a positive feeback loop increasing ROS production (237). We also felt that as the cells were immortalized and removed from the dietary environment, diet and smoking history should not alter ROS production; but this is an assumption.

3.1C:Enhanced chemiluminescence for the assay of ROS production

Enhanced CL is a simple and real time measure of ROS production, but the chemicals used to enhance detection are not inert in the process of ROS formation. As discussed in the introduction lucigenin, which is the most frequently used CL probe, was not sensitive enough to detect lymphoblast ROS production. CLA was used in place of lucigenin. High background luminescent levels were detected. The high background is thought to be due to traces of transition metals in the experimental solutions (204) and can be abolished by addition of DETC. Although it is thought that CLA can act as an antioxidant (238;239) other authors have shown that it correlates well with electron spin trapping to assay ROS production (204). Certainly our ROS production results with CLA were in keeping with results obtained using luminol and isoluminol.

We chose to use isoluminol as well as luminol, because some authors have shown that luminol attenuates immunoglobulin priming of ROS production in neutrophils (208). Luminol proved able to detect differences between stimulated NT and HT lymphoblasts ROS production, and there was no apparent attenuation of the respiratory burst as compared with CLA CL. The attenuation in ROS production assayed by luminol experienced by Faldt *et al* (208) was unlikely to be a problem in lymphoblasts, as they are divorced from previous priming influences.

3.1D:Lymphoblast agonists TPA and AA

TPA is thought to stimulate NADPH oxidase to produce ROS indirectly by activation of protein kinase C (240), and AA directly activates NADPH oxidase (139). The use of 2 agonists, as well as demonstrating that the results are reproducible, shows that direct activation of NADPH oxidase with AA also results in the excess production of ROS in the HT cell lines. This suggests that some of the increased production is due to a difference in NADPH oxidase activity, rather than a pathway activating NADPH oxidase. This cannot be used as an argument against priming of NADPH oxidase, as the final step of priming is thought to be phosphorylation of $p47^{phox}$, and AA can act synergistically with the phosphorylated $p47^{phox}$ (101).

The dose of AA is supra physiological and is higher than most other authors who tend to use 10μM. We did perform dose response curves on 5 cell lines, that seemed to plateau at 50μM, but we are aware that at this level AA may be having non-specific membrane effects.

3.1E:Limitations of the Experimental Methods

3.1E(i):Lymphoblasts

The limitations of Lymphoblasts have already been discussed in Chapter 3.1A.

3.1E(ii):Limitations of Chemiluminescence

The system we used to assay ROS production could not detect a difference in basal ROS production. It is not clear if this is a false negative result or a true result. As we could only detect a difference in stimulated ROS production between the 2 groups, there needs to be a trigger for ROS production in vivo, for this to be clinically significant.

The Chemiluminescence detects ROS production but does not confirm the source of ROS. The abolition of stimulated ROS production with DPI (an inhibitor of flavoproteins), but only a minimal reduction with rotenone (inhibitor of mitochondrial NADH), suggests a non-mitochondrial flavoprotein, such as NADPH oxidase, is the source of the stimulated ROS production.

3.1E(iii):Limitations of PHOX Subunit Analysis

Lymphoblasts have been used to study the NADPH oxidase subunits in chronic granulomatous disease. The cytosolic components ($p47^{phox}$, $p67^{phox}$, and rac) are preserved in the transforming process whilst the membrane components are depleted. Difference in the membrane components in the HT group will

suggest in vivo differences, but it would not be possible to make a numerical extrapolation.

3.1E(iv):Limitations of Priming Studies

The inhibitor studies were carried out, based on the concensus concentration of agent to be used. This could potentially introduce errors as most of the previous work has been with neutrophils and ideally I should have performed dose response curves for each of the inhibitors.

When planning the experiments inhibiting possible priming pathways we decided not to inhibit PKC or G proteins. G protein activity is probably increased in HT lymphoblasts, but we believe it only becomes apparent when a G protein agonist is present (171). Neither TPA nor AA activates NADPH oxidase through G proteins and we found no other NADPH oxidase agonist that activated through G proteins. As G proteins are not being activated in our model, inhibiting G proteins alone should have no differential effect on ROS production. This assumption may not hold, as Siffert et al (171) found increased turnover of the HT lymphoblasts in culture due to a factor in foetal calf serum that was activating the HT upregulated G proteins.

