Advanced Glycation Endproducts and oxidative stress from the Neutrophil Respiratory Burst.

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CONTENTS

ABSTRACT

Advanced Glycation Endproducts (AGEs), resulting from the non-enzymatic reaction of reducing sugars with proteins, accumulate in patients with diabetes mellitus and with advancing age and are implicated in the pathogenesis of vascular disease.

Oxidative stress also participates in vascular pathology and has also been reported in the context of diabetes and ageing. This study set out to explore the contribution of AGEs to oxidant stress generation, particularly by examining their effects on the respiratory burst of neutrophils and lymphoblasts.

Using chemiluminescence to detect reactive oxygen species (ROS), AGEs did not stimulate the neutrophil respiratory burst directly, but caused a dose-dependent enhancement of the neutrophil respiratory burst in response to a mechanical stimulus (up to 265% ± 42%, $p=0.022$) or chemical stimulation with fMLP (formylleucylphenylalanine) 100nM (up to 218% ± 19%, $p<0.001$). This phenomenon was immediate and reversible, and depended on the simultaneous presence of AGEs with the additional stimulus; hence AGEs appear to act as neutrophil 'co-agonists'. The in vivo correlates of mechanical and chemical stimulation may be vascular shear stress and microbial exposure respectively, especially since some acute vascular events have been correlated with infective episodes.

The 'co-agonist' effect of AGEs on the neutrophil respiratory burst appears to involve upregulation of the NADPH oxidase enzyme, as evidenced by a DPI-dependent suppression of basal and augmented ROS output. This in turn is dependent upon the generation of arachidonic acid (which may potentiate NADPH oxidase subunit function), via cytosolic phospholipase A_2 ($cPLA_2$) activation. The whole process is sensitive to adjustments of the intracellular redox status, implying a role for upstream redox signalling.

The lymphoblast model demonstrated that cell lines derived from hypertensive individuals exhibited greater stimulated ROS production than those derived from normotensive individuals, but beyond this they did not respond to AGEs with any enhancements in respiratory burst activity.

Abbreviations:

AA – Arachidonic acid

AGEs – Advanced Glycation Endproducts

ARDS – Acute respiratory distress syndrome

CML – Carboxymethyl-lysine

DAG – Diacylglycerol

DPI - Diphenylene iodonium

EBV – Epstein Barr virus

EDRF – Endothelium-derived relaxing factor

fMLP - Formyl-methionylleucylphenylalanine

GDI - Guanine nucleotide dissociation inhibitor

GDP – Guanine dinucleotide phosphate

GTP – Guanine trinucleotide phosphate

GM-CSF - Granulocyte-Macrophage-Colony Stimulating Factor

Hb – Haemoglobin

HBSS - Hanks balanced salt solution

HELSS - Haloenol-lactone suicide substrate

HPLC – High performance liquid chromatography

Hrp – Horse radish peroxidase

HSA – Human serum albumin

HT – Hypertensive

ICAM-1 - Intercellular cell adhesion molecule-1

IHD – Ischaemic heart disease

IP_3 - Inositol 1,4,5-triphosphate

LDL – Low density lipoprotein

LDCL - Lucigenin-derived chemiluminescence

LPS – Lipopolysaccharide

MAFP - Methyl-arachidonylfluorophosphonate

MAPK – Mitogen activated protein kinase

MM-Albumin – Minimally modified albumin

MSR – Macrophage scavenger receptor

NAC – N-acetylcysteine

NADPH oxidase – Nicotinamide Adenine Dinucleotide Phosphate oxidase

NF-κB - Nuclear Factor-κB

NO- Nitric oxide

NOS - Nitric oxide synthase

NT – Normotensive

PA – Phosphatidic acid

PAF – Platelet activating factor

PBS – Phosphate buffered saline

Phox – Phagocyte oxidase component

PI3K – Phosphatidyl-inositol-3-kinase

PIP_2 - Phosphatidyl-inositol-diphosphate

PIP_3 - Phosphatidyl-inositol-triphosphate

PKA – Protein kinase A

PKC – Protein kinase C

$cPLA_2$ – Cytosolic phospholipase A_2

$iPLA_2$ – Calcium-independent phospholipase A_2

$sPLA_2$ – Secretory phospholipase A_2

PLC – Phospholipase C

PLD – Phospholipase D

PTKs – Protein tyrosine kinases

RAGE- Receptor for Advanced Glycation Endproducts

RLU – Relative light units

ROS – Reactive oxygen species

SAPK – Stress-activated protein kinase

SOD – Superoxide dismutase

SOD-M – Superoxide dismutase mimetic

sRAGE – soluble form of the Receptor for Advanced Glycation Endproducts

SHRs – Spontaneously hypertensive rats

TF - Tissue factor

TNF - Tumour necrosis factor

TPA - 12-O-Tetradecanoylphorbol-13-acetate

VCAM-1 - Vascular cell adhesion molecule-1

VEGF - Vascular endothelial growth factor

Chapter 1 - INTRODUCTION

1.1 Advanced Glycation Endproducts - Background

Advanced Glycation Endproducts (AGEs) are products of protein and sugar which were first described almost 100 years ago by the food chemist, Louis Camille Maillard; hence the formative processes often being referred to as Maillard reactions. Maillard's work was derived from observing alterations to the physicochemical properties of protein-containing foods that were heated with sugars. The result was the generation of products that were yellow-brown in colour with changes also in flavour and odour. Latterly, it has been increasingly recognised that not only may such physicochemical changes to proteins also occur within living organisms, but that these AGEs may be mediators of a wide spectrum of pathologies.[1,2]

In specific chemical terms, AGEs are a heterogeneous group of irreversible protein-carbohydrate adducts resulting from the multi-step, non-enzymatic reaction of amino acids with reducing/aldose sugars (glucose, galactose, maltose, lactose). Despite fructose being a ketose sugar, its capacity to isomerise to glucose allows similar participation in this reaction. A consequence of AGE formation occurring through non-enzymatic processes is that accumulation is time-dependent, taking from weeks to months. However, it is also dependent upon temperature and the prevailing sugar and protein concentrations, which accounts for their increased accumulation in patients with diabetes mellitus and with ageing.

In Maillard reactions, the participating amino acids may be components of other structures such as lipoproteins and chromatin as well as pure peptides. Lysine is the amino acid most frequently involved in this reaction due to the presence of a free amino group at the terminal end of its aliphatic side chain but in vivo, proteins with low turnover rates are also very susceptible to glycation due to the time-dependent formation of AGEs. These include structural components of the connective tissue matrix and basement membrane, such as collagen, but also a myriad of other proteins including lens crystalline, nerve sheath myelin, tubulin, complement, plasminogen activator and fibrinogen.

The first stage in the non-enzymatic reaction of an aldose sugar with an amino acid involves a condensation reaction between the carbonyl group of the sugar and the free amino group of the amino acid containing compound, resulting in the formation of a Schiff base. This product is unstable and undergoes spontaneous rearrangement in the form of a dehydration reaction followed by isomerisation resulting in a more stable glycoprotein adduct or Amadori compound. Up to this stage, all reactions are potentially reversible, but subsequent dehydration, elimination and condensation reactions, with or without accompanying oxidation, yield either the final irreversible cross-links of the Advanced Glycation Endproduct or reactive intermediates called dicarbonyl compounds which may also act as precursors for AGE formation.
The presence of oxidation accelerates the glycation process[3] with the resultant products termed glycoxidation products (e.g. Carboxymethyl-lysine [CML], Pentosidine) as opposed to the pure glycation products (e.g. Pyrraline, Imidazolone) generated in the absence of any reactive oxygen species

(ROS). This enhanced formation and accumulation of AGEs through oxidative means is also indirectly demonstrated in vivo. Thus streptozotocin-induced diabetic rats typically show an increase in glycated-haemoglobin within 18 weeks, but when additionally treated with high doses of the antioxidant vitamin E, these increases in glycated-haemoglobin are suppressed, implicating an enhancing role of oxidation in this process.[4]

It is therefore evident that the formation of glycoxidation products is dependent upon the ROS levels as well as concentrations of the carbohydrate and protein precursors. This can sometimes lead to a self-perpetuating phenomenon as exemplified by the glycoxidation product CML. The latter is known to be able to generate binding sites for divalent metals and facilitate redox activity. This occurs through the Fenton reaction, in which the reaction of a transition metal with hydrogen peroxide generates highly reactive hydroxyl radicals that may subsequently contribute to further glycoxidation,[5] with the whole process being retarded by antioxidants such as ascorbate.[5] It is demonstrated explicitly by observing that rat tail tendons implanted into the peritoneal cavity of diabetic rats developed a greater CML content and hence copper content over tail tendons that had been implanted into the peritoneal cavities of control rats. Moreover, the CML and copper-rich tendons were able to oxidise ascorbate to a far greater degree than the control tendons.[6] The oxidative stress that results from such metal-catalysed reactions may not only be directed towards the original CML-bearing glycoxidation product, but also towards exogenous substrates, generating a cycle of further AGE/Amadori product formation if the substrates happen to be sugar and protein-based, or else contributing to other oxidative processes such as lipid peroxidation. In

the context of the latter, Sakurai et al demonstrated enhanced induction of lipid peroxidation by glycated polylysine in the presence of ferric iron. The involvement of free radicals was again substantiated by the fact that the addition of either Vitamin E or the metal chelating agent, desferrioxamine, suppressed this lipid peroxidation. [7]

Oxidative stress is thus seen to enhance AGE formation, but the presence of another reactive metabolite, the dicarbonyl compound, may also further AGE production leading to another concept of 'dicarbonyl stress.' Reactive dicarbonyls may form under a range of circumstances which include both oxidative and non-oxidative reactions. Non-oxidative formation has already been alluded to in the context of generation from the rearrangement and decomposition of Amadori products and may also occur through decomposition of intermediates in the glycolytic pathway.[8] They may also arise from the oxidation (metal-catalysed) of carbohydrates, proteins or even polyunsaturated fatty acids[9, 10] resulting in the generation of compounds such as glyoxal, methylglyoxal and 3-deoxyglucasone. Notwithstanding the different mechanisms of dicarbonyl formation, these compounds, bearing two -*C*=*O* groups, are highly reactive intermediates which in turn may interact with lysine, arginine or cysteine residues on proteins giving rise to an alternative mode of AGE generation.

In addition to the synthesis of AGEs via the various mechanisms described, exogenous sources such as food and tobacco smoking may also contribute to the accrual of AGEs. Tobacco smoking probably results in the formation of carbonyl-derived AGE compounds[11] with increased levels of these being

detected in lens crystalline and in the vasculature of smokers[12] whereas food-derived AGEs may originate through heat treatment and non-enzymatic reactions of protein amino groups with reducing sugars in the cooking process or as part of natural food spoilage. Under normal circumstances, the bioavailability of such ingested AGEs is thought to be less than 10%[13] owing to the inability to digest AGE cross-links and poor gastrointestinal absorption. However, AGE elimination partly depends upon renal excretion and therefore greater levels of AGEs are found in patients with uraemia, allied to the tendency of carbonyl compounds to accumulate in uraemia.[2] Indeed, the imputed deleterious consequences of uraemic AGE accumulation has led to food-derived AGEs being labelled as "glycotoxins".[13] As well as renal elimination, the degradation and metabolism of AGEs is probably dependent upon specific AGE-receptors on tissue macrophages of the reticuloendothelial system.[14] Such receptor-mediated uptake and processing may be facilitated by insulin possibly through activation of the phosphatidyl-inositol-3-kinase pathway, leading to lysosomal degradation of AGEs, exocytosis and subsequent renal clearance of small AGE-peptides. [15]

By virtue of the diversity of substrates and pathways leading to their formation, AGEs are a heterogeneous class of compound, with the precise structural features of dominant "native" AGEs being uncertain. Currently the most common methods used for detection have been High Performance Liquid Chromatography (HPLC), ELISA and immunohistocytochemistry using polyclonal antibodies generated to proteins browned by glucose. An important feature of such anti-AGE antibodies generated against AGEs prepared in vitro under physiological conditions, has been the realisation that there is a cross-

reactivity of AGE-epitopes that is common to proteins modified by AGEs in vivo.[16] Apart from the commonality of such epitope recognition however, there are defining characteristics for glycated proteins that are not based on structure and which most AGE products fulfil. Thus they are classically described as being (i) yellow-brown in colour, (ii) exhibiting typical fluorescence spectra, (iii) possessing cross-linking ability, and (iv) interacting with AGE-specific receptors. [2]

Like AGEs, AGE-binding molecules also have an assortment of structures but additionally serve diverse purposes. The first AGE-recognition molecules to be identified were isolated from cells of monocyte/macrophage lineage and found to belong to the class of receptors known as scavenger receptors.[17] Since then, non-enzymatically glycated collagen has been found to be an avid ligand for the Macrophage Scavenger Receptor (MSR).[18] As well as promoting the attachment of cells to the underlying extracellular matrix, such receptors predominantly facilitate the binding, endocytosis and degradation of modified LDL, although their role may also encompass the removal of other effete cellular components and foreign particles.

Most recently a 32 kDa AGE-binding protein exhibiting high-affinity binding for AGE ligands compared with that of other carbohydrates has been isolated, called galectin-3.[19] It is expressed on macrophages and a range of other cell types, being expressed minimally at the glomerular/mesangial level under normal conditions, but demonstrating increasing expression with age. The diabetic milieu also enhances its expression, which occurs earlier and to a greater extent than in matched non-diabetic subjects.[20] When diabetic

glomerular disease is studied in galectin-3 knockout mice which have been rendered diabetic with streptozotocin, the galectin-3-deficient mice are shown to develop accelerated glomerulopathy compared to their diabetic wild-type counterparts, despite similar metabolic excursions. Thus the galectin-3-regulated pathway appears to serve as a protective mechanism towards AGE-induced injury. [21]

In between, a variety of other AGE-receptors have also been discovered; OST-48 bears some homology to proteins present in the endoplasmic reticulum[22] and may act with a protective role similarly to galectin-3,[23] whilst a receptor called 80K-H is an efficient substrate for Protein Kinase C (PKC)[24] and through its ligation, may activate a signal transduction pathway of undetermined function.

However the best-characterised AGE-binding receptor is probably the receptor for Advanced Glycation Endproducts (RAGE). Using immunohistochemical and in-situ hybridisation techniques, the presence of RAGE has been confirmed on monocytes and in a variety of tissues, including neural tissue, skeletal and smooth muscle.[25] It is a 35 kDa member of the immunoglobulin superfamily of cell surface molecules with an extracellular portion comprising one variable-type domain and two common-type domains, a single spanning transmembrane portion and a very short, highly charged cytoplasmic tail. The locus of the main structural determinants mediating AGE-recognition and binding, lie in the variable-type domain of the extracellular portion, whilst the intracellular domain is most akin to the CD20 molecule and being very short, is likely to recruit signal transduction

molecules in the cytoplasm to effect cellular actions once the receptor is ligated.[26] Placement of RAGE in the immunoglobulin superfamily suggests that RAGE might participate in the host response to environmental perturbation, rather than solely functioning as an AGE-scavenging system. Consistent with this concept has been the discovery that the RAGE gene is located amongst a cluster of genes on chromosome 6 that code for the major histocompatibility complex receptors.[27] Furthermore, analysis of the RAGE gene has identified probable binding sites for nuclear factor-κB (NF-κB) in its promoter sequences.[28] The transcription factor NF-κB has a role in activating genes involved in inflammatory responses and is stimulated by the local redox environment. Hence RAGE expression appears to be responsive to local environmental changes.

The tissue expression of RAGE is a more complex phenomenon. Within humans and rodents, RAGE exhibits high expression during early development, predominantly in the central nervous system. Its principal ligand at this stage appears to be a molecule called amphoterin, with its engagement probably mediating neurite outgrowth in developing neurons.[29] However as animals mature, RAGE expression decreases to low levels in a range of cells including smooth muscle cells, endothelium, mononuclear phagocytes, pericytes, neurons, cardiac myocytes and hepatocytes. Subsequently, when particular pathological processes intervene, RAGE expression is upregulated[30] with the evidence pointing to cellular activation processes upon the binding of ligands to this RAGE receptor.[31, 32] Indeed, a striking feature is the co-localisation of this expressed RAGE with RAGE-ligands (including

AGEs) at pathological sites such as atherosclerotic plaques or at sites of Amyloid-β (Aβ) deposition in the Alzheimer's brain.

1.2 AGE accumulation in the clinical setting

From what has been previously said, the formation of AGEs may be summarised as a glycative/glycoxidative post-translational modification of protein-containing structures. The first in-vivo evidence for glucose-mediated post-translational modification of proteins came from structural studies on human Haemoglobin (Hb), in which the variant HbA1c was found to carry a glucose-Amadori product at its N-terminal valine.[33] Erythrocytes from diabetic patients have a greater HbA1c component than erythrocytes from non-diabetic individuals and since the amount of this AGE-precursor Amadori product is related by equilibrium to the glucose concentration, the measurement of HbA1c has now become a routine indicator of glucose control in the clinical assessment of diabetic patients.

In addition to equilibrium shifts favouring the formation of Amadori products in the diabetic state, quantitation of the irreversible AGE-adducts themselves reveals the markedly enhanced accumulations that can occur with diabetes mellitus in vivo. Thus when rats are examined, in which diabetes has been experimentally-induced by streptozotocin, the AGE content is seen to increase in lens tissue, kidney and aorta after only five weeks;[34, 35] moreover, compared to control rats, there exists a sixteen-fold greater renal AGE content at this stage.[35] In terms of the time taken for such accumulation to be associated with pathological changes, it has been demonstrated that a five

month intravenous administration of exogenous AGE to non-diabetic animals in concentrations sufficient to achieve the equivalent plasma levels of a diabetic animal, is capable of elevating renal AGE content to levels that are 50% greater than in control animals, with demonstrable abnormalities in renal histology including a 50% expansion of glomerular volume, increased mesangial matrix expression and glomerular basement membrane thickening.[36]

In humans, under normal conditions, Haemoglobin-AGE (Hb-AGE) accounts for approximately 0.4% of circulating haemoglobin, though this may increase up to 0.75% in diabetic subjects.[37] With regards to AGEs in general, levels may be approximately doubled in diabetes mellitus correlating with the severity of diabetic microvascular disease.[38,39] Indeed, even adolescents with diabetic microvascular disease, especially proliferative retinopathy, have significantly elevated serum AGE levels compared to non-diabetic subjects, again demonstrating that even in youth, AGE accumulation can be considerable in diabetic individuals.[40] Nonetheless, the time dependency of AGE formation also results in rising concentrations with increasing age[41] and higher levels of tissue AGEs may be found in older versus younger animals at a number of different organ sites including lung and aortic tissue.[42,43]

In vivo, it is likely that a combined glycoxidative process is more pertinent to AGE formation than pure glycation alone[44] with additional influence being conferred by the oxidant-status of the tissue microenvironment. Thus, in situations where the local redox potential has been shifted to favour oxidant stress, such as at inflammatory loci, or systemically in patients with uraemia,

AGE formation is substantially enhanced. Indeed, although a multitude of different AGE-structures may exist in vivo, the glycoxidatively formed AGEs carboxymethyl-lysine (CML) and pentosidine contribute predominantly to this burden, with a lesser contribution from the pure glycated-product, pyrraline.[45,46] Such glycoxidated-AGEs have been detected in vivo in atherosclerotic tissues, tissue collagen of the skin, the lens and the kidney (renal cortex, mesangium and glomerular basement membrane).[45,47]

Local oxidant stress may explain the occurrence and formation of AGEs in the atherosclerotic tissue of non-diabetic, non-uraemic patients, although protein turnover may also be relevant in this scenario with the trapping of macromolecules in the expanded neointima of the atherosclerotic vasculature.[48] Studies of phagocytes at sites of inflammation reveal that they are able to generate CML through myeloperoxidase, an enzyme which is released from the secretory granules of activated phagocytes.[49] This enzyme has a heme co-factor and may act through a metal catalysed oxidative process to generate the AGE-CML. The relative independence of such pathways from hyperglycaemia may thus explain the presence of AGEs in the atherosclerotic lesions of non-diabetic subjects as well as the presence of AGEs at various loci in patients with inflammatory conditions such as rheumatoid arthritis.[50]

As mentioned previously, under conditions of renal dysfunction, the failed renal clearance of AGE degradation peptides assists the accumulation of glycation products.[48,51] Allied to the increased oxidant stress in uraemia and the accumulation of reactive dicarbonyl precursor compounds in this

condition, marked elevations of AGE-modified plasma proteins (such as apoB-LDL) have been noted in patients with uraemia (of both diabetic and non-diabetic origin) compared to subjects with normal renal function.[52]

Finally, the effect of reduced protein turnover is also relevant to in vivo AGE generation, as exemplified by changes related to the tissue accumulation of amyloidogenic polypeptides. Dialysis-related amyloidosis occurs in patients on long-term haemodialysis and results from the accumulation in joint tissues of amyloid largely composed of β2-microglobulin. The latter demonstrates an abundance of AGE-modification due to the combined effects of delayed macromolecule turnover and uraemic oxidative stress.[53] In Alzheimer's disease meanwhile, AGE-modification of components of the intracellular neurofibrillary tangles[54] and of extracellular amyloid-β peptide (Aβ)[55] has also been observed in patients without diabetes, consistent with the potential for AGE-modification of macromolecules whose turnover has been delayed by other pathological processes.

1.3 AGEs and vascular disease

It has already been established that almost all tissues may be subject to modification by spontaneously-forming AGEs during normal development and ageing and to an accelerated degree with diabetes mellitus. A clinical correlation is seen to exist between the degree of AGE accumulation in these states and the increased vascular burden commonly associated with these conditions. That this AGE accumulation is not merely an epiphenomenon of the vascular pathology has been amply demonstrated by in vivo evidence on the direct pathogenic influence of AGEs. Thus in animal studies, the short-

term infusion of in vitro-prepared AGE-albumin can reproduce vascular and renal defects similar to those associated with experimental diabetes, including vascular leakage and unresponsiveness to vasodilatory agents.[56] Similarly, when euglycaemic rats were administered an eight week course of AGEs, they exhibited a resultant increase in glomerular and arteriolar basement thickening, mesangial expansion and glomerulosclerosis, with proteinuria and vascular dysfunction.[36,56] Significant AGE deposits were also detected in the retinal vasculature of AGE-infused rats, whilst the short term administration of in vitro prepared AGE-albumin produced vascular defects such as vascular permeability and leakage, and subendothelial mononuclear recruitment.[57,58] As well as the predominantly microvascular changes seen with exogenous AGE administration over the short to medium term, the long term infusion of physiological amounts of AGE-modified serum albumin into non-diabetic rabbits also resulted in macrovascular abnormalities, with resultant AGE accumulation in aortic tissues associated with intimal changes and increased cellular adhesion.[59] In similar animals placed additionally on a cholesterol-rich diet, these AGE-induced changes were markedly enhanced, consisting of multifocal atheroma containing foam-like cells and massive lipid droplets[59] and demonstrating the potential for interaction between various vascular risk factors in the development of atherosclerosis.

Support for a causal role of AGEs in the induction of vascular change has come from studies where attempts have been made to reduce the in vivo accessibility of AGE ligands. In a diabetic rodent model (the streptozotocin-treated rat), the increased vascular leakage that is a well-known feature of

diabetic microvasculature, was almost completely abrogated by ligation of circulating AGEs with a truncated, soluble form of the RAGE receptor (sRAGE); these reductions in vascular permeability, as measured by tissue-blood isotope ratios, occurred at a variety of tissue sites including the intestine, skin and kidney.[60] Furthermore, in another animal model, the atherosclerosis-prone mouse (homozygous for the deletion of the apolipoprotein E gene), which was again rendered diabetic by streptozotocin, treatment with sRAGE resulted in a dose-dependent reduction in the quantity of atheroma formation compared to that occurring in diabetic mice who were not treated with sRAGE.[61] This effect was independent of the lipid and glycaemic status of the mice and again underscores the potential for adverse interaction between AGEs and the vasculature with respect to atherogenesis.

1.4 Mechanisms of AGE-induced vasculopathy

The relationship of AGEs to vascular pathology may be explained by a number of different mechanisms that work independently or in concert with each other. The most established processes are described below:

i) Protein crosslinking.

A characteristic feature of AGE proteins is their ability to crosslink protein structures[62] predominantly through the involvement and interaction of lysine residues. This is supported by in vitro work using the agent phenacyl thiozolium bromide (PTB) which is able to cleave chemical crosslinks between two lysine residues.[63] The first proteins shown to be crosslinked in vivo and to be correlated with a pathological change were lens crystallines. Incubation in

vitro of lens crystalline proteins with glucose or glucose-6-phosphate results in the formation of high molecular weight protein aggregates that diffract light and bestow an opacification on the solution. Solutions of crystalline proteins in the absence of added sugar remain clear. Analysis of these protein aggregates reveals that the light scattering elements are crosslinked by disulphide and non-disulphide bonds.[64] This enhanced accumulation of glucose-derived cross-links is consistent with the observation that senile cataracts occur at a younger age in diabetic patients.

As a result of AGE formation and cross-linking of proteins, certain physicochemical changes take place on connective tissue in diabetes and ageing. AGE-mediated crosslink formation leads to a decrease in solubility and susceptibility of proteins to enzymatic digestion,[65] which in turn affects the process of tissue remodelling. These properties are particularly relevant to the function of connective tissue components such as collagen and elastin. Characteristically they are associated with increases in thickness[36] and rigidity,[43] changes typically observed in diabetic and ageing tissues. This may be manifest in the kidney as glomerulosclerosis and thickening of the capillary basement membrane. Elsewhere, post-mortem histological studies show a correlation between AGE tissue accumulation and aortic stiffness[66] and this provides an explanation for the stiffening of the heart and vasculature, otherwise known as arteriolosclerosis, that occurs with ageing.[67] In addition, AGE moieties formed on matrix components, such as on the vessel wall and kidney, retain their ability to cross-link and trap a variety of plasma proteins, most notably lipoproteins,[2] leading to reduced cholesterol efflux from the

vessel wall, lipoprotein accumulation and ultimately contributing to macrovascular disease.

ii) Influences on cellular adhesion and migration.

In the last fifteen years, it has been discovered that AGEs may exert specific effects on cellular function contingent upon the engagement of a variety of diverse cell surface molecules,[68] some of which have previously been mentioned. Ligation of the best characterised receptor, Receptor for AGE (RAGE), results in well-documented cellular perturbations which have purported roles in vascular pathophysiology. Interruption of AGE/RAGE interaction by a variety of means (antisense RAGE DNA, anti-RAGE antibodies or excess sRAGE) has been shown to attenuate AGE-induced upregulation of Vascular cell adhesion molecule-1 (VCAM-1), Vascular Endothelial Growth Factor (VEGF) and Tissue Factor (TF) expression,[69,70,71] factors which all play roles in the development of vascular pathology. Thus VCAM-1 is the adherence molecule associated with the early phase of experimental atherosclerosis and increased VCAM-1, along with Intercellular cell adhesion molecule-1 (ICAM-1), may be responsible for the increased interaction of the endothelium with circulating leucocytes[72] which may be a prelude to atherogenic processes; meanwhile VEGF upregulation may contribute to the new vessel formation that is a component of diabetic microvascular disease.[73] The AGE-induced enhancement of TF expression may help to sway the dynamic endothelial balance from an anti-coagulant to a pro-coagulant state resulting in local thrombosis and vasoconstriction[53] which may further be aided by the induction of a number of inflammatory cytokines such as Tumour Necrosis Factor (TNF) and interleukin-6 (IL-6).[74]

Through their interaction with RAGE, AGEs are thus a powerful instigator for the accumulation of macrophages and inflammatory cells in vivo. The development of such local inflammation by AGEs is furthermore aided by the fact that AGEs are themselves potently chemotactic to mononuclear phagocytes; this is dependent on the function of the RAGE receptor, with blockade of the latter by anti-RAGE IgG or the addition of excess sRAGE, decreasing macrophage chemotaxis towards a source of soluble AGEs in vitro.[75] The potency of this attraction is clearly demonstrated by experiments utilising a chemotaxis chamber in which AGE-albumin is adsorbed to the upper chamber and is able to retard macrophages from migrating down to the lower chamber where another chemotactic stimulus such as the bacterial cell wall peptide, formyl-methionylleucylphenylalanine (fMLP), resides. [75]

iii) Induction of oxidative stress.

Reduction-oxidation (redox) reactions involve the transfer of electrons or hydrogen atoms from one reactant (the "oxidised" substrate) to another (the "reduced" recipient). Oxidative stress reflects an imbalance towards oxidative processes due either to the excess ROS production or to a deficiency in anti-oxidant defence mechanisms and may potentially lead to tissue damage.[76] In vivo, many oxidant species are free radicals, molecules with unpaired electrons in their outer orbit. Their high reactivity relates to the instability of this electron configuration, leading to the extraction of electrons from other sources to pair with and stabilise the lone electron. Classical ROS include the superoxide and hydroxyl radicals, but also encompass hydrogen peroxide (which though itself is not a free radical, is eagerly receptive of two more

electrons making it a cytotoxic oxidant), hypochlorous acid (produced from oxygen by the neutrophil enzymes NADPH oxidase and myeloperoxidase), peroxynitrite and even nitric oxide, as all of these have oxidising effects and are derived from molecular oxygen. Biological systems have developed ways of countering the toxic effects of ROS. Foremost are the antioxidants, molecules that avidly combine with and annihilate active oxygen species,[77] such as ascorbic acid (Vitamin C) and α-tocopherol (Vitamin E). In addition cells have developed catalytic means of eliminating ROS including the superoxide dismutase (SOD) and catalase family of enzymes which catalyse the dismutation of superoxide and breakdown of hydrogen peroxide respectively.

A state of increased oxidative stress has been reported in the diabetic and ageing populations and may provide a link between the ischaemic prevalences in these two groups and the associated increases in AGE accumulation. Several clinical studies have shown increases in levels of oxidative stress in type 1 and type 2 diabetes when compared to healthy age-matched subjects, as measured by the accumulation of oxidatively-modified lipids and DNA.[78-81] Similarly, increased oxidative modification of collagen in diabetes has also been observed.[82] Meanwhile, Stadtman and co-workers have studied oxidative modifications to proteins in human and rat tissues by means of protein carbonyl measurement, and found them to be elevated two to threefold in old age compared to the young.[83]

It has already been seen that AGEs may contribute directly to oxidative stress as a consequence of the chemical processes leading to their formation,

including the formation of reactive carbonyl compounds and the auto-oxidation of glucose. Additionally, glycoxidative products resulting from these processes may bind transition metals and catalyse the production of further glycoxidative moieties through the Fenton reaction.

The ability of AGEs to increase the local inflammatory burden at a given site by virtue of receptor-mediated increases in the expression of leucocyte adhesion molecules such as VCAM-1 and the intrinsic chemotactic properties of AGEs has also been discussed. The resultant influx of inflammatory cells will comprise at least a significant component of phagocytic cells which liberate ROS according to their role in anti-microbial and inflammatory processes. Even in states where glycation products do not abound systemically, there may be regional accumulation of AGEs in pathological regions such as atheromatous aorta, leading to a local influx of inflammatory cells to such areas and subsequent increases in local oxidant stress.

Oxidative stress contributes to the pathogenesis of ischaemic diseases via a number of proposed mechanisms, including alterations in vascular tone and calibre, changes in vascular permeability, alteration of vascular endothelial adhesive properties, oxidation of lipids and interference with intracellular signalling processes. Oxidative stress impairs endothelium-dependent vasodilatation[84,85] through nullification of nitric oxide (NO), the body's endogenous Endothelium-Derived Relaxing Factor (EDRF). Similarly in the coronary vessels of diabetic animals, the addition of SOD or the pre-treatment of animals with α-tocopherol, restores function to an otherwise impaired endothelium-dependent relaxation.[86] Interestingly, it has also been

demonstrated that AGEs are able to quench NO and contribute to impaired vasodilatory responses in a similar fashion.[87]

Quenching of NO by free radicals however, not only affects vasomotor tone but additionally the anti-thrombotic properties of endothelium, since NO inhibits the expression of adhesion molecules (VCAM-1, ICAM-1) and the proliferation of vascular smooth muscle cells.[88] Work from several laboratories has also shown the presence of chronically activated T-lymphocytes within human atheroma[89,90] which may be implicated in plaque rupture. The inflammatory cells inhibit collagen synthesis and may therefore participate in the conversion of a stable to unstable plaque that ruptures under appropriate haemodynamic stress. ROS are produced in great quantity by this influx of inflammatory cells which then adversely affects endothelial integrity.[91] The ability of superoxide to neutralise NO has already been mentioned and the resultant vasospasm exacerbates all of this, but the direct effect of phagocyte-derived ROS also promotes platelet accumulation, aggregation and activation.[92,93] The breach in endothelial integrity promoted by ROS and inflammatory cell influx then enlarges the interstitial space, inducing leakage of blood constituents into the subendothelial layers. Furthermore, ROS may trigger activated inflammatory cells to release elastase, other proteolytic enzymes[94] and arachidonic acid metabolites, which cause endothelial and smooth muscle injury and capillary leakage.[95]

As well as acting through vasoactive intermediates, there is substantial evidence that oxidant stress also directly increases vascular endothelial permeability. The perfusion of hydrogen peroxide into isolated rabbit lungs

results in an increased capillary filtration coefficient within 30 minutes leading to pulmonary oedema[96] and this is not associated with increases in capillary pressure, implying that the oxidant-mediated tissue oedema is attributable to increased vascular endothelial permeability. The structural basis of such ROS-mediated increases in endothelial permeability may relate to alterations in the intercellular gap junctions, and cellular shape changes[97] that reflect gross reorganisation of the actin cytoskeleton;[98] this may be due to the direct oxidation of actin subunits, interfering with actin subunit interaction[99] or may be indirectly mediated through the activity of phospholipase enzymes.[100]

Atherogenesis is also promoted by the oxidation of Low Density Lipoprotein (LDL) to oxidised-LDL (ox-LDL) which is recognised by scavenger receptors on macrophages and internalised to form so-called foam cells.[101] In contrast to the uptake of native LDL by the LDL receptor on macrophages, the uptake of ox-LDL by the scavenger receptor pathway is not subject to negative feedback regulation resulting in massive uptake of cholesterol by the macrophages.[102] Ox-LDL is known to be able to transform macrophages and smooth muscle cells into foam cells in addition to modulating growth factor and cytokine expression, thus leading to the formation of atherosclerotic plaques.[103,101] In addition, ox-LDL is directly chemotactic to monocytes[104] and stimulates the binding of monocytes to the endothelium.[105] Once monocytes cross the endothelial layer, they become trapped in the subendothelial space, partly because ox-LDL inhibits their egress from the arterial wall.[106] Ox-LDL is also cytotoxic to vascular cells,[107] promoting the release of lipids and lysosomal enzymes into the intimal extracellular space and enhancing the progression of atherosclerotic lesions.[108] Supportive correlations are seen

between ox-LDL levels and the absence or presence of an ischaemic burden. Thus patients with carotid atherosclerosis have higher levels of autoantibodies to ox-LDL than do age-matched normal subjects[109] and plasma concentrations of immunoreactive ox-LDL are higher in patients with acute myocardial infarction than in normal subjects.[110]

Quite separately, ROS themselves have also been recognised to possess messenger roles within certain biological pathways.[111] However, their function within such pathways may again result in deleterious cellular responses when overactivated. In this context, the downstream activation of cellular activating events following the engagement of RAGE by AGE ligands has been shown to rely upon oxidant signalling. RAGE ligation by AGEs activates a G-protein, $p21^{ras}$, leading in turn to activation of an intracellular enzyme Mitogen-Activated Protein Kinase (MAPK) and ultimately to nuclear translocation of the transcription factor Nuclear Factor κB (NF-κB).[112] The latter is classically involved in oxidant-triggered gene transcription, many of which are involved in inflammatory and reactive processes. The AGE-dependent activation of $p21^{ras}$ has been shown to be dependent on the intracellular redox status with depletion of intracellular glutathione by buthionine sulfoximine enhancing its activation. It has further been discovered that a cysteine residue within $p21^{ras}$ is a target for free radicals. When experiments were performed in cells with a mutant form of $p21^{ras}$, in which this cysteine residue was replaced by serine, the AGE-RAGE dependent activation of MAPK was blocked.[112] This provides indirect evidence for ROS generation contingent upon AGE-RAGE interaction

and is consistent with the presence of an oxidant-sensing mechanism that mediates inflammatory processes.

There is to date however, little evidence for any direct activation of cellular ROS production following AGE stimulation. Moreover, little is known about the direct effects of AGEs on neutrophil function, one of the protagonists of ROS production in the vascular compartment. If AGEs are able to directly activate cellular ROS production, it would be important to know whether this could interact with other adverse vascular phenotypes in a synergistic or additive manner to further enhance intravascular ROS production with deleterious sequelae. A number of investigators have already demonstrated increased ROS production in the hypertensive phenotype pertaining to enhanced activation of the neutrophil respiratory burst,[113,114] and this has been attributed to an increase in the quantity of some of the enzyme subunits involved in the respiratory burst process.[115] Therefore the clinical synergism with which the phenotypes of diabetes and hypertension combine to produce ischaemic vascular insult, could conceivably result from any combined dysregulated ROS production of these individual phenotypes.

1.5 The role of neutrophils in vascular disease

A substantial body of clinical and epidemiological evidence implicates the leucocyte in the pathogenesis of atherosclerotic disease. The white cell count has been shown in many epidemiological studies to be a powerful and consistent predictor of cardiovascular events. Thus in the large Multiple Risk Factor Intervention Trial (MRFIT) and in a separate Japanese cohort, there

were strong correlations between the white cell count and the prevalence of coronary artery disease, risk of non-fatal myocardial infarction and the risk of sudden cardiac death, with this being independent of other risk factors such as cholesterol levels, blood pressure, sex or smoking.[116,117] A positive correlation is also seen between the white cell count and both sudden death and re-infarction in patients who have sustained a prior myocardial infarction and when the differential white cell count is examined, the most important cell type predicting ischaemic events in general is the neutrophil.[118] Indeed such predictive properties are not restricted to coronary disease, but also extend to the risk of thrombotic stroke and re-thrombosis following peripheral vascular grafting.[119,120]

There are several possible explanations for the involvement of white cells in the pathogenesis of vascular disease, including direct microvascular occlusion, interaction with other blood constituents such as platelets, and the release of toxic metabolites. The current focus is on the latter process, as during the course of phagocytosis and bacterial killing, neutrophils transform molecular oxygen to various toxic metabolites, including oxygen-derived free radicals, utilising a membrane-bound enzyme, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This process is known as the respiratory burst and it is these free radicals which may directly initiate tissue damage in several diseases where they are produced in excess.

1.6 The leucocyte NADPH oxidase

The leucocyte NADPH oxidase catalyses the one-electron reduction of molecular oxygen to produce the superoxide anion (O_2^-) using NADPH as the electron donor:

$$2O_2 + NADPH \rightarrow 2\ O_2^- + NADP^+ + H^+$$

The resultant superoxide is dismutated to hydrogen peroxide and in the presence of another enzyme, myeloperoxidase, present in neutrophil secretory granules, the peroxide oxidises chloride ions to form the highly reactive hypochlorous acid (HOCl) which plays a key role in microbicidal activity.

The NADPH oxidases are a group of plasma membrane-associated enzymes found in a variety of cells of mesodermal origin and are the major source of superoxide in vascular cells and myocytes.[121,122] Accumulating evidence suggests that NAD(P)H oxidase may be responsible for excessive superoxide generation in cardiovascular diseases. Both basal and NAD(P)H-stimulated superoxide production is significantly elevated in rats with heart failure secondary to myocardial infarction, and treatment with SOD improves endothelium-dependent vasorelaxation markedly in these rats.[123] NAD(P)H oxidase activity has also been reported to be increased, along with vascular superoxide production in Spontaneously Hypertensive Rats (SHRs),[124] whilst enhancement of the leucocyte respiratory burst of hypertensive patients has also been identified.[113-115] Furthermore, segments of saphenous veins, harvested from patients undergoing coronary artery bypass surgery have

revealed an association between increased NAD(P)H oxidase-dependent superoxide production and the presence of diabetes and/or hypercholesterolaemia.[125] In this context, the association of increased vascular NAD(P)H oxidase activity with clinical risk factors suggests an important role for NAD(P)H oxidase-mediated superoxide production in human atherosclerosis.

The neutrophil NADPH oxidase is found in phagocytes and B-lymphocytes. It comprises five 'phox' (phagocyte oxidase) components: $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, $p22^{phox}$ and $gp91^{phox}$. In the resting cell, three of these five components, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$, reside in the cytosol as a complex. The other two components, $p22^{phox}$ and $gp91^{phox}$ are located in the membranes of intracellular organelles called secretory vesicles and specific granules and are known in their dimeric combination as flavocytochrome b558. The composite cytochrome contains prosthetic heme and flavin groups. The latter is largely responsible for the initial function of the NADPH oxidase, acting as the electron receiver and resulting in the reduced form of NADPH which is then able to donate the electron onwards to substrates such as molecular oxygen.[121] The oxidase is inhibited by flavin antagonists such as deaza-FAD and diphenylene iodonium (DPI). Such selective antagonism of the oxidase system has upheld the notion that DPI-sensitive mechanisms are synonymous with a flavin-mediated and particularly NADPH oxidase-mediated mechanism. [126]

In the resting cell, the separation of the oxidase components into two compartments maintains the enzyme in an inactive state. When the cell is exposed to any of a wide variety of stimuli, the cytosolic component $p47^{phox}$ becomes heavily phosphorylated and the entire cytosolic complex migrates to the membrane where it associates with flavocytochrome b558, forming the active oxidase.[121] The subunit $p47^{phox}$ is chiefly responsible for transporting the cytosolic complex to the membrane during oxidase activation. This is evident because neutrophils lacking $p47^{phox}$ are unable to transfer $p67^{phox}$ from the cytosol to the membrane during activation, although $p67^{phox}$-deficient neutrophils are still able to transfer $p47^{phox}$ in a normal fashion.[127] Before the cytosolic oxidase components can be transferred to the membrane however, a pre-requisite phosphorylation of $p47^{phox}$ must take place, this being one of the characteristic events of oxidase activation. Protein phosphorylation by kinase enzymes is a common activation motif in biological systems. $P47^{phox}$ has the potential to be extensively phosphorylated, with 8 to 9 serine residues in the C-terminal quarter of the molecule acquiring phosphates.[128] Some but not all of these residues must be critically phosphorylated to endow the oxidase with full catalytic activity.[129] The effect of $p47^{phox}$ is to tighten by nearly 100-fold, the binding of each of the other cytosolic proteins to the assembled oxidase.[130] The function of $p67^{phox}$ meanwhile is more uncertain. Like $p47^{phox}$, it is also phosphorylated under the influence of cellular activating agents such as phorbol esters and the bacterial cell wall peptide, formylmethionylleucylphenylalanine (fMLP).[131] There is also an obligatory

requirement for $p67^{phox}$ to facilitate electron transfer to the flavin of cytochrome b558 and hence enable oxidase activity.[132]

Activation of the oxidase complex is aided by the participation of two low molecular weight guanine nucleotide-binding proteins (G-proteins): rac2, which in the resting cell is located in the cytoplasm in a dimeric complex with rho-GDI (guanine nucleotide dissociation inhibitor) and rap1A, which is located in the membranes.[121] Rac2 appears to have some similarity of function to $p47^{phox}$ insofar as it may be involved, but not essential to the translocation of the cytosolic complex to the membrane during oxidase activation; this occurs however through interaction with $p67^{phox}$ rather than $p47^{phox}$.[133] Rap1A belongs to the ras family, which has general roles in regulating cell proliferation. In experiments where rap1A was locked in either a GTP or GDP-bound form, phorbol ester-induced oxidase activation was inhibited.[134] Thus oxidase functioning appears dependent on rap1A cycling from a GTP to a GDP-bound state.

Quite separately, $p40^{phox}$ may play an inhibiting regulatory role in oxidase functioning. Cells expressing $p40^{phox}$ along with other recombinant oxidase components produce only about half the amount of O_2^- generated by cells in which $p40^{phox}$ expression is absent.[135] $P40^{phox}$ is phosphorylated in the resting cell, taking up additional phosphate when the NADPH oxidase is activated and losing the additional phosphate when the oxidase is deactivated, emphasising again the importance of phosphorylation mechanisms in the regulation of the oxidase complex. Overall, such a

complex array of subunit interactions allows the activity of the oxidase enzyme to be regulated in a precise fashion in response to multiple activating and inhibitory influences.

1.7 Modulation of neutrophil NADPH oxidase activity

A change takes place in neutrophil reactivity between its basal and activated states. However this transition is not a straightforward switch. When neutrophils from patients with stable ischaemic heart disease are compared with those from age-matched controls, stimulation with the bacterial cell wall peptide, fMLP, results in a far greater neutrophil response in those neutrophils from patients with stable ischaemic heart disease (IHD) than those from normal subjects. This applies to a number of neutrophil effector functions including chemotaxis and ROS generation.[136] The hyperactive potential of neutrophils in patients with stable IHD may relate to an upregulation of their effector functions in anticipation of a further stimulus. When stable heart disease progresses and an acute myocardial infarction supervenes, even the basal neutrophil ROS generation is found to be exaggerated.[137] Thus a serial enhancement in the oxidative capacity of neutrophils is seen that may translate into a mechanism for the pathophysiological progression of normal arteries to stable IHD and then to unstable IHD.

One well-described system of upregulating neutrophil effector function in response to a given stimulus is 'priming'. A priming stimulus does not elicit any effector function on its own, but instead potentiates a future elevated response to another challenging stimulus. This is important as normal

circulating neutrophils do not exhibit anything like their full microbicidal capacity when challenged with a variety of chemical and physical stimuli de novo. Following a priming stimulus however, effector function, such as the release of superoxide, may occur at a much more rapid rate and with a shorter time lag after exposure to the subsequent challenging stimulus. Hence the neutrophil respiratory burst that occurs in response to a secretagogue agonist and results in the release of superoxide anions may be enhanced up to 20-fold by prior exposure of cells to a priming agent.[138] Similarly, substantial priming of agonist-induced degranulation and the generation of lipid mediators (principally arachidonic acid, leukotriene B4 and platelet activating factor) has also been described.[139,140] By definition such priming agents must be presented to the cells for a variable period before the cell is exposed to the subsequent activating stimulus. Certain agents may have compound roles, behaving as priming agents at low concentrations and activating stimuli at higher concentrations.[141]

Although originally described as an in vitro phenomenon, many priming agents possess biological relevance in vivo, and are released in response to a range of insults such as infection, inflammatory diseases, trauma and ischaemia. Furthermore, primed neutrophils have been identified in the peripheral blood of patients after trauma, ARDS, bacterial infection and in the joints of patients with rheumatoid arthritis.[141] As these primed neutrophils manifest an augmented release of oxygen metabolites following stimulation, priming might enhance anti-microbicidal activity yet predispose the host to increased oxidative tissue damage. Neutrophil priming has been shown to be critical for the induction of endothelial injury both in vitro and in vivo. Activated

neutrophils produce minimal damage to endothelial monolayers in vitro, but when first primed with low concentrations of Lipopolysaccharide (LPS), endothelial damage resulted.[142] Likewise, in a rabbit model, intravascular administration of low concentration LPS, followed by the bacterial cell wall peptide fMLP, greatly enhanced neutrophil vascular sequestration within the lung with consequent damage, an effect that was not seen when either agent was used alone.[143]

A large number of neutrophil priming agents, physiological, pathological and pharmacological, have already been identified (Table 1.71). Priming may additionally be induced by physical stimuli such as osmotic swelling.[144] It is widely presumed that the various priming factors either act through modification of one or more components of the cellular signal transduction mechanisms mediating agonist-induced neutrophil activation, or through a general increase in the catalytic activity of the NADPH oxidase or perhaps even a combination of these two processes. With the existence of a great diversity of priming agents and differential potencies (exemplified by inositol hexakisphosphate being a much weaker priming agent than TNF-α[141]), it is possible that there is no single priming mechanism. Nonetheless, it is likely that common themes exist and evidence for a variety of mechanisms may be briefly examined.

The duration of pre-exposure to a priming agent in order to induce priming is highly variable, ranging from 1 minute for Substance P[141] to 120 minutes for GM-CSF.[141] Despite this delay, it is unlikely that priming involves de novo protein synthesis or post-translational modifications of proteins; for one, in the

case of Substance P, the time interval is all too brief but in addition, experiments on primed azotemic neutrophils have shown that the presence of inhibitors of protein synthesis such as actinomycin D and cycloheximide, which block transcription and translation respectively, do not abolish the ability of uraemic serum to prime neutrophils harvested from normal serum. Also, the finding that priming of the oxidative burst is rapidly reversible after incubation of normal neutrophils in uraemic serum, suggests that the priming process does not involve fixed posttranslational modifications of normal cellular proteins.[145]

Table 1.71

Established Neutrophil Priming Agents:

Atrial Natriuretic Peptide (ANP),
Substance P,
Inositol hexakisphosphate,
Platelet Activating Factor (PAF),
Tumour Necrosis Factor-α (TNF-α),
Interleukin-8 (IL-8),
Orthovanadate,
Influenza A virus,
Lipopolysaccharide (LPS),
Granulocyte-Macrophage-Colony Stimulating Factor (GM-CSF),
Interferon-γ (IF-γ).

One proposed action of priming agents is to cause upregulation of receptors to neutrophil activating agonists which would have the effect of augmenting subsequent responses to that particular secretagogue agonist. In particular, GM-CSF, TNF-α and Lipopolysaccharide have all been shown to upregulate

neutrophil chemoattractant receptors with demonstrable increases in fMLP receptor density on neutrophil membranes.[146-148] In fact, not only the receptors themselves, but receptor-coupled G-proteins have also been shown to be up-regulated in this fashion. Caution is required before assuming that these changes are sufficient to enable priming however and are not just epiphenomena; thus some authors have suggested that this increase may simply reflect the surface mobilisation of granule-associated fMLP receptors which are normally resident in the cytosol, with this mobilisation taking place as part of stimulated exocytosis.[141] Thus while TNF-α increases fMLP binding to neutrophils, this effect lags behind the cytokine's ability to prime fMLP-induced degranulation and hence cannot be responsible for the priming effect.[147] Similar arguments prevail against a role for receptor-coupled G-protein increases as the mediators of priming, though additional evidence comes from work demonstrating an absence of subsequent G-protein-linked signalling events (notably inositol 1,4,5-triphosphate [Ins(1,4,5)P_3] generation) in primed cells.[149]

As neutrophil priming is conferred by a vast array of disparate agents, acting on a wide range of different types of transmembrane cellular receptors, it is plausible that the multiple receptor-mediated pathways eventually converge on common intracellular pathways linked with the neutrophil NADPH oxidase.[150] This is suggested in studies of neutrophils from azotemic individuals which exist in a primed state and cannot be further upregulated by the addition of TNF-α and vice versa, even though the proximal pathways by

which azotemic serum and TNF-α are believed to initiate priming are vastly different.[151]

If there is a final common pathway on which the various priming mechanisms might converge, then several candidate systems may be examined, beginning with the neutrophil NADPH oxidase itself. Activation of the oxidase has been seen to involve translocation of its cytosolic and secretory granule components to the plasma membrane which is in turn dependent upon phosphorylation processes and also changes to the intracellular cytoskeleton that facilitate such intracellular trafficking. Lipopolysaccharide (LPS) is one priming agent that has been found to affect such intracellular trafficking; neutrophil priming with LPS increases membrane translocation of the cytochrome b558 component and also the cytosolic units $p47^{phox}$, $p67^{phox}$ and rac2. The latter components translocate to the membrane in small quantities with LPS exposure, but in even larger and more significant quantities when LPS pre-treated neutrophils are further challenged with fMLP and the latter is associated with a significantly enhanced neutrophil respiratory burst compared to non-LPS-exposed neutrophils.[152] As the priming agent PAF, has been shown to augment f-actin assembly in response to subsequent fMLP stimulation[153] it seems likely that cytoskeletal changes are behind some of the movements in intracellular proteins seen following LPS-exposure and it may be through such cytoskeletal reorganisation that priming agents mediate some of their effects.

As $p47^{phox}$ activation is mediated through phosphorylation processes, and a variety of protein kinases [Protein Kinase C (PKC), Protein Kinase A (PKA), Mitogen Activated Protein Kinase (MAPK)] are all capable of phosphorylating $p47^{phox}$ with overlapping predilection for the various critical phosphoserine residues,[128] differential kinase activation might also mediate some aspects of the priming mechanism. Indeed, a priming role has already been ascribed to Phosphoinositide 3-Kinase (PI3K). Many neutrophil secretagogue agonists stimulate PI3K with resultant formation of phosphatidylinositol 3,4,5-triphosphate [PtdIns(3,4,5)P_3] from PtdIns(4,5)P_2. Wortmannin is a relatively specific and irreversible inhibitor of neutrophil PI3K activity and is able to abolish both fMLP and phagocytosis-induced respiratory burst activity.[154] Furthermore wortmannin has been shown to be able to inhibit both GM-CSF and TNF-α priming of the fMLP-induced respiratory burst, with the latter demonstrating that the time course of PtdIns(3,4,5)P_3 accumulation mirrored that of superoxide generation.[149,155] In the experiments with TNF-α, it appeared that priming exerted a direct action on PI3K activity and did not serve to increase the substrate PtdIns(4,5)P_2 from which PtdIns(3,4,5)P_3 is formed.

Finally, tyrosine phosphorylation has emerged as another possible candidate linking diverse priming mechanisms in a final common pathway because it is associated with neutrophil activation per se and also with exposure to a variety of priming agents including fMLP, PAF, Complement, GM-CSF and TNF-α.[150] It has been discovered too that manipulation of the phosphotyrosine state experimentally, via inhibition of either tyrosine phosphatase (with

orthovanadate) or tyrosine kinase (with genistein) activity, results in enhancement or attenuation of such priming effects respectively.[156] Moreover, it appears that only a few (less than 8) protein substrates are significantly tyrosine phosphorylated under such circumstances and of these, perhaps only two are consistently found to be tyrosine phosphorylated in primed cells (with associated molecular weights 40-44 and 72-74kDa).[157,158] Although the identity of these target proteins has not yet been elucidated, the 40-44kDa protein has been speculated to be a member of the MAP Kinase family, with tyrosine phosphorylation of MAP Kinase being associated with chemoattractant activation of the NADPH oxidase.[159] MAP Kinases are also typically activated in response to a variety of stressor stimuli and have already been seen to be potentially involved in $p47^{phox}$ phosphorylation. There is however a paucity of consistent evidence for MAP Kinase involvement in neutrophil priming mechanisms from experiments involving specific inhibitors of p38MAP Kinase (SB203580) and p44MAP Kinase (PD98059) which are unable to inhibit neutrophil priming responses from a number of classical agents.[160,161]

The uncertainties regarding the component mechanisms of neutrophil priming underscore its complexities. Nevertheless, the fact that neutrophil responses, especially the respiratory burst, may be subject to control by exogenous agents, points towards ways whereby the vascular redox state may be regulated in vivo. The current investigations are an attempt to determine the contribution of Advanced Glycation Endproducts to the vascular oxidant status through any effects on the neutrophil respiratory burst.

1.8 Hypothesis and aims

Given the contribution of oxidative stress to vascular pathophysiology and the increasing evidence that AGEs may contribute also to the development of atherosclerotic vascular disease, it may be hypothesised that AGEs are capable of directly inducing cellular oxidant stress. To date, there has not been a great deal of experimental evidence that demonstrates such a phenomenon, although surrogate markers, like the activation of redox-sensitive pathways,[112] have hinted at this possibility.

The hypertensive state is also associated with enhanced oxidative stress and it is conceivable that AGEs might interact with this in an additive or synergistic fashion, thus generating even greater oxidant stress. This could provide a model for the deleterious cardiovascular effects seen when the hypertensive and diabetic states co-exist.

Following on from these hypotheses, the aims of the study are as follows:

1) To examine the effect of AGEs on neutrophil respiratory burst function, as the latter results in a significant and measurable generation of ROS and neutrophil activity is positively correlated to vascular events per se.

2) To see whether AGEs are specifically capable of modulating neutrophil NADPH oxidase activity, because this enzyme complex, responsible for effecting the respiratory burst, is known to be subject to modulation by a variety of other chemical mediators.

3) To test the hypothesis that AGEs interact synergistically or additively with the putatively enhanced oxidative burst of 'hypertensive' lymphoblasts, thus generating even greater oxidative stress.

4) To study the mechanisms underlying any detected effects of AGEs on respiratory burst function.

Chapter 2 - MATERIALS AND METHODS

2.1 Reagents

Arachidonic acid (AA), BAPTA-AM, Bis-N-Methylacridinium Nitrate (Lucigenin), Butanol, Butan-2-ol, Catalase (Bovine liver), Chelerythine, Digitonin, Diphenyleneiodonium (DPI), EGTA, Fetal calf serum, Formyl-methionylleucylphenylalanine (fMLP), Fura 2-AM, Genistein, Glucose, Herbimycin A, Histopaque 1077, Horse radish peroxidase type II (HRP), Indomethacin, Luminol, N-acetylcysteine, Platelet Activating Factor, Percoll, RPMI 1640, 12-O-Tetradecanoylphorbol-13-acetate (TPA), Tissue Culture Media 199 (TC199), Tiron, Tumour Necrosis Factor-α (TNFα - human recombinant), Tyrphostin A25, Wortmannin and Y-27632 were all purchased from Sigma-Aldrich.

20% Endotoxin-free Human Serum Albumin (HSA) was from BioProducts Laboratory.

Isoluminol was purchased from Fluka.

Haloenol-lactone suicide substrate (HELSS), Manoalide and Methyl-arachidonylfluorophosphonate (MAFP) were purchased from Biomol.

Mepacrine (also known as quinacrine), MK-886, PD98059, Ro-31-8220 and SB 203580 were from Calbiochem.

SOD mimetic (MnTMPyP) was from Alexis.

^{3}H-AA, Anti-rabbit and anti-mouse IgG conjugated to horse radish peroxidase were obtained from Amersham Life Science.

The polyclonal rabbit p44/42 MAP kinase antibody was purchased from Cell Signalling Technologies. The monoclonal mouse phosphotyrosine antibody was supplied by Transduction Laboratories (USA).

2.2 Cell and media preparation

Preparation of AGE-Albumin: AGE-Albumin and Minimally Modified Albumin (MM-Albumin) were prepared by pre-incubating endotoxin-free 20% Human Serum Albumin (HSA) with 1M or 30mM Glucose respectively, at 37°C for 12 weeks in 100 mmol/l phosphate (pH 7.4). At the end of the incubation, AGE-Albumin/MM-Albumin was dialysed against Phosphate Buffered Saline (PBS) for 24 hours and 0.9% Sodium Chloride for 12 hours. Nonglycated HSA was used as a negative control.

Tissue Culture: Lymphoblast cultures were established according to the protocol of Ng et al.[162] A study group of 12 hypertensive (HT) patients and 12 age and sex-matched normotensive (NT) subjects was selected for investigation. The lymphoblasts derived from these subjects were maintained in RPMI 1640 growth medium containing glutamine, penicillin, 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 5% CO_2 and O_2. Aliquots were taken on the day of experiment and cells were recovered by centrifugation (1400g for 10 minutes). The cells were spun through Histopaque 1077 at 1400g for 10 minutes to isolate the viable cells and washed in TC199 before being suspended in 1-5mls of TC199. The cells were incubated at 37°C for 20 minutes prior to the commencement of experiments,

in a rotating hybridisation oven (Appligene) to reduce the likelihood of cell clumping and adherence. Cell numbers were estimated using a Coulter counter (Beckman).

Neutrophil Isolation: Neutrophils were prepared according to the method of Baron et al.[163] 20 ml fresh citrated blood was obtained from healthy adult volunteers after informed consent, added to Dextran solution [300mg in 5 ml of Hanks Balanced Salt Solution (HBSS) containing: 140mM NaCl, 4mM $NaHCO_3$, 0.3mM Na_2HPO_4 (anhydrous), 5mM KCl, 0.4mM KH_2PO_4 (anhydrous), 5.5mM Glucose, 0.4mM MgSO4.7H_2O] and allowed to sediment for 30 minutes. The plasma (containing platelets and leucocytes) was removed and centrifuged for 10 minutes at 482g. The resultant cell pellet was vortexed with dilute HBSS (1 in 5 dilution with distilled water to a total volume of 5mls) for 10 seconds to lyse contaminating erythrocytes, before osmolality was rapidly restored by the addition of an excess volume of HBSS (20mls). A mixed leucocyte pellet was then obtained by further centrifugation at 482g for 10 minutes, with the resultant pellet resuspended in 1-2mls of TC199 (pH 7.4) and layered onto a pre-prepared continuous density gradient of Percoll (65% Percoll, 10% 10 x TC199, 1.6% 1M HEPES, 0.4% 5M NaCl and 23% sterile water, pH 7.4). Following centrifugation at 21982g for 15 minutes at 4°C, the dense neutrophil band was recovered, washed in TC199 and incubated at 37°C for 20 minutes prior to use, in a rotating hybridisation oven (Appligene) to prevent neutrophil clumping. The viability of neutrophils isolated by this method was more than 95%, as determined by the trypan blue dye exclusion

test. The subsequent counting of cells was performed on a Coulter counter (Beckman).

Coating of Microplate wells: For those experiments where coated luminometer plates were required, the wells of Microlite-2 plates were filled with 100µl PBS containing 500µg AGE-Albumin/Albumin, covered and left overnight on a plate shaker. They were then washed three times with PBS and left to dry prior to use.

2.3 Chemiluminescence

Detection of Reactive Oxygen Species: ROS detection was based on the chemiluminescent technique described by Liu et al[164] with chemiluminescence recorded predominantly on a microplate luminometer (Berthold LB96V) and some comparative measurements being made on a tube luminometer (Berthold LB953). For experiments undertaken on the LB96V microplate luminometer, uncoated Microlite-2 plates (Dynex) were used as the substrate for measurements.

Three different chemiluminescent probes were examined: Lucigenin 50µM, Isoluminol 50µmol/l and Luminol 5µmol/l. Each of these three probes were made up in a balanced salt solution containing 140mM NaCl, 15mM HEPES, 5mM KCl, 5mM Glucose, 1.8mM $CaCl_2.2H_20$ and 0.8mM $MgSO_4.7H_2O$ (pH 7.4). The solutions of Isoluminol and Luminol contained in addition 6 units Horse Radish Peroxidase (HRP)/ml. All experiments were performed in triplicate with 10^5 cells per well and were carried out at 37°C. The three

different chemiluminescent probes were tested to see if there might be cell or stimulus-specific differences in ROS detection. Thus ROS detection from neutrophils and lymphoblasts was measured following mechanical stimulation of the cells and chemical stimulation with one of two agents, fMLP and TPA.

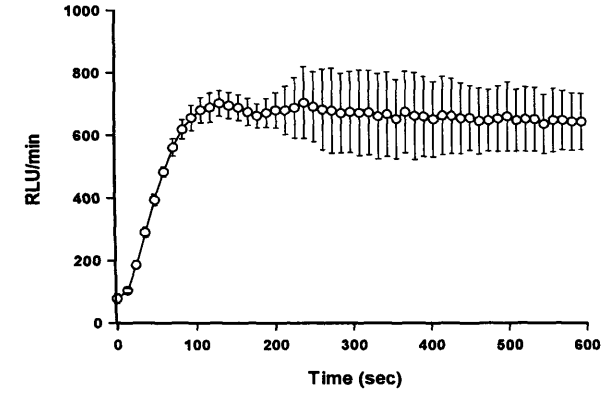
Using chemiluminescent techniques, ROS production was assayed in real time from neutrophils and lymphoblasts following stimulation by a variety of modalities and using the three different chemiluminescent probes; representative findings from these experiments are displayed in Figures 2.31-2.38. In neutrophils, it was apparent that ROS were most sensitively detected with Lucigenin, and this was true for all modes of cell stimulation (Figures 2.31-2.34), with Isoluminol being the next most effective chemiluminescent probe for neutrophil ROS detection. An evaluation of the total ROS production (determined by calculating the area under the curve at 6 minutes), confirmed Lucigenin as the most sensitive probe with regards to neutrophil stimulation (Figure 2.34). With all probes, in the presence of AGE but absence of cellular stimulation, no chemiluminescent response was detected, negating any suggestion of interfering autofluorescence from the reaction media or AGEs themselves (data not shown).

With Lymphoblasts however, Luminol and Isoluminol were superior to Lucigenin for ROS detection (predominantly following TPA stimulation), with Luminol being slightly more sensitive than Isoluminol (Figures 2.35-2.38) which was again confirmed on analysis of the cumulative ROS produced (Figure 2.38). Luminol and Isoluminol were much more sensitive at detecting ROS production following the stimulation of any cell by chemical means but

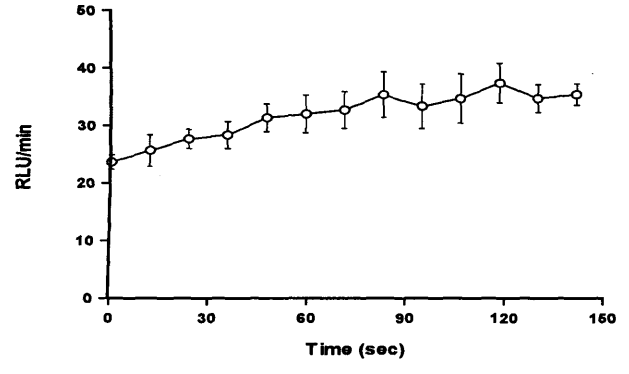
not by mechanical means (Figures 2.31-2.38). Finally, lymphoblasts appeared to produce less ROS overall compared to neutrophils for an equivalent cell number and also produced ROS less readily upon stimulation with the exception of chemical stimulation with TPA (Figures 2.34-2.38). As a result of these observations, Lucigenin was adopted as the chemiluminescent probe of choice for assaying ROS production from neutrophils, whilst Luminol was chosen for the detection of ROS from lymphoblasts in all subsequent experiments. Similarly, as chemical stimulation of lymphoblasts with fMLP did not elicit a response of greater magnitude compared to chemical stimulation with TPA, analysis of the lymphoblast response to chemical stimulation was confined to an analysis of ROS production consequent upon TPA stimulation only.

Figure 2.31

A.



B.



C.

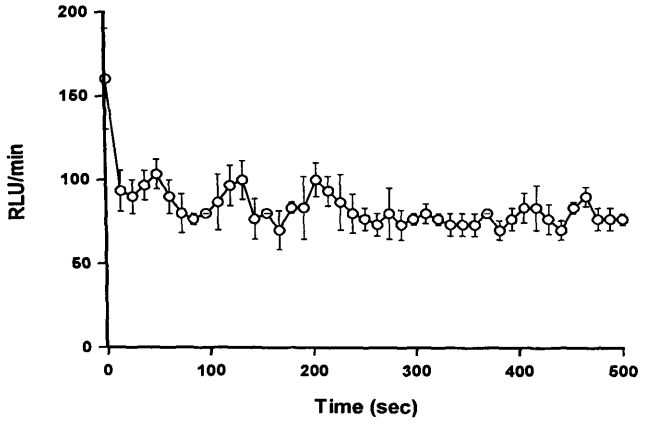
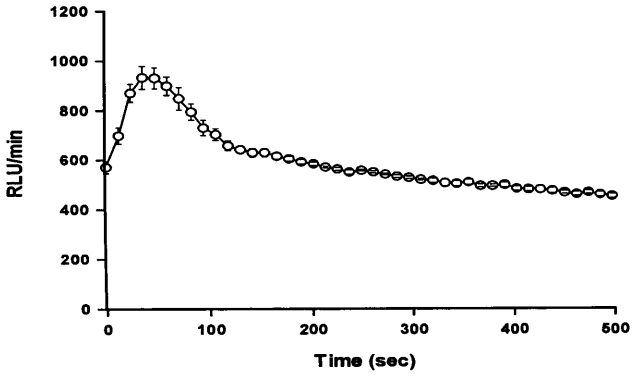


Figure 2.31 The effect of mechanical stimulation on neutrophil ROS production as detected by three different chemiluminescent probes: (A) Lucigenin 50µmol/l (B) Isoluminol 50µmol/l (C) Luminol 5µmol/l.

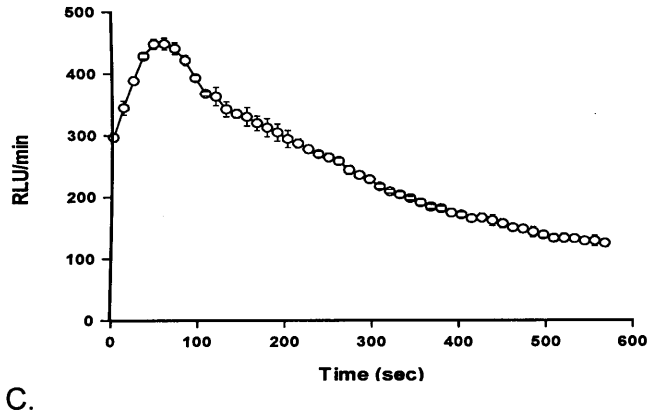
Results are representative of multiple experiments and each result represents the mean of 3 separate aliquots of 10^5 cells recorded simultaneously. All depicted results were obtained on the same day using the same isolate of neutrophils. RLU, Relative Light Units. Where error bars are not seen, SEMs are less than the size of the symbol.

Figure 2.32

A.



B.



C.

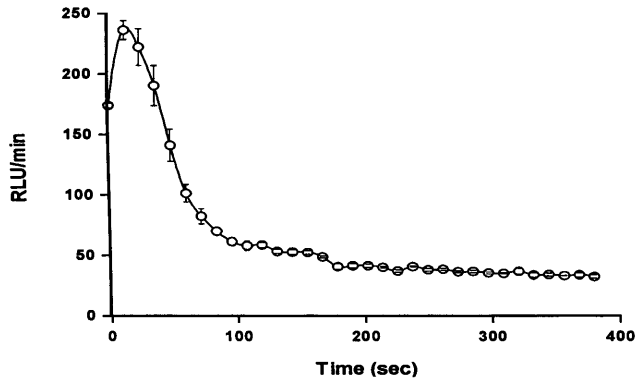
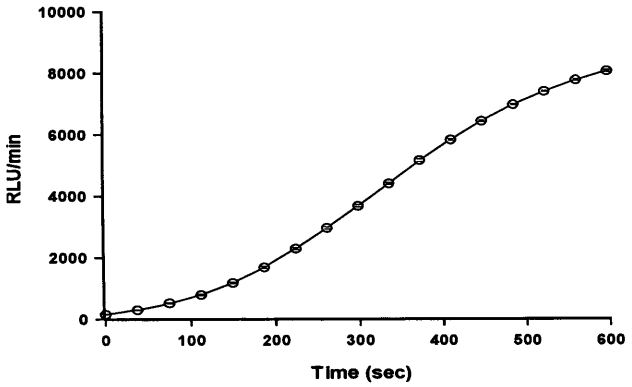


Figure 2.32 The effect of neutrophil stimulation with the chemoattractant peptide, fMLP, on ROS production as detected by three different chemiluminescent probes: (A) Lucigenin 50µmol/l (B) Isoluminol 50µmol/l (C) Luminol 5µmol/l.

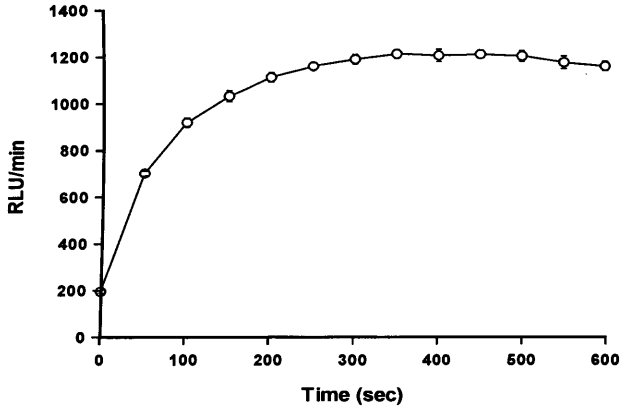
Results are representative of multiple experiments and each result represents the mean of 3 separate aliquots of 10^5 cells recorded simultaneously. All depicted results were obtained on the same day using the same isolate of neutrophils. RLU, Relative Light Units. Where error bars are not seen, SEMs are less than the size of the symbol.

Figure 2.33

A.



B.



C.

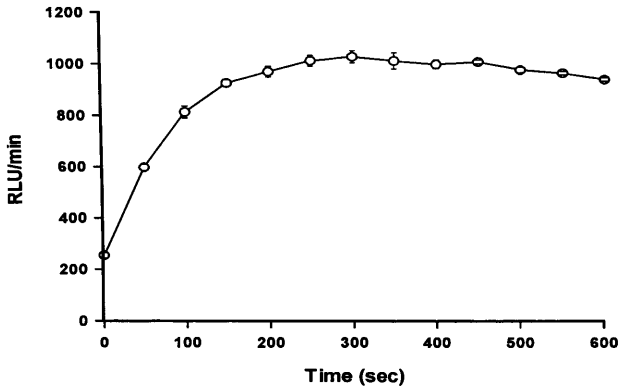
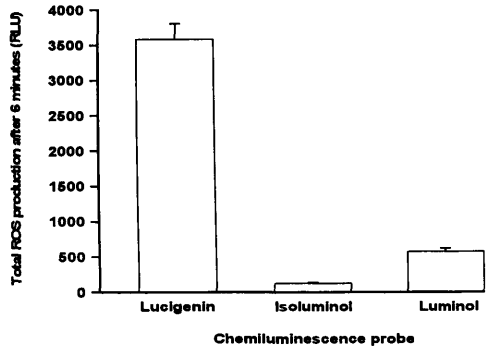


Figure 2.33 The effect of neutrophil stimulation with the phorbol ester, TPA, on ROS production as detected by three different chemiluminescent probes: (A) Lucigenin 50µmol/l (B) Isoluminol 50µmol/l (C) Luminol 5µmol/l.

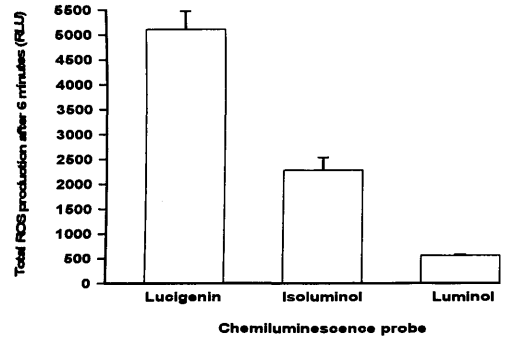
Results are representative of multiple experiments and each result represents the mean of 3 separate aliquots of 10^5 cells recorded simultaneously. All depicted results were obtained on the same day using the same isolate of neutrophils. RLU, Relative Light Units. Where error bars are not seen, SEMs are less than the size of the symbol.

Figure 2.34

A.



B.



C.

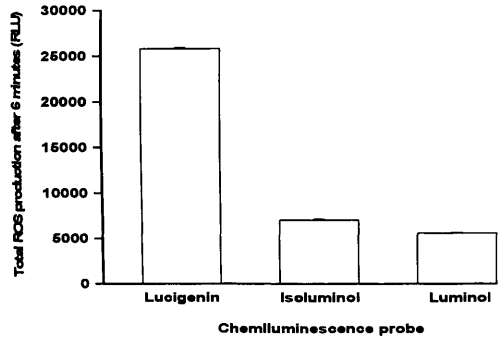
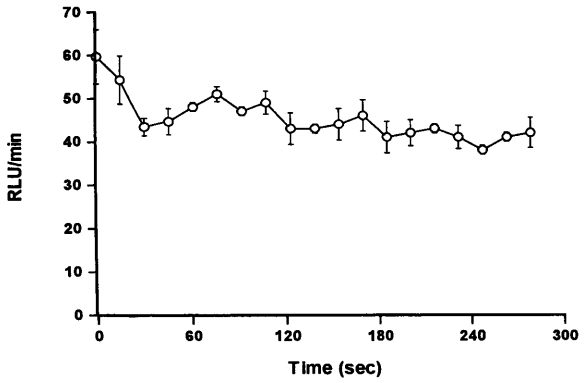


Figure 2.34 Cumulative ROS production over 6 minutes by neutrophils exposed to a variety of stimuli, as detected by chemiluminescence with three different chemiluminescent probes (Lucigenin 50µmol/l, Isoluminol 50µmol/l, Luminol 5µmol/l). (A) ROS production following mechanical stimulation of neutrophils ($p<0.001$ between groups by ANOVA). (B) ROS production following chemical stimulation of neutrophils with fMLP ($p<0.001$ between groups by ANOVA). (C) ROS production following chemical stimulation of neutrophils with TPA ($p<0.001$ between groups by ANOVA).

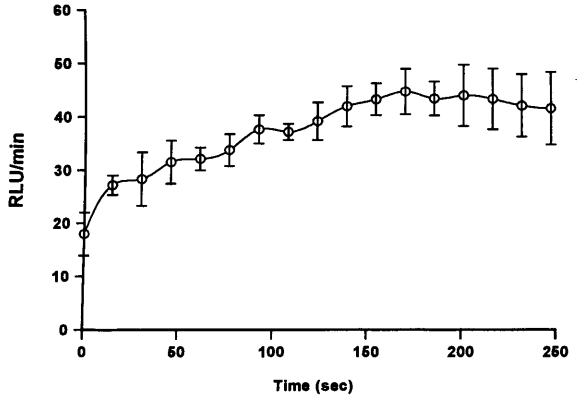
All results are derived from experiments with the same isolate of neutrophils carried out in parallel and are the means of 3 repetitions of the experiment. Each analysis was based on measurements from 100,000 neutrophils. RLU, Relative Light Units. Error bars denote SEMs.

Figure 2.35

A.



B.



C.

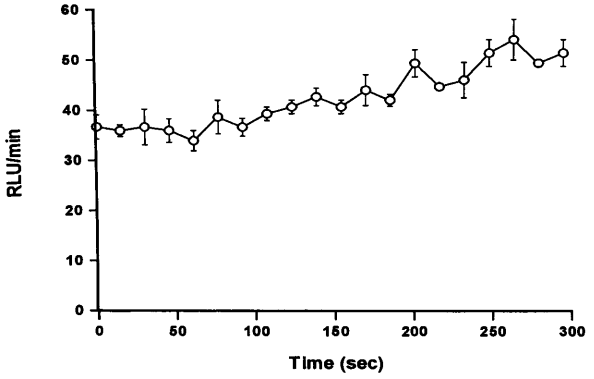
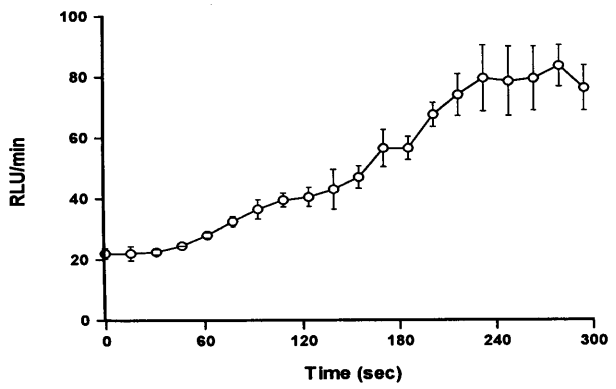


Figure 2.35 The effect of mechanical stimulation on ROS production by lymphoblasts as detected by three different chemiluminescent probes: (A) Lucigenin 50µmol/l (B) Isoluminol 50µmol/l (C) Luminol 5µmol/l.

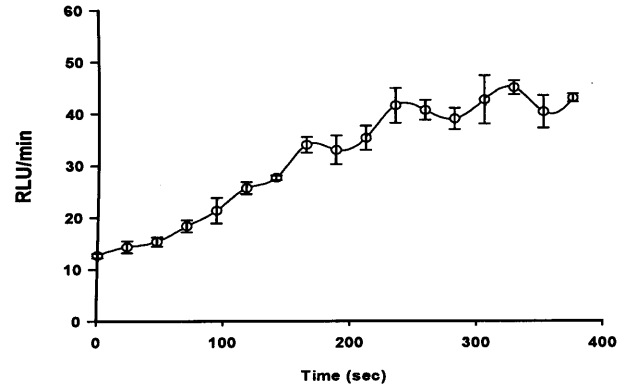
Results are representative of multiple experiments and each result represents the mean of 3 separate aliquots of 10^5 cells recorded simultaneously. All depicted results were obtained one the same day using the same isolate of lymphoblasts. RLU, Relative Light Units. Where error bars are not seen, SEMs are less than the size of the symbol.

Figure 2.36

A.



B.



C.

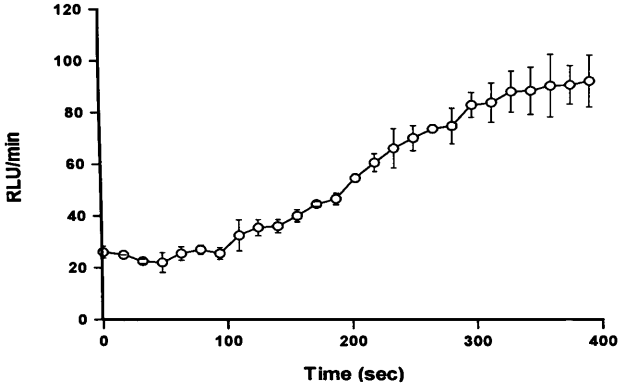
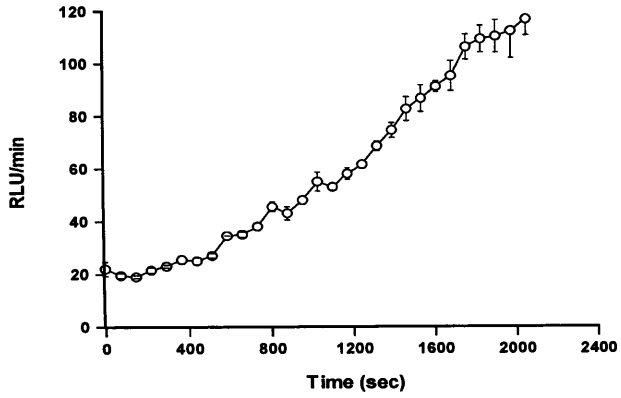


Figure 2.36 The effect of stimulating lymphoblasts with the chemoattractant peptide, fMLP, on ROS production as detected by three different chemiluminescent probes: (A) Lucigenin 50µmol/l (B) Isoluminol 50µmol/l (C) Luminol 5µmol/l.

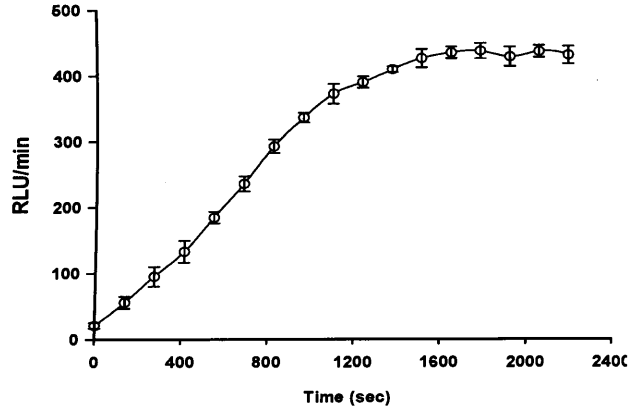
Results are representative of multiple experiments and each result represents the mean of 3 separate aliquots of 10^5 cells recorded simultaneously. All depicted results were obtained on the same day using the same isolate of lymphoblasts. RLU, Relative Light Units. Where error bars are not seen, SEMs are less than the size of the symbol.

Figure 2.37

A.



B.



C.

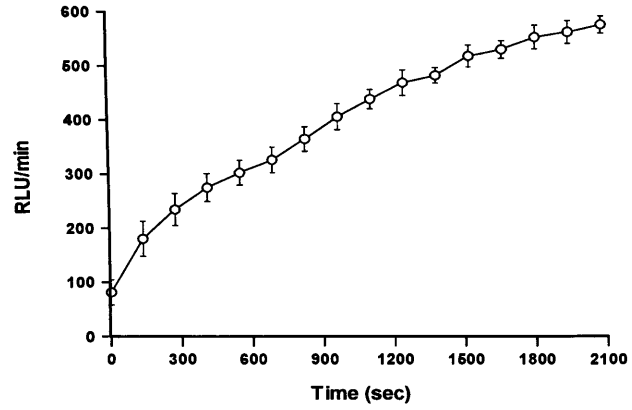


Figure 2.37 The effect of stimulating lymphoblasts with the phorbol ester, TPA, on ROS production as detected by three different chemiluminescent probes: (A) Lucigenin 50µmol/l (B) Isoluminol 50µmol/l (C) Luminol 5µmol/l.