The literature on mechanism of action of TPA is old, but suggests TPA stimulates PKC (123). We felt inhibition of PKC would not give any meaningful results if TPA activates PKC alone. 4 alpha-phorbol 12 beta-myristate 13 alpha-acetate (4 alpha-PMA) is a phorbol ester that does not activate PKC. 4 alpha-PMA could have been used as a control. If 4 alpha-PMA had no effect on ROS

production it would suggest, but not exclude, other pathways of NADPH oxidase activation by TPA. Inhibition of PKC with staurosporine or Ro318220 almost completey abolishes the ROS production induced by TPA (241-243).

3.2:Discussion of results

3.2A:Chemiluminescence experiments

3.2A(i):Baseline ROS production in NT and HT lymphobiasts

The high baseline readings detected using CLA have already been commented on earlier in the discussion. We were unable to detect any difference in baseline ROS production between NT and HT cell lines using CLA, luminol or isoluminol. We are unable to say if this apparent equality between NT and HT unstimulated ROS production is a true effect or just a reflection of the limits of sensitivity to detect ROS. As mentioned earlier in the introduction, methods and discussion, TPA stimulated lymphoblast ROS production is 5% of neutrophils stimulated ROS production.

Electron spin resonance can be used to assay ROS production but we did not have access to the technology. Utilising spectrofluorometry, amplex red is able to detect as little as 5pmol of H_2O_2 (244). Zhou suggests that amplex red is able to detect H_2O_2 production from as few as 2000 neutrophils, whereas Liu estimated that luminol and isoluminol based detection required a minimum of 9000 neutrophils (212). Amplex red may therefore have had some additional

benefit in detecting a difference in non-stimulated ROS production in NT and HT groups.

The in vivo significance of not being able to detect a difference in basal ROS production in NT and HT cell lines is difficult to interpret. As the lymphoblasts are thought to produce 5% of the corresponding neutrophil ROS production, it is still possible there is a clinically significant difference. It is also possible that there will not be a difference in basal ROS production between the 2 groups. The reason why stimulated ROS production may be clinically significant is discussed in the following section.

3.2A(ii):Kinetics of AA and TPA stimulation

AA produced a rapid rise in ROS production that likewise decayed very quickly, whilst TPA ROS production slowly rose to a maximum over 5 to 30 minutes, sometimes longer. These traces are consistent with the direct action of AA on NADPH oxidase, whilst TPA activates NADPH oxidase through PKC.

3.2A(iii):Effect of DPI, rotenone, tiron and catalase on ROS production

DPI abolished ROS production whilst rotenone had no effect on ROS production, as detected by luminol and CLA CL. DPI inhibits flavoproteins and has been shown to inhibit ROS production from un-stimulated macrophage mitochondria (225). As the ROS production was inhibited by DPI but not rotenone, this shows that a non-mitochondrial flavoprotein is responsible for ROS production. In the context of the agonists used, this strongly suggests that

NADPH oxidase produces the increased ROS seen in HT. More specific methods for downregulating NADPH phox subunits, such as use of RNA silencing (double stranded RNA leading to sequence-specific repression of gene expression) could now be employed, but was not available at the time this work was performed (245).

Rotenone reduced TPA stimulated ROS production detected by isoluminol, but interestingly not the AA stimulated ROS production detected by isoluminol. Rotenone had no effect on CLA or luminol detected ROS production, both of which assay intra and extracellular ROS production. It would suggest that mitochondrial ROS production has little to do with the total stimulated ROS production (intra and extracellular). As isoluminol detects extracellular ROS production, it would suggest that the rotenone alters the distribution between the intra and extracellular pools of ROS.