Results are representative of multiple experiments and each result represents the mean of 3 separate aliquots of 10^5 cells recorded simultaneously. All depicted results were obtained on the same day using the same isolate of lymphoblasts. RLU, Relative Light Units. Where error bars are not seen, SEMs are less than the size of the symbol.

Figure 2.38

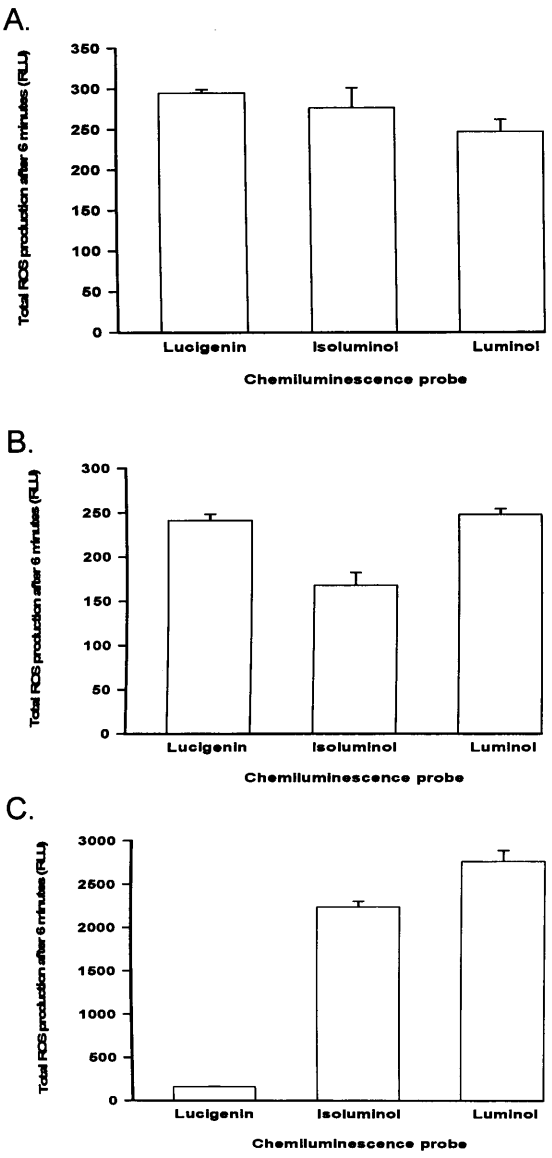


Figure 2.38 Cumulative ROS production over 6 minutes from lymphoblasts exposed to a variety of stimuli, as detected by chemiluminescence with three different chemiluminescent probes (Lucigenin 50µmol/l, Isoluminol 50µmol/l, Luminol 5µmol/l). (A) ROS production following mechanical stimulation of lymphoblasts ($p=0.342$ between groups by ANOVA). (B) ROS production following chemical stimulation of lymphoblasts with fMLP ($p=0.002$ between groups by ANOVA). (C) ROS production following chemical stimulation of lymphoblasts with TPA ($p<0.001$ between groups by ANOVA).

All results are derived from experiments with the same isolate of lymphoblasts, carried out in parallel and are the means of 3 repetitions of the experiment. Each analysis was based on measurements from 100,000 lymphoblasts. RLU, Relative Light Units. Error bars denote SEMs.

2.4 Cell stimulation protocols

Neutrophil stimulation: Mechanical stimulation of neutrophils was by multiple passages through a standard 1 ml Gilson pipette tip. Figure 2.39 demonstrates the effect on neutrophil ROS production as detected by chemiluminescence of a variable number of passages through a standard 1ml pipette tip; all measurements were undertaken from neutrophils that were suspended in a solution of Lucigenin (50μM) and Human Serum Albumin (200μg/ml). Providing that neutrophils were subject to more than one passage through the pipette tip, the same amplitude of neutrophil ROS production appeared to be induced despite variations in the actual number or intensity of passages. A similar threshold effect of shear stress on other aspects of neutrophil behaviour has been previously observed.[165] Nonetheless, a set number of 3 passages was undertaken for all the experiments in order to ensure conformity.

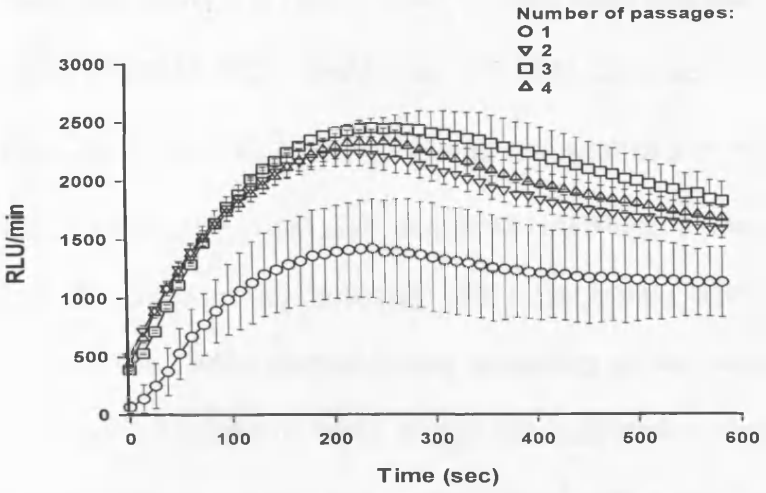
The chemical stimuli used in this study were formyl-methionylleucylphenylalanine (fMLP) (100nM) a bacterial cell-wall peptide that activates neutrophils via specific G-protein-coupled cell-surface receptors[166] and 12-O-Tetradecanoylphorbol-13-acetate (TPA) (500nM), a phorbol ester that is a direct activator of intracellular Protein Kinase C (PKC).[167]

Lymphoblast stimulation: Mechanical stimulation of lymphoblasts was by multiple passages through a standard 1 ml pipette tip as described above. Three passages were undertaken as standard for each experiment. The chemical stimuli used for lymphoblasts were again fMLP (100nM) and TPA (500nM).

Inhibitor protocols (pre-incubation): For experiments in which potential signalling pathways were investigated through the use of specific enzyme inhibitors or anti-oxidants, neutrophils/lymphoblasts were incubated for varying lengths of time with the enzyme inhibitor/anti-oxidant of interest in tissue culture medium (TC199, pH7.4) and the addition of 1% HSA at 37°C (see Results for individual inhibitor/anti-oxidant incubation times). The cells were then washed in further TC199 devoid of inhibitor, before being resuspended in a solution of the relevant chemiluminescent probe (Lucigenin 50μM, Isoluminol 50μmol/l or Luminol 5μmol/l). Stimulation and recording of ROS output then took place according to the previously described chemiluminescence protocol.

Figure 2.39

A.



B.

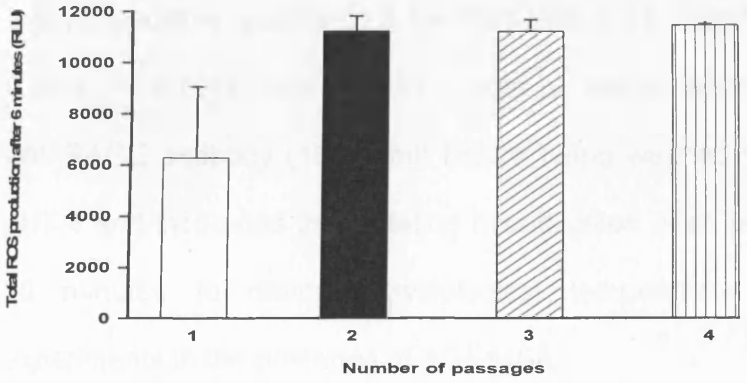


Figure 2.39 The effects of mechanical stimulation on neutrophil respiratory function as determined by chemiluminescence-detected ROS output. (A) Neutrophils were suspended in a solution of Lucigenin (50μM) and Human Albumin Solution (200μg/ml) and then subjected to one or more passages through a standard 1ml Gilson pipette tip, before being injected into a microplate well for chemiluminescent detection of ROS output over a period of time. (B) Total neutrophil ROS output, as defined by the cumulative ROS output over the first 6 minutes following release of neutrophils into the microplate well, was calculated for neutrophils which had been subjected to varying numbers of passages through the 1ml Gilson pipette tip. $p<0.001$ between groups by ANOVA. Compared to a single passage, ROS output with any number of multiple passages is significantly greater with $p<0.001$ for each comparison (post-hoc Tukey's test).

The results are the average of 3 simultaneous experiments, with 100,000 neutrophils being used per microplate well and are representative of multiple experiments. RLU, Relative Light Units. The mean SEMs are shown for each figure.

Electropermeabalisation of neutrophils: Neutrophils were purified as previously described before being washed twice with ice-cold permeabilisation buffer (140mM KCl, 10mM Hepes, 10mM Glucose, 1mM $MgCl_2$, 0.193mM $CaCl_2$ and 1mM EGTA, pH7.2) and resuspended in the same buffer along with 100µM GTP, 1mM ATP and 2mM NADPH to a final concentration of 10^7 cells/ml (cell counting performed with a Beckman Coulter counter) and stored on ice. They were permeabilised according to the method of Grinstein and Furuya[168]. Aliquots of 800µl of this cell suspension were placed in a 0.4cm electroporation cuvette and permeabilised with a Bio-Rad gene pulser using two consecutive pulses of 3.75kV/cm with a 25 microfarad capacitor (t=0.3-0.5ms). The cells were then incubated on ice for 30 minutes with or without anti-RAGE antibody (100µg/ml) before being washed three times in TC199, pH7.4 and incubated in a rotating hybridisation oven (Appligene) at 37°C for 20 minutes to restore physiological temperature prior to stimulation experiments in the presence of AGE/HSA.

Fluorimetric analysis of intracellular calcium concentrations: Neutrophils were incubated in TC199 containing Fura2-AM (1µM) for 1 hour at 37°C on a rotating hybridisation oven to prevent neutrophil clumping. After this incubation, the cells were washed in TC199 without Fura2-AM and aliquots of cells, equal in volume and cell number (as determined on a Coulter counter), were added to the cuvette of a Fluorometer with continuous stirring. Fluorescence was measured at an emission wavelength of 510nm and with dual excitation wavelengths of 340 and 380nm. After equilibration of the baseline fluorescence intensity, AGE or HSA were added to give a final

concentration of 200µg/ml. After equilibration of fluorescence intensity again, Digitonin 100µM was added to the cuvette for cell lysis in order to measure total binding of Fura2 with extracellular calcium. Once this had equilibrated, EGTA 15mM was then added to chelate the aforementioned calcium and render Fura2 totally unbound. The concentration of intracellular calcium, $[Ca^{++}]_i$, was then calculated using the ratiometric equation of Grynkiewicz.[169] To quantitatively characterize the relative concentrations of Ca^{2+}-bound and Ca^{2+}-free dye, and thus measure $[Ca^{2+}]_i$, it is sufficient to calculate the ratio of the intensities of fluorescence at the two excitation wavelengths. The measurement of $[Ca^{2+}]_i$ concentration is based on the analytical expression:

$$[Ca^{2+}]_i = K_d * (R-R_{min})/(R_{max}-R) * (S_{f2}/S_{b2})$$

where R is the ratio of the fluorescence intensities obtained with excitation at 340 nm and 380 nm; R_{min} and R_{max} are the limiting ratios, and S_{f2} and S_{b2} are the limiting 380 nm fluorescence intensities in the absence of Ca^{2+} and in the presence of saturating amounts of Ca^{2+} respectively.

Neutrophil Fractionation: Membrane and cytosol fractions were prepared according to the method of Levy and Malech.[170] Following the isolation of neutrophils as detailed previously, neutrophils were centrifuged, resuspended in relaxation buffer (10mM KCl, 3mM NaCl, 3.5mM $MgCl_2$, 1.25mM EGTA, 10mM HEPES – pH7.4, containing 1mM PMSF, 100µM leupeptin, 10µg/ml aprotinin, 1mM $NaVO_3$ and 25mM NaF), snap frozen in liquid nitrogen, defrosted and sonicated three times for 15 s on ice using a Soniprep 150 sonicator (MSE) on a 10 micron amplitude setting. The post-nuclear

supernatant was centrifuged at 150,000g for 60 minutes (Beckman Optima Max Ultracentrifuge) to obtain a cell membrane pellet and cytosol supernatant. Membrane pellets were then resuspended in the above relaxation buffer.

Detection of Arachidonic Acid (AA) Production: Labelling of neutrophils with [^{3}H]-AA and the release of radiolabeled AA were performed according to the method of DiPersio et al.[171] The cells were incubated with 2.5μCi/ml [^{3}H]-AA for 45 minutes at 37°C. Those cells requiring further treatment with the $cPLA_2$ inhibitor, Methyl-arachidonylfluorophosphonate (MAFP), or N-acetylcysteine were incubated for a further 60 minutes in TC199 with 1% HSA as an additive. Cells were then washed three times in TC199 and incubated for 30 minutes with AGE-albumin or albumin at 200μg/ml in TC199. Following this final incubation, cells were centrifuged and an aliquot of the supernatant was counted using liquid scintigraphy.

Adhesion Assay: Neutrophil adhesion was assessed using a fluorescence technique performed in 12-well Perspex plates (Amersham). Cells were loaded with BCECF [2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein] by incubation with 4μM BCECF for 1 hour at 37°C in a rotating hybridisation oven to prevent cell clumping. Following this, the neutrophils were washed with TC199 devoid of BCECF and resuspended in TC199 containing AGE or HSA at 200μg/ml. The number of cells in each solution was kept the same using a Coulter counter to adjust for the same number of cells per millilitre. The cells in solution of TC199 (plus AGE or HSA) were then added to the 12-well plates and after 1-10 minutes at 37°C, they were emptied, washed once

with PBS and lysed with 500µl of detergent-containing (1% Triton X-100) PBS-buffer. The plates were then left to equilibrate on a plate shaker for 5 minutes before the contents of each well (500µl volume) were serially analysed for fluorescence intensity in a fluorimeter.

2.5 Protein analysis

Antibodies: Polyclonal antibodies to the RAGE-receptor and $p47^{phox}$ were developed in-house. An epitope corresponding to the intracellular domain of RAGE was chemically conjugated to keyhole limpet haemocyanin using the heterobifunctional cross linker (EMCS, Calbiochem, Nottingham, UK). $p47^{phox}$ was produced in SF9 insect cells infected with recombinant baculovirus expressing $p47^{phox}$ (a gift from Dr David Lambeth, Emory University, Atlanta, USA) and purified by ion exchange chromatography as described by Leto et al.[172] Antisera to the RAGE-receptor and $p47^{phox}$ components were raised in rabbits by monthly subcutaneous injections of 500µg of the various proteins. The antibodies were purified by protein A columns and eluted with 100mM Glycine, pH2·5. The specificity of the $p47^{phox}$ antibody was determined by performing 2 Western blots, one probed with the antibody alone (at a concentration of 1µg/ml) and the other probed with a combination of the antibody and the antigen (at a concentration of 10µg/ml). The band of interest was taken as the band whose intensity was decreased in the antigen-blocked blot.

SDS-Polyacrylamide Gel Electrophoresis: The technique was based on that described by Laemmli.[173] 50-500µg protein extracts from either

neutrophils or the lymphoblast cell lines were resolved on 7.5-15% SDS polyacrylamide gels. The resolved proteins were Western blotted onto nitrocellulose, and different antibody probes were used to detect the presence of the various proteins as follows:

a) *RAGE-receptor:* Following resolution of cellular proteins on a 10% (w/v) SDS-polyacrylamide gel, the blots were blocked in 2.5% BSA in PBS with 0.1% Tween-20, then washed and labelled with the primary antibody (polyclonal anti-RAGE antibodies - 50µg/ml with 5% horse serum) in PBS with 0.1% Tween-20 and 5% dried milk powder, for one hour. After further washing of the blot, this was in turn labelled with anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:1500 in PBS with 0.1% Tween-20 and 5% dried milk powder, for one hour.

b) *Cytosolic Phospholipase A_2 (cPLA$_2$):* Cytosol and membrane fractions (100µg protein) were resolved on 10% (w/v) SDS-polyacrylamide gels. The resolved proteins were blotted to nitrocellulose which was then blocked in 2.5% BSA in PBS with 0.1% Tween-20. After washing, the blots were incubated for 1 hour with mouse monoclonal anti-cPLA$_2$ antibody (Santa Cruz) diluted 1:500 in PBS with 0.1% Tween-20 and 1% goat serum. After extensive washes, the blot was incubated for a further 1 hour in a 1:2000 dilution (in PBS with 0.1% Tween-20) of peroxidase-conjugated anti-mouse antibody (Sigma).

c) *Tyrosine-phosphorylated proteins:* After resolution of cell lysates on a 10% (w/v) gel and Western blotting onto nitrocellulose, the blots were

incubated for 1 hour with an anti-phosphotyrosine antibody (anti-PY20 – Calbiochem) at a concentration of 1µg/ml in PBS with 0.1% Tween-20 and 1% goat serum as a blocking agent. After washing, the primary antibody was labelled for one hour with an anti-mouse secondary antibody (1:2000 dilution in PBS with 0.1% Tween-20) conjugated with horse radish peroxidase (Sigma).

d) *Phosphorylated p44MAP Kinase:* A 10% (w/v) SDS-polyacrylamide gel was used to resolve the proteins prior to Western blotting. The nitrocellulose was then incubated with a mouse monoclonal antibody to phospho-p44MAPK (Calbiochem) (1µg/ml – diluted in PBS with 0.1% Tween-20) and 1% goat serum for one hour and following washing, secondary labelling was carried out with a peroxidase-conjugated anti-mouse antibody (1:2000 dilution in PBS with 0.1% Tween-20) for one hour.

e) *$p47^{phox}$:* A 10% (w/v) SDS-polyacrylamide gel was used to resolve the proteins prior to Western blotting. The nitrocellulose was then incubated with polyclonal antibody to $p47^{phox}$ (at a concentration of 1µg/ml) for one hour before washing the nitrocellulose and undergoing a second one hour incubation with anti-rabbit IgG conjugated to horseradish peroxidase diluted 1:1500 in PBS with 0.1% Tween-20 and 5% dried milk powder.

For all the above Western blots, antibody incubation took place at room temperature and the protein bands were detected using enhanced

chemiluminescence kits from Amersham. Band intensities were analysed on a BioRad Densitometer.

2.6 Data analysis

Chemiluminescent values are presented as Relative Light Units per minute (RLU/min) (means with SEMs). Analysis of the total ROS production (RLU) was derived from a calculation of the area under the curve (cut-off at 6 minutes). Two-tailed t-tests were used for comparisons of two groups and one-way ANOVA with Tukey's post hoc analysis for between-group analyses involving more than two groups (SPSS Inc).

Chapter 3 - RESULTS

3.1 The effect of AGEs on the Neutrophil Respiratory Burst

3.11 INTRODUCTION

An increased accumulation of Advanced Glycation Endproducts (AGEs) occurs classically in a number of clinical states including diabetes mellitus, ageing and uraemia. In this study, the contribution of AGEs to intravascular oxidant stress was investigated, as this has possible involvement in the development of atherogenic processes. The action of AGEs on the neutrophil respiratory burst was examined through the real-time visualisation of cellular ROS production following AGE-exposure.

3.12 RESULTS

AGE-albumin enhances the neutrophil respiratory burst induced by another stimulus. The direct effect of AGEs on neutrophil ROS production was assessed through the addition of AGE-Albumin or unmodified Albumin (50-800μg/ml) to 5 x 10^5 neutrophils which had been allowed to settle onto microplate wells. The subsequent integrated ROS production over 15 minutes was measured and compared to baseline ROS production (also over 15 minutes) as measured by Lucigenin-enhanced chemiluminescence. Although the addition of both AGE-Albumin and Albumin resulted in slight increases of neutrophil ROS production over basal levels, there was no significant difference between them (comparing the two groups over the given dose-range, p=0.852) [Figure 3.121].

However, neutrophils subjected to a secondary stimulus (mechanical or chemical) in the presence of AGE-Albumin, responded with a respiratory burst which was significantly enhanced compared to activation in the presence of unmodified Albumin (Fig. 3.123), with the magnitude of enhancement dependent on the AGE concentration (Fig. 3.124). Both mechanical and fMLP stimulation resulted in a two-phase neutrophil ROS burst, a primary peak and a secondary trough phase, with AGE-Albumin able to enhance both these aspects (Figs. 3.123A and B). The intensity of the fluorescence spectra of the Albumin and AGE-modified Albumin samples used in this study give some quantitative idea to the relative degrees of AGE modification of these entities (Fig. 3.122). Minimally Modified-Albumin (MM-Albumin) was also able to augment peak neutrophil ROS release following mechanical or fMLP stimulation, albeit with a much reduced gain compared to AGE-Albumin (Figs. 3.123A and B). However, when evaluating the total ROS production (cumulative ROS production over 6 minutes), MM-Albumin no longer conferred any significant augmentation effect following fMLP stimulation (Figs. 3.123E). Thus at 400μg/ml (an optimum concentration for both AGE-Albumin and MM-Albumin), AGE-Albumin and MM-Albumin enhanced peak ROS production in response to mechanical stimulation by an average of 362% ($p<0.001$) and 157% ($p=0.011$) respectively and total ROS production by an average of 282% ($p<0.001$) and 169% ($p=0.009$) respectively ($p<0.001$ between groups in both cases) [Figs. 3.123A and D]. But with fMLP stimulation, although both AGE-Albumin and MM-Albumin increased peak ROS production (Fig. 3.123B) [by an average of 177% ($p<0.001$) and 119% ($p=0.021$) respectively], only AGE-Albumin enhanced total ROS production

significantly (as determined by the area under the curve at 6 minutes), and this was by an average of 145% ($p<0.001$) [Fig. 3.123E].

The kinetics of TPA-induced ROS production were different to those following mechanical or fMLP stimulation, with a rise to maximum ROS output, which then slowly declined. Nonetheless, total ROS output was far greater with TPA-stimulation compared to fMLP-stimulated cells with the same number of cells being analysed (Figure 3.123F). On average, AGE-Albumin enhanced peak and total ROS production following TPA stimulation by 116% ($p=0.006$) and 145% ($p=<0.001$) respectively, with MM-Albumin having no statistically significant effect on the enhancement of ROS production following this stimulus (enhancement of peak ROS production by MM-Albumin was 110%, $p=0.10$, and enhancement of total ROS production by MM-Albumin was 104%, $p=0.40$) [Figs. 3.123C and F]. Because AGE-Albumin had less of an enhancing effect on the TPA-induced compared to the fMLP-induced neutrophil ROS burst, further analyses on the enhancing effect of AGE-Albumin were confined to fMLP-mediated responses.

Even within each class of AGE modification there was a dose response relationship. Analysis of this relationship was conducted by recording the peak rather than total ROS production as MM-Albumin was shown not to elicit a significant augmentation of total ROS production yet was able to enhance the peak ROS burst to a limited degree (see earlier and Figs. 3.123B and E). For AGE-Albumin, optimum responses were achieved at concentrations of AGE-Albumin above 200μg/ml for both mechanical and fMLP stimulation (for both forms of stimulation, $p<0.001$ between groups) [Figs. 3.124A and B]. At

progressively higher doses of AGE-Albumin, this relative difference was maintained although the absolute chemiluminescent intensity from the samples diminished. The dose response for MM-Albumin was much less marked although still significant for both forms of stimulation (for both, $p<0.001$ between groups) [Figs. 3.124C and D] and a similar pattern to AGE-Albumin was observed. Thus there was little response below a concentration of 400μg/ml, but at higher concentrations (800μg/ml and above), it was also not possible to detect any possible small differences in ROS production (Figs. 3.124C and D).

That there appeared to be no requirement for significant pre-incubation of neutrophils with AGE-Albumin to elicit these responses was explicitly demonstrated by the fact that longer pre-incubation with AGE-Albumin exerted no greater effect than immediate suspension of cells in AGE-Albumin prior to stimulation (Figs. 3.125A and B). In fact, longer incubation times were unable to compensate for suboptimal doses of AGE-Albumin (Figs. 3.125C and D).

The effect of temporally reversing the two processes of AGE-Albumin addition and stimulation was also investigated. When neutrophils had been mechanically stimulated first, prior to the subsequent addition of AGE-Albumin or control, there was no differential enhancement of ROS generation (total or peak) in the cells exposed to AGE-Albumin ($p=0.603$ comparing total ROS production) [Fig. 3.126A]. Mechanically stimulating neutrophils and then releasing them onto AGE-Albumin or Albumin-coated microplate wells also did not result in any major difference in neutrophil ROS production (total or peak)

(p=0.319 comparing total ROS production) [Fig. 3.126B]. Chemical stimulation with fMLP prior to the addition of AGE-Albumin did not demonstrate any increase in ROS production over the addition of ordinary Albumin to fMLP-stimulated cells either (p=0.885 comparing total ROS production) [Fig. 3.126C]. However, if neutrophils were allowed to settle onto microplate wells that had been pre-coated with AGE-Albumin or Albumin, and then were subsequently stimulated chemically with fMLP, an enhancement in ROS production was seen from the AGE-Albumin wells (p<0.001 comparing peak and total ROS production) [Fig. 3.126D].

It is well recognised that the cellular enzyme NADPH oxidase is responsible for phagocytic ROS production[174] and it could be inferred that any increase in neutrophil ROS output is attributable to increased NADPH oxidase activity. As confirmation, neutrophils were pre-incubated with the flavoprotein inhibitor diphenyleneiodonium (DPI)[175] for 30 minutes and at various concentrations, before addition of AGE-Albumin/Albumin (200μg/ml) and application of a mechanical or chemical stimulus, all in the continuing presence of DPI. The result was a dose-dependent abrogation of ROS release, albeit maintaining the relative differential production of ROS between experimental and control groups (for both, p<0.001 between groups) [Figs. 3.127A and B]. In contrast, the specific mitochondrial flavoprotein inhibitor rotenone (100nM-10μM) had little effect on neutrophil ROS output in this situation (data not shown).

Figure 3.121

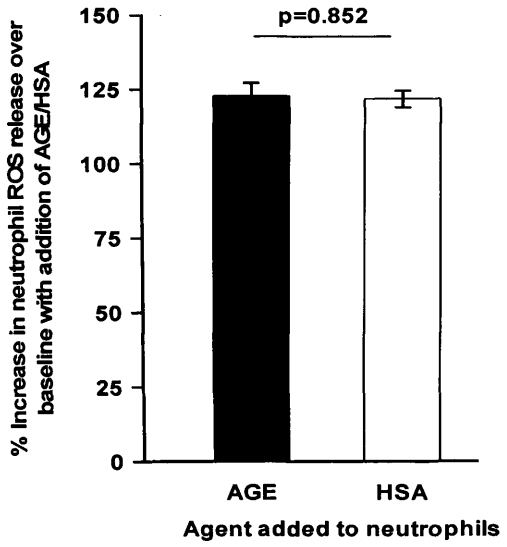


Figure 3.121 The effect of direct addition of AGE-Albumin or HSA (100µg/ml) on ROS production when added to a population of neutrophils which had already been allowed to settle onto microplate wells. Results are shown as the cumulative increase in ROS production over 15 minutes, expressed as a percentage of the baseline ROS production, from the cells on the microplate, with ROS being measured by chemiluminescent response. Results are from four separate experiments with analysis by ANOVA.

Figure 3.122

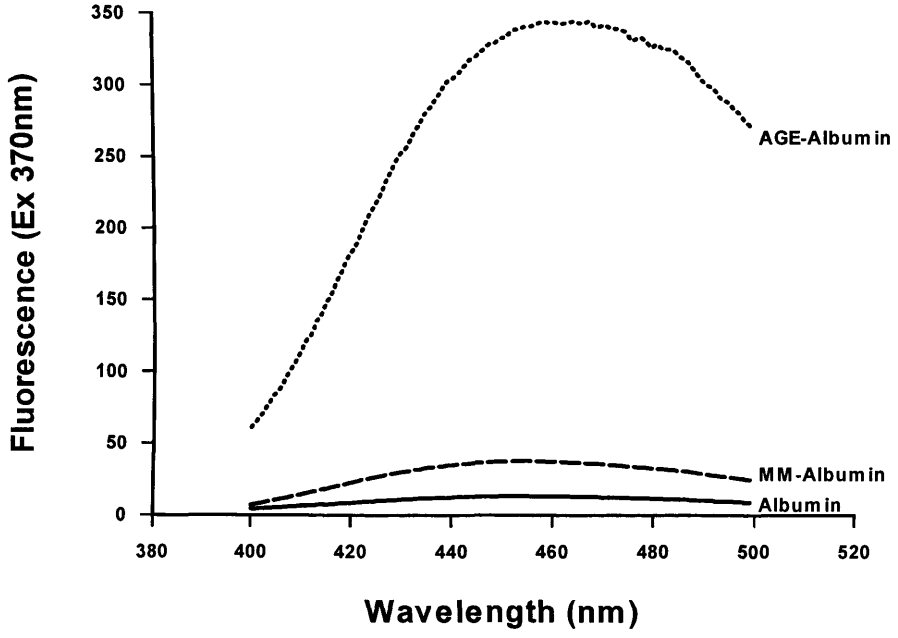
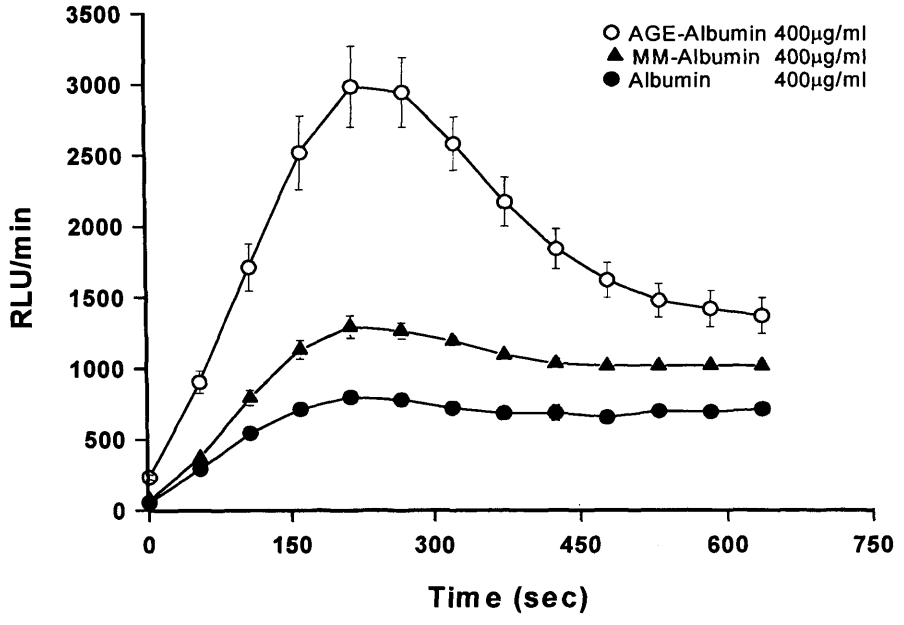


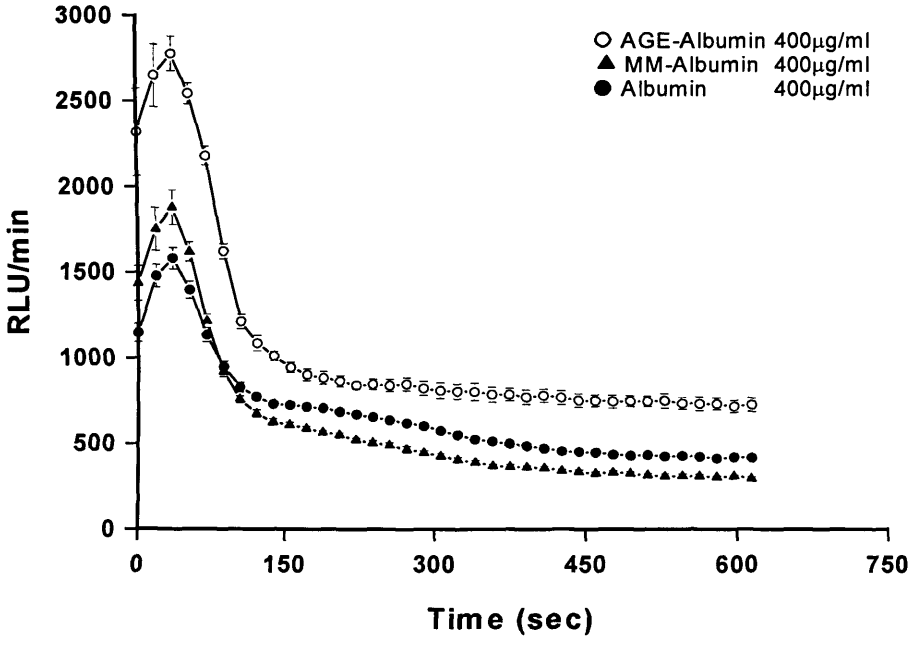
Figure 3.122 Fluorescence spectra of AGE-Albumin, Minimally Modified Albumin and Albumin. The excitation wavelength is 370nm.

Figure 3.123

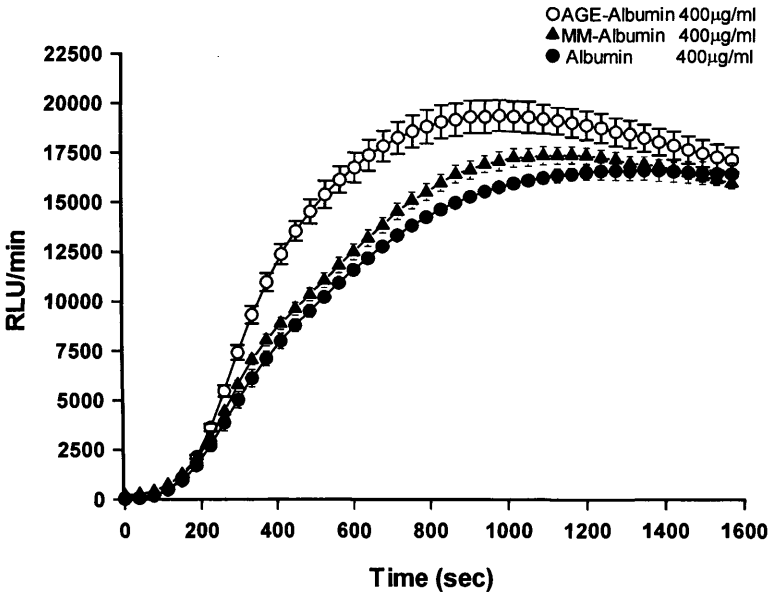
A.



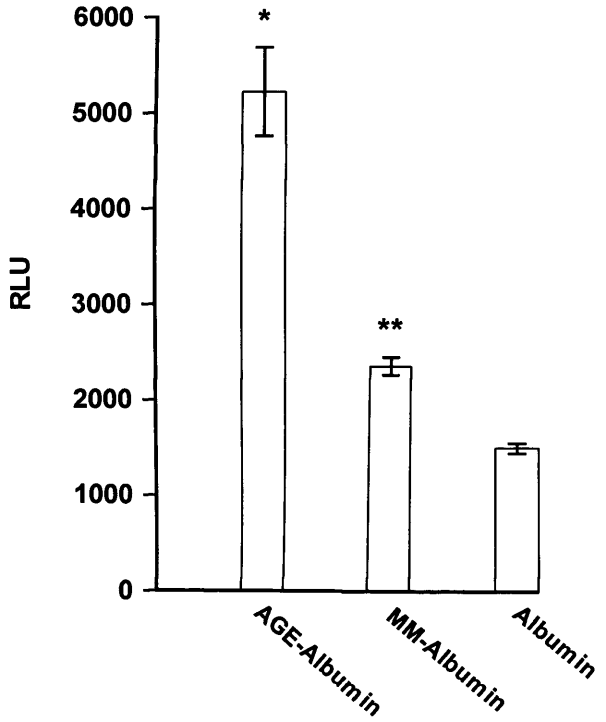
B.



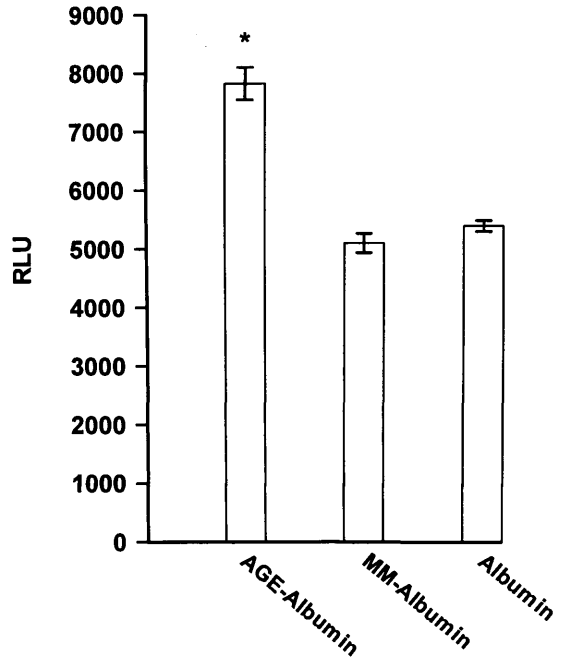
C.


OAGE-Albumin 400µg/ml
MM-Albumin 400µg/ml
Albumin 400µg/ml
RLU/min
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
0
200
400
600
800
1000
1200
1400
1600
Time (sec)

D.


RLU
6000
5000
4000
3000
2000
1000
0
*
**
AGE-Albumin
MM-Albumin
Albumin

E.



F.

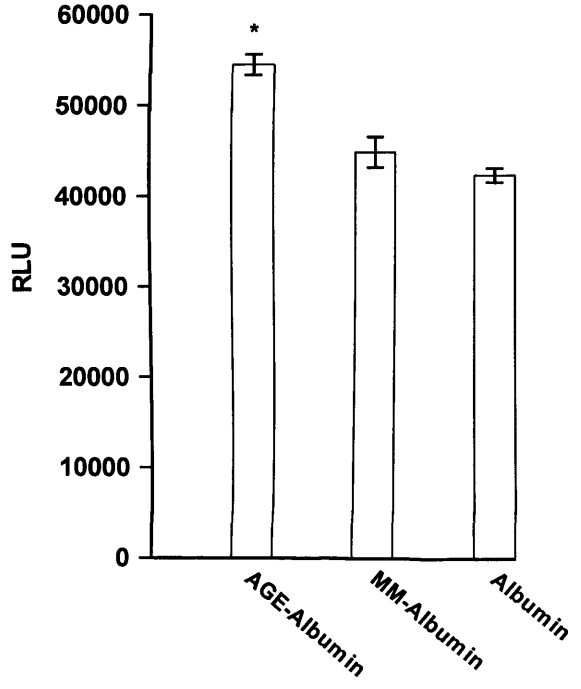


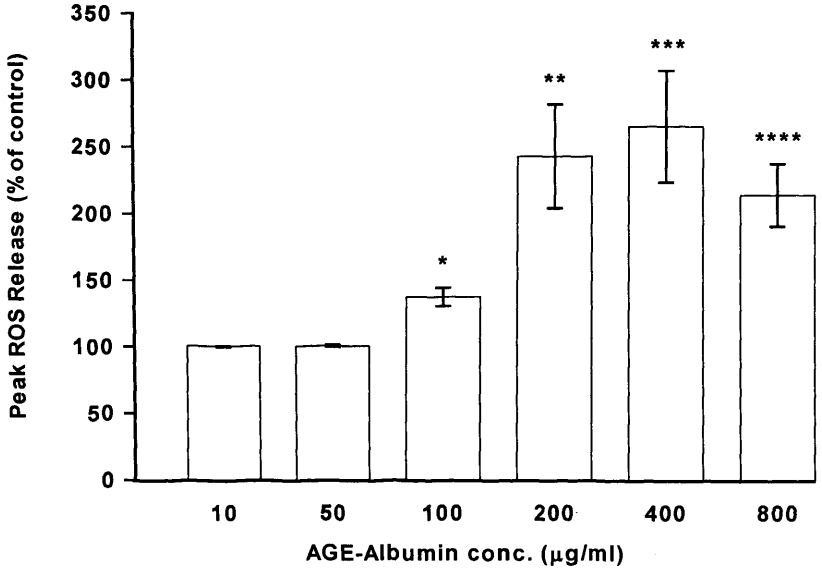
Figure 3.123 Acute neutrophil ROS production as detected by Lucigenin-enhanced chemiluminescence in the presence of AGE-Albumin, Minimally Modified Albumin (MM-Albumin) or Albumin, all at 400μg/ml. (A) Response to a mechanical stimulus ($p<0.001$ between groups by ANOVA). (B) Response

to stimulation with fMLP ($p<0.001$ between groups by ANOVA). (C) Response to stimulation with TPA ($p=0.006$ between groups by ANOVA). (D) Total ROS production over 6 minutes after mechanical stimulation ($p<0.001$ between groups by ANOVA). For comparisons with control, *$p<0.001$ and **$p=0.009$ (post hoc Tukey's analysis). (E) Total ROS production over 6 minutes after stimulation with fMLP ($p<0.001$ between groups by ANOVA). For comparisons with control, *$p<0.001$(post hoc Tukey's analysis between groups). (F) Total ROS production over 6 minutes after stimulation with TPA ($p<0.001$ between groups by ANOVA). For comparisons with control, *$p<0.001$ (post hoc Tukey's analysis between groups).

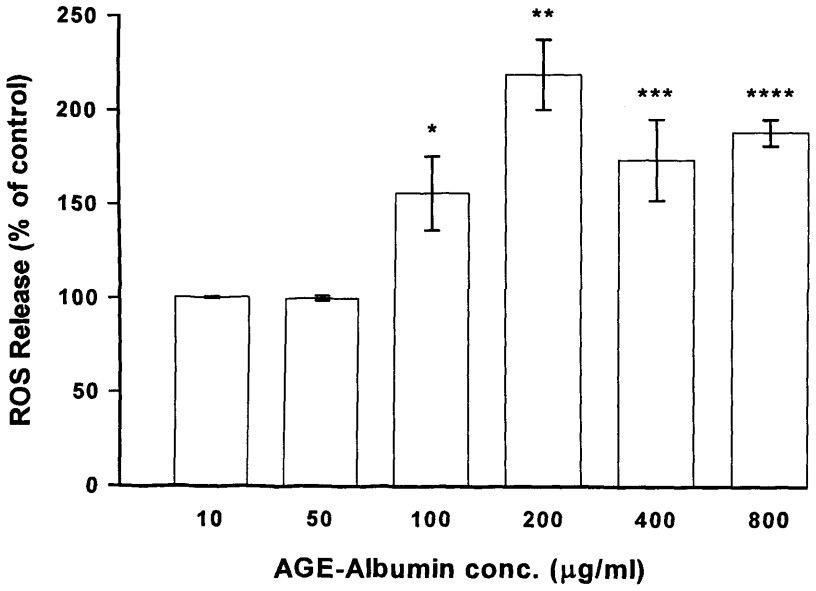
Results are representative of multiple experiments and each result represents the mean of 3 separate aliquots of 10^5 cells recorded simultaneously. RLU, Relative Light Units. Where error bars are not seen, SEMs are less than the size of the symbol.

Figure 3.124

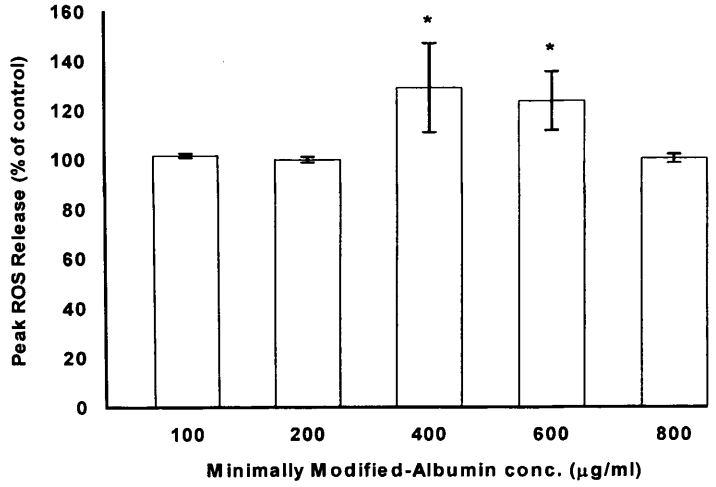
A.



B.



C.



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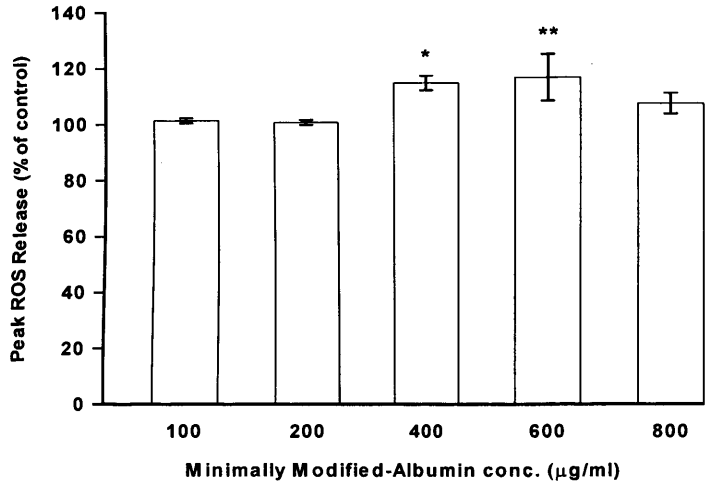
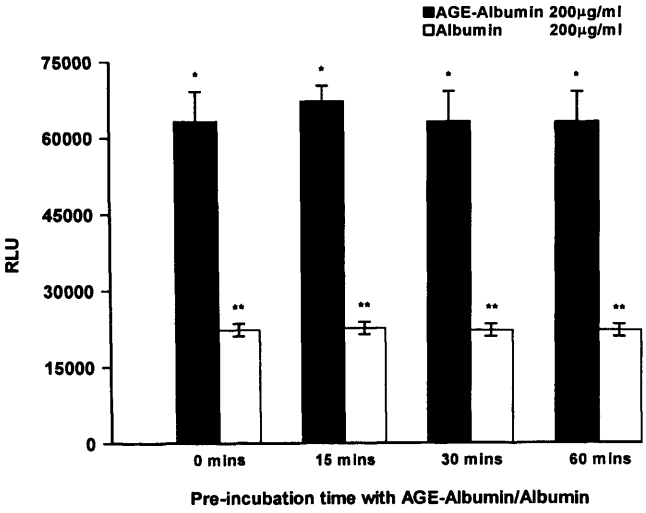


Figure 3.124 Dose-responses for enhancement of the peak neutrophil ROS release by AGE-modified Albumin. Results are expressed as the percentage of the peak neutrophil ROS release achieved by control (albumin-exposed) cells. (A) Peak ROS release following mechanical stimulation in the presence of varying doses of AGE-Albumin ($p<0.001$ by ANOVA between groups. With post hoc Tukey's analysis comparing to control, *$p=0.047$, **$p=0.013$, ***$p=0.022$, and ****$p=0.002$). (B) Peak ROS release following stimulation with fMLP in the presence of varying concentrations of AGE-Albumin ($p<0.001$ by ANOVA between groups. With post hoc Tukey's analysis comparing to control, *$p=0.03$, **$p<0.001$, ***$p=0.003$ and ****$p=0.032$). (C) Peak ROS release following mechanical stimulation in the presence of varying doses of MM-Albumin ($p<0.001$ by ANOVA between groups. With post hoc Tukey's analysis comparing to control, *$p<0.001$). (D) Peak ROS release following stimulation with fMLP in the presence of varying concentrations of MM-Albumin ($p<0.001$ by ANOVA between groups. With post hoc Tukey's analysis comparing to control, *$p=0.009$ and **$p<0.001$).

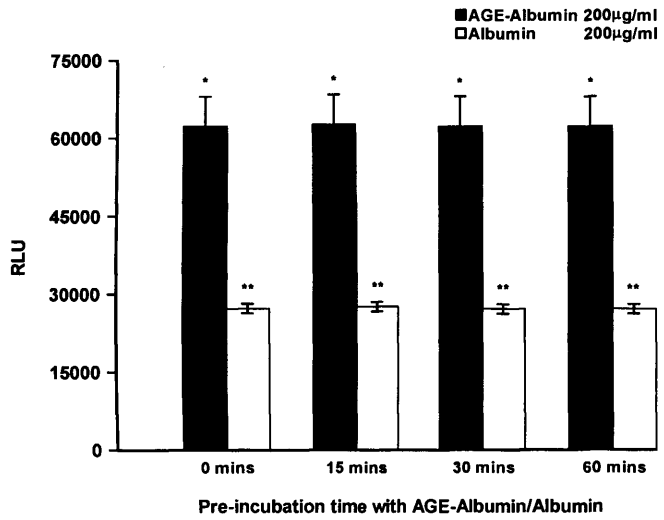
The results for each dose are the mean results from 10 different sets of neutrophils, with 10^5 cells being used each time..

Figure 3.125

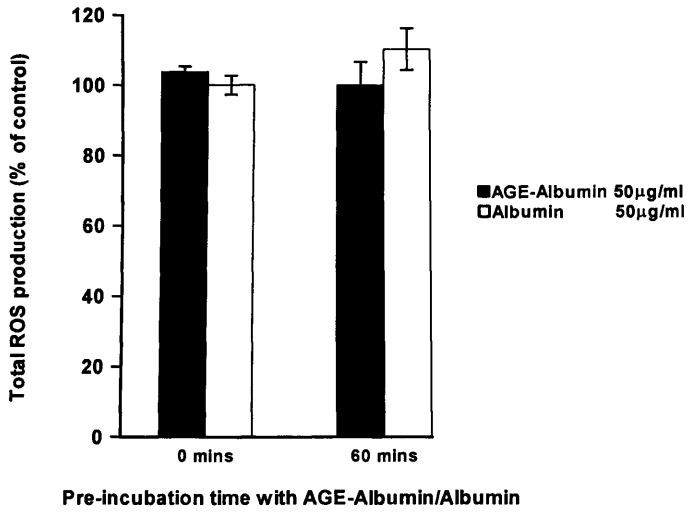
A.



B.



C.



D.

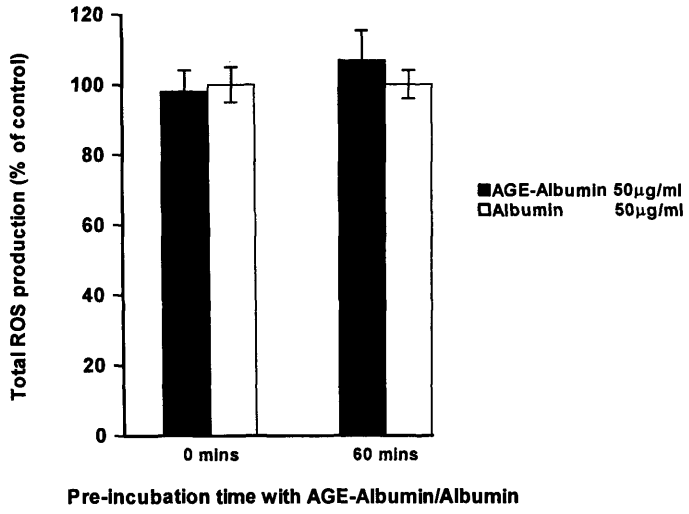


Figure 3.125 The effect of varied pre-incubation times of neutrophils with AGE-Albumin on the augmentation of the neutrophil ROS burst. This study was both performed at optimal levels of AGE-Albumin required for an augmentation response and also at sub-optimal levels. (A) Total ROS production after mechanical stimulation and following prior incubation with AGE-Albumin or Albumin for the various specified lengths of time. AGE-Albumin used at a dose of 200µg/ml. * p=0.932 by ANOVA, for comparisons between the AGE groups pre-incubated for different time intervals. ** p=0.99 by ANOVA, for comparisons between the Albumin groups pre-incubated for different time intervals. (B) Total ROS production after fMLP (100nM) stimulation and following prior incubation with AGE-Albumin or Albumin for the various specified lengths of time. AGE-Albumin was used at a dose of 200µg/ml. * p=1.0 by ANOVA, for comparisons between the AGE groups pre-incubated for different time intervals. ** p=0.932 by ANOVA, for comparisons between the Albumin groups pre-incubated for different time intervals. (C) Total ROS production expressed as a percentage of the baseline production following incubation with suboptimal concentrations of AGE-Albumin (50µg/ml) for 1 hour compared to immediate exposure. ROS generation was measured following mechanical stimulation of neutrophils. P=0.085 between groups by ANOVA. (D) Total ROS production expressed as a percentage of the baseline production following incubation with suboptimal concentrations of AGE-Albumin (50µg/ml) for 1 hour compared to immediate exposure. ROS production was measured following stimulation of neutrophils with fMLP100nM. p=0.375 between groups by ANOVA.

In the case of each figure, the results are the mean values from 4 different sets of neutrophils, with 10^5 cells being used each time.

The effect of AGE-albumin on the neutrophil respiratory burst is a reversible process. The potential for reversibility of the AGE-effect on neutrophils was examined. Neutrophils were incubated in AGE-Albumin or control Albumin (both 200μg/ml) for a period of 30 minutes prior to being washed, and then activated immediately either mechanically or with fMLP. With both stimuli, the differential enhancement of ROS production previously induced by AGE-Albumin was abrogated. Thus for mechanical stimulation, total ROS production was 108% ± 13% of control values (p=0.71) and following stimulation with fMLP, total ROS production was 105% ± 4% of control values (p=0.12).

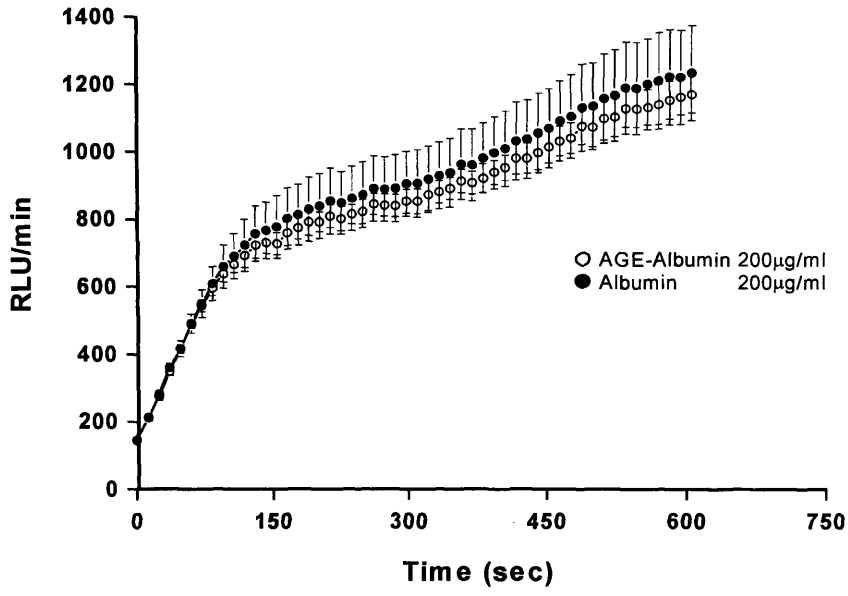
The effect of AGE-albumin is complementary to the effect of neutrophil priming agents in enhancing neutrophil respiratory burst capacity. A variety of other agents have also been documented to possess the capacity for increasing neutrophil ROS production. One such group of agents, collectively termed 'neutrophil priming agents'[150] and including two well-studied agents TNFα and PAF, differs from AGE-Albumin in requiring significant pre-incubation time with cells in order to achieve an effect. In investigating the possibility of shared cellular upregulation mechanisms between AGEs and priming agents, the effects of combined exposure to these agents was studied. Neutrophils were pre-incubated with TNFα, PAF or control vehicle for the required priming time (30 minutes) with the subsequent addition of AGE-Albumin or Albumin (both at 200μg/ml) and further stimulation of the cells either mechanically or by fMLP (Figs. 3.128A-D). Both TNFα and PAF appeared to exert even more potent augmentation effects on the

neutrophil respiratory burst than AGE-Albumin alone, and the effects appeared to be complementary when AGE-Albumin was combined with them. For mechanical stimulation, there was an approximately 10-fold increase in the absolute values of total and peak ROS production in both AGE-Albumin and Albumin groups when TNFα was added, generally maintaining the differential increase in ROS production between the AGE-Albumin and Albumin groups at 150-200%, whether assessed by peak or total ROS production (Fig. 3.128A). With a combination of TNFα priming and fMLP stimulation, there was an approximately 6-fold increase in peak ROS and 11-fold increase in total ROS production, with a maintenance of the differential increase in ROS production (peak or total) between 150-160% (Fig. 3.128B).

PAF priming led to a lesser rise in ROS production than TNFα. Allied to mechanical stimulation, there was an approximately 2-fold rise in peak ROS and 1.5-times increase in total ROS production, maintaining a 200-230% differential increase (in peak or total ROS production) in AGE-Albumin compared to Albumin groups (Fig. 3.128C). With fMLP stimulation, there was only a 1.5-times increase in peak ROS production but no real change in total ROS production, due principally to lower secondary trough phase ROS production in the PAF groups (data not shown), negating the effect of the higher peak ROS values in these groups. There was nonetheless still a differential increase in peak and total ROS production to the level of 130-180% in the AGE-Albumin compared to Albumin groups (Fig. 3.128D).

Figure 3.126

A.



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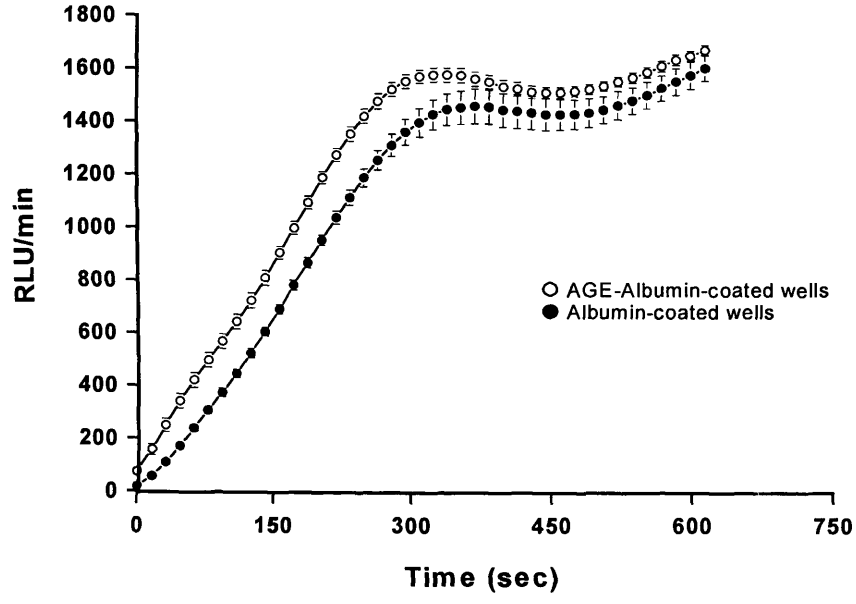

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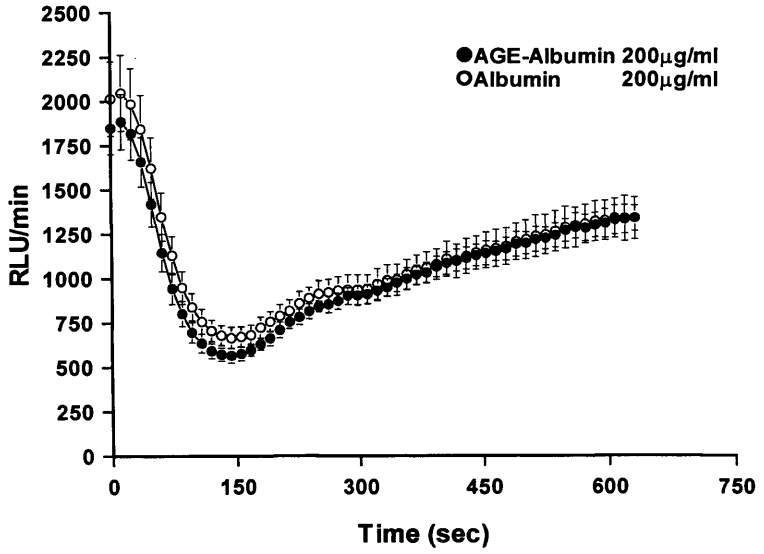


D.

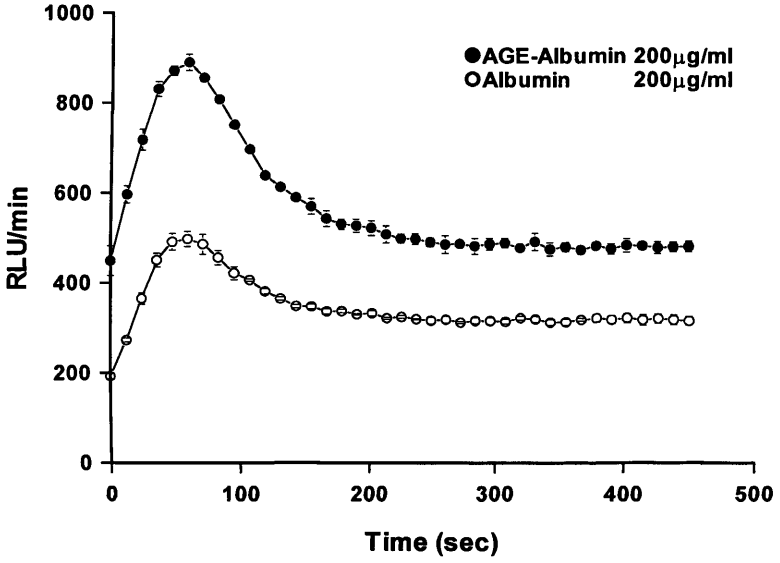
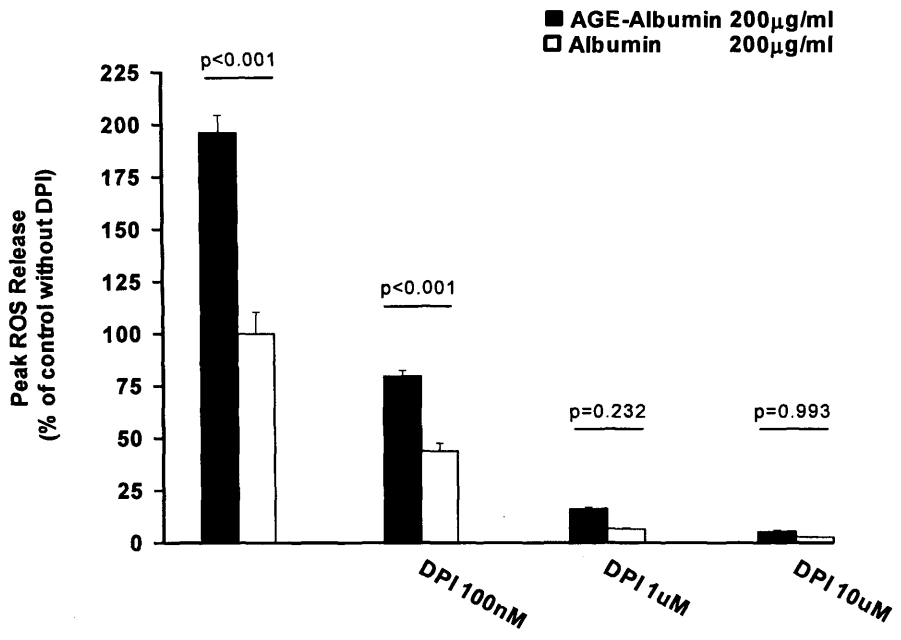


Figure 3.126 Acute neutrophil ROS production detected by Lucigenin-enhanced chemiluminescence when subjected to a reversal in the order of AGE-Albumin addition and mechanical stimulation. (A) Neutrophils were subjected to a mechanical stimulus first, before subsequent addition of AGE-Albumin or Albumin. (B) Neutrophils were mechanically stimulated and then released onto microplate wells coated with AGE-Albumin or Albumin. (C) Neutrophils were stimulated with fMLP before the rapid subsequent addition of AGE-Albumin of Albumin. (D) Neutrophils were allowed to settle on microplate wells coated with AGE-Albumin or Albumin and then stimulated with fMLP.

Results are representative of multiple experiments and each result is the mean of 3 aliquots of 10^5 cells recorded simultaneously. RLU/min, Relative Light Units per minute. Where error bars are not seen, SEMs are less than the size of the symbol.

Figure 3.127

A.



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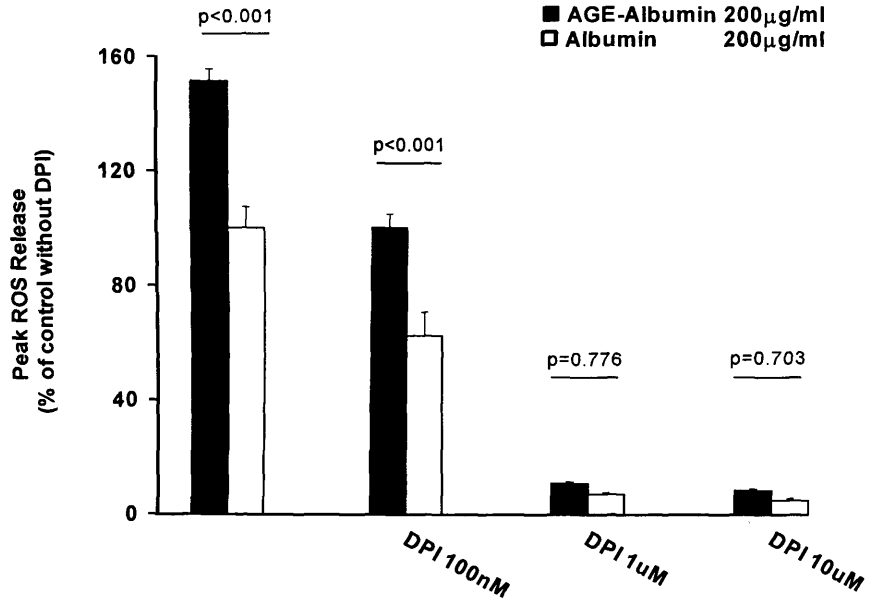
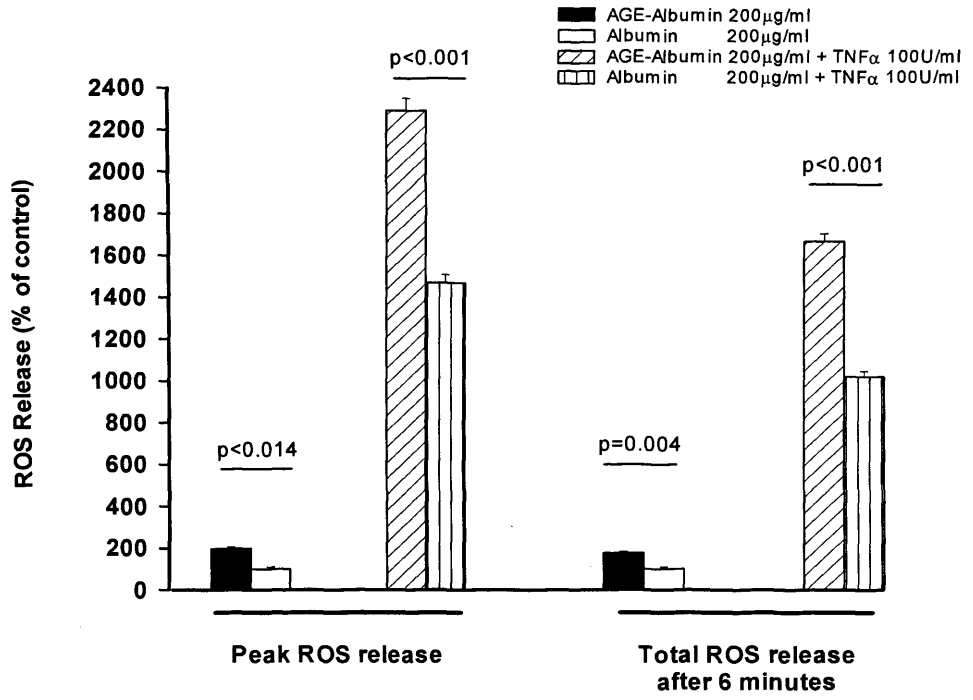


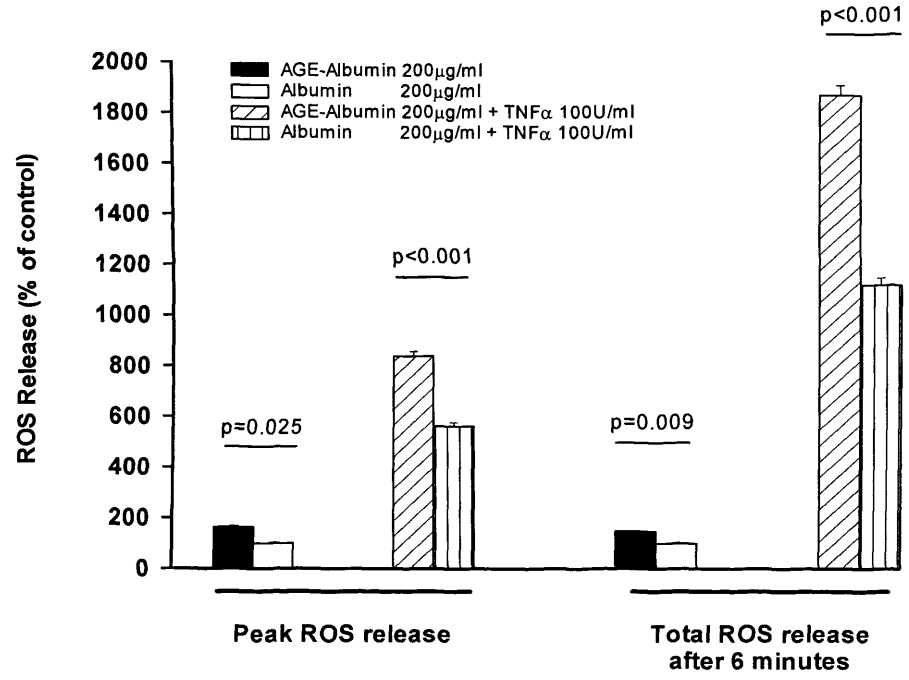
Figure 3.127 Peak neutrophil ROS release with exposure to AGE-Albumin or control Albumin, under conditions of increasing DPI concentration. (A) ROS release following mechanical stimulation ($p<0.001$ between groups by ANOVA). (B) ROS release following stimulation with fMLP ($p<0.001$ between groups by ANOVA). Peak ROS release is expressed as a percentage of peak ROS release with Albumin control in the absence of DPI.

Figure 3.128

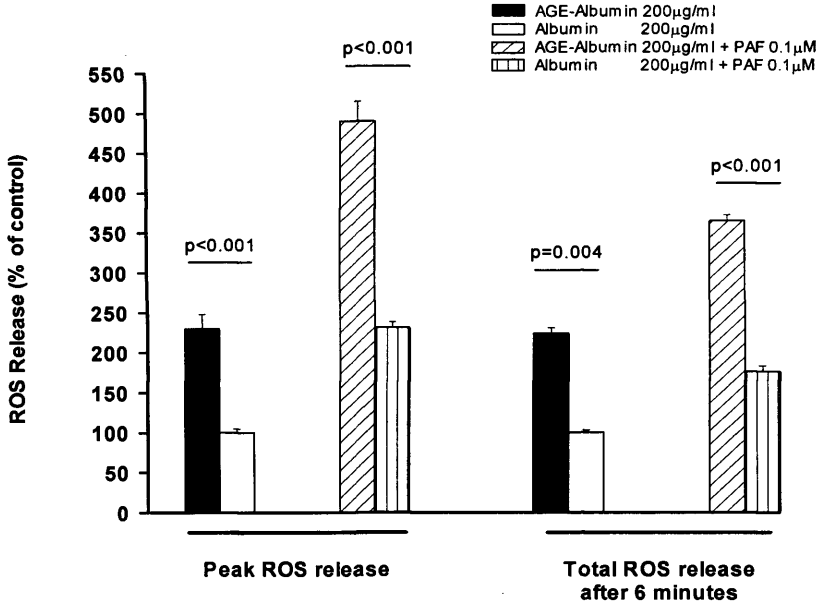
A. Mechanical Stimulation



B. fMLP Stimulation



C. Mechanical Stimulation



D. fMLP Stimulation

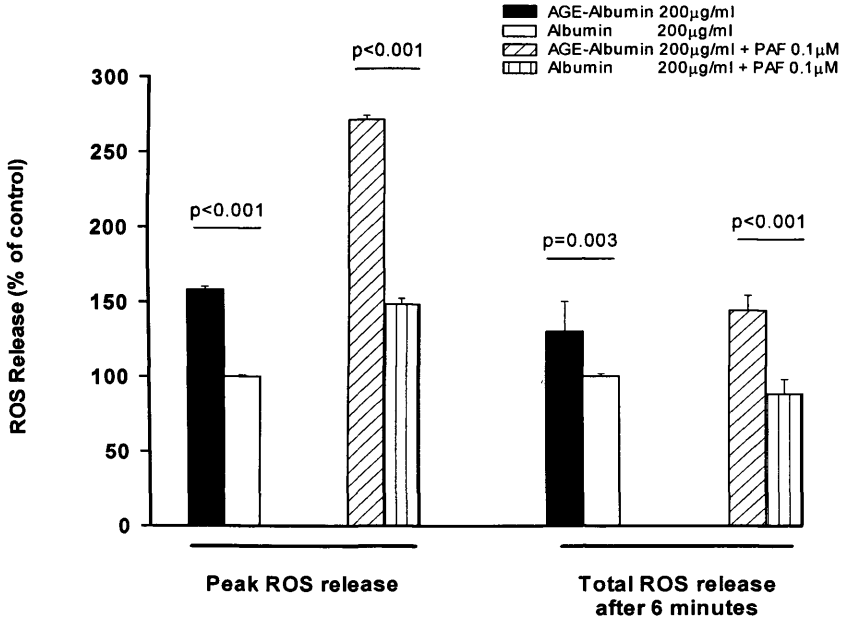


Figure 3.128 AGE-Albumin complements both Tumour Necrosis Factor-α (TNF α) and Platelet Activating Factor (PAF) to further augment neutrophil ROS production. Neutrophils were pre-incubated with TNF α (100 U/ml), PAF (0.1µM) or DMSO vehicle for 30 minutes before addition of AGE-Albumin or Albumin (both at 200µg/ml). They were then subjected to a mechanical stimulus or activation by fMLP in the continuing presence of TNF α (A and B respectively) or PAF (C and D respectively). Each recording is the mean of 3 separate aliquots of 10^5 cells measured simultaneously and the results are representative of multiple experiments. For (A) – (D), $p<0.001$ between groups by ANOVA.

3.13 DISCUSSION

It has been demonstrated that AGEs do not exert any directly measurable effect on the neutrophil respiratory burst when acting alone. However, in concert with another neutrophil stimulus, whether mechanical or chemical, there appears to be a significant amplification of the neutrophil respiratory burst, as detected by chemiluminescence techniques. A bell-shaped dose-response curve appears to exist for this effect (seen with both AGE-Albumin and MM-AGE), although the diminished ROS detection at higher AGE concentrations is likely to reflect impairment of light transmission by higher protein concentrations.[176]

The effect of AGEs does not entail any appreciable interaction time with neutrophils. Indeed, when doses of AGE-Albumin were used that had been previously demonstrated to be suboptimal in terms of enhancing the stimulated neutrophil response, longer incubation times with such suboptimal doses did not imbue AGE-Albumin with any enhancing properties. Thus no evidence of a 'priming' effect was noted with AGE-Albumin. However, the evidence clearly points to a prerequisite for the presence of AGEs in the immediate neutrophil milieu, prior to or simultaneously with the stimulus in order that augmentation of the neutrophil ROS burst may take place. Extending this notion, it has also become apparent that the effect of AGEs on neutrophils is a reversible phenomenon with no imprinting of any prior interaction on cell memory and response. In this respect, AGEs are acting as cellular agonists, but because a second cell stimulus is required before this

agonist action is manifest, AGEs appear to have the novel property of being co-agonists of the neutrophil NADPH oxidase.

The inhibitor studies indicate that the ultimate effect of AGEs is expressed through activity of the neutrophil NADPH oxidase enzyme and it is likely that AGEs trigger a set of processes which eventually result in upregulation of the activity of this enzyme.

3.2 An examination of the effect of AGEs on the Respiratory Burst of Lymphoblasts and on their cooperation with the hypertensive phenotype

3.21 INTRODUCTION

Within the vascular compartment, although phagocytic cells are the dominant ROS-producing leucocytes, a number of other cell types are also capable of generating oxidant stress including other leucocytes, fibroblasts and vascular smooth muscle cells. EBV-immortalised lymphoblasts provide another model for examining leucocyte ROS production despite the ROS levels they generate being substantially less than from phagocytic cells.[115] ROS generation from lymphoblasts derived from hypertensive (HT) and normotensive (NT) subjects was examined following stimulation by a variety of means including AGEs. Previous studies have demonstrated that elements of the hypertensive phenotype can persist in EBV-immortalised lymphoblasts.[162] Such cells also express components of the NADPH oxidase[177] and like neutrophils, they are also capable of mounting a respiratory burst in response to a chemical stimulus, albeit with a less dramatic effect. These cells may therefore be used as models for investigating the production of ROS, free from the influences of environmental factors present in vivo (such as the consumption of exogenous antioxidants and other pharmacological therapies) and free from any effect that the neutrophil extraction process might incur on cell behaviour.

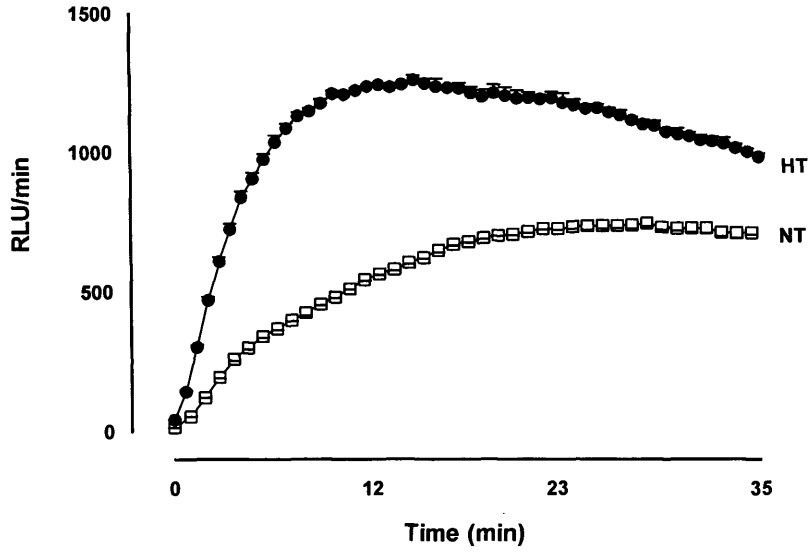
Clinically there is a noted synergy between the presence of hypertension and diabetes mellitus for the development and progression of atherosclerotic disease. There is a correlation between increased ROS production and hypertension in animal models[178] that might partially explain vascular risk in hypertension. The effect of AGE addition to HT and NT lymphoblasts was therefore examined for evidence of synergy of ROS production that might explain the combined pathological consequences of hypertension and diabetes in the clinical setting.

3.22 RESULTS

Lymphoblasts exhibit differential ROS production according to their phenotype. Lymphocytes taken from subjects, who were either hypertensive or normotensive and otherwise matched for age, sex and other cardiovascular risk factors, were immortalised to generate the lymphoblast cell lines. A representative sample of 12 normotensive (NT) and 12 hypertensive (HT) cell lines was examined. ROS production was found to be higher from the HT as compared to the NT cell lines following stimulation with the phorbol ester TPA. Thus peak ROS production in the NT cell line was 745.7 ± 12.3 RLU/min and in the HT cell line was 1258.7 ± 17.8 RLU/min (Figure 3.221). On the other hand, the response from lymphoblasts following mechanical or fMLP stimulation was of very low magnitude (see RLU/min in Figs. 2.35-2.36) which is in accordance with their known properties.

Figure 3.221

A.



B.

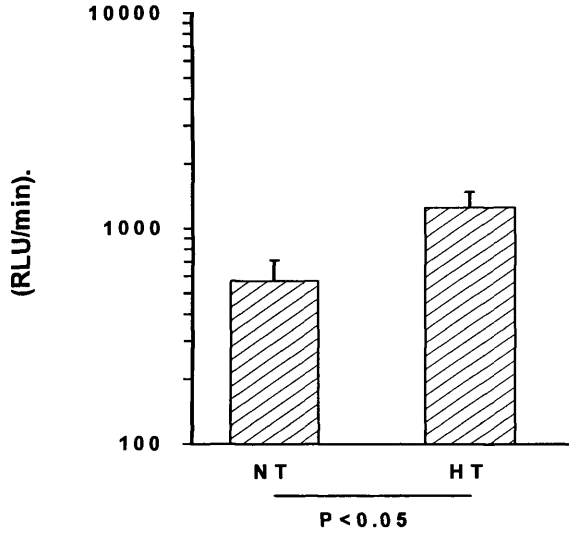


Figure 3.221 (A) Luminol enhanced chemiluminescence of 12-O-tetradecanoylphorbol-13-acetate (TPA) (500nM) stimulated cell lines. The results are from a single HT and NT cell line. The results for each cell line are the mean of three separate aliquots containing 10^5 cells recorded simultaneously; they are representative of multiple similar experiments. HT, hypertensive. NT, normotensive. (B) Peak luminol enhanced chemiluminescence, stimulated with 500nM 12-O-tetradecanoylphorbol-13-acetate (TPA). The results are the mean of 12 NT and 12 HT cell lines and are derived from measurements undertaken on aliquots of 10^5 cells of each cell line. NT, normotensive. HT, hypertensive. RLU, relative light units.

Advanced glycation endproducts do not exert a direct stimulatory effect on lymphoblasts. Attempts were made to see if AGEs were directly capable of stimulating lymphoblast respiratory burst activity. Lymphoblasts which had been aliquoted into microplate wells were subject to the addition of AGE or Albumin and any ensuing ROS production was calculated as the integrated RLU emission over a 6 minute time frame. This was repeated at a variety of different concentrations of AGE/Albumin, although the chemiluminescent counts at the higher concentrations of AGE-albumin and Albumin were exceedingly small. Nonetheless, it could be seen that there was no significant increase in ROS production with either AGE or Albumin addition at any of the concentrations used, and this applied to both the HT and NT lymphoblast phenotypes (Figure 3.222).

Advanced glycation endproducts are unable to enhance ROS production further in stimulated lymphoblasts. Lymphoblasts (HT and NT phenotypes) were suspended in AGE/Albumin and then aliquoted into multiple microplate wells, either with a prior mechanical stimulus or a proceeding chemical stimulus with fMLP or the phorbol ester TPA. Unlike neutrophils, lymphoblasts display a minimal response to mechanical stimulation and fMLP, in terms of respiratory burst activity (Figures 3.223A and B), with only a very slow rise to very low levels of ROS production. However they do respond to stimulation with TPA with gradually increasing respiratory burst activity that builds up to a significant plateau of activity (Figure 3.221A). Despite this, the presence of AGEs prior to lymphoblast stimulation with TPA did not result in any significant enhancement of ROS production over lymphoblast stimulation in the presence

of Albumin. In this way lymphoblasts are seen to differ fundamentally from the response of neutrophils. This lack of augmentation of the TPA-stimulated respiratory burst in lymphoblasts by AGE, held true over a wide dose range of AGE/Albumin tested, and importantly, held true for both lymphoblast phenotypes (Figures 3.224A and B). Likewise, AGEs did not impart any ability to lymphoblasts to enhance ROS production with mechanical or fMLP stimulation over a wide range of AGE-Albumin doses tested (Figures 3.224E and F). There were no significant differences between lymphoblasts of either phenotype that had been pre-incubated in AGE or Albumin at each of the concentrations examined. There was however an overall lower level of ROS detection at the highest concentration (400µg/ml) of AGE/HSA examined.