The tiron (chelator of superoxide) when used with CLA, luminol and isoluminol, abolished detectable ROS production. CLA CL is abolished by SOD (228) and therefore our results with tiron are in keeping with the previous findings. Although tiron catalyses the break down of superoxide, previous studies have shown that SOD has abolished luminol CL in dose dependent manner (226;227;229). Catalase only partially reduces luminol CL, which again fits with previous works (229) that suggest that the concentration of luminol may be important. CLA CL previously has been shown to be unaffected by catalase, but our results showed about a 40% reduction in stimulated CL (203). A possible cause for the discrepancy could be due to catalase reducing the high base line

readings we experienced. Ultimately the chelator results suggest that the lymphoblast produce a variety of ROS upon stimulation.

3.2A(iv):Stimulated ROS production and hypertension

We have demonstrated increased AA stimulated ROS production in HT cell lines compared to NT cell lines with CLA, luminol and isoluminol. When stimulating the lymphoblasts with TPA, we were able to detect significantly more ROS in HT cell lines with CLA and luminol but not Isoluminol. There are a number of ways of interpreting these results. Firstly HT lymphoblasts do produce more ROS, but on TPA stimulation the ROS are predominantly intracellular and therefore not detected by isoluminol. Secondly it is possible it could be a type 2 error. The isoluminol detects extracellular ROS and even with the AA stimulation the peak ROS production detected is numerically less than with the other methods of CL. There is approximately a 70% increase in the TPA stimulated ROS production between NT and HT cell lines as detected by isoluminol. The ROS data are not normally distributed and therefore there is more chance of not detecting a significant result, despite the study being theoretically appropriately powered to detect a 50% difference.

3.2A(v):Correlation of ROS production with BP

We demonstrated a significant correlation between DBP and SBP with AA stimulated ROS production as measured by CLA, luminol and isoluminol. TPA stimulated ROS production correlated with SBP and DBP as assayed by CLA

and luminol. TPA stimulated ROS production assayed by isoluminol did not correlate with DBP (R=0.20, P=0.34) or SBP (R=0.26, P=0.22). The lack of significant correlation seems to be a combination reduced magnitude of difference as Isoluminol only assays extra cellular ROS and small group size.

3.2A(vi):ROS production and family history of hypertension

Lacy *et al* (37) found that neutrophil ROS production was not determined by BP, but by family history of hypertension. We therefore performed a subgroup analysis of ROS production and family history of hypertension. In the NT group, non of the subjects were know to have a family history of hypertension, whilst in the HT group 50% (6 subjects) had a family history of hypertension (HT FH). We were unable to detect a difference in TPA or AA stimulated ROS production as assayed by CLA or luminol between HT and HT FH groups (Table 6). Isoluminol was not included in this subgroup analysis as no difference was found between the NT and HT groups. Caution must be used in interpreting these groups as the numbers are small and the study was not powered to look at this sub group. The results at least suggest that the increase in stimulated ROS production between NT and HT groups is not entirely due to a family history of hypertension.

3.2A(vii): ROS production and age

As with the family history of hypertension, the aim behind looking at variation of ROS with age was to try and ensure the small variation in age in the NT an HT groups was not the real cause of the observed difference. Previous work has suggested a reduction in ROS production with age. The study was not

powered to formally study the effect of age on ROS production but we could detect only a small non significant correlation with age (r=-0.05 to 0.35). Based on this we can say that the 7 year difference in age between the groups is probably not confounding the results but we cannot comment on the effect of age on ROS. As most study suggest that ROS output declines with age, the older HT group would offset any observed difference.

3.2A(viii):Comments on ROS production in NT and HT lymphoblasts

The results add to the growing body of evidence in favour of an association between ROS production and hypertension. The reason why it is stimulated ROS production and not basal ROS production that is associated with HT is not clear. One possible reason is that as the lymphoblasts produce 5% the amount of ROS that neutrophils emit and as the baseline ROS production is small, any differences in basal ROS production are below the threshold of CL detection with our equipment. Another possibility is that the primed or upregulated NADPH oxidase predisposes to HT but requires another trigger to unmask the phenotype. This is rather like a two hit hypothesis, where 2 factors need to be present to cause a condition to develop. A possible trigger for increased ROS production is angiotensin II (8;246). The increased stimulated ROS production may also have implications in the development of atheroma. Atherosclerosis is thought by many to be an inflammatory process (247); it is conceivable that inflammatory mediators could activate the respiratory burst. It has been found that small changes in C-reactive protein have a strong inverse

correlation with endothelial function (248); increased ROS production could mediate the decreased endothelial function. Stimulated ROS production may also be the link that associates chronic infections with endothelial dysfunction (249), and with the increased incidence of ischaemic heart disease.