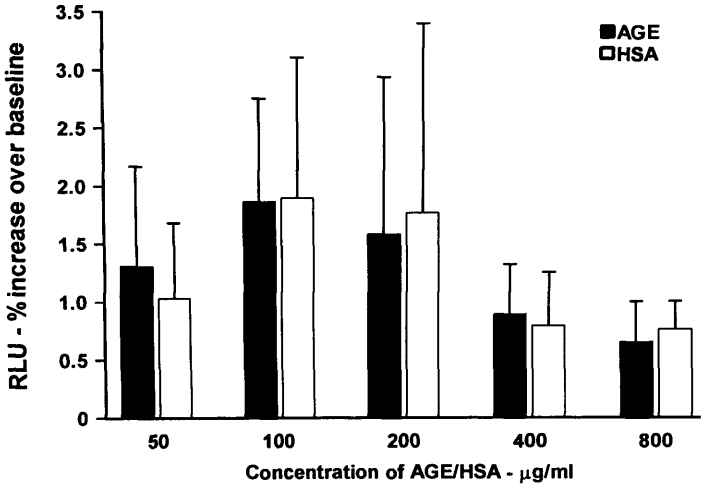
Lymphoblasts are incapable of responding to advanced glycation endproducts with an enhanced respiratory burst activity despite prior interaction time. AGEs have been shown to be able to act as neutrophil co-agonists, not requiring any significant pre-incubation time to effect augmentation of the neutrophil respiratory burst in response to an additional stimulus; such a relationship however does not hold true for AGEs and lymphoblasts. Yet it remained a possibility that given sufficient interaction time, AGEs might be able to exert an effect on lymphoblast activity. A range of different concentrations of AGE/Albumin was pre-incubated with lymphoblasts of HT and NT phenotype for varying time periods before subsequent lymphoblast activation by another mode of stimulation. Neither the HT nor NT cell lines appeared susceptible to a range of AGE concentrations over varying durations of pre-incubation time. These responses are shown in Figure 3.225

for total ROS output following cell stimulation by TPA, though no significant enhancements were seen either following stimulation by mechanical means or fMLP.

The Receptor for Advanced glycation endproducts (RAGE) is expressed on lymphoblast membranes. In view of the largely insignificant effect of AGEs on lymphoblast respiratory burst activity, evidence for the existence of the RAGE receptor was sought in lymphoblasts, with this presumed to be a likely mediating factor in AGE-related cellular responses. Lymphoblast lysates from both HT and NT cell lines were run on SDS-PAGE gels and subject to Western blotting with monoclonal antibodies to the RAGE receptor. The presence of the RAGE receptor was clearly identified when compared with control protein (Figure 3.226).

Figure 3.222

A.



B.

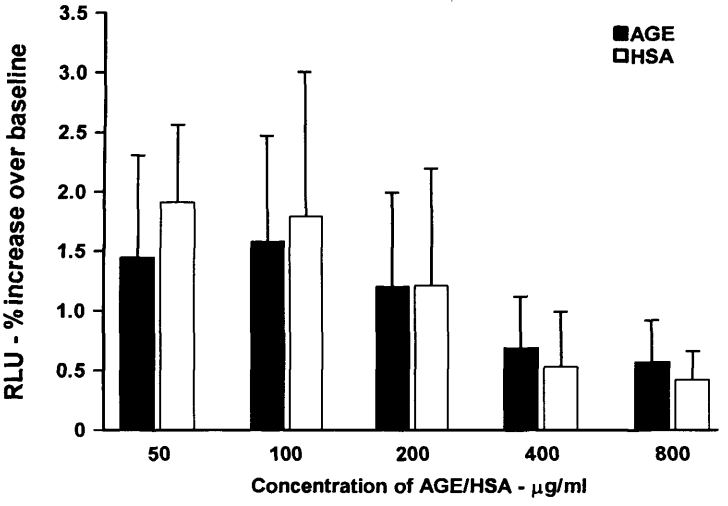
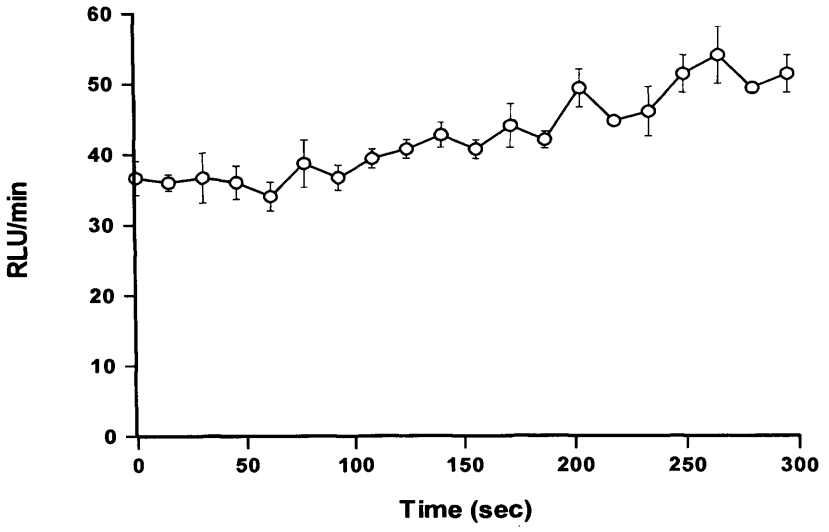


Figure 3.222 (A) The direct effects of AGE/HSA on ROS production by HT lymphoblasts as detected by luminol enhanced chemiluminescence. The cumulative increase in ROS production (detected as Relative Light Units) over the 6 minutes following addition of AGE/HSA to the cells in the microplate (100,000 cells per well) expressed as a percentage of the baseline ROS generation ($p=0.114$ between groups by ANOVA). (B) The direct effects of AGE/HSA on ROS production by NT lymphoblasts as detected by luminol enhanced chemiluminescence. The cumulative increase in ROS production (detected as Relative Light Units) over the 6 minutes following addition of AGE/HSA to the cells in the microplate (100,000 cells per well) expressed as a percentage of the baseline ROS generation ($p=0.467$ between groups by ANOVA).

For each figure, experiments were conducted at five different concentrations of AGE/HSA and error bars show the SEMs. The results for each concentration are averaged from three repetitions of the experiment.

Figure 3.223

A.



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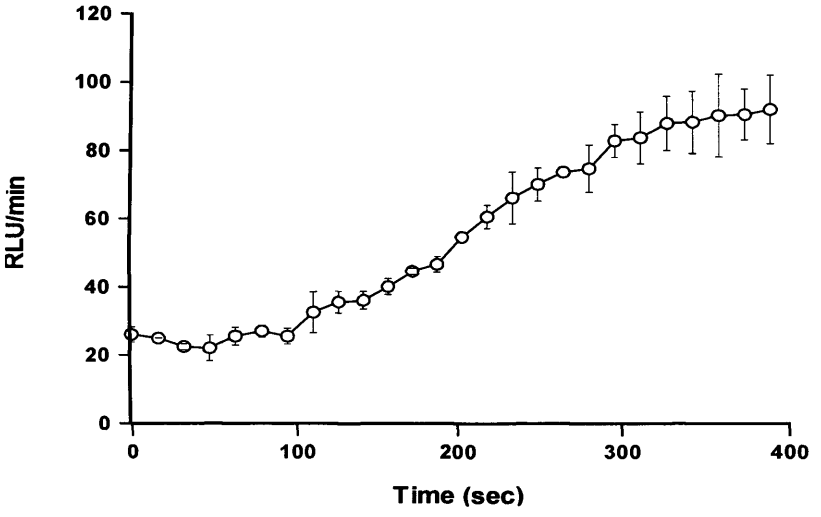
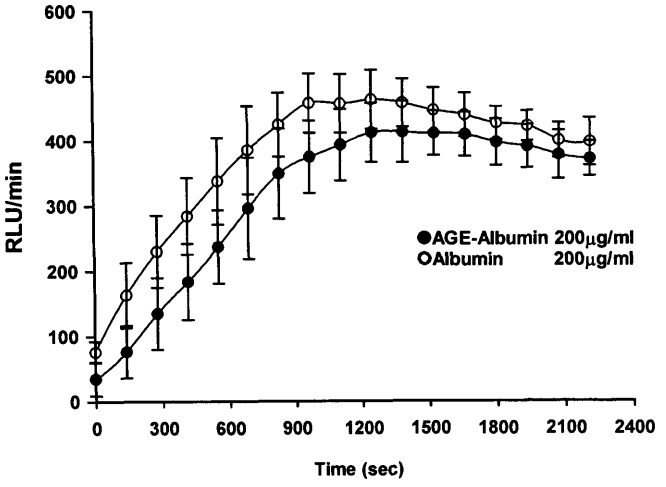


Figure 3.223 (A) ROS production from an NT lymphoblast in response to mechanical stimulation. (B) ROS production from an NT lymphoblast following stimulation with the peptide fMLP (100nM). ROS production is measured in terms of Relative Light Units (RLU) of chemiluminescence with luminol as the chemiluminescent probe.

Both experiments were carried out in triplicate (SEMs demonstrated) with 10^5 cells per microplate well, and are representative of ROS production under similar conditions in a range of NT and HT lymphoblasts.

Figure 3.224

A.



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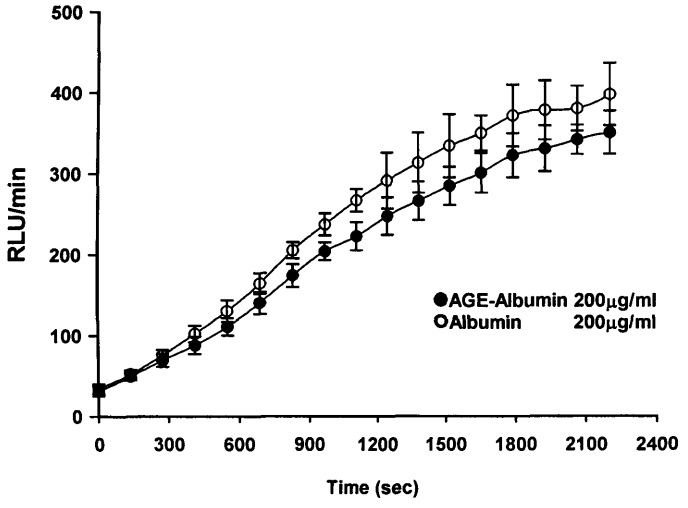

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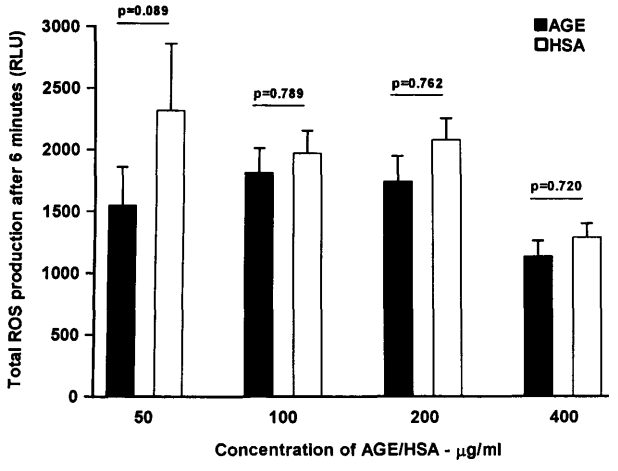
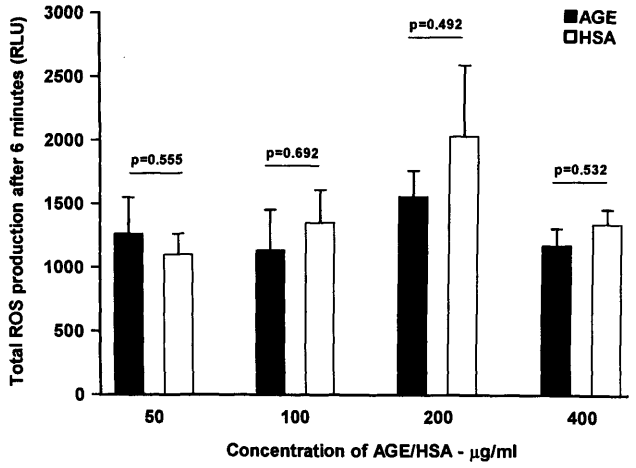
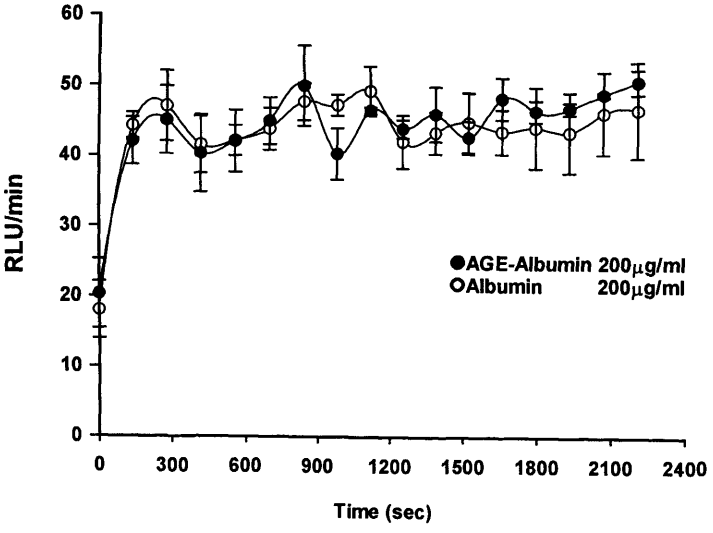


D.


Total ROS production after 6 minutes (RLU)
3000
2500
2000
1500
1000
500
0
p=0.555
p=0.692
p=0.492
p=0.532
AGE
HSA
50
100
200
400
Concentration of AGE/HSA - µg/ml

E.


RLU/min
60
50
40
30
20
10
0
0
300
600
900
1200
1500
1800
2100
2400
Time (sec)
AGE-Albumin 200µg/ml
Albumin 200µg/ml

F.

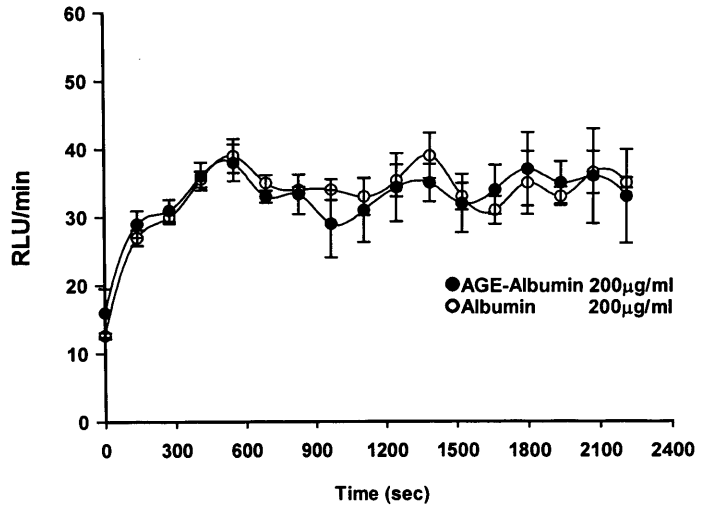


Figure 3.224

A) ROS production from HT lymphoblasts following TPA stimulation in the presence of AGE or HSA. (B) ROS production from NT lymphoblasts following TPA stimulation in the presence of AGE or HSA.

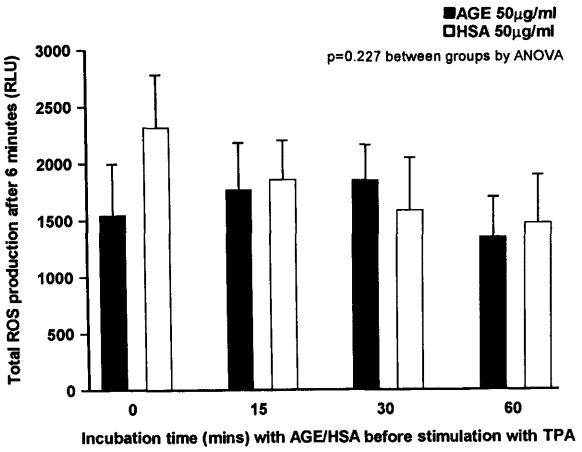
Both experiments were carried out in triplicate (SEMs shown) with 10^5 cells per microplate well and are representative of ROS production under similar conditions in a range of NT and HT lymphoblasts.

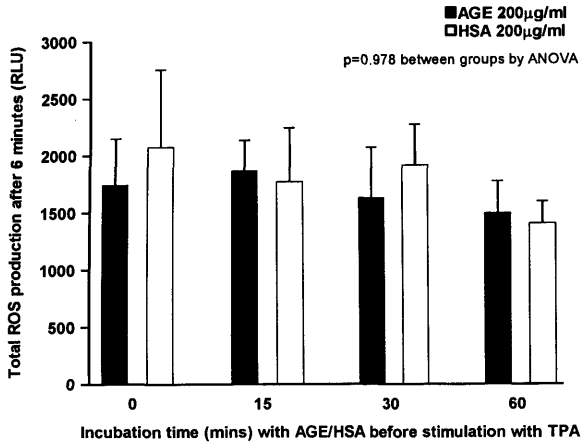
(C) Cumulative ROS production over the first 6 minutes following TPA stimulation in HT cell lines prior exposed to AGE/HSA at the variety of concentrations shown. The results are averaged from triplicate recordings of one representative cell line and SEMs are displayed. (D) Cumulative ROS production from NT cell lines stimulated with TPA in the presence of varying concentrations of AGE/HSA. The results are averaged from triplicate recordings of one representative cell line and SEMs are displayed. (E) ROS production from HT lymphoblasts in response to mechanical stimulation and in the presence of AGE or HSA. The real time ROS production from HT lymphoblasts is shown but is representative of the effect produced from NT lymphoblasts as well. (F) ROS production from HT lymphoblasts in response to stimulation with the bacterial cell wall peptide fMLP, in the presence of AGE or HSA. The real time ROS production from HT lymphoblasts is shown but is representative of the effect produced from NT lymphoblasts as well.

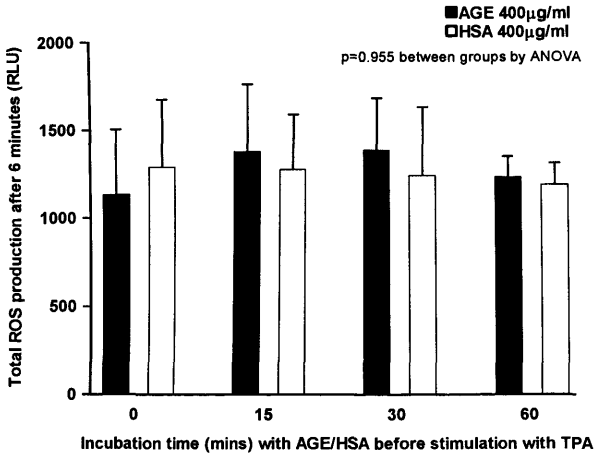
ROS detection in each of these experiments utilised luminol as the chemilunescent probe.

Figure 3.225

A.







B.

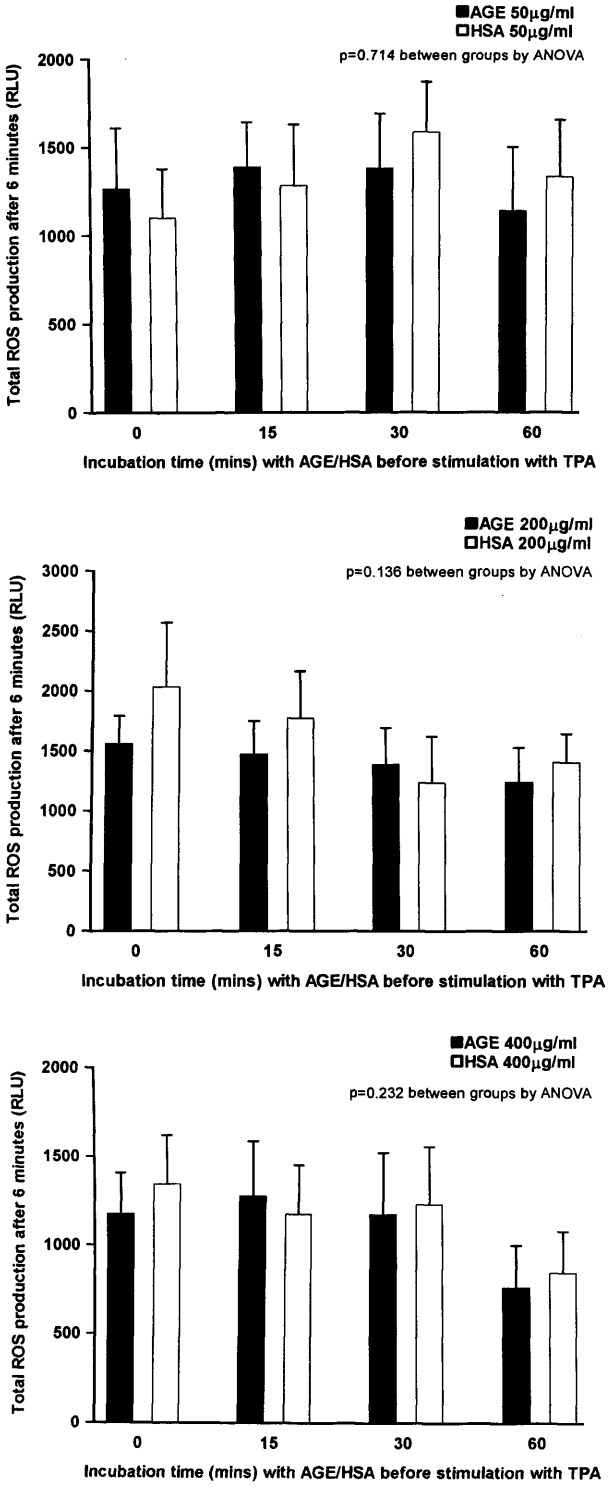


Figure 3.225 The effect of longer pre-incubation times with AGE-albumin on the responses of lymphoblasts to stimulation with TPA. (A) HT lymphoblasts were stimulated with TPA having been pre-incubated with the varying concentrations of AGE/HSA specified and for the varying amounts of time detailed in each graph. The results are the mean cumulative ROS production

(over 6 minutes post-stimulation with TPA) from triplicate recordings of a representative cell line (10^5 cells used per microplate well). SEMs are shown. (B) NT lymphoblasts were stimulated with TPA having been pre-incubated with the varying concentrations of AGE/HSA specified and for the varying amounts of time detailed in each graph. The results are the mean cumulative ROS production (over 6 minutes post-stimulation with TPA) from triplicate recordings of a representative cell line (10^5 cells used per microplate well). SEMs are shown.

ROS detection in each of these experiments utilised luminol as the chemilunescent probe.

Figure 3.226

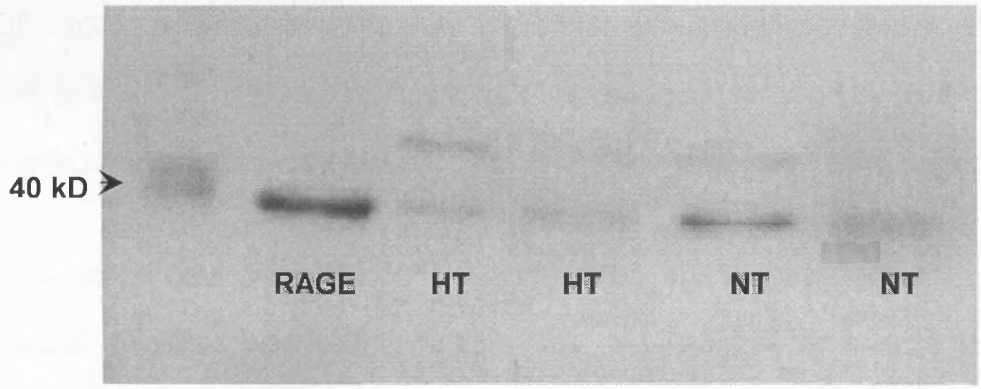


Figure 3.226 Representative lymphoblast lysates derived from hypertensive (HT) and normotensive (NT) subjects and probed with an antibody to the RAGE receptor. Purified RAGE protein was used in the control lane. For both the HT and NT lanes, the lysates were derived from different lymphoblast cell lines and protein loading per lane was variable (100-500µg).

3.23 DISCUSSION

Despite demonstrating expression of the RAGE receptor in lymphoblasts, no evidence was found for an effect of AGEs on the lymphoblast respiratory burst. As was the case with neutrophils, there certainly did not appear to be any direct capacity for AGEs to stimulate an oxidative response, but neither were AGEs able to augment the respiratory burst in response to a fellow stimulus. This was unlikely to be due to dose insufficiency as demonstrated by testing a wide variation of doses of AGE/Albumin with similar findings. In fact, there was a reduction in chemiluminescent counts with higher AGE/Albumin concentrations in a similar manner to that seen in neutrophils and again this may be attributed to light quenching by the high protein load. The possibility of AGEs acting as priming agents in lymphoblasts was also discounted on the basis of any lack of increase in the lymphoblast respiratory burst following variable pre-incubation with AGEs.

Compared to neutrophils, lymphoblasts therefore appear relatively inert in terms of oxidative species generation. The possibility of a hypertensive phenotype translating into a state of increased oxidative species production was raised by the observation of higher ROS production in TPA-stimulated HT lymphoblasts compared to NT lymphoblasts. Unfortunately, any possibility of studying synergy of ROS production between hypertensive and hyperglycaemic states was rendered unattainable due to the inability of AGEs to produce a response from the lymphoblast cell type.

3.3 Advanced Glycation Endproducts Stimulate an Enhanced Neutrophil Respiratory Burst Mediated through the Activation of Cytosolic Phospholipase A_2 and Generation of Arachidonic Acid

3.31 INTRODUCTION

AGEs behave as neutrophil co-agonists, augmenting the respiratory burst induced by a secondary stimulus, but the mechanisms governing this effect are unknown. Messenger systems and intracellular signalling are likely to play a role in mediating this response rather than a simple direct oxidative action of the AGEs themselves. Although AGEs are well known to possess direct oxidative capacity, they have already been shown in these studies to lack a direct contribution to the ROS produced by activated neutrophils.

It is probable that the initiation of such processes is receptor-mediated. Receptors capable of binding AGEs have been described, of which the Receptor for Advanced Glycation Endproducts (RAGE) is the best characterised, with a common feature of its ligation being the activation of redox-sensitive pathways. Also of particular relevance to the co-agonist action of AGEs on the neutrophil NADPH oxidase, are similar reports ascribing a facilitatory effect to arachidonic acid (AA) on NADPH oxidase activation.[179,180] The focus of the current study was to explore the mechanisms underlying AGE co-agonist activity, including the possible roles of AA, an unsaturated fatty acid produced by phospholipase A_2 (PLA_2) enzymes, and the RAGE receptor.

3.32 RESULTS

AGE-augmentation of the stimulated neutrophil respiratory burst is abolished by inhibitors of cytosolic phospholipase A_2 ($cPLA_2$) and by N-acetylcysteine. In seeking to establish the mechanisms by which AGEs augment the neutrophil respiratory burst, the effect of manipulating both AA production and intracellular redox status on this particular property of AGEs was examined. AA is hydrolysed from membrane glycerophospholipids by PLA_2[181] of which different isoforms exist. As shown previously, the respiratory burst was augmented in the presence of AGE-albumin compared to control albumin, following mechanical or chemical (fMLP) stimulation (Figs. 3.321A-E).[182] This augmented response was significantly attenuated by a 30 min pre-incubation of neutrophils with the minimum effective dose of either of two $cPLA_2$ inhibitors, MAFP 50nM[183] and mepacrine 10μM[184] (Figs. 3.321A and B), and attenuated to a lesser degree by a 60 min pre-incubation of neutrophils with the intracellular glutathione precursor N-acetylcysteine 10mM (Fig. 3.321E). For the latter, AGE-augmented ROS output diminished from 201% to 138% ($p<0.01$) with mechanical stimulation and from 169% to 138% ($p<0.01$) with fMLP stimulation. A variety of other direct free radical scavengers were also examined for effects on reducing either total ROS output or in reducing the AGE-augmented ROS output. However, a 60 min pre-incubation with the direct free radical scavengers acetylsalicylic acid, ascorbic acid and tiron, or pre-incubation with radical-metabolising enzymes (catalase and superoxide dismutase mimetic [SOD-M]), produced negligible effects. In fact, when acetylsalicylic acid was pre-incubated with neutrophils for 60 minutes at a variety of concentrations, it was found that very high doses

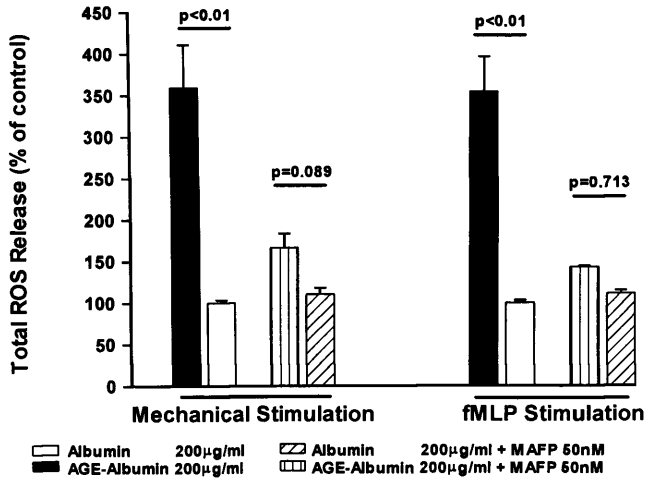
(10μM) actually resulted in an overall increase in ROS production by neutrophils, both from control and from AGE-stimulated cells. This was statistically significant and was accentuated when neutrophils were stimulated with fMLP. Lower doses of acetylsalicylic acid neither enhanced ROS detection nor did they make any impact on reducing ROS output per se (figures 3.321F and G). With the scavenger ascorbate, there was a similar enhancement of ROS detection at high doses of scavenger (again with a prior 60 minute pre-incubation), though this was only statistically significant following stimulation with fMLP again. As with acetylsalicylic acid, the lower doses again had a negligible impact on reducing ROS production or attenuating the AGE-augmented increases in ROS production (figures 3.321H and I). The only free radical scavenger that was able to impact on overall ROS production, albeit not affecting the augmentation resulting from AGE-stimulation, was Tiron, with the highest dose (10mM) being the most effective (figures 3.321N and O). The effect of ROS-disposing enzymes was also examined, in particular looking at the effect of catalase, with its ability to catalyse the breakdown of hydrogen peroxide, and a superoxide dismutase mimetic (SOD-M), the latter dismutating superoxide to hydrogen peroxide. Both enzymes demonstrated an ability to reduce the oxidative stress burden through a reduction in detected ROS species when incubated with neutrophils and left in the reaction media during neutrophil stimulation. There was a dose-dependent effect for both enzymes, but again, although the overall ROS output was diminished, there was little effect on specifically reducing the augmentation induced by AGE-stimulation (figures 3.321J-M).

Finally, inhibition of other PLA_2 isoforms ($sPLA_2$ by Manoalide 1μM[185] and $iPLA_2$ by Haloenol-lactone suicide substrate [HELSS] 100 nM[186] – both being minimal effective doses) did not result in significant reduction of the AGE-augmented neutrophil respiratory burst (Figs. 3.321C and D).

AGE-exposed neutrophils exhibit enhanced liberation of arachidonic acid which is abrogated by inhibitors of cytosolic phospholipase A_2 and N-acetylcysteine. To strengthen the inference that $cPLA_2$ inhibition reduced AGE co-agonist activity by means of reducing AA production, the formation of AA following AGE-exposure was assessed directly. Identical numbers of neutrophils, pre-labelled with ^{3}H-AA, were incubated for 30 minutes in AGE-albumin or albumin (200μg/ml), at the end of which AA release was assayed. AGE-exposed neutrophils exhibited a higher rate of AA release compared to albumin-exposed neutrophils (151% ± 16%, $p<0.01$) [Fig. 3.322]. This enhancement was abrogated by pre-incubating neutrophils for 30 minutes with a minimum effective dose (50nM) of MAFP, an inhibitor of $cPLA_2$, prior to AGE-albumin/albumin exposure (Fig. 3.322).

Figure 3.321

A.



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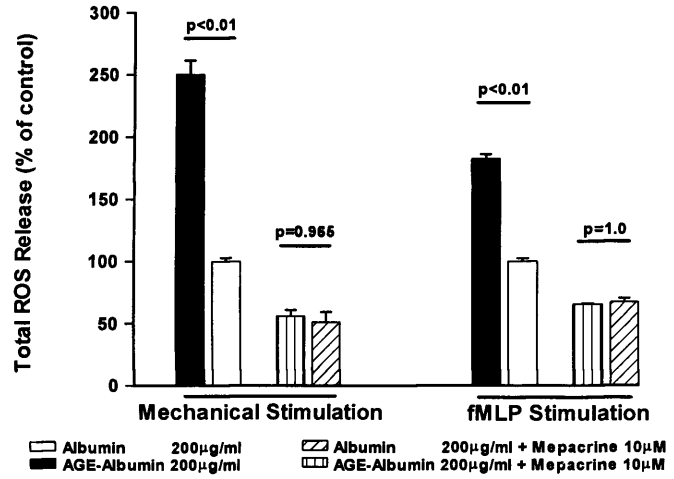

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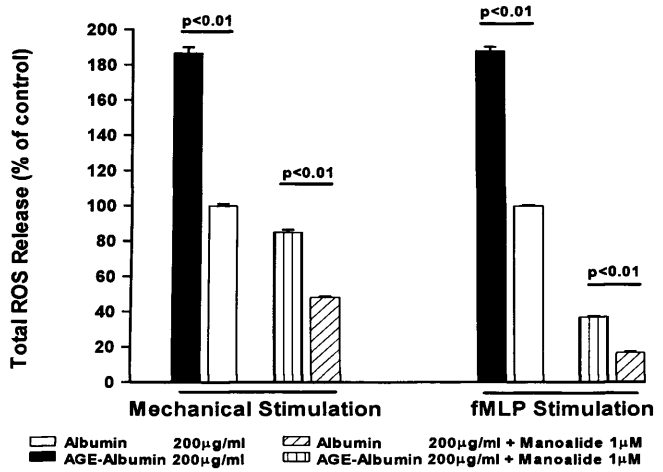
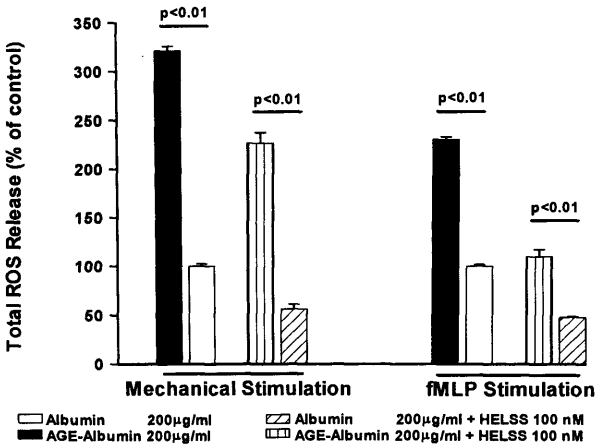
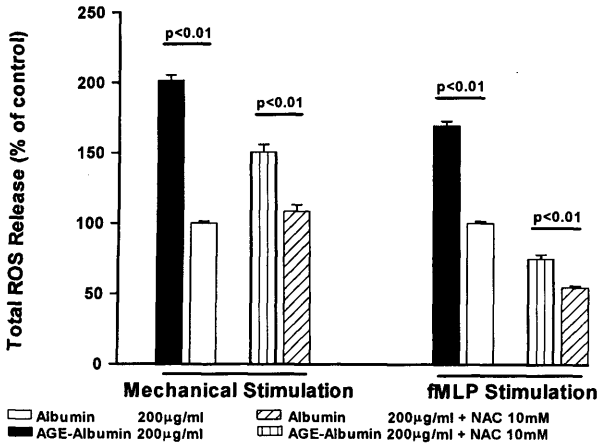


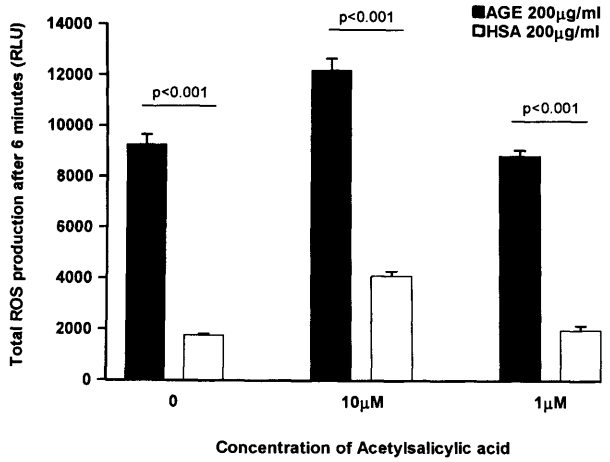
D.


Total ROS Release (% of control)
350
300
250
200
150
100
50
0
p<0.01
p<0.01
p<0.01
p<0.01
Mechanical Stimulation
fMLP Stimulation
Albumin 200µg/ml
AGE-Albumin 200µg/ml
Albumin 200µg/ml + HELSS 100 nM
AGE-Albumin 200µg/ml + HELSS 100 nM

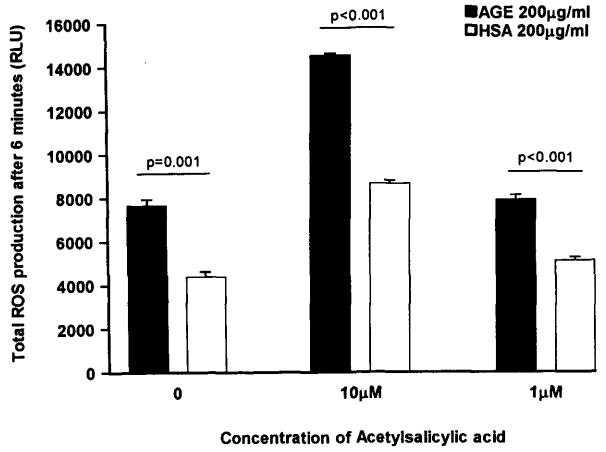
E.


Total ROS Release (% of control)
250
200
150
100
50
0
p<0.01
p<0.01
p<0.01
p<0.01
Mechanical Stimulation
fMLP Stimulation
Albumin 200µg/ml
AGE-Albumin 200µg/ml
Albumin 200µg/ml + NAC 10mM
AGE-Albumin 200µg/ml + NAC 10mM

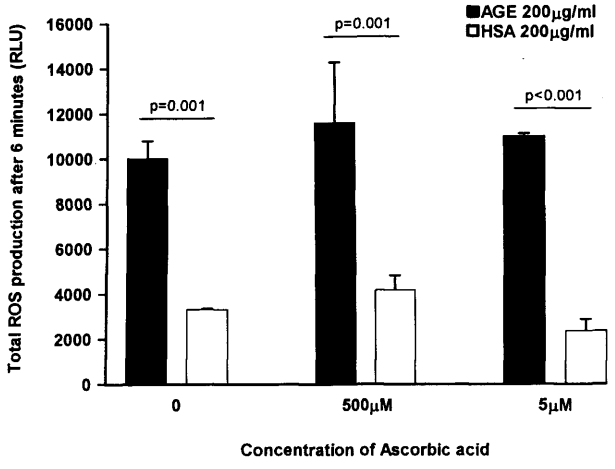
F.


Total ROS production after 6 minutes (RLU)
14000
12000
10000
8000
6000
4000
2000
0
p<0.001
p<0.001
p<0.001
AGE 200µg/ml
HSA 200µg/ml
0
10µM
1µM
Concentration of Acetylsalicylic acid

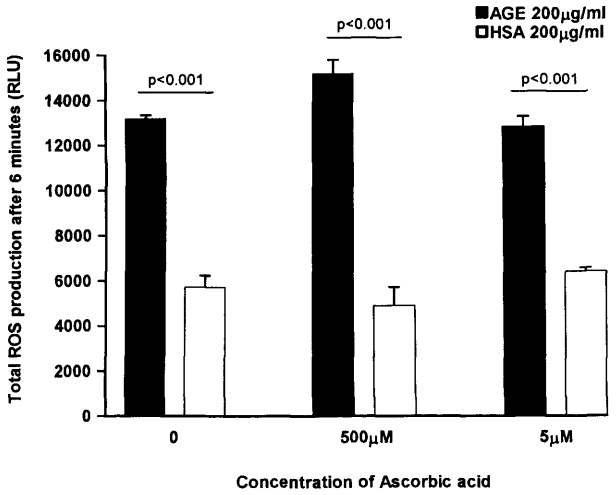
G.


Total ROS production after 6 minutes (RLU)
16000
14000
12000
10000
8000
6000
4000
2000
0
p<0.001
p=0.001
p<0.001
■AGE 200µg/ml
□HSA 200µg/ml
0
10µM
1µM
Concentration of Acetylsalicylic acid

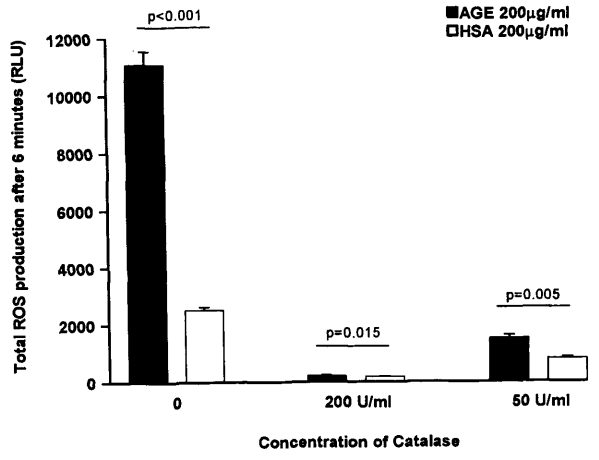
H.


Total ROS production after 6 minutes (RLU)
16000
14000
12000
10000
8000
6000
4000
2000
0
p=0.001
p=0.001
p<0.001
■AGE 200µg/ml
□HSA 200µg/ml
0
500µM
5µM
Concentration of Ascorbic acid

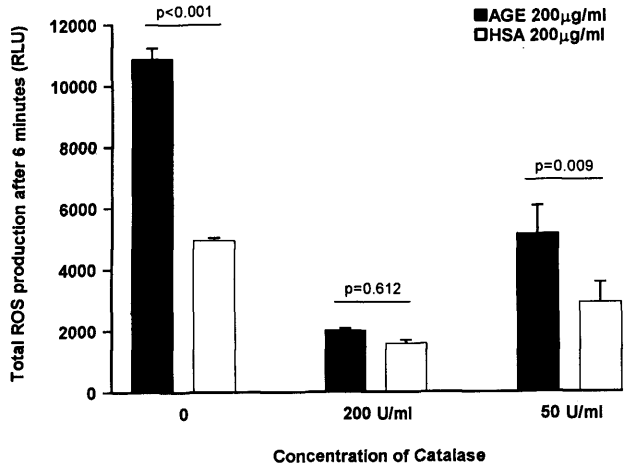
I.


Total ROS production after 6 minutes (RLU)
16000
14000
12000
10000
8000
6000
4000
2000
0
p<0.001
p<0.001
p<0.001
■AGE 200µg/ml
□HSA 200µg/ml
0
500µM
5µM
Concentration of Ascorbic acid

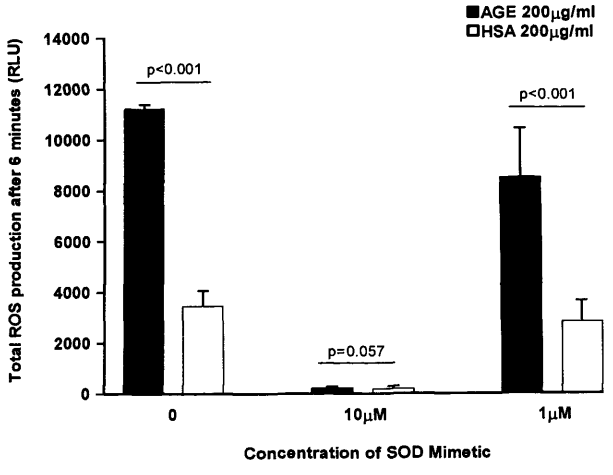
J.


■AGE 200µg/ml
□HSA 200µg/ml
p<0.001
p=0.015
p=0.005
Total ROS production after 6 minutes (RLU)
12000
10000
8000
6000
4000
2000
0
0
200 U/ml
50 U/ml
Concentration of Catalase

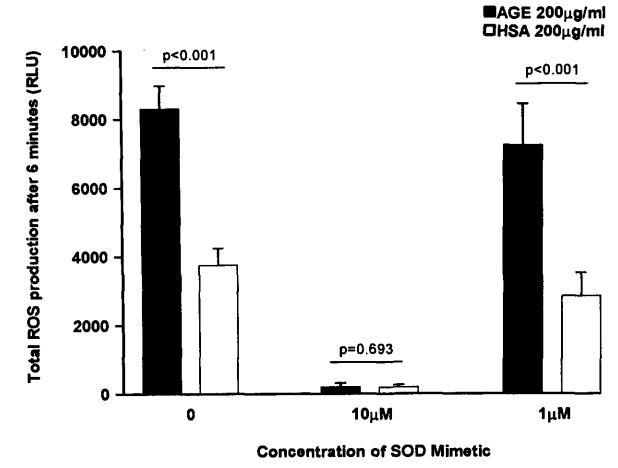
K.


■AGE 200µg/ml
□HSA 200µg/ml
p<0.001
p=0.612
p=0.009
Total ROS production after 6 minutes (RLU)
12000
10000
8000
6000
4000
2000
0
0
200 U/ml
50 U/ml
Concentration of Catalase

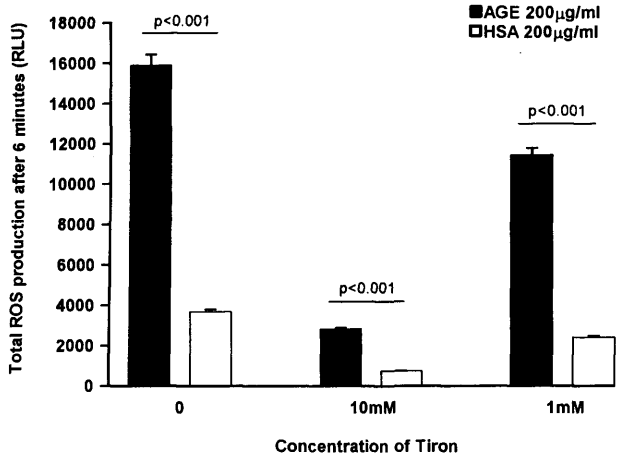
L.


■AGE 200µg/ml
□HSA 200µg/ml
p<0.001
p=0.057
p<0.001
Total ROS production after 6 minutes (RLU)
14000
12000
10000
8000
6000
4000
2000
0
0
10µM
1µM
Concentration of SOD Mimetic

M.



N.



O.

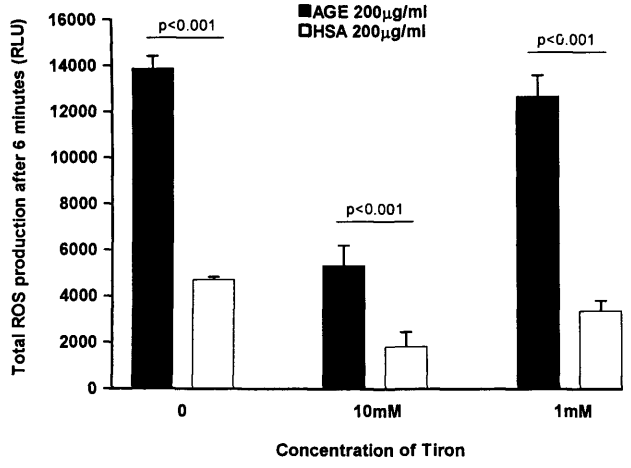


Figure 3.321 Total neutrophil ROS production (over 6 minutes), detected by Lucigenin-enhanced chemiluminescence, in the presence of AGE-albumin/albumin (200µg/ml) and in response to mechanical or chemical (fMLP) stimuli. ROS release is expressed as a percentage of the output from

control (albumin-exposed) neutrophils. Effect of pre-incubation with various compounds on ROS production from neutrophils exposed to AGE-Albumin/Albumin: (A) Methyl-arachidonylfluorophosphonate (MAFP) 50nM ($p<0.01$ between groups by ANOVA). (B) Mepacrine 10μM ($p<0.01$ between groups by ANOVA). (C) Manoalide 1μM ($p<0.001$ between groups by ANOVA). (D) Haloenol-lactone suicide substrate (HELSS) 100 nM ($p<0.01$ between groups by ANOVA). (E) N-acetylcysteine (NAC) 10mM ($p<0.001$ between groups by ANOVA).

The effect of free radical buffering systems on detected neutrophil ROS output and the AGE-augmented neutrophil ROS output. Neutrophils were pre-incubated for 60 minutes with one of several free radical scavengers at various doses (acetylsalicylic acid, ascorbic acid and tiron), the enzyme catalase, or a superoxide dismutase mimetic (SOD-M) enzyme. In each case, the investigated compound was left to remain in the neutrophil media whilst the cells were undergoing stimulation and whilst ROS detection was taking place. (F) ROS output following mechanical stimulation of neutrophils and pre-incubation with acetylsalicylic acid at different doses. (G) ROS output following fMLP stimulation of neutrophils and pre-incubation with acetylsalicylic acid at different doses. (H) ROS output following mechanical stimulation of neutrophils and pre-incubation with ascorbic acid at different doses. (I) ROS output following fMLP stimulation of neutrophils and pre-incubation with ascorbic acid at different doses. (J) ROS output following mechanical stimulation of neutrophils and pre-incubation with catalase at different doses. (K) ROS output following fMLP stimulation of neutrophils and pre-incubation with catalase at different doses. (L) ROS output following mechanical stimulation of neutrophils and pre-incubation with SOD-M at different doses. (M) ROS output following fMLP stimulation of neutrophils and pre-incubation with SOD-M at different doses. (N) ROS output following mechanical stimulation of neutrophils and pre-incubation with Tiron at different doses. (O) ROS output following fMLP stimulation of neutrophils and pre-incubation with Tiron at different doses.

Results are representative of multiple experiments with each result derived from the mean of 3 separate aliquots of 10^5 cells recorded simultaneously.

As the interaction of AGEs with some cellular receptors has been noted to activate redox-sensitive pathways,[112] an important aim was to establish whether redox-sensitive pathways might govern the AGE-induced increases in neutrophil AA production. The intracellular levels of reduced glutathione, a factor in the intracellular redox status, were manipulated using a 60 min pre-incubation of neutrophils with N-acetylcysteine (NAC); the latter is a thiol-antioxidant and synthetic precursor of glutathione. Subsequently exposing neutrophils to AGE-albumin did not result in any significant increases in AA production over control cells (104% ± 17%, p=0.94) [Fig. 3.322].

AGEs mediate activation of cytosolic phospholipase A_2. Activation of the enzyme $cPLA_2$ requires its phosphorylation and translocation from the cytosol to the plasma membrane.[187] Therefore it could be hypothesised that if AGE-albumin is an activator of $cPLA_2$, it might induce similar changes. After transient exposure to AGE-albumin/albumin followed by resuspension in relaxation buffer, neutrophils were rapidly fractionated into membrane and cytosolic components. Comparing AGE-albumin and albumin-exposed neutrophils, immunoblots revealed increased $cPLA_2$ in the plasma membrane fractions of the former, with a corresponding decrease in the cytosolic fractions (Fig. 3.323 and Table 3.321). Additionally, the presence of a phosphorylated $cPLA_2$ moiety in the membrane fraction derived from cells exposed to AGE-albumin was revealed through reduced electrophoretic mobility or gel-shift (Fig. 3.323). Such AGE-induced $cPLA_2$ phosphorylation and increased membrane translocation was abolished by a 60 minute pre-incubation of neutrophils with 10mM N-acetylcysteine (Fig. 3.323 and Table 3.321).

Figure 3.322

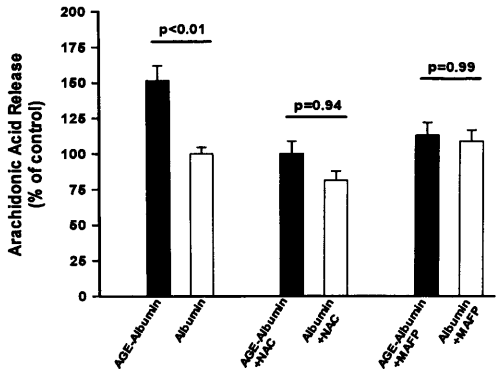


Figure 3.322 Neutrophil AA release over 30 minutes following the addition of AGE-albumin (black bars) or albumin (white bars), both at 200µg/ml and with prior incubation in N-acetylcysteine or MAFP.

Results are expressed as the percentage increase in AA release over the control (albumin group) and are the mean of 5 experiments. Analysis was by one way ANOVA with Tukey's test ($p<0.01$ between groups).

Table 3.321 Quantitative comparison of $cPLA_2$ (non-phosphorylated) in different neutrophil fractions after exposure to AGE-albumin/albumin. Measurements are in arbitrary densitometric units with SEMs shown in parentheses. The phosphorylated $cPLA_2$ band (P-$cPLA_2$) is only seen in the membrane fraction of neutrophils exposed to AGE-albumin. Quantitation was made by analysis of 5 experiments, with the significance of the differences analysed by Tukey's test following ANOVA.

* For both the cytosolic and membrane fractions, $p<0.01$ between groups by ANOVA.

	CYTOSOL*		MEMBRANE*	
	AGE-albumin	**Albumin**	**AGE-albumin**	**Albumin**
$cPLA_2$	0.53 (0.02)	0.75 (0.05)	2.53 (0.17)	0.51 (0.06)
Significance	P=0.018		P=0.024	
	AGE-albumin + NAC	**Albumin + NAC**	**AGE-albumin + NAC**	**Albumin + NAC**
$cPLA_2$	0.62 (0.06)	0.72 (0.02)	1.01 (0.05)	0.89 (0.10)
Significance	P=0.086		P=0.762	

Figure 3.323

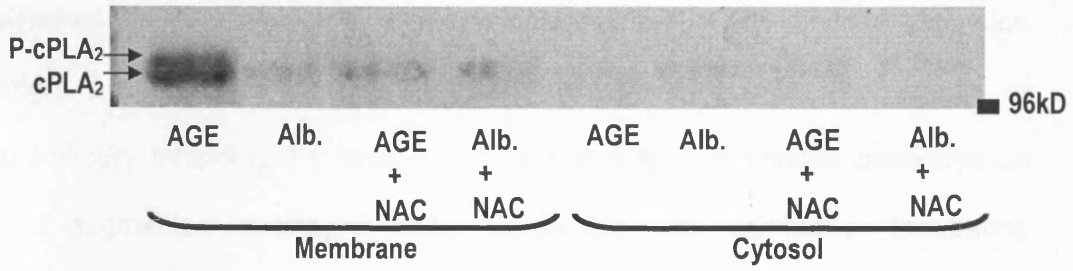


Figure 3.323 Qualitative assessment of $cPLA_2$ in subcellular neutrophil fractions. Neutrophils exposed to AGE-albumin (AGE) and albumin (Alb) with or without N-acetylcysteine (NAC), were fractionated into membrane and cytosol fractions. $cPLA_2$ was detected by Western blotting. P-$cPLA_2$ represents phosphorylated $cPLA_2$, which has reduced electrophoretic mobility and is gel-shifted. The 96kD marker is shown. The immunoblot shown is representative of 5 experiments.

Arachidonic acid mimics AGE-induced augmentation of the stimulated neutrophil respiratory burst. As $cPLA_2$ inhibitors nullified AGE-induced AA release and AGE-enhanced ROS release from stimulated neutrophils, an investigation was made into neutrophil ROS production following the direct addition of AA. Over the dose range tested, neutrophil ROS release (both total and peak) in response to a secondary stimulus, was enhanced in the presence of AA at concentrations greater than or equal to 50nM (average enhancement of total ROS production by 157% with mechanical stimulus and 163% with fMLP stimulus) and this enhancement was of similar magnitude to that induced by AGE-Albumin itself (average enhancement of total ROS production by 168% with mechanical stimulus and 152% with fMLP stimulus) [Figs. 3.324A and B] although direct addition of AA to unstimulated neutrophils at the concentrations evaluated, did not result in ROS release (data not shown).

Arachidonic acid and not one of its metabolites, is responsible for augmenting the stimulated neutrophil respiratory burst. To determine whether the facilitatory role of AA in phagocytic NADPH oxidase activation might be mediated through its downstream metabolites, the effects of specifically inhibiting the cyclo-oxygenase and lipo-oxygenase pathways on AGE-augmented neutrophil ROS production was assessed. Incubating neutrophils for 60 minutes in the presence of various doses of the cyclo-oxygenase inhibitor indomethacin[185] or the lipo-oxygenase inhibitor MK-886[188] did not attenuate the differential enhancement of ROS production caused by AGE-albumin in response to mechanical/chemical stimuli (Figs. 3.325A and

B), although at higher doses there was widespread attenuation of ROS output from both AGE-albumin and control groups in fMLP-activated cells.

Figure 3.324

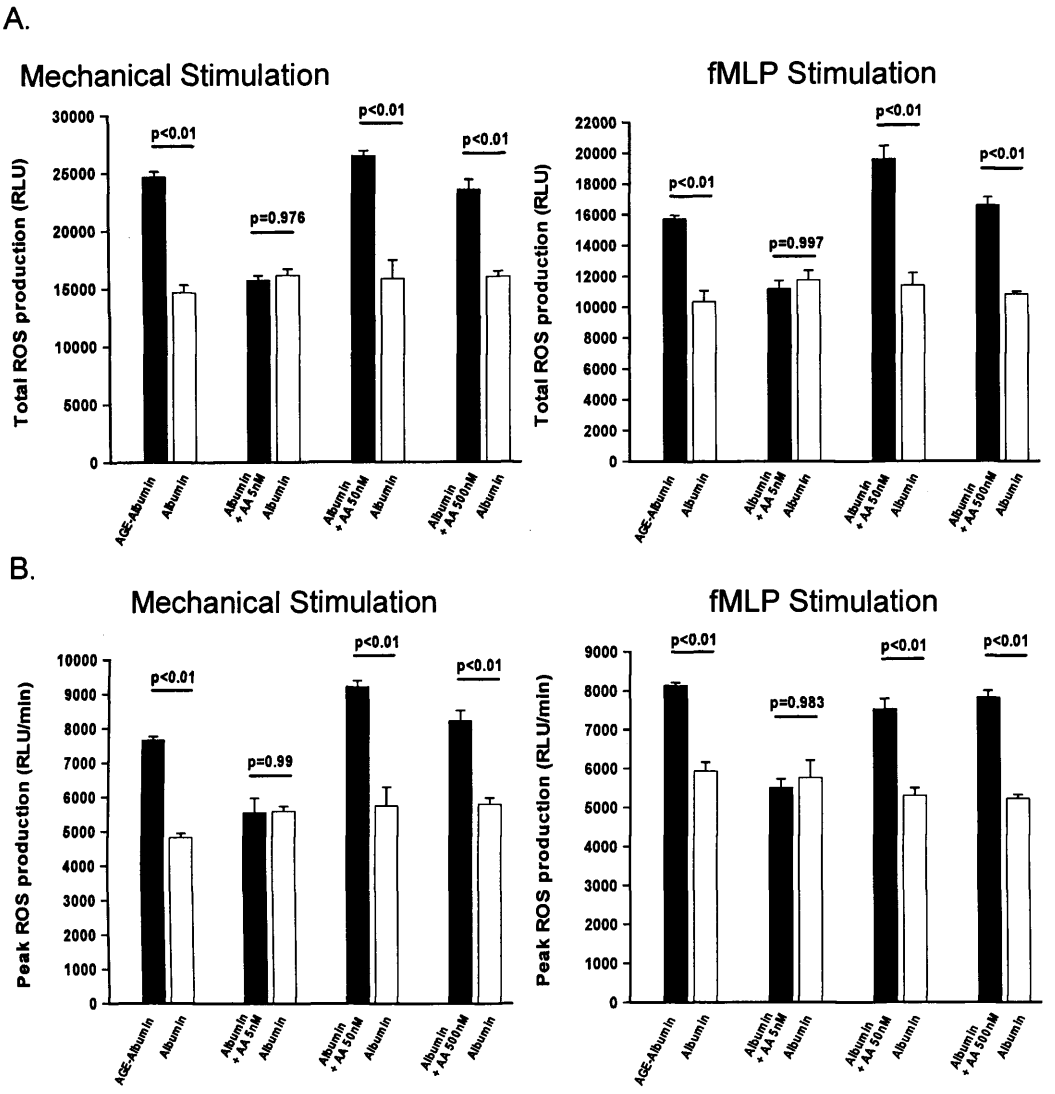
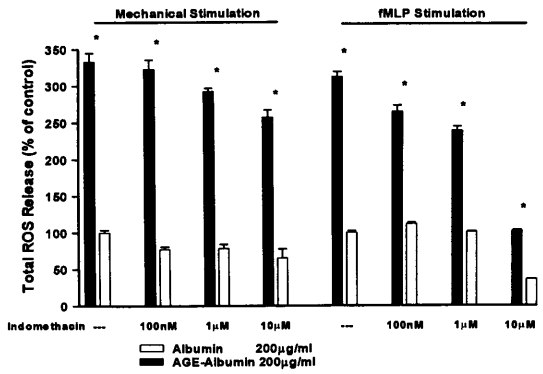


Figure 3.324 Acute neutrophil ROS production, detected by Lucigenin-enhanced chemiluminescence, in the presence of AGE-Albumin/Albumin (200µg/ml) and/or varying doses of AA, and in response to mechanical or chemical (fMLP) stimuli. (A) Total neutrophil ROS production (over 6 minutes) after stimulation in the presence of AGE-albumin (200µg/ml), albumin (200µg/ml) or albumin (200µg/ml) plus varying doses of AA ($p<0.01$ between groups by ANOVA). (B) Peak neutrophil ROS production after stimulation in the presence of AGE-albumin (200µg/ml), albumin (200µg/ml) or albumin (200µg/ml) plus varying doses of AA ($p<0.01$ between groups by ANOVA).

Each recording is the mean of 3 separate aliquots of 10^5 cells measured simultaneously and the results are representative of multiple experiments. Total ROS production measured in RLU (Relative Light Units) and Peak ROS production measured in RLU/min.

Figure 3.325

A.



B.

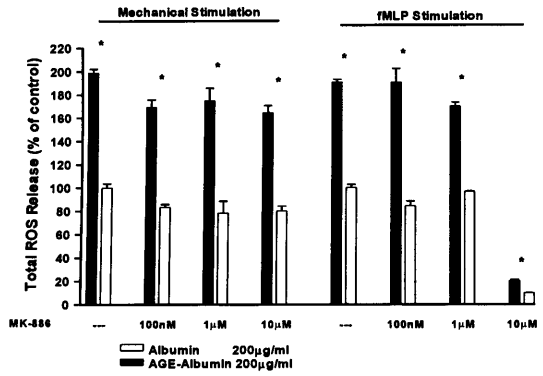


Figure 3.325 Acute neutrophil ROS production, detected by Lucigenin-enhanced chemiluminescence, in the presence of AGE-albumin/albumin (200μg/ml) and/or varying doses of Indomethacin or MK-886, and in response to mechanical or chemical (fMLP) stimuli. ROS production is expressed as a percentage of the ROS output from control cells (neutrophils exposed to albumin 200μg/ml, in the absence of inhibitor and stimulated accordingly by mechanical means or with fMLP). (A) Total neutrophil ROS production (over 6 minutes) after stimulation in the presence of AGE-albumin/albumin (200μg/ml) with or without varying doses of Indomethacin ($p<0.01$ between groups by ANOVA). (B) Total neutrophil ROS production (over 6 minutes) after stimulation in the presence of AGE-Albumin/Albumin (200μg/ml) with or without varying doses of MK-886 ($p<0.01$ between groups by ANOVA).

Each recording is the mean of 3 separate aliquots of 10^5 cells measured simultaneously and the results are representative of multiple experiments.

*
$p<0.01$ by post-hoc Tukey's analysis of ANOVA.

3.33 DISCUSSION

The ability of AGEs to augment the activated neutrophil respiratory burst has already been demonstrated and has been shown to be not simply due to AGE-autofluorescence.[182] It has now been shown that this effect involves the activity of the enzyme $cPLA_2$, which is activated by prior phosphorylation and translocation of the phospho-enzyme from the cytosol to the plasma membrane. Both the phosphorylation and intracellular translocation of $cPLA_2$ were shown clearly on Western blotting, and the importance of $cPLA_2$ was confirmed by the inhibiting effect of two different $cPLA_2$ inhibitors on the AGE-augmentation of the neutrophil respiratory burst.

At the plasma membrane, phospholipids are hydrolysed by $cPLA_2$, yielding AA. It is the latter which possesses similar characteristics to AGEs in terms of enhancing neutrophil NADPH oxidase activity and is hence likely to be responsible for mediating the effect of AGEs. Moreover, the absence of any interference with this process by inhibitors of cyclo-oxygenase and lipo-oxygenase, suggest that AA per se and not one of its metabolites, is responsible for this process.

Furthermore, the direct production of AA in response to AGE-stimulation of neutrophils was demonstrated using radiolabeled techniques and such production was also abrogated by $cPLA_2$ inhibition. It was also diminished by the intracellular antioxidant NAC, explaining the ability of NAC to attenuate the action of AGEs on the neutrophil NADPH oxidase. This sequence of events hints at a

role for redox activation further upstream of $cPLA_2$ activation and indicates the presence of a multi-step signalling pathway that is required to mediate the effects of AGE on the neutrophil respiratory burst.

3.4 An exploration into additional signalling and signal-transduction mechanisms mediating the AGE-augmented neutrophil respiratory burst

3.41 INTRODUCTION

The evidence for involvement of cytosolic phospholipase A_2 ($cPLA_2$), arachidonic acid and the intracellular redox potential in the mediation of AGE co-agonist activity has been explored, but the involvement of other systems in the signalling cascade is still unknown. In particular, information is lacking on the upstream activators of $cPLA_2$ itself, and the mode of AGE-ligation employed by neutrophils in this process.

Although co-agonism as a concept differs from 'priming', both involve upregulation of the neutrophil response and it is possible that they may have one or more pathways in common with each other. It is also possible that co-agonism shares common pathways with established agonists of the neutrophil NADPH oxidase.

The downstream signaling pathways of fMLP and phorbol esters, the two main agonists employed in the current study, are summarised in Figure 3.411. Pathways which have been definitively or putatively linked to the phosphorylation and activation of $cPLA_2$, phosphorylation and/or activation of NADPH oxidase subunits and enhancements of aspects of neutrophil function (chemotaxis, adhesion, cytoskeletal activation) have been emphasised.

For most of these enzymes and messengers, evidence already exists for their involvement in cellular activation and/or priming processes:

1) **Protein Kinase C (PKC):** PKC is a key intracellular enzyme involved in the activation of the neutrophil NADPH oxidase enzyme. Its direct activation by phorbol esters, (which function as analogues of the native PKC activator, diacylglyerol [DAG]) results in the initiation of the neutrophil respiratory burst.[189] Additionally, some authors have linked the upregulation of PKC to priming mechanisms.[190] Notable substrates of PKC action include the $p47^{phox}$ subunit of the NADPH oxidase and p42/44 Mitogen Activated Protein Kinase (MAPK), making them potential candidates for mediating any PKC-driven effect.

2) **Phosphatidyl-inositol-3-kinase (PI3K):** Clear evidence already exists for the role of PI3K in facilitating priming responses from neutrophils with the effects of the classical priming agent, TNF-α, being mediated by this intracellular enzyme.[149] In particular, PI3K plays a pivotal role in G-protein-linked receptor signalling cascades, such as those involving the fMLP-receptor. PI3K activity results in the accumulation of PIP_3 and subsequent adhesion, proliferation, cytoskeletal and activation responses, which also require the cooperation of a number of other downstream kinases.

3) **Protein tyrosine kinases (PTKs):** Much attention has focused on the potential roles of protein tyrosine kinases in enabling priming responses in

neutrophils. These signalling systems are often receptor-mediated, which make them favourable candidates to mediate any AGE-receptor signal transduction. They have been shown to facilitate PI3K activation and the ensuing phosphatidyl-inositol-triphosphate (PIP_3) production can also positively feedback onto tyrosine kinases, resulting in further PIP_3 production. It has also been observed that tyrosine phosphorylation of intracellular proteins occurs following cellular exposure to a number of classical priming agents such as fMLP, PAF, complement, GM-CSF and TNF-α.[150]

4) **Phospholipase D (PLD):** PLD is a key enzyme that hydrolyses membrane phospholipids yielding phosphatidic acid (PA). The latter is capable of interacting with many protein kinases, some of which have been implicated in intracellular signalling and in particular, priming mechanisms.[191,192] PA is additionally a substrate for the formation of DAG,[193] which in turn may activate PKC[194] and link into other potential priming mechanisms.

5) **Mitogen Activated Protein Kinases (MAPKs):** The MAPK enzymes play a central role in cellular responses by various stress stimuli such as cell proliferation, apoptosis, migration, or gene expression,[195] making them logical candidates for assessment in cell stimulatory processes, especially those resulting in an inflammatory response. The Stress Activated Protein Kinases (SAPK) represent a sub-group of the MAPK superfamily, with the stressors that normally activate these enzymes including variables such as temperature and osmotic changes.[196] It has already been discovered that the activation of

p42/44MAPK may occur consequent to ligation of the RAGE receptor by AGEs.[197] The RAGE receptor is the most characterised receptor for AGE ligands. Moreover, its activation has already been shown to lead to proliferative responses which might well encompass free radical production.

6) **Calcium:** This well-established intracellular second messenger is important for mediating neutrophil activation responses.[198] It has both an established role in PKC activation and putative role in phosphorylation of NADPH oxidase subunits. Transient elevation of intracellular calcium certainly occurs following stimulation by fMLP, which takes place via a phospholipase C (PLC)-dependent mechanism.

7) **Rho-kinase:** Rho-kinase is involved in the transduction of integrin-mediated cell responses which mediate changes in cell shape and structure that occur in physiological and pathological states.[199] Such changes involve cytoskeletal regulation and may influence such activities as phagocytosis and the respiratory burst. As neutrophil adhesion to the substratum may involve integrin ligation and activation,[200] any activator of neutrophil function may potentially entail interplay with Rho-kinase.

8) **NADPH oxidase subunit translocation:** A non-enzymatic mechanism of upregulating neutrophil respiratory function, which has already been mentioned in the context of priming with lipopolysaccharide, is to preferentially upregulate NADPH oxidase assembly in the target cells by means of

increasing the cytosol to membrane translocation of $p47^{phox}$ so that activation of the cell takes place on a semi-assembled enzyme complex.[152]

Using such established processes as a basis for investigation, the current studies have explored additional candidate pathways that might be involved in the co-agonist action of AGEs.

Figure 3.411

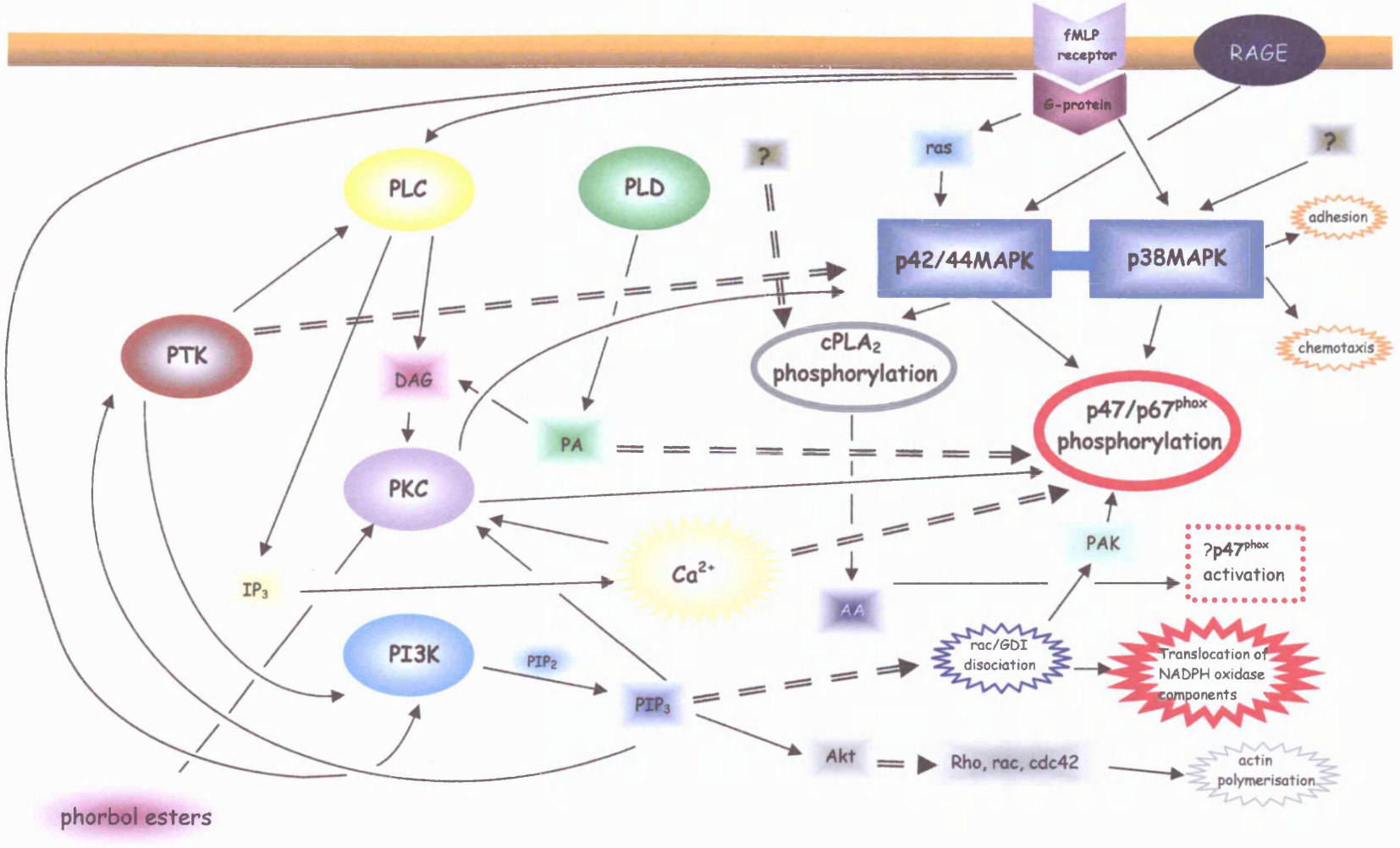
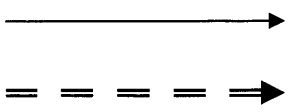

fMLP receptor
RAGE
G-protein
ras
?
?
PLC
PLD
p42/44MAPK
p38MAPK
adhesion
chemotaxis
PTK
DAG
cPLA2 phosphorylation
PA
p47/p67phox phosphorylation
PKC
PAK
?p47phox activation
IP3
Ca2+
AA
PI3K
PIP2
rac/GDI disociation
Translocation of NADPH oxidase components
PIP3
Akt
Rho, rac, cdc42
actin polymerisation
phorbol esters

Figure 3.411 - An overview of cellular signalling pathways with roles in neutrophil NADPH oxidase activation and upregulation of aspects of neutrophil function.



Abbreviations: AA, arachidonic acid; **Akt**, a serine threonine protein kinase homologue of the viral oncogene v-akt; **cPLA$_2$**, cytosolic phospholipase A$_2$; **DAG**, diacylglycerol; **fMLP**, formyl-methionyl-leucyl-phenylalanine; **GDI**, GDP-dissociation inhibitor; **IP$_3$**, inositol triphosphate; **MAPK**, mitogen-activated protein kinase; **p47/p67^{phox}** – NADPH oxidase subunits; **PA**, phosphatidic acid; **PAK**, p21-activated kinase; **PI3K**, phosphatidyl-inositol-3-kinase; **PIP$_2$**, phosphatidyl-inositol-diphosphate; **PIP$_3$**, phosphatidyl-inositol-triphosphate; **PKC**, protein kinase C; **PLC**, phospholipase C; **PLD**, phospholipase D; **PTK**, protein tyrosine kinase; **RAGE**, receptor for AGE; **ras**, a monomeric GTPase; **rho, rac, cdc42** – small GTP-binding proteins of the *rho*-family.

3.42 RESULTS

Inhibition of Protein Kinase C (PKC) attenuates overall ROS production by neutrophils but has no effect on AGE-induced augmentation of the neutrophil respiratory burst. Two general PKC inhibitors (chelerythine and Ro-31-8220), active against the various isoforms of the enzyme, were pre-incubated with neutrophils for varying lengths of time and at a variety of inhibitor concentrations before the addition of AGE/HSA (200μg/ml) and subsequent activation by mechanical stimulation or with the bacterial cell wall peptide, fMLP, with the cumulative ROS production over the first 6 minutes post-stimulation being calculated.

The optimum pre-incubation time with chelerythine was 30 minutes, with longer pre-incubation times tending to result in greatly diminished responses. The results of a 30 minute pre-incubation with chelerythine on stimulated ROS production in neutrophils are summarised graphically in Figure 3.421. Higher concentrations of chelerythine (10μM) resulted in a similar gross attenuation of response even with brief pre-incubation times. Lower concentrations (1μM and 100nM) led to a general reduction in ROS production, though this appeared to be more likely following mechanically-stimulated ROS production than after cell stimulation by fMLP.

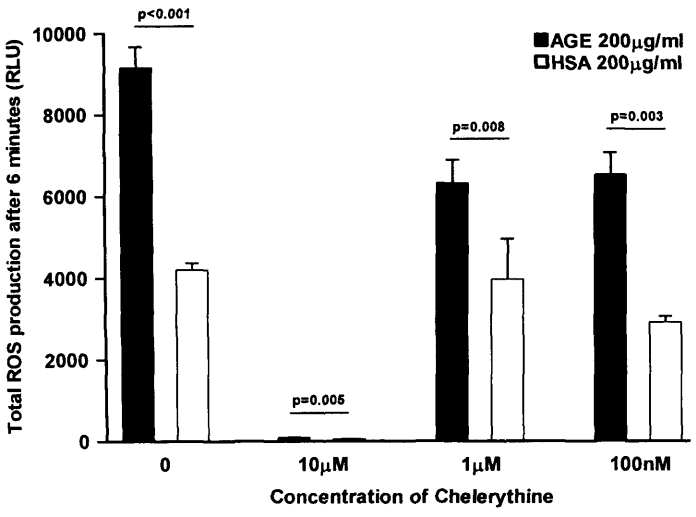
The PKC inhibitor Ro-31-8220 was examined for confirmation of these results, and was also noted to abolish almost any response when pre-incubated for longer than 30 minutes or when the higher dose (10μM) was used (Fig. 3.422).

Lower concentrations caused a general reduction in ROS production (1μM proving more effective than 100nM). Nonetheless, there remained a superiority of ROS production in the AGE-exposed neutrophils and this was true following both mechanical and fMLP-stimulation (Figure 3.422).

The neutrophil respiratory burst is dependent upon Phosphatidylinositol-3 Kinase (PI3K), though its inhibition does not attenuate the AGE-induced augmentation of the neutrophil respiratory burst. The potent PI3K inhibitor, wortmannin, was used to assess the role of PI3K in AGE-mediated responses. As with the PKC inhibitors, pre-incubation intervals exceeding 30 minutes also resulted in meagre ROS generation and the results shown (Figure 3.423) have been derived from 30 minute incubations with a range of wortmannin concentrations. After pre-incubation with wortmannin, AGE or HSA (200μg/ml) were added to the neutrophils and the cells were subsequently activated by mechanical stimulation or chemically, with fMLP. The outcome measured was the total ROS production over 6 minutes. Both 100nM and 50nM concentrations of wortmannin were still highly potent in suppressing any response after 30 minutes of pre-incubation, with even the lower dose of 10nM significantly compromising the neutrophil ROS burst (Figure 3.423). Nonetheless, a relatively greater ROS output was maintained in the AGE-treated cells and this was true whether cellular stimulation was by mechanical or chemical means (Figure 3.423).

Figure 3.421

A.



B.

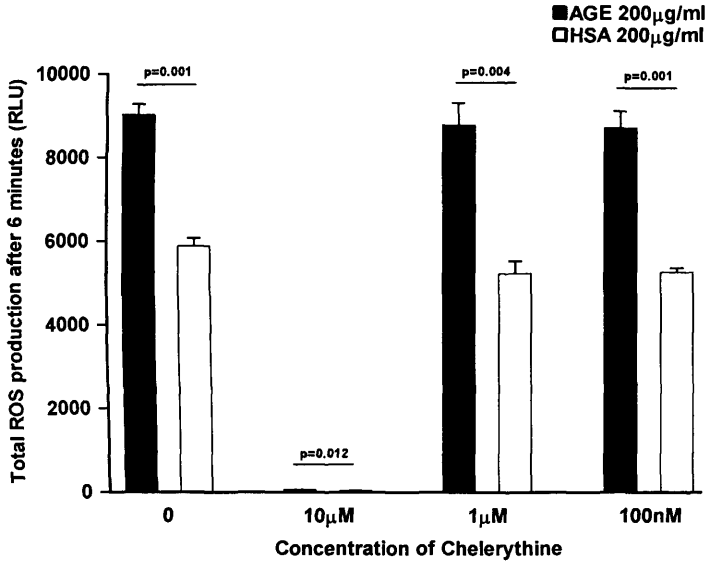
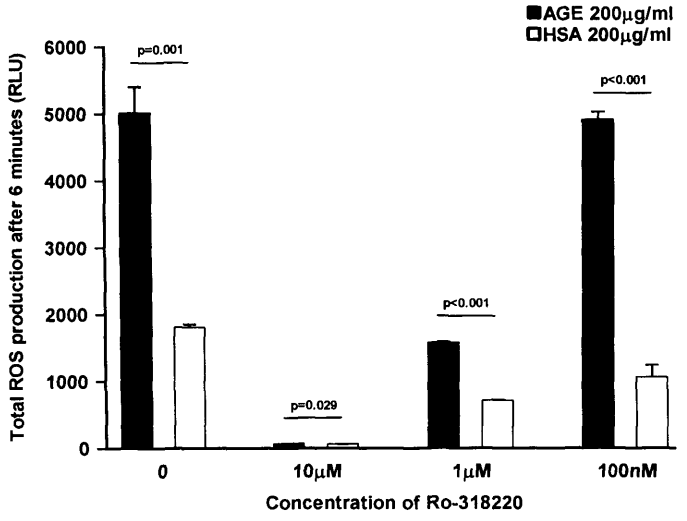


Figure 3.421 The effect of the protein kinase C inhibitor (Chelerythine) on the ROS burst from AGE-exposed and control neutrophils. (A) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with chelerythine at a range of concentrations. (B) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with chelerythine at a range of concentrations.

ROS output is expressed as the cumulative Relative Light Units (RLUs) over 6 minutes, as detected by Lucigenin-enhanced chemiluminescence. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

Figure 3.422

A.



B.

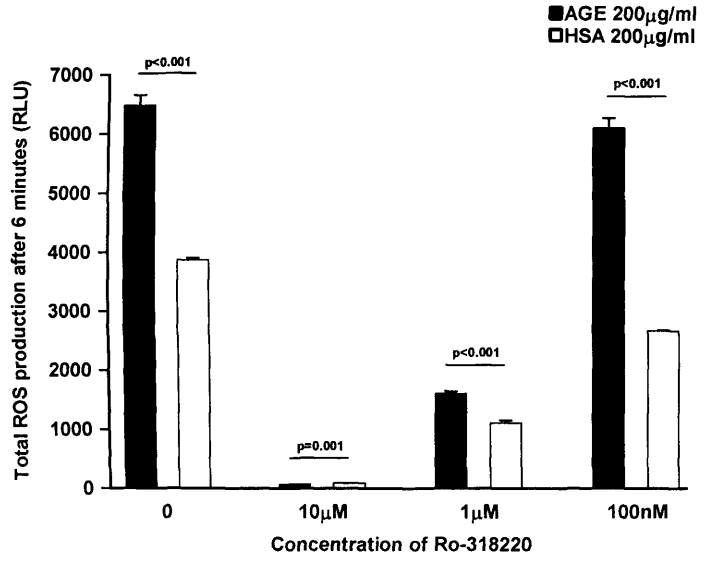
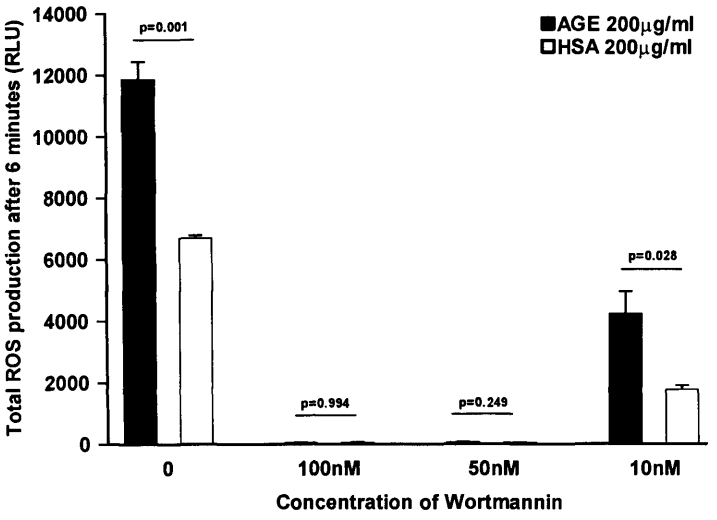


Figure 3.422 The effect of the protein kinase C inhibitor (Ro-31-8220) on stimulated neutrophil ROS production from AGE and HSA-exposed cells. (A) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with Ro-31-8220 at a range of concentrations. (B) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with Ro-31-8220 at a range of concentrations.