As the increased stimulated ROS production of HT cells persists in transformed cells cultured in-vitro, we believed this could be due to a genetically predetermined ability to produce increased ROS. However if priming can exert an effect that persists post transformation, then an acquired defect would be apparent in the cultured cells.

3.2B:NADPH oxidase subcomponent analysis

3.2B(i):p47^{phox}, p67^{phox}, gp91^{phox} and Rac2 subunits

We could demonstrate no increased expression of p47^{phox}, p67^{phox}, gp91^{phox} and Rac2 subunits in the HT lymphoblasts. Other groups have found that there have been increased levels of gp91^{phox} and p67^{phox} but we could find no evidence of this in the lymphoblast model. As the lymphoblasts are poor sources of p22^{phox} and gp91^{phox}, compared with neutrophils, it might be possible to miss a small difference in gp91^{phox} between NT and HT lymphoblasts.

3.2B(ii):p22^{phox} subunit

We have found increased levels of p22^{phox} in the HT lymphoblasts which is in keeping with 2 groups that have shown increased production of p22^{phox} mRNA

in animal models of hypertension (145;250). The presence of $p22^{phox}$ is known to be critical for ROS production in x-linked chronic granulomatous disease (251) and in experimental models where $p22^{phox}$ is depleted (250). $p22^{phox}$ interacts with an SH3 domain of $p47^{phox}$ allowing electron transfer to occur and therefore the reduction of oxygen (103). The amount of $p22^{phox}$ may be a rate-determining step in the production of ROS.

3.2B(iii):Multiple regression analysis

The small increase in $p22^{phox}$ is probably insufficient alone to account for all the variance in ROS production. The multiple regression analysis we performed is flawed because of the small sample size, but suggests that the increase in $p22^{phox}$ accounts for approximately 30% of the increased stimulated HT ROS production.

3.2B(iv):Other factors altering subcomponent activity

One possible cause of increased ROS production is polymorphisms of $p22^{phox}$; the C242T polymorphism has been associated with increased ROS production (146) and may be associated with ischaemic heart disease (149;150;153) and stroke (155). Gardemann *et al* have also found a possible correlation between the $p22^{phox}$ polymorphism A640G and ischaemic heart disease (151).

NADPH oxidase can also be primed. Priming describes a complex process, often initiated by cytokines or infections, to enhance stimulated ROS production (168;169). The priming of NADPH oxidase increases serine phosphorylation of the phox subunits or can induce conformational changes of the phox subunits. Tyrosine kinases are thought to play a key role in priming. We therefore looked at tyrosine phosphorylation and inhibitors of second messengers thought to be involved in priming.

3.2C:Tyrosine phosphorylation

3.2C(i):Basal tyrosine phosphorylation

We found at least 6 tyrosine phosphorylated bands at approximately 110, 60, 44, 42, 38 and 28kDa. There was only one band with increased basal tyrosine phosphorylation in the HT lines and that would be consistent with p44/p42 MAP kinase. We therefore analysed the protein extracts with a specific tyrosine threonine phopsho p44/p42 MAPK antibody.

3.2C(ii):Basal p44/p42 MAPK phosphorylation

It has previously been shown that total p44/p42 MAPK levels are equal in the NT and HT groups (230). We demonstrated increased tyrosine /threonine phosphorylated p44/p42 MAPK in the unstimulated 12 HT lymphoblasts compared to the 12 NT lymphoblasts. This suggests that the increase is due to increased phosphorylation rather than increased total amounts of p44/p42 MAPK.

3.2C(iii):Stimulated p44/p42 MAPK phosphorylation

Upon stimulation of the lymphoblasts with TPA we found increased phosphorylation of p44/p42MAPK but there was no difference in phosphorylated p44/p42MAPK between the stimulated HT and NT cell lines. To see if the changes in basal tyrosine phosphorylation have an impact on stimulated ROS production, a variety of inhibitors were used to see if there was a differential effect on ROS production.