ROS output is expressed as the cumulative Relative Light Units (RLUs) over 6 minutes, as detected by Lucigenin-enhanced chemiluminescence. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

Figure 3.423

A.



B.

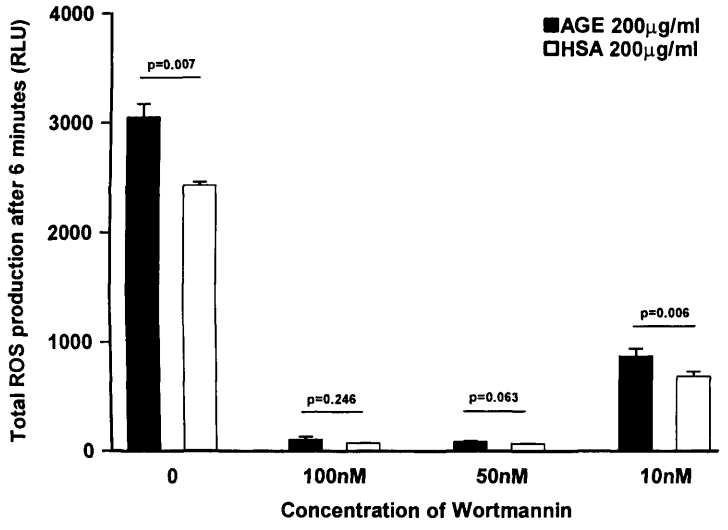


Figure 3.423 The effect of the PI3 Kinase inhibitor, Wortmannin, on the neutrophil ROS burst and the AGE-augmented neutrophil ROS burst. (A) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with wortmannin at varying doses. (B) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with wortmannin at varying doses.

ROS output is expressed as the cumulative Relative Light Units (RLUs) over 6 minutes, as detected by Lucigenin-enhanced chemiluminescence. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

Protein tyrosine kinases do not play a significant role in mediating AGE co-agonist stimulation of the neutrophil respiratory burst. Three different tyrosine kinase inhibitors were used to test for the involvement of tyrosine phosphorylation in mediating the AGE co-agonist stimulation of the neutrophil respiratory burst. Two general tyrosine kinase inhibitors, genistein[201] and the synthetic inhibitor tyrphostin A25,[202] were each incubated with neutrophils for varying lengths of time and at varying concentrations before the addition of AGE or HSA (200μg/ml) and subsequent activation by mechanical means or with fMLP. The outcome was the cumulative ROS production over 6 minutes, as measured by chemiluminescence. Incubation with either inhibitor for more than 15 minutes resulted in greatly diminished cellular response, in terms of ROS production. This was also evident with higher doses of genistein and shorter pre-incubation periods. Lower doses of genistein (10μM and 1μM) resulted in an overall diminution of ROS production from both the AGE and control (HSA) groups, although the superiority of ROS production from the AGE-exposed cells upon stimulation by either mechanical or chemical methods was maintained (Figure 3.424).

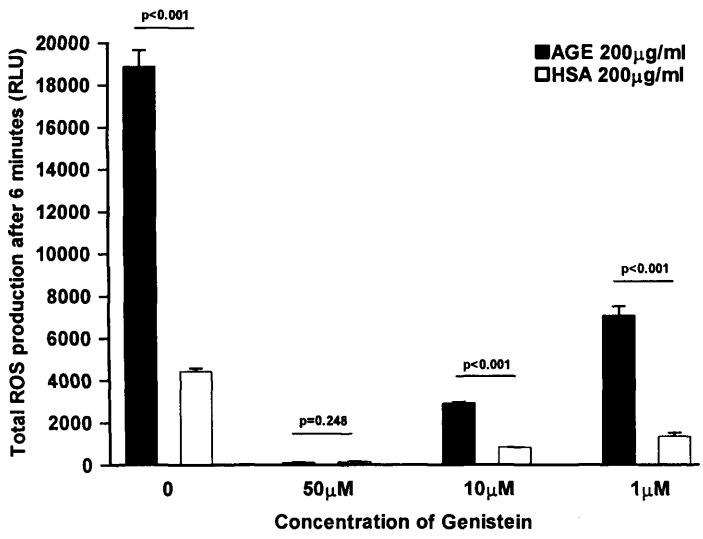
Almost identical pictures were seen with the synthetic tyrosine kinase inhibitor tyrphostin (Figure 3.425) and with herbimycin (Figure 3.426). The latter has a greater predilection for the inhibition of src-type non-receptor-linked tyrosine kinases.[203] It was incapable of attenuating the AGE-induced augmentation of the neutrophil respiratory burst, except at higher concentrations whereupon ROS generation was generally suppressed in both the AGE and control cells (Figure

3.426). An apparent increase in ROS production from herbimycin-treated neutrophils that were activated by AGEs and mechanical stimulation was not statistically significant (Figure 3.426A).

An alternative investigation into the prospective role of protein tyrosine phosphorylation in the mediation of AGE co-agonist activity was made through the examination of intracellular protein tyrosine phosphorylation events directly after cells had been incubated in the presence of AGE compared to HSA. Neutrophils were extracted following the usual protocol and then allowed to settle in a rotating incubator at 37°C for 30 minutes to allow any stimulatory influences from the extraction process to subside. The neutrophils were then suspended in a solution of AGE or HSA (at 200μg/ml) and after 1 minute, the cells were 'flash-frozen' in liquid nitrogen. Cellular lysates from these two sets of neutrophils were run on SDS-PAGE gels and probed with an anti-phosphotyrosine antibody (anti-PY20) with an anti-goat secondary antibody. Although the presence of multiple bands was observed in both specimens, most notably at molecular weights 30, 38, 44 and 70 kDa (Figure 3.427A), there was no significant difference in the density of such bands between the two samples when analysed formally with densitometry (Figure 3.427B).

Figure 3.424

A.



B.

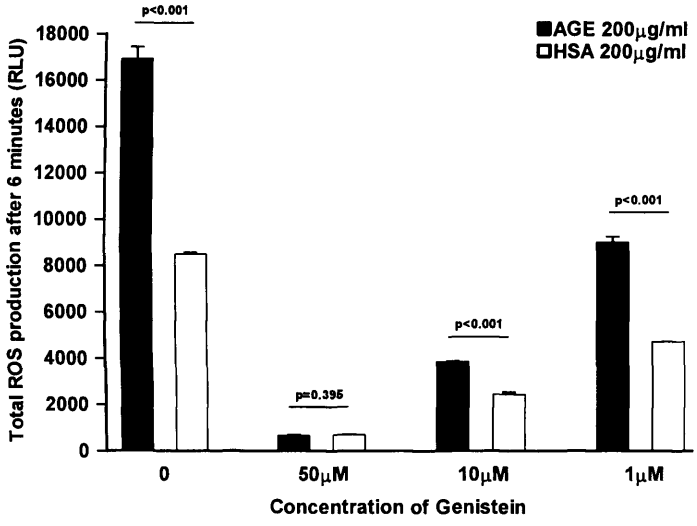
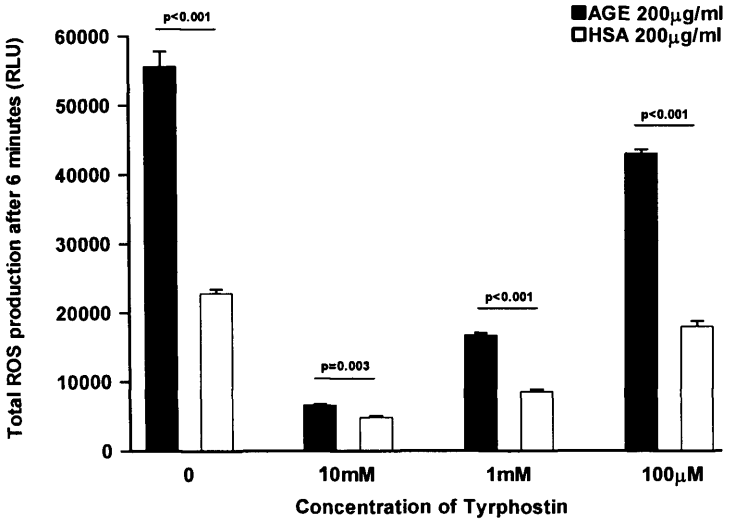


Figure 3.424 The effect of the Tyrosine Kinase inhibitor, Genistein, on the neutrophil ROS burst and the AGE-augmented neutrophil ROS burst. (A) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with genistein (for 15 minutes) at a range of concentrations. (B) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with genistein (for 15 minutes) at a range of concentrations.

ROS output is expressed as the cumulative Relative Light Units (RLUs) over 6 minutes, as detected by Lucigenin-enhanced chemiluminescence. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

Figure 3.425

A.



B.

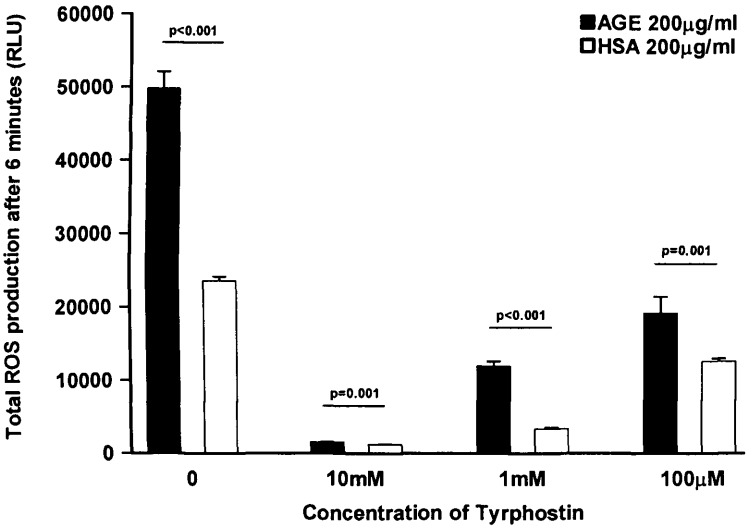
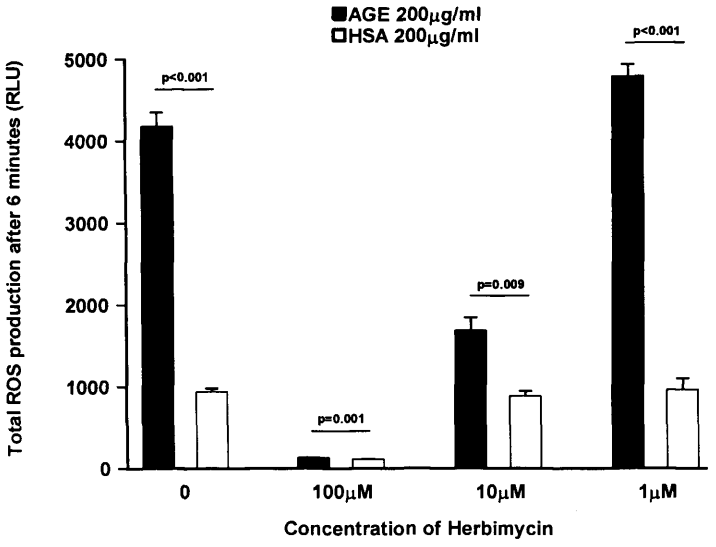


Figure 3.425 The effect of the Tyrosine Kinase inhibitor, Tyrphostin, on the neutrophil ROS burst and the AGE-augmented neutrophil ROS burst. (A) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with tyrphostin (for 15 minutes) at a range of concentrations. (B) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with tyrphostin (for 15 minutes) at a range of concentrations.

ROS output is expressed as the cumulative Relative Light Units (RLUs) over 6 minutes, as detected by Lucigenin-enhanced chemiluminescence. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

Figure 3.426

A.



B.

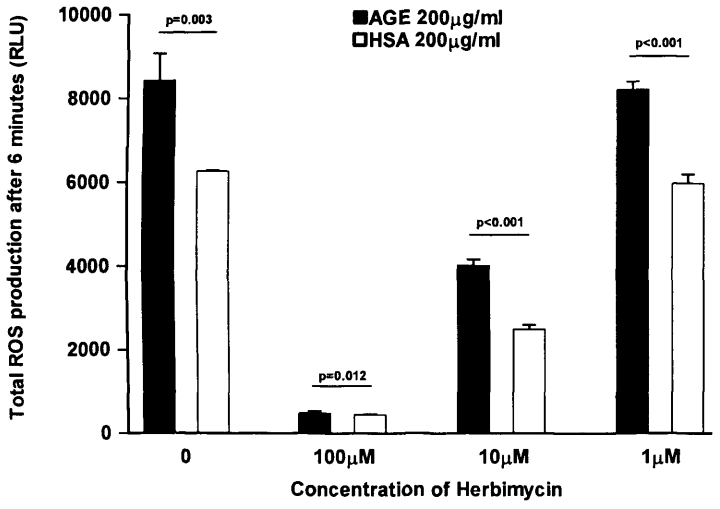
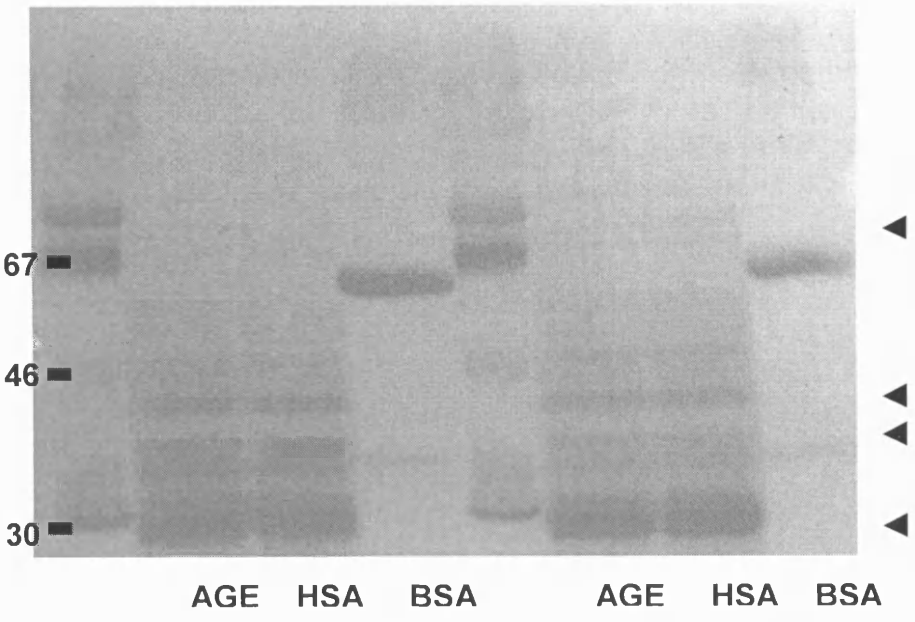


Figure 3.426 The effect of the Tyrosine Kinase inhibitor, Herbimycin, on the neutrophil ROS burst and the AGE-augmented neutrophil ROS burst. (A) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200μg/ml) and pre-incubated with herbimycin (for 15 minutes) at a range of concentrations. (B) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200μg/ml) and pre-incubated with herbimycin (for 15 minutes) at a range of concentrations.

ROS output is expressed as the cumulative Relative Light Units (RLUs) over 6 minutes, as detected by Lucigenin-enahnced chemiluminescence. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

Figure 3.427

A.



B.

MW (kDa)	AGE	HSA	p value
30	1.532 (0.016)	1.565 (0.028)	0.33
38	0.895 (0.020)	0.889 (0.011)	0.85
44	1.024 (0.008)	1.014 (0.014)	0.83
70	0.642 (0.019)	0.625 (0.015)	0.76

Figure 3.427 (A) Neutrophil lysates after having been suspended in a solution of AGE/HSA at 200µg/ml. Lysates were run onto SDS-PAGE gels and probed with anti-phosphotyrosine antibody (Anti-PY20). Arrowheads denote bands subject to subsequent densitometric comparison. (B) Densitometric analysis of the

highlighted bands, with given molecular weights (MW) in kDa (from Figure 3.427A) when neutrophil lysates from neutrophils exposed to AGE/HSA were probed with an anti-phosphotyrosine antibody. Measurements are in arbitrary units of optical density (± SEM in parentheses).

Figure 3.427A is representative of multiple repetitions of the experiment and the results in Figure 3.427B are the mean of 4 blots with the same cell lysates. The significance between bands from cells incubated with AGE or HSA is denoted by the p value.

Phospholipase D is not a mediator of AGE co-agonist stimulation of neutrophil ROS generation. The effect of manipulating PLD was examined through its inhibition by the alcohol butan-1-ol and an assessment of its effects on stimulated neutrophil ROS production. As a control, equivalent strength alcohol butan-2-ol was used in a parallel arm of the experiment. Long incubation times with butanol adversely affected cellular responses and a representation from a 10 minute pre-incubation is shown (Figure 3.428). The effect was a diminution of ROS output from both active and control cells, with a greater predilection for inhibiting the peak ROS output following fMLP stimulation (data not shown) although the AGE-specific augmentation in ROS production was maintained. Greater strengths than 0.03% alcohol caused a general degrading of ROS production to a degree that it was not possible to discern much output at all.

MAP kinases are not involved in mediating the enhancing effect of AGEs on the neutrophil respiratory burst. The potential role of MAPKs in mediating the effects of AGEs on neutrophil ROS production was first examined through inhibition of p42/44MAPK activity with PD 98059. Following a 60 minute pre-incubation of the cells with inhibitor, there was minimal impact on ROS production generally, even at high inhibitor concentrations (Figures 3.429A and B), and the differential stimulated ROS output from AGE compared to HSA exposed neutrophils, was maintained at all concentrations of inhibitor used and for both methods of cellular stimulation (Figures 3.429A and B). Furthermore, when neutrophil lysates were run onto SDS-PAGE gels and probed with

antibodies to p44/p42MAPK, there was no increase in the quantity of phospho-MAPK in the lysates of AGE-exposed compared to HSA-exposed neutrophils (Figures 3.429C and D).

The presence of AGEs may be considered to exert a local osmotic effect on cells. However, inhibiting the activation of SAPK2 (alternatively known as p38MAPK) with the specific inhibitor SB203580, did not attenuate any augmenting effect of AGEs on neutrophil ROS production (Figure 3.4210). The optimum pre-incubation time for this inhibitor was 30 minutes and whilst higher concentrations did reduce ROS production from cells exposed to AGE and HSA to similar levels, this was only because ROS output was almost totally abolished in both mechanically and fMLP-stimulated cells. In fact, at non-inhibiting concentrations of SB203580, there was a slight potentiation of ROS production from both active and control cells, with maintenance of the AGE-augmented difference between the two samples.

Neutrophils do not exhibit differential $p47^{phox}$ intracellular traffic in response to AGE-exposure. Neutrophils which had been incubated in the presence of AGE or albumin for 30 minutes were 'snap-frozen' in liquid nitrogen to arrest all cellular processes. After thawing, the cell pellets were washed in PBS and then subjected to high frequency sonication to disrupt the plasma membranes. After the cytosol and nuclear pellet had been spun down and separated, the cell membrane pellet was obtained by further high speed centrifugation and was then solubilised and run on an SDS-PAGE gel before

being probed with antibodies to $p47^{phox}$ (Figure 3.4211A). The quantity of $p47^{phox}$ in the membranes from the two cell conditions, as analysed by densitometry of the bands, showed no significant difference (Figure 3.4211B).

Inhibition of the AGE-augmented neutrophil respiratory burst is not attainable by exposing electropermeabilised neutrophils to antibodies directed against the intracellular domain of RAGE. The presence of the RAGE receptor has been confirmed in neutrophils (Figure 3.4212) and the role of the AGE/RAGE interaction in facilitating upregulated ROS output from stimulated neutrophils was examined. An antibody against the cytoplasmic terminal of the RAGE receptor was raised but in order to effect interference with RAGE signalling, this antibody required access to the cytoplasmic space, crossing the plasma membrane; this was attempted by subjecting the neutrophils to electroporation. Neutrophils were suspended in electroporation buffer in the presence of antibody to the RAGE receptor and for control purposes a separate neutrophil sample was also kept in the presence of denatured anti-RAGE antibody. They were then pulse electroporated and harvested before undergoing mechanical or fMLP-stimulation in the presence of AGE or HSA (both at 200μg/ml), with ROS output determined by chemiluminescence. As additional comparators, the experiment was performed without anti-RAGE antibody or electroporation, and stimulation was also performed without electroporation but in the presence of anti-RAGE antibody in order to exclude any extraneous influences from either incubation in the presence of anti-RAGE or from the

electroporation process itself. The electroporation protocol had been previously shown to be effective on neutrophils, using FITC-labelled IgG to assess uptake of IgG into the cytoplasm. Incubation with anti-RAGE antibody alone did not have any effect on either stimulated ROS production per se or on the augmented ROS production from AGE-exposed neutrophils, and this held true for both mechanically-stimulated and fMLP-stimulated neutrophils (Figures 3.4213A and B). Unexpectedly, electroporation of neutrophils with anti-RAGE antibody did not attenuate the AGE-induced increase in ROS production with either mode of neutrophil stimulation, and electroporation in the presence of denatured antibody was likewise without significant effect (Figures 3.4213A and B). However, it was noted overall that the level of ROS production from neutrophils in the presence of AGE or albumin was significantly higher in the electroporated cells whatever the form of stimulation and whatever the type of antibody present. Although electroporation in the presence of denatured anti-RAGE antibody appeared to give slightly higher ROS production with stimulation than from electroporated cells in the presence of functional anti-RAGE antibody (Figures 3.4213A and B), this difference was not statistically significant.

The Rho-kinase inhibitor Y-27632 is unable to impair the augmenting effect of AGEs on the stimulated neutrophil respiratory burst. Attempts to reduce Rho-kinase activity were made using the specific Rho-kinase inhibitor Y-27632. Neutrophils were pre-incubated with Y-27632 for 15 minutes prior to the experiments; this was the optimum period of pre-incubation as longer times

incurred greater toxicity. When neutrophils were stimulated mechanically, the highest dose of Y-27632 used (10μM) resulted in an overall impairment of ROS production from both AGE-exposed and control neutrophils. With mechanical stimulation at the lowest concentration of inhibitor used (100nM), there was in fact a further augmentation of the ROS response from both control and active groups although this was not statistically significant. Overall, the AGE-exposed group still demonstrated a correspondingly superior response (Figure 3.4214A). Following fMLP stimulation however, although the higher concentration of Y-27632 again impaired ROS production, the lower concentration surprisingly enhanced ROS production significantly from both AGE and control cells such that the combination of AGE and Y-27632 was at least additive in terms of ROS production (Figure 3.4214B).

The results of an investigation into the manipulation of intracellular calcium on the stimulated respiratory burst from AGE-exposed neutrophils. Neutrophils were incubated in the presence of the intracellular calcium chelator, BAPTA-AM. This incubation was carried out at a variety of concentrations of BAPTA-AM and for varying lengths of time in an attempt to achieve potential inhibition of response without inducing cellular toxicity. Following a 30 minute pre-incubation, BAPTA-AM chelation of intracellular calcium stores resulted in a marked reduction of ROS production in response to both mechanical and fMLP stimulation (Figures 3.4215A and B). This occurred over a wide range of BAPTA-AM concentrations, and inhibited output from AGE and HSA-exposed neutrophils

proportionately, indicating a general sensitivity to this intracellular calcium depletion. Moreover, the kinetics of ROS production from fMLP-stimulated neutrophils were slightly altered, such that the diminished peak of ROS production also occurred with a slight time lag compared to cells stimulated in the absence of BAPTA-AM (Figure 3.4216B). In contrast BAPTA-AM did not affect the kinetics of ROS production following mechanical stimulation of neutrophils (Figure 3.4216A). Despite these effects, no impact was made on reducing the enhancements in stimulated ROS output derived from AGE-exposure (Figure 3.4216).

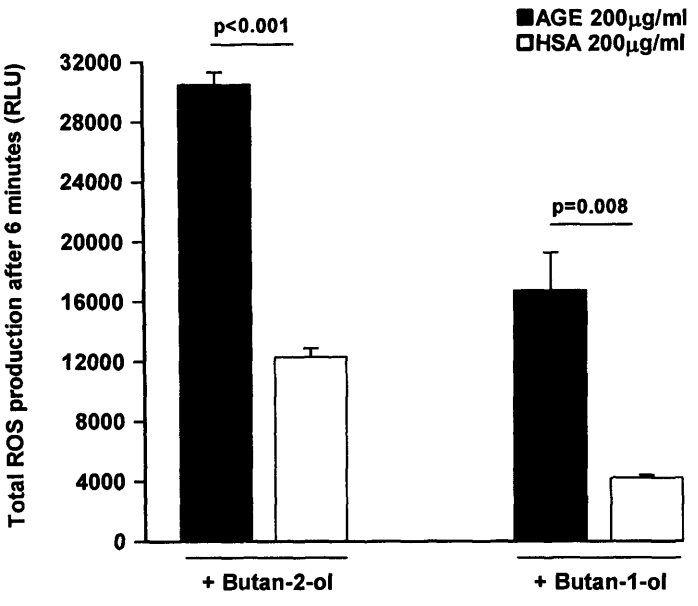
The effect of reducing intracellular calcium flux from extracellular sources was examined through the actions of EGTA, a calcium chelator which does not have access to the intracellular space. Incubation of neutrophils with EGTA also took place over a wide concentration range and for varying time periods. For demonstarion of effect, neutrophil were pre-incubated with EGTA for 30 minutes. Following mechanical stimulation there was less of a tendency for EGTA to reduce neutrophil ROS production (Figure 3.4215C) as compared to the effects of intracellular chelation with BAPTA-AM, though at the highest concentration used (10μM), the reduction in ROS generation was obviously greater. On the other hand, there was marked reduction in the generation of ROS following fMLP stimulation when neutrophils had been previously incubated with EGTA and this was present over a wide range of concentrations of EGTA (Figure 3.4215D). Unlike chelation by BAPTA-AM, EGTA did not affect the kinetics of ROS

production by neutrophils which had been stimulated either mechanically or with fMLP (Figures 3.4216C and D).

The effect of AGEs on intracellular calcium was independently assessed fluorometrically with the fluorescent calcium probe Fura2-AM. Neutrophils which had been pre-loaded with the fluorophore Fura2-AM were studied at an emission wavelength of 510nm following the addition of either AGE or HSA and excitation at both 340 and 380nm. From this a two-wavelength ratiometric analysis of intracellular calcium flux was determined in neutrophils following the addition of either AGE or HAS (200µg/ml). No significant difference was found following the addition of either substance with the influx following the addition of AGE being 133.67 ± 6.70 nM of calcium and the influx following the addition of HSA being 134.66 ± 15.31 nM of calcium (p=0.890).

Figure 3.428

A.



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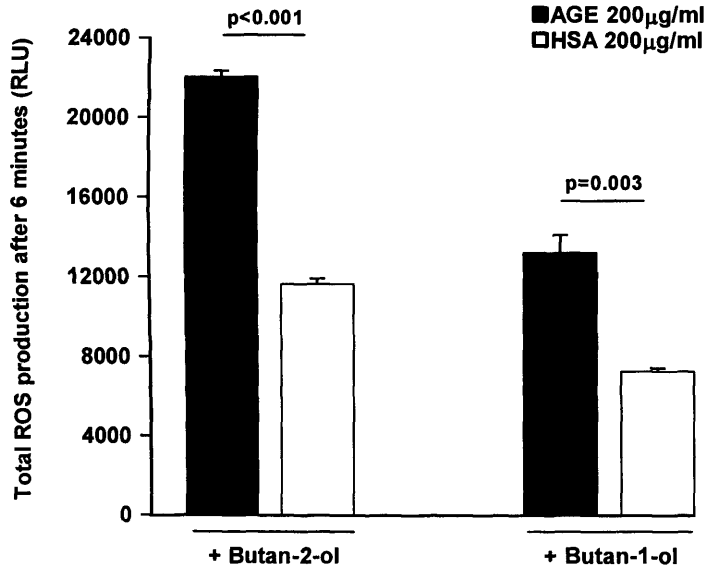
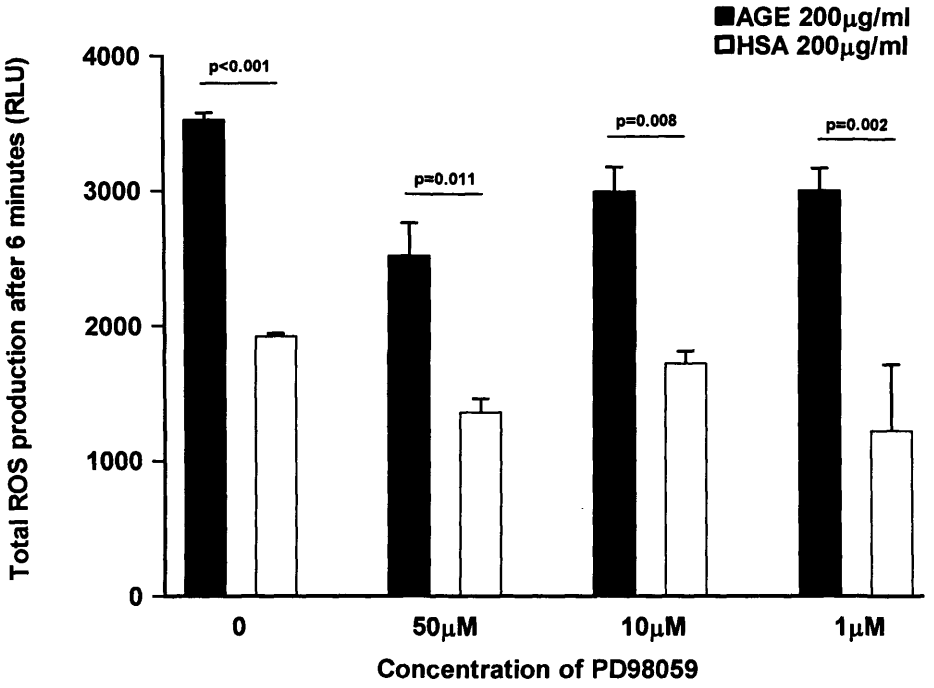


Figure 3.428 The effect of inhibiting phospholipase D on the respiratory burst from AGE and HSA-exposed neutrophils. Neutrophils were preincubated with butan-1-ol (0.3%) or control (butan-2-ol 0.3%) for 10 minutes before the addition of AGE and subsequent mechanical stimulation (A) or stimulation with fMLP (B). ROS production was detected by Lucigenin-enhanced chemiluminescence. The cumulative RLU (Relative Light Units) over the first 6 minutes post stimulation was calculated. The results shown are the means from triplicate experiments (10^5 cells per microplate well) with SEMs shown and are representative of multiple experiments carried out.

Figure 3.429

A.



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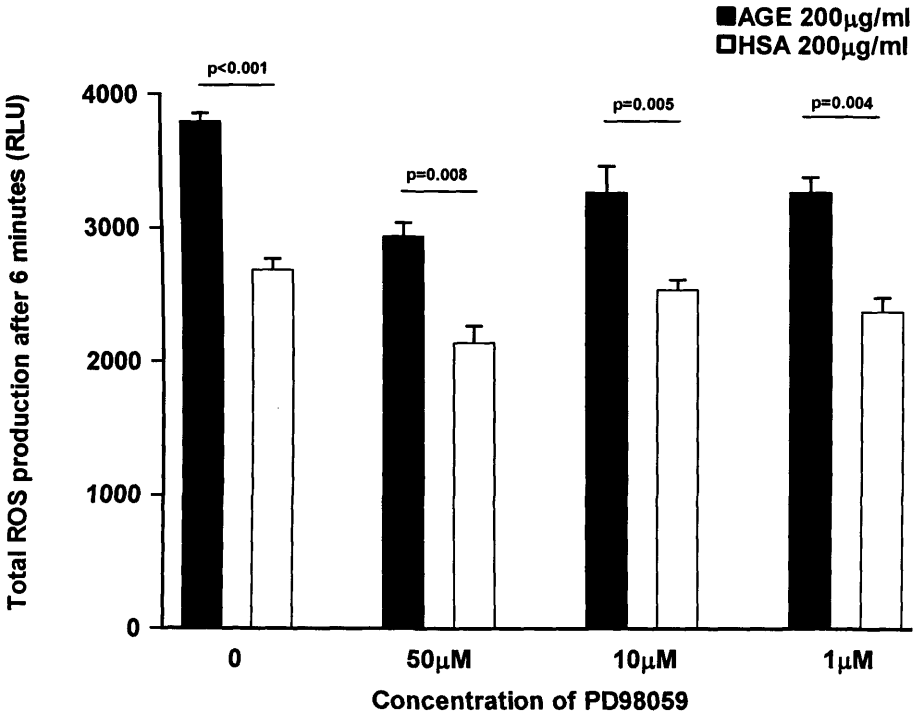
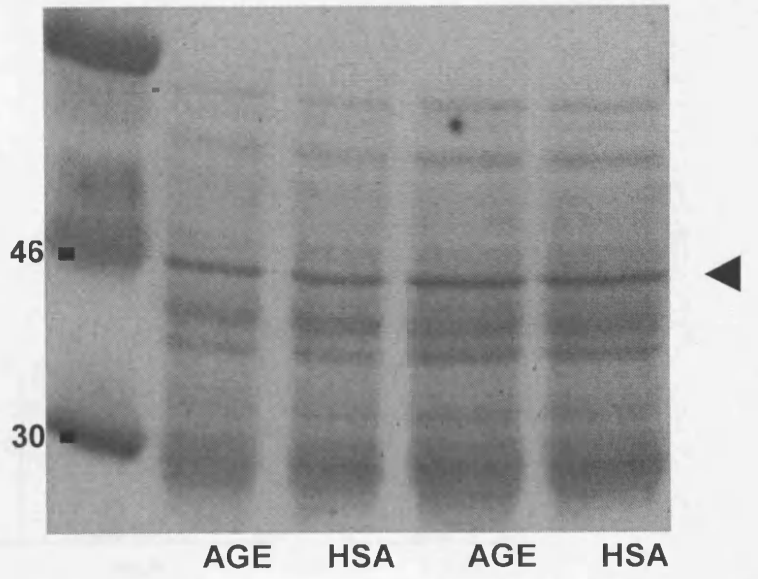


C.



D.

	AGE	HSA	p value
OD	1.513 (0.016)	1.528 (0.013)	0.596

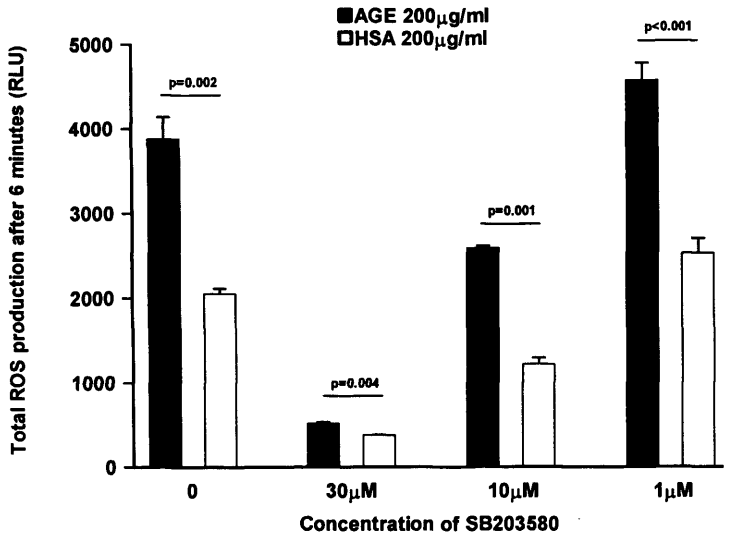
Figure 3.429 The effect of the MAPK Kinase inhibitor, PD90859, on the neutrophil ROS burst and the AGE-augmented neutrophil ROS burst. (A) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with PD 98059 at a range of concentrations. (B) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with PD 98059 at a range of concentrations.

ROS output was detected by Lucigenin-enhanced chemiluminescence and expressed as the cumulative Relative Light Units (RLUs) over 6 minutes. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

(C) Western blot of lysates from neutrophils exposed to AGE or HSA and probed with an anti-p44phosphoMAPK antibody. The relevant molecular weight marker is shown and an arrowhead denotes the band of interest. The blot is representative of multiple experiments. (D) Densitometric analysis of the phospho-p44MAPK band with the results expressed as arbitrary optical density units (± SEMs in parentheses). The results are the mean of 3 blots, all performed with the same cellular lysates and the significance is denoted by the p value.

Figure 3.4210

A.



B.

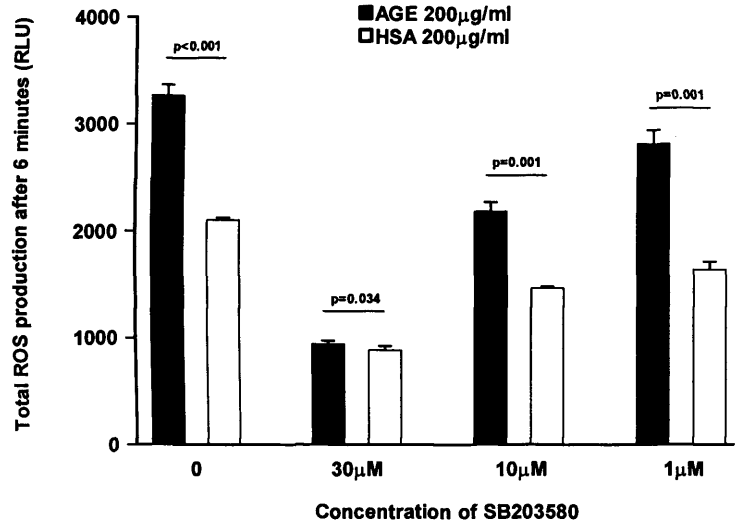
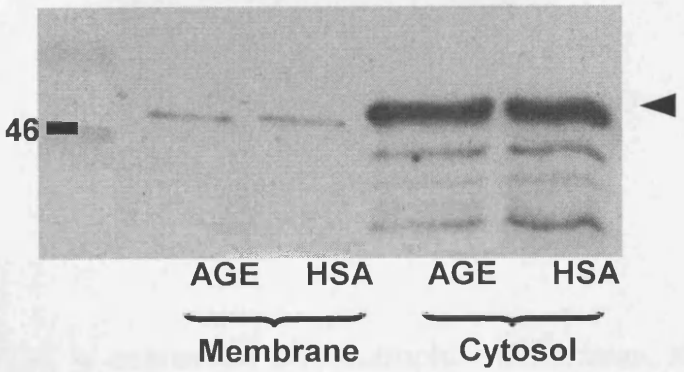


Figure 3.4210 The effect of the SAPK2/p38MAPK inhibitor, SB203580, on the neutrophil ROS burst and the AGE-augmented neutrophil ROS burst. (A) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with SB203580 at a range of concentrations. (B) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with SB203580 at a range of concentrations.

ROS output was detected by Lucigenin-enhanced chemiluminescence and expressed as the cumulative Relative Light Units (RLUs) over 6 minutes. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

Figure 3.4211

A.



B.

	AGE	HSA	p value
Cytosol	2.92 (0.05)	2.97 (0.04)	0.76
Membrane	0.55	0.605	0.14

Figure 3.4211 The presence of the $p47^{phox}$ subunit of the NADPH oxidase in cytosolic and membrane subfractions of neutrophils that have been exposed to AGE or HSA. (A) Western blot of cytosol and membrane fractions, derived from lysates of neutrophils that had been exposed to AGE/HSA 200µg/ml. The arrowhead denotes the $p47^{phox}$ band and the relevant molecular weight marker (in kDa) is shown. (B) Densitometric analysis of the $p47^{phox}$ band with optical density of the bands expressed in arbitrary units (± SEMs in parentheses). The blot is representative of multiple repetitions of the experiment and the quantitative results represent the mean of 3 experiments carried out with the same neutrophil lysate. The significance between results is denoted by the p value.

Figure 3.4212

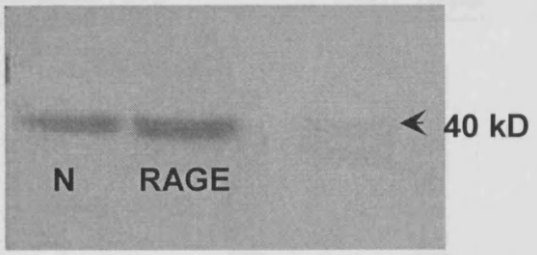
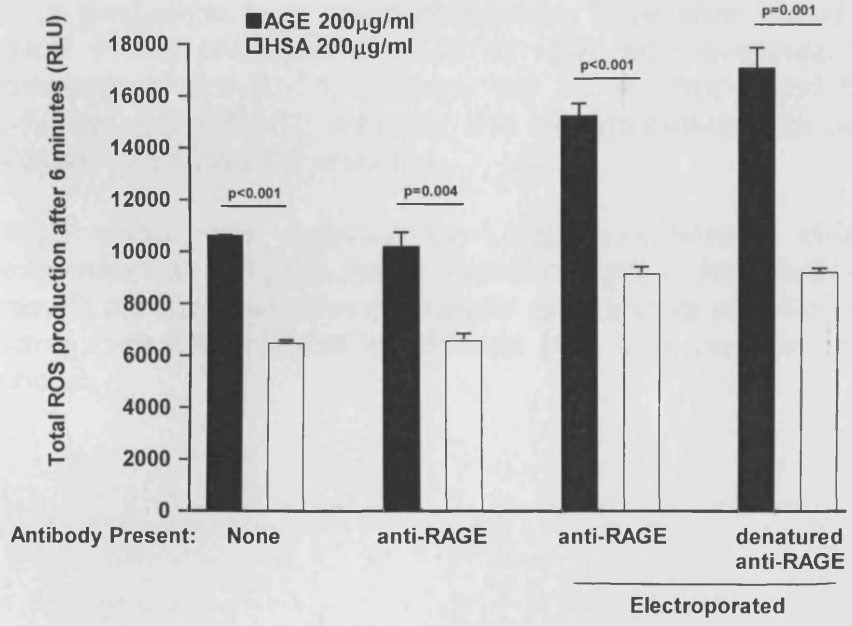


Figure 3.4212 RAGE is expressed on neutrophil membranes. Neutrophil lysate (N) was run on a polyacrylamide gel along with purified RAGE protein as a positive control. Following Western blotting, the blots were probed with anti-RAGE antibody.