3.2D:Inhibitor experiments

3.2D(i):Effect of all inhibitors on basal ROS production

None of the tyrosine kinase inhibitors, PD98059 (inhibitor of p44/p42 MAPK), SB203580 (inhibitor of p38 MAPK), wortmannin (inhibitor of PI3K) and the inhibitors of PLA_2 had any apparent effect on basal ROS production as assayed by luminol. As basal CL levels were low it is not possible to exclude a subtle undetectable reduction in ROS production.

3.2D(ii):Inhibition of tyrosine phosphorylation

We have found that the tyrosine kinase inhibitors genistein and tyrphostin A25 reduce stimulated ROS production in NT and HT lymphoblasts by about 70%, and herbimycin A produces about a 40% reduction. The percentage reduction was the same in NT and HT cell lines. These results suggests that receptor linked tyrosine kinases have a larger role to play in lymphoblast ROS production than src type tyrosine kinases. Although there is increased basal tyrosine phosphorylation in the HT cells, the tyrosine kinase inhibitor induced reduction in TPA stimulated ROS production is most likely due to blocking of the stimulatory pathway of NADPH oxidase. The reduction is less likely to be due to inhibition of priming, as a larger percentage inhibition of ROS production would be expected in primed (HT) than unprimed (NT) samples (221). Although this finding reveals little about HT priming of lymphoblasts it alters the conventional understanding of the mechanism of TPA stimulation of NADPH oxidase as shown in figures 1 and 2.

Our results suggest that tyrosine kinases/tyrosine phosphorylation have a near essential role in the TPA stimulation pathway of NADPH oxidase in human lymphoblasts. Tyrosine kinase inhibition blocks TPA stimulation of macrophages (252), but the role of tyrosine kinases in TPA stimulation of neutrophils is not clear. Dusi (253) found that TPA stimulation of neutrophils was associated with increased tyrosine phosphorylation of p44/p42 MAPK and a 75kDa protein, but the tyrosine phosphorylation was not essential for TPA activation of NADPH

oxidase. In light of this and as inhibition of p44/p42 MAPK only produced about a 20% reduction in ROS production, we decided not to perform assays of p44/p42 MAPK phosphorylation after inhibition with tyrosine kinase inhibitors. Similarly genistein (a non-specific tyrosine kinase inhibitor) does not inhibit the TPA stimulation of neutrophil NADPH oxidase (254). Bennett et al (255) and Mitsuyama et al (256) found that tyrosine kinase was upstream of PKC, and therefore of TPA stimulation.

3.2D(iii):Inhibition of p44/p42MAPK

The p44/p42 MAPK inhibitor PD98059 reduced TPA stimulated ROS production by less than 25%, and there was not an increased reduction in the stimulated HT ROS production. This suggests that p44/p42MAPK is not involved in priming of the HT lymphoblasts NADPH oxidase. The results would be consistent with p44/p42 MAPK having a non-essential role in signaling TPA stimulated lymphoblasts ROS production.

3.2D(iv):Inhibition of p38 MAPK

The p38 MAPK inhibitor SB203580 had no detectable effect on TPA stimulated ROS production assayed by luminol. These results suggest that p38MAPK does not have a role in priming of HT lymphoblast NADPH oxidase, and is not involved in signaling TPA stimulated ROS production in lymphoblasts.

3.2D(v):Inhibition of PI3K

The PI3K inhibitor wortmannin reduced TPA stimulated ROS production by less than 25%, and there was no difference in the reduction seen in the NT and HT groups. These results suggest that PI3K is not involved in priming of HT lymphoblast NADPH oxidase. The results would be consistent with PI3K having a non-essential role in the TPA stimulated lymphoblast ROS production.

3.2D(vi):Inhibition of $cPLA_2$

The non-specific PLA_2 inhibitor mepacrine reduced TPA stimulated lymphoblast ROS production by approximately 35%, with no significant difference in the reduction between the NT and the HT cell lines. MAFP, a specific $cPLA_2$ inhibitor, reduced TPA stimulated lymphoblast ROS production by approximately 20%, with no significant difference in the reduction between the NT and the HT cell lines. These results suggest that PLA_2 is not involved in priming of HT lymphoblast NADPH oxidase. The results would be consistent with PLA_2 having a non-essential role in the TPA stimulated lymphoblast ROS production.

The modest reductions in ROS production with the inhibitors of PLA_2, are in keeping with those found Daniels (221); however Dana found that PLA_2 activity is essential for stimulation of NADPH oxidase (135). The non-specific PLA_2 inhibitor, mepacrine, is a more potent inhibitor of ROS production than MAFP, an inhibitor of $cPLA_2$, which is consistent with the results of Daniels et al (221). These results with MAFP and mepacrine, despite suggesting an isoform other

than $cPLA_2$ is involved in TPA stimulation pathway, are in apparent conflict with the results of Dana et al (135) who show convincingly an essential requirement for $cPLA_2$. MAFP is structurally very similar to AA, and although it inhibits $cPLA_2$, it could be acting as a partial agonist for NADPH oxidase, hence explaining the inconsistent results.

3.2D(vii):Inhibition of other phospholipases

NADPH oxidase can also be primed to produce increased ROS by PLD and PLC (168). PLD can be inhibited by 1-butanol and 1% ethanol. Both are crude agents that probably have other cellular effects and therefore we did not include these inhibitors in our studies inhibiting stimulated ROS production. PLC can be inhibited by neomycin and U73122, neither have been tried on the lymphoblasts (256;257).

3.2E:Conclusions of tyrosine phosphorylation and inhibitor experiments

Our results are consistent with tyrosine kinases having an essential role in signalling TPA stimulated ROS production, and p44/p42 MAPK, PI3K, PLA_2, but not p38 MAPK, having small non-essential roles in TPA stimulation of lymphoblast ROS production. In neutrophils there is no consensus for the roles of p44/p42 MAPK, p38 MAPK or PI3K in TPA stimulation.

A simple interpretation of these results would suggest that tyrosine kinase is down stream from PKC. Once tyrosine kinase is activated it has multiple actions activating p44/p42 MAPK, PI3K and PLA_2, explaining why inhibition of

the individual components does not abolish ROS production. The multiple actions of tyrosine kinases are compatible with previous work but its position down stream of PKC seems unlikely (see figure 2). Another possibility is a positive feedback mechanism. Endogenous ROS can activate a wide variety of tyrosine kinases in neutrophils (258). DPI, an inhibitor of flavoproteins such as NADPH oxidase, can abolish tyrosine phosphorylation in TPA stimulated macrophages (259). It is possible that the TPA stimulated tyrosine phosphorylation is down stream of NADPH oxidase, and is involved in a positive feed back mechanism. This may also explain the slow rise in lymphoblast ROS production after stimulation with TPA.

Although the increased basal phosphorylation of p44/p42 MAPK may represent increased priming of certain processes within the HT cells, we could not demonstrate any increased basal ROS production by NADPH oxidase in these cells compared to the NT cell lines. The inhibitor experiments strongly suggested that tyrosine kinase activity and p44/p42 MAPK activity were not priming NADPH oxidase to produce more ROS in HT subjects.

3.2F:Possible other causes of excess ROS production in HT

As described earlier polymorphism in $p22^{phox}$ may increase NADPH oxidase ROS output and there is some evidence they are associated with ischaemic heart disease.

We have not studied priming through PLD or PLC, but both could be part of a priming cascade (168).

ROS levels will be a balance of production and destruction; therefore decreased levels of superoxide dismutase, catalase and glutathione could increase the ROS pool. Previous work suggests levels of antioxidant defenses possibly rise in response to increased ROS production (191-193), rather than low levels being a cause of increased ROS.

3.3:Summary of results

In summary we have shown increased stimulated ROS production in lymphoblasts from HT patients. As the increase in ROS production is in cultured lymphoblasts it is suggestive of a genetic predisposition. NADPH oxidase is probably responsible for the increased ROS production in HT lymphoblasts, in part because of over expression of the $p22^{phox}$ subunit.