Figure 3.4213

A.



B.

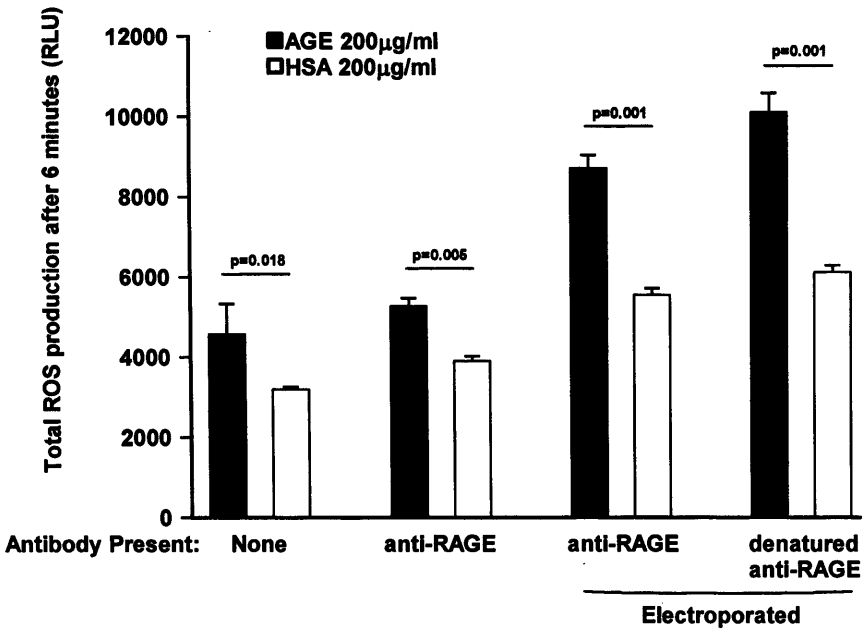
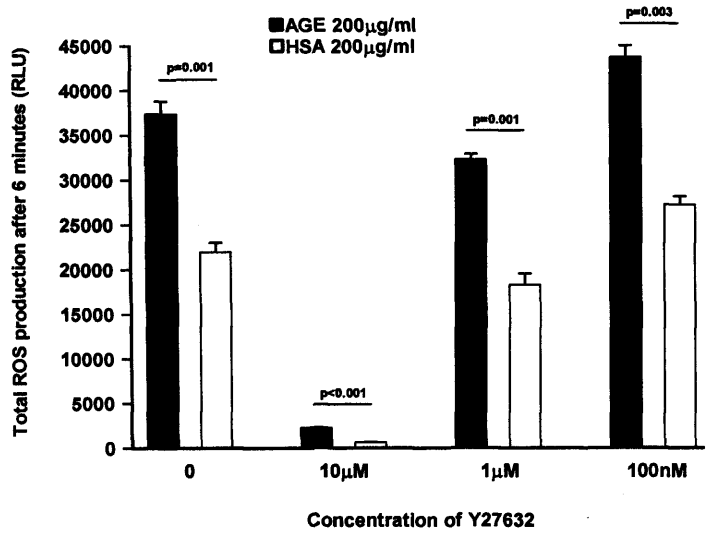


Figure 3.4213 The effect of attempting RAGE-blockade with electroporation of neutrophils and subsequent exposure to anti-RAGE antibody, on the neutrophil ROS burst and the AGE-augmented neutrophil ROS burst. (A) ROS production from neutrophils which have been mechanically stimulated in the presence of AGE or HSA are compared to stimulation in the presence of anti-RAGE antibody per se, electroporated cells with subsequent addition of anti-RAGE antibody and electroporation with subsequent addition of denatured anti-RAGE antibody. (B) ROS production from neutrophils which have been stimulated with the peptide fMLP in the presence of AGE or HSA are compared to stimulation in the presence of anti-RAGE antibody per se, electroporated cells with subsequent addition of anti-RAGE antibody and electroporation with subsequent addition of denatured anti-RAGE antibody.

ROS output was detected by Lucigenin-enhanced chemiluminescence and expressed as the cumulative Relative Light Units (RLUs) over 6 minutes. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

Figure 3.4214

A.



B.

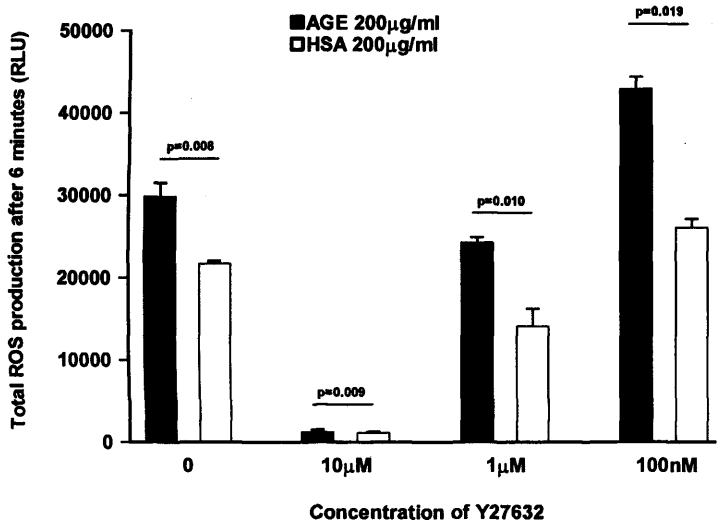
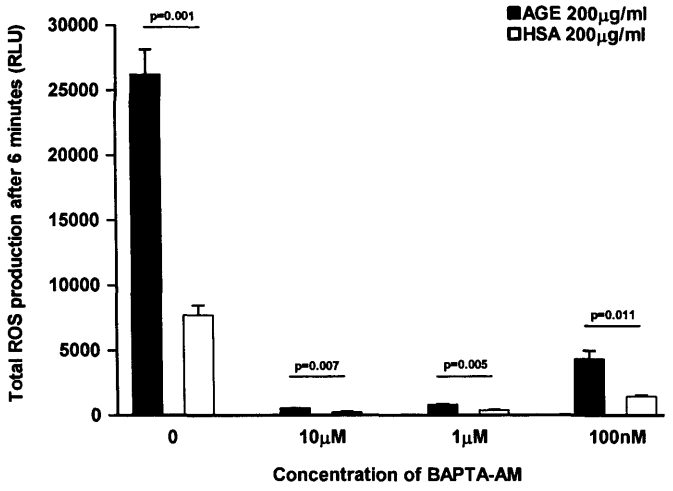


Figure 3.4214 The effect of the Rho-kinase inhibitor (Y27632) on the neutrophil ROS burst and the AGE-augmented neutrophil ROS burst. (A) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with Y27632 at a range of concentrations. (B) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with Y27632 at a range of concentrations.

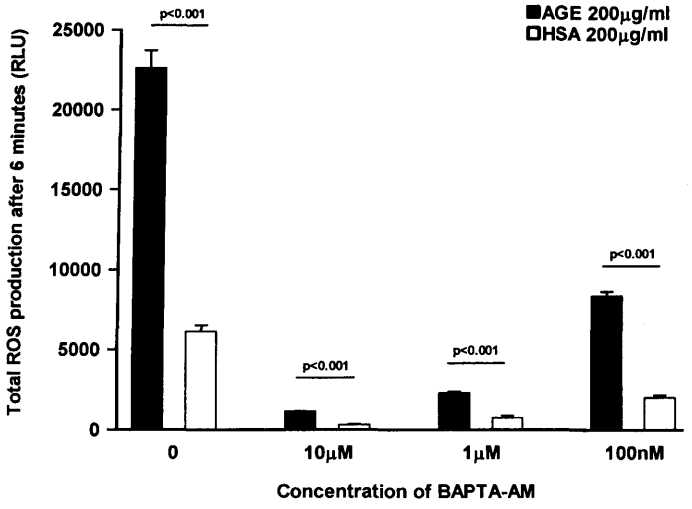
ROS output was detected by Lucigenin-enhanced chemiluminescence and expressed as the cumulative Relative Light Units (RLUs) over 6 minutes. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

Figure 3.4215

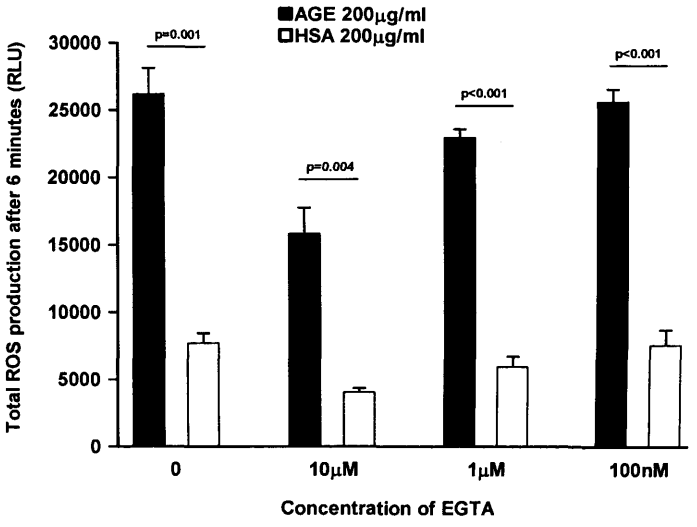
A.



B.



C.



D.

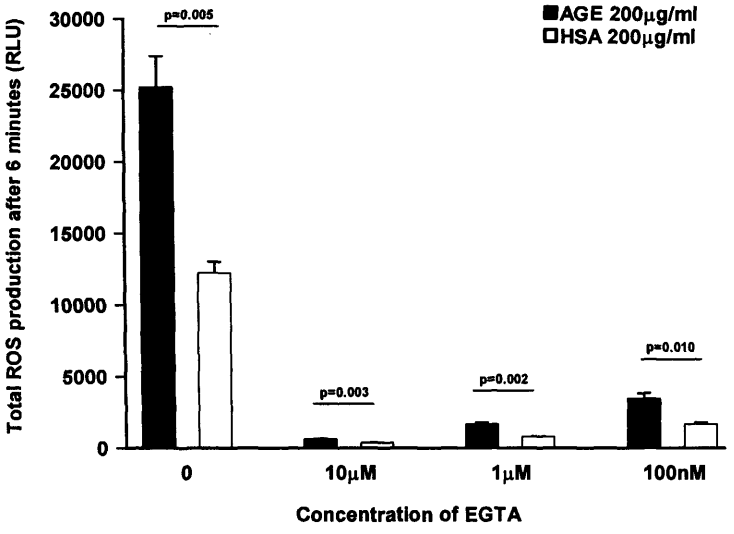
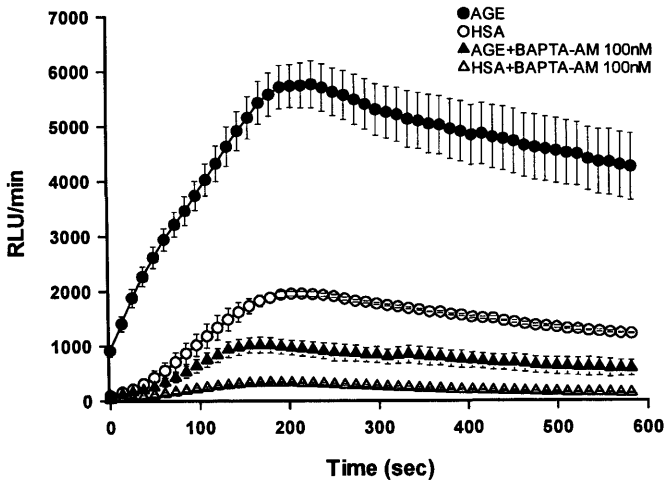


Figure 3.4215 The effect of manipulating intracellular calcium on the neutrophil ROS burst and the AGE-augmented neutrophil ROS burst. (A) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with the intracellular calcium chelator, BAPTA-AM, at a range of concentrations. (B) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with BAPTA-AM at a range of concentrations. (C) Mechanically-stimulated ROS production from neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with the extracellular calcium chelator, EGTA, at a range of concentrations. (D) ROS production following fMLP-stimulation of neutrophils that have been co-stimulated with AGE or HSA (200µg/ml) and pre-incubated with EGTA at a range of concentrations.

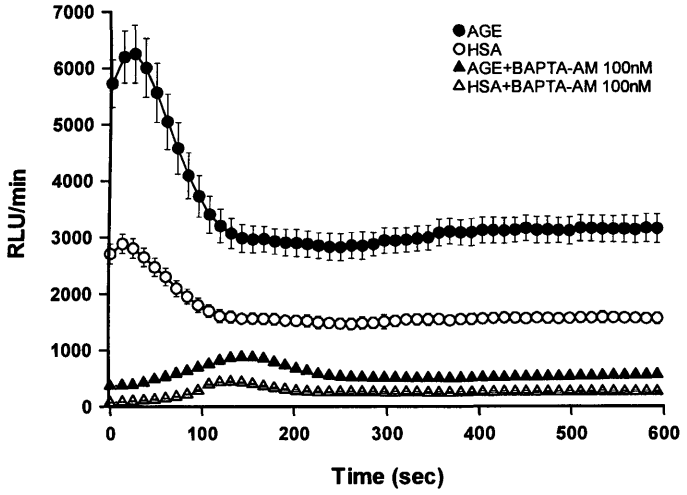
ROS output was detected by Lucigenin-enhanced chemiluminescence and expressed as the cumulative Relative Light Units (RLUs) over 6 minutes. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

Figure 3.4216

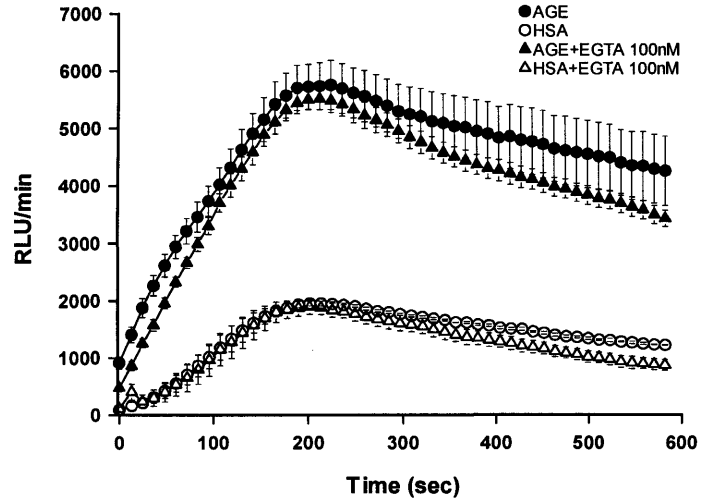
A.



B.



C.



D.

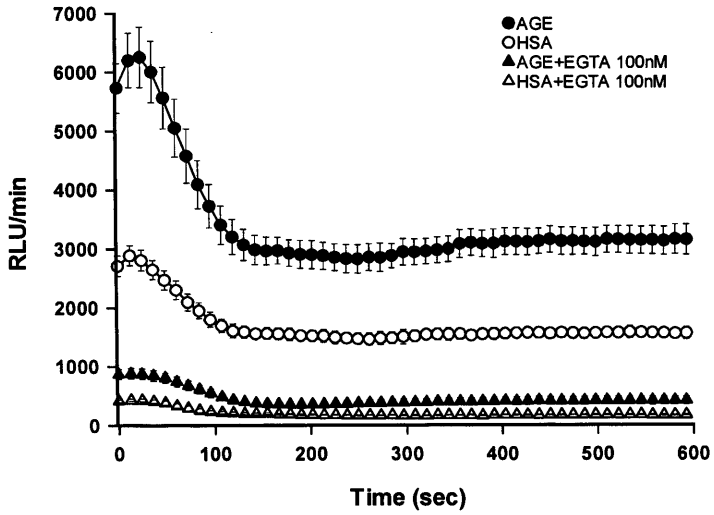


Figure 3.4216 The kinetics of the neutrophil ROS burst and the AGE-augmented neutrophil ROS burst following manipulation of intra- and extracellular calcium. (A) ROS production from neutrophils which have been mechanically stimulated in the presence of AGE or HSA, and preincubated with or without the intracellular calcium chelator BAPTA-AM (100nM). (B) ROS production from neutrophils which have been preincubated with or without the intracellular calcium chelator BAPTA-AM (100nM) and then stimulated with the peptide fMLP with co-stimulation by AGE or HSA. (C) ROS production from neutrophils which have been mechanically stimulated in the presence of AGE or HSA, and preincubated with or without the extracellular calcium chelator EGTA (100nM). (D) ROS production from neutrophils which have been preincubated with or without the extracellular calcium chelator EGTA (100nM) and then stimulated with the peptide fMLP with co-stimulation by AGE or HSA.

ROS production was detected by Lucigenin-enhanced chemiluminescence and expressed as RLU (Relative Light Units) per minute. All results are representative of multiple experiments and are the mean values of the same cells being tested in triplicate (10^5 cells per microplate well) with SEMs shown.

3.43 DISCUSSION

In attempting to unravel the signalling cascade behind the AGE-effect on the neutrophil NADPH oxidase, a number of intracellular kinases with reputed roles in agonist or priming responses were visited; these included PKC, PI3K, PTKs, MAPKs, PLD and Rho-kinase. Direct inhibition of these enzymes was the predominant means of determining their roles. In the case of p42/44MAPK, indirect inhibition was achieved through the use of PD 98059 which inhibits MAPK kinase directly and hence results in reduced phosphorylation and reduced activity of p44/p42MAPK.[204] In all these studies however, it was noticeable that the relative enhancement of ROS production in the neutrophils stimulated with AGEs, was maintained. Therefore there is no evidence for involvement of any of the above kinases in mediating the effect of AGEs on the neutrophil respiratory burst. With almost all the inhibitors, there was a great attenuation of ROS output with prolonged preincubation times, or when higher doses of inhibitor were used. This is likely to reflect cytotoxicity as many of the 'specific' inhibitors still exert a range of effects, especially at higher doses. In addition to the inhibitors, there was evidence from the analysis of neutrophil lysates which, specifically, supported an absence of protein tyrosine phosphorylation or phosphoMAPK activation with AGE exposure.

Translocation of $p47^{phox}$ was investigated as it is a method of NADPH oxidase upregulation employed by lipopolysaccharide in the priming of neutrophils, but it too was not contributory to the AGE-related NADPH oxidase upregulation and neither was there a role for calcium in this process, as shown with both chelation

studies and calcium fluorophore analysis. Intracellular calcium chelation appeared to exert some slight effects on the kinetics of ROS production but these were minor compared to the main role of calcium in facilitating NADPH oxidase activation per se.

Finally, attempts at blocking RAGE signalling with anti-RAGE antibodies were inconclusive. Electroporation was employed as a technique to allow the anti-RAGE antibody to gain access to the RAGE receptor, since the epitope it was raised against lay in the cytoplasmic domain of the receptor. With this method, no attenuation of the AGE-effect was seen which may indicate a number of possibilities including a failure of the electroporation technique itself, denaturing of the antibody by the process of electroporation, or an inability of the antibody to block receptor-mediated events. However, electroporation did cause an overall increase in ROS production which may relate to it exerting a stimulating effect in general.

3.5 An examination of cell-adhesion as a mediator for AGE-augmentation of the neutrophil respiratory burst

3.51 INTRODUCTION

Neutrophil activation in vivo often entails a prior sequence of events resulting in the sequestration of neutrophils at a focus of inflammation. This may involve adherence of the chemoattracted neutrophils to the vascular endothelium and subsequent diapedesis through to the tissue spaces. In arresting neutrophil movement and initiating neutrophil adhesion, much depends upon the interaction between leucocyte β2-integrins (particularly CD11b/CD18 or Mac-1) and their endothelial cell ligand, intercellular adhesion molecule-1 (ICAM-1).[205] Certain priming agents such as ANP, TNF-α and GM-CSF are able to attenuate the neutrophil chemotactic response to fMLP across a cellulose-nitrate membrane[206] and this has been proposed to relate to enhancements in neutrophil integrin expression.[207] Thus in certain situations, priming may act to recruit a greater number of neutrophils to an inflamed locus, with an increase in neutrophil adhesion to a target substrate being one method of upregulating neutrophil responses. The effects of AGEs on neutrophil adhesion were therefore examined as an alternative explanation for the ability of AGEs to augment the stimulated neutrophil respiratory burst.

3.52 RESULTS

The kinetics of neutrophil ROS production, as determined by chemiluminescence, vary according to the conditions under which they are measured. It was observed that the kinetics of the neutrophil respiratory burst as measured by chemiluminescent techniques differed between experiments performed in a tube luminometer versus experiments carried out on a microplate system. When neutrophils in lucigenin suspension were loaded into test tubes in a tube luminometer to a total volume of 5 mls and ROS production was observed following mechanical stimulation, the rate of rise of ROS production was found to be more gradual and did not achieve a peak or plateau until at least 15 minutes (Figure 3.521B). However, when performed on a microplate, the total volume of lucigenin in which the neutrophils were suspended was 200µl and the resultant ROS generation was more rapid, achieving a typical peak and plateau response well within 6 minutes (Figure 3.521A).

Neutrophil chemiluminescence was also examined on microplates that had been irradiated to yield a negatively charged, hydrophilic surface, repellent to neutrophil adhesion. ROS production under these circumstances, in the presence of AGE or albumin, was compared to ROS production from neutrophils tested on non-irradiated microplates (Figure 3.522A and B). After mechanical stimulation, the rise of ROS production occurred more gradually on the irradiated microplates and exhibited different kinetics, so that there was an absence of a peak and plateau response within the same time frame taken by neutrophils on normal microplates to achieve such responses (Figure 3.522A). Following

stimulation with fMLP, the kinetics of ROS production were similar on the two media, though there was diminished ROS production on the irradiated microplate overall and the late secondary rise in ROS generation from AGE-exposed cells did not occur on this medium.

Overall, the levels of ROS generation were significantly lower on the hydrophilic medium after both mechanical and fMLP stimulation (Figures 3.522A and D) but despite this, the differential ROS production in AGE-exposed compared to albumin-exposed neutrophils was maintained, with the ratio of this differential being essentially similar on the two different media following both modes of cell stimulation (Figures 3.522A and D).

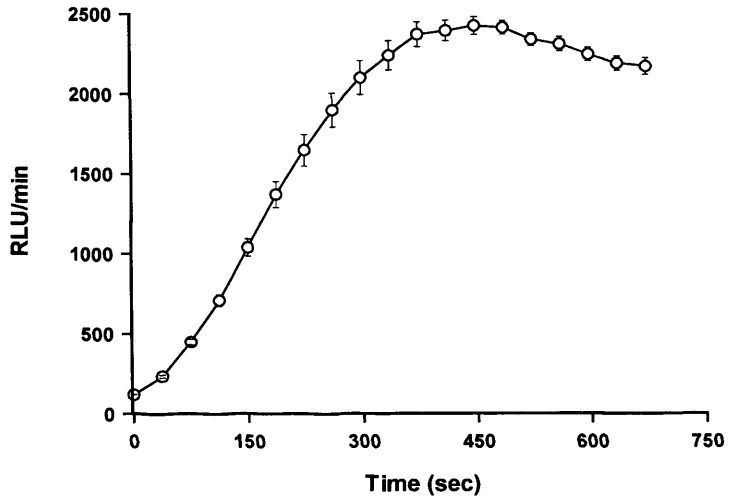
AGE-exposed neutrophils do not demonstrated increased adhesion to their underlying substrate. The importance of the interaction of neutrophils with their substrate for initiating ROS generation has been demonstrated. Potentially therefore, the enhancement in stimulated ROS production of AGE-exposed compared to albumin-exposed neutrophils, might be explained by an enhanced interaction or adhesion of such neutrophils with their underlying substrate.

An investigation was made to see if AGE-exposed neutrophils exhibited greater adhesive properties compared to albumin-exposed control cells. The numbers of neutrophils adherent to 6-plate wells in the presence of AGE as opposed to HSA was determined by fluorescence measurement of cells that had been pre-labelled with the fluorescent indicator BCECF. After deposition of such labelled cells (in

the presence of AGE/HSA) into the 6-plate wells, the contents of the wells were emptied at regular time intervals and the number of remaining adherent cells calculated by fluorescence measurements once the remaining cells had been lysed. Of note, after the non-adherent cells and cell medium had been discarded at each time interval, the 6-plate wells were washed lightly on a plate shaker to remove further non-adherent cells and the plates were similarly agitated with the ensuing lysis medium to ensure more uniform lysis of the remaining adherent cells. From this there was no significant difference in neutrophil adherence at any of the time intervals in cells exposed to AGE versus HSA (Figure 3.523).

Figure 3.521

A.



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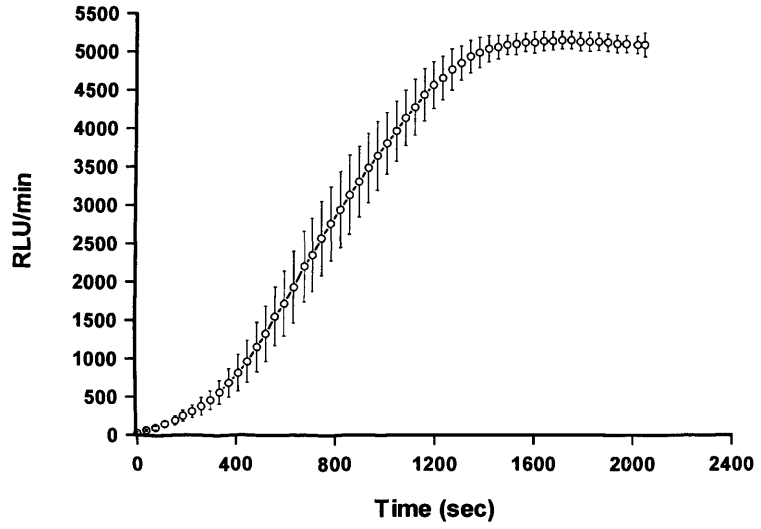


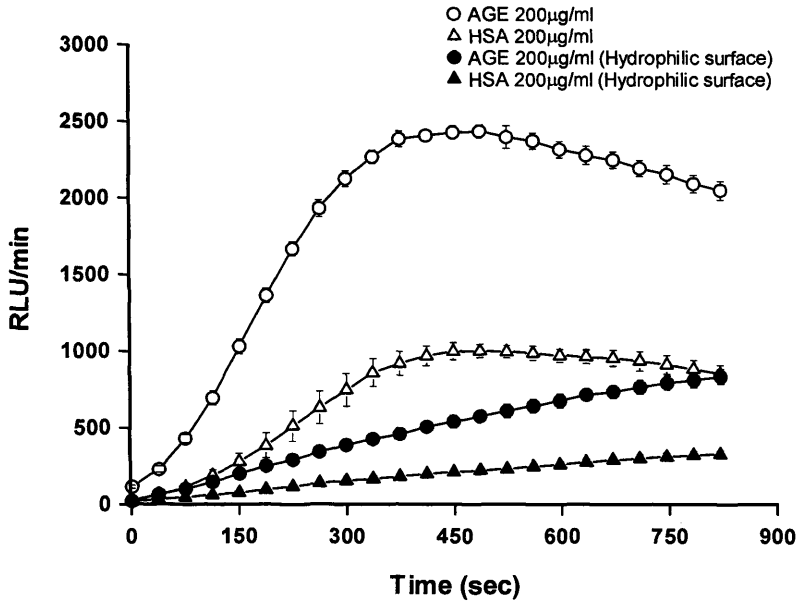
Figure 3.521 A comparison of the kinetics of neutrophil ROS generation, as measured by Lucigenin-enhanced chemiluminescent techniques following mechanical stimulation, in: (A) a small volume microplate well, compared to: (B) a large volume test tube. The difference in the time axes between the comparators is noted.

ROS production is measured in arbitrary Relative Light Units (RLU) per minute, and the scales for the two luminometers are different, resulting in different absolute amounts of ROS generated between the two samples. Both recordings are representative of multiple experiments and are the mean results of three consecutive experiments with SEMs included.

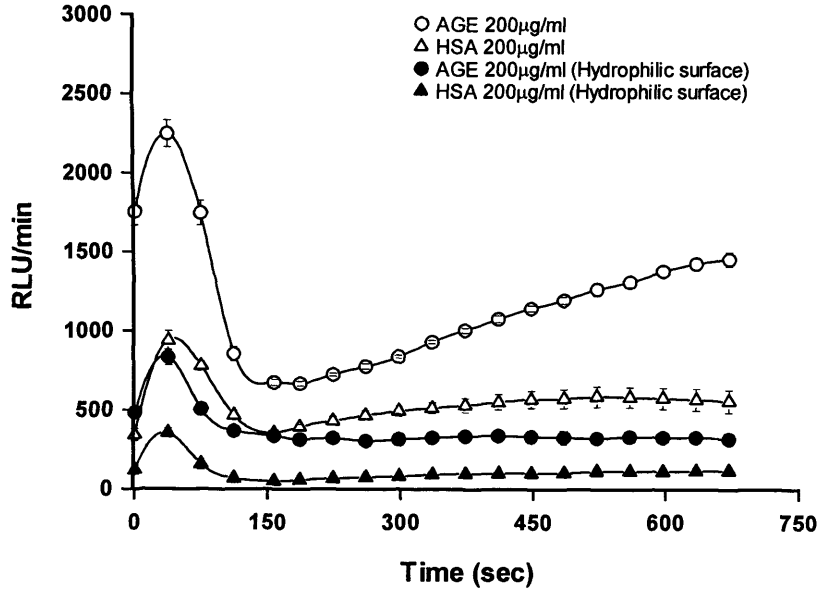
For (A) 10^5 cells per microplate well were used and for (B), 5 X 10^5 cells were used per tube.

Figure 3.522

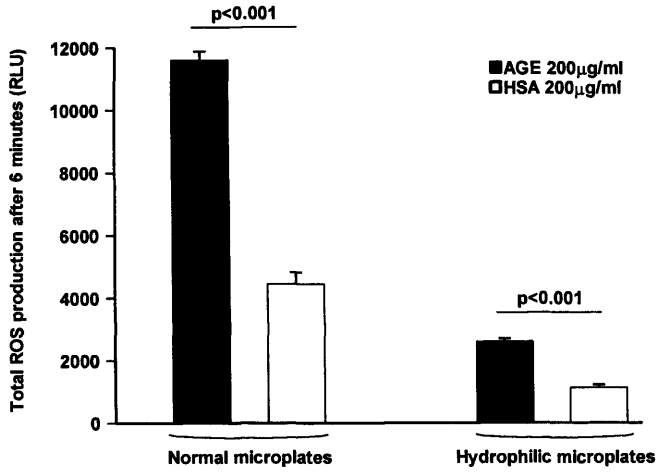
A.



B.



C.



D.

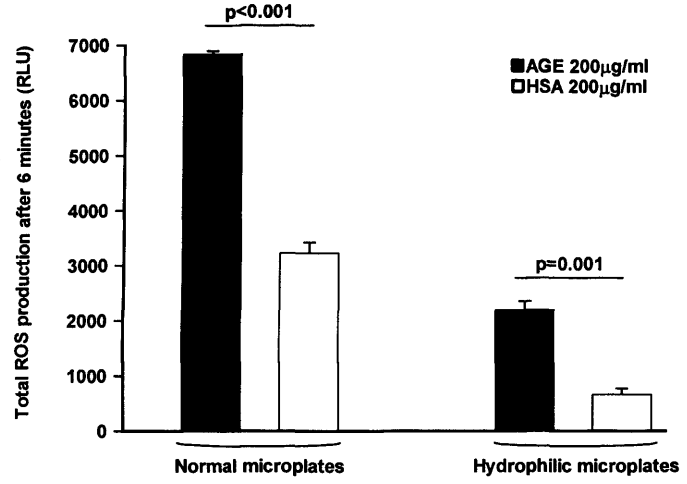


Figure 3.522 The effects of interfering with neutrophil adhesion, by the use of a hydrophilic medium, on the neutrophil ROS response. ROS production was compared between neutrophils that had been stimulated in the presence of AGE/HSA (200µg/ml) on irradiated, hydrophilic microplates versus normal microplates. (A) A comparison of the kinetics of ROS production from the two media following mechanical stimulation. (B) A comparison of the kinetics of ROS production from the two media following fMLP stimulation. (C) A comparison and quantitation of total ROS produced from the two media following mechanical stimulation. (D) A comparison and quantitation of total ROS produced from the two media following fMLP stimulation.

ROS production was detected by Lucigenin-enhanced chemiluminescence and recorded as the total RLU (Relative Light Units) over 6 minutes. The kinetic tracings shown are representative of multiple experiments and are the mean of three consecutive experiments (10^5 cells per microplate well) with SEMs included.

Figure 3.523

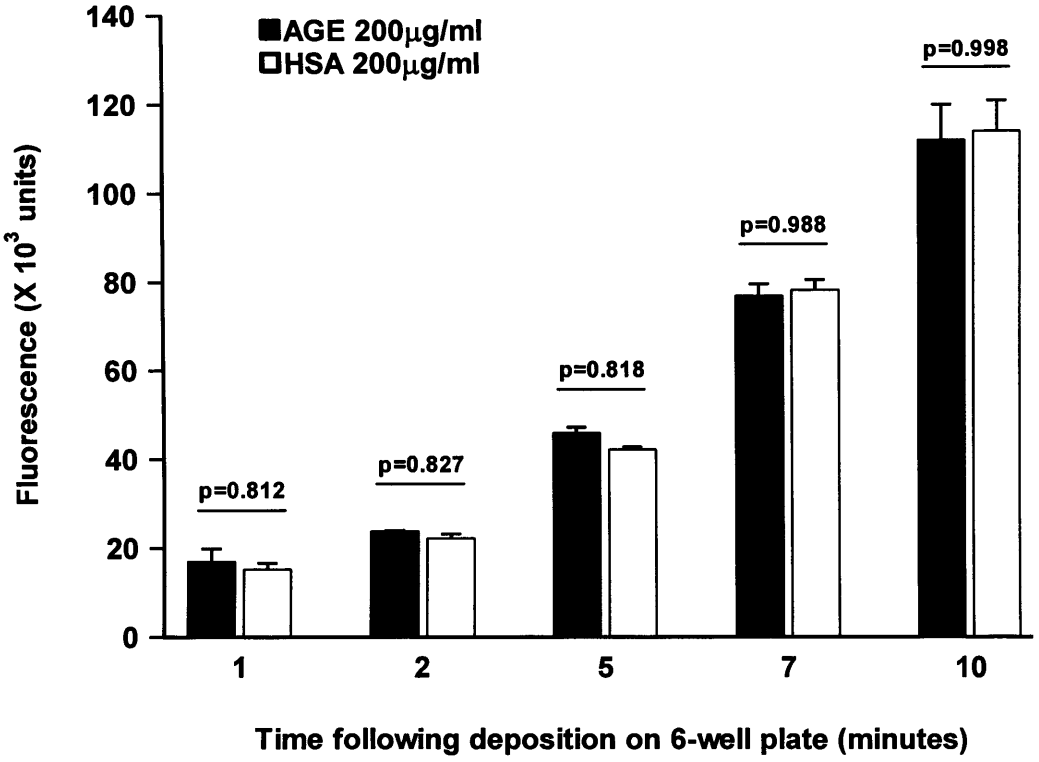


Figure 3.523 The effect of AGEs on neutrophil adhesion to a plastic substrate. Neutrophils in equal suspensions of AGE or Albumin (200µg/ml) were allowed to settle in plastic 6-well plates for varying lengths of time before the number of adherent cells was analysed. Analysis of adherent cells was by fluorescence counting of lysed, BCECF pre-loaded cells which were lysed after the excess cells and AGE/Albumin had been discarded. The results are the mean of 6 experiments with SEMS included.

3.53 DISCUSSION

The observation that the rate of neutrophil ROS production was affected by the volume of cellular suspension and the size of vessel, in which measurement was undertaken, indicate that the deposition and adhesion of neutrophils to their substrate is vital to the initiation of ROS production in these experiments. Thus with a larger volume of cellular suspension, the time taken for neutrophils to engage with the substrate is greater, accounting for the slower ROS production when measurements were conducted in the larger volume tube luminometer. This phenomenon was corroborated by observing lower levels of ROS generation on the hydrophilic (and hence 'neutrophil-repellant') medium after neutrophil stimulation.

Despite this, stimulated ROS production was still superior in AGE-exposed compared to albumin-exposed neutrophils and the relative degree of superiority was not affected by the hydrophilic media. Therefore retarding neutrophil adhesion does not impact upon the ability of AGEs to augment the neutrophil respiratory burst and makes adhesion an unlikely mediator of the ROS-amplifying effects of AGEs.

Chapter 4 – DISCUSSION

4.1 The identification of a novel mechanism for AGE-induced vascular dysfunction

Premature macrovascular disease is a prevalent accompanying pathology in diabetes mellitus[208] and in renal failure,[209] whilst macrovascular disease itself is increasingly common with advancing age. The increased rate of accumulation of AGEs in these conditions is recognised as a potential contributor to this pathology.[210] AGEs have putative pathogenic effects on the vasculature ranging from the cross-linking of proteins that results in arteriolosclerosis and cardiac diastolic dysfunction,[66,67] being chemoattractive to inflammatory leucocytes[75] and also promoting endothelial adhesion and permeability via the induction of adhesion molecules and Vascular Endothelial Growth Factor (VEGF) expression respectively.[69,70] The latter processes involve the interaction of AGEs with the RAGE receptor; experimentally, extracellular ligation of AGEs by the intravenous administration of excess soluble RAGE receptor (a method of depleting free circulating AGEs) has been shown to decrease endothelial permeability and atheroma formation in diabetic rats.[60]

Oxidative stress is also increased in the aforementioned conditions of diabetes, uraemia and ageing, and is a contributor to the atherogenic process.[211] Its effects include neutralisation of the endogenous endothelial-derived relaxing factor, Nitric Oxide (NO), which leads to unopposed vasoconstriction and an increase in

vascular resistance and blood pressure.[84,85] Vascular oxidants also modify endothelial prostacyclin production. The latter is a vasodilator and potent antiplatelet agent that inhibits both platelet and neutrophil aggregation and adhesion to the endothelium.[212] Platelet aggregation and endothelial permeability are two other factors that are enhanced directly by free radical release too.[92,93] Most notably however, oxidative modification of LDL (ox-LDL) results in its uptake by macrophage scavenger receptors and the resultant generation of foam cells which contribute directly to atheromatous plaque formation. Inflammation in atherosclerotic plaques is intensified by the fact that ox-LDL is recognised by unique lectin-like receptors on human arterial endothelial cells called LOX-1 receptors. Incubation of endothelial cells with ox-LDL upregulates the expression of LOX-1 and results in the further formation and release of ROS, serving as a potent chemoattractive force for further inflammatory cell influx.[213-215]

Work from several laboratories has shown the presence of chronically activated leucocytes within human atheroma[89,90] which may have important implications in plaque rupture. The inflammatory cells inhibit collagen synthesis which allied to ROS production may participate in the conversion of a stable to unstable plaque that ruptures under appropriate haemodynamic stress.[91] The neutralisation of NO and the resultant vasospasm exacerbates all of this, whilst the direct effect of phagocyte-derived ROS also promotes platelet accumulation, aggregation and activation.[92,93] The breach in endothelial integrity promoted by ROS and inflammatory cell influx then enlarges the interstitial space, inducing leakage of blood constituents into the subendothelial layers. Further activation of

inflammatory cells takes place which elicits the production of elastase, other proteolytic enzymes and arachidonic acid metabolites, resulting in endothelial and smooth muscle injury and also capillary leakage;[95] this process is aided by the fact that the release of ROS per se also stimulates the release of proteolytic enzymes.[94] In addition to the involvement of free radicals in the pathogenesis of occlusive arterial disease, biochemical events that occur during ischaemia also result in the generation of free radicals on reperfusion which may further exacerbate the previously mentioned problems. Overall there is evidence of free-radical pathology in a wide range of cardiovascular diseases, corroborated by the presence of free-radical products such as lipid peroxides in aortic atherosclerotic lesions[216] and increased levels of these same products in the serum of patients with occlusive arterial disease.[217]

As well as AGEs and oxidative stress both being positively correlated with vasculopathic processes, AGEs themselves may contribute directly to the oxidative burden. This can occur through the process of glucose auto-oxidation, one of the pathways to AGE formation, and through the participation of AGEs with transition metals in the Fenton reaction,[5] as well as through the activation of redox-sensitive intracellular signalling pathways. With respect to the latter, a state of increased oxidative stress has been reported upon ligation of the RAGE receptor by AGEs,[218-221] albeit inferred indirectly from observing the activation of intracellular pathways normally regulated by oxidant stress and the abrogation of AGE/RAGE responses by antioxidants.[219, 220] One study did report the direct detection of hydrogen peroxide release when AGEs were allowed to interact with

the RAGE receptor on endothelial cells. Although peroxide release was a direct response in this case, it occurred as a slow, integrated accumulation measured over a period of 60 minutes,[221] but generally the potential for direct AGE-induced free radical release by inflammatory cells has until now received little attention.

In the current studies, further evidence for AGE-induced oxidant stress has been outlined. There is no evidence for any direct, rapid induction of oxidant stress upon the exposure of neutrophils to AGE-albumin. However, the presence of AGE-albumin allows for a dose-dependent enhancement of the ROS burst imparted by a secondary stimulus, whether it is mechanical