We found increased p44/p42 MAPK tyrosine phosphorylation in basal HT lymphoblasts. We however found no evidence for priming of stimulated lymphoblast NADPH oxidase activity through pathways involving tyrosine kinases, p38 MAPK, p44/p42 MAPK, PKC, PLA_2 or PI3K. We have found that inhibition of tyrosine kinases, particularly the receptor linked tyrosine kinases, reduces stimulated ROS production of lymphoblasts, both HT and NT, by approximately 70%. We believe the tyrosine kinase inhibition blocks the stimulatory pathway of NADPH oxidase activation, rather than the priming pathway of lymphoblasts stimulated by TPA. p44/p42 MAP kinase, PLA_2 and PI3K have a small and non-essential role in the stimulatory, but not the priming, pathway of lymphoblast NADPH oxidase, when stimulated by TPA. Thus, the primed phenotype of the HT derived lymphoblasts is not dependent on tyrosine kinase linked pathways, despite the demonstrated increase in p44/p42 MAP kinase phosphorylation in these cells lines.

3.4:Clinical significance of results

The clinical significance of increased ROS production is not entirely clear. Increased ROS production does cause impaired endothelial function and rises in blood pressure that can be ameliorated by simple antioxidants. There are a number of theoretical reasons why the increased ROS burden in hypertension may accelerate atherosclerosis (as outlined in the introductory chapter). Unfortunately outcome studies of antioxidants generally produce no effect on all cause mortality or cardiovascular mortality. Most of the negative studies have been undertaken on a group of patients who will have established but silent atherosclerosis. Therefore a possible reason for this apparent lack of benefit could be that antioxidants are unable to reverse atherosclerosis once it is established.

If ROS production is genetically predetermined and this increase in ROS can be detected prior to development of hypertension it may be possible to develop simple preventative strategies. An appealing preventative strategy would be antioxidants. Although use of antioxidants later in life did not prevent cardiovascular disease, early use may prevent the development of atheroma and reduce morbidity and mortality.

3.5:Further work

To confirm the baseline ROS production is the same in HT and NT lymphoblasts, more sensitive amplex red spectrofluorometry or even electron spin resonance experiments could be performed. It is thought that amplex red is more sensitive at detecting H_2O_2.

More specific methods for downregulating NADPH phox subunits, such as use of RNA silencing (double stranded RNA leading to sequence-specific repression of gene expression) are now available and could be used to confirm that lymphoblast ROS production originates from NADPH oxidase (245).

To get statistically more meaningful results about family history of hypertension and its role in ROS production further subjects would need to be recruited to both the HT group and the FH HT group.

To establish the clinical significance of the increased lymphoblast ROS production in HT, it would be interesting to compare neutrophil and lymphoblast ROS production from the same subjects. It would also be of interest to measure endothelial ROS production and endothelial function in the same group, if possible, to see what the relationship is between white cell and endothelial ROS production.

To further elucidate the mechanism of increased stimulated ROS production in the HT cell lines it would be interesting to perform analysis of the $p22^{phox}$ polymorphisms particularly looking for C242T polymorphism.

To validate if lymphoblasts are suitable for studying priming, a variety of primers need to be applied to lymphocytes and the lines then transformed, ROS

production could then be compared to paired unprimed controls. If evidence of priming persisting post tranformation then further investigation of lymphoblast priming could be undertaken. To look at priming further, the final step of priming (which is thought to be phosphorylation of serine residues on $p47^{phox}$) should be examined further. To simply assess serine phosphorylation of $p47^{phox}$ we tried to develop a phosphoserine specific antibody but this proved technically difficult. Commercially developed phosphoserine antibodies may be of use in this area. In addition, to compare amounts of serine phosphorylation of $p47^{phox}$ in NT and HT cell lines, radioactive isotope studies could be utilised. If these experiments yield positive results it would be worth then assaying PLD and PLC activity.

Analysis of the antioxidant defences (superoxide dismutase, catalase and glutathione) would be a different avenue to pursue but would probably be inappropriate given that previous studies have suggested increased levels. Such increased levels could be a response to the increased oxidative stress.

Our experiments showed that receptor linked tyrosine kinases are involved in activation of NADPH oxidase but the position in the activation pathway was not clear. To see if tyrosine kinase is down stream of NADPH oxidase and has a positive feed back role we could compare tyrosine phosphorylation after stimulation with TPA, with and without DPI (an inhibitor of flavoproteins and therefore NADPH oxidase).

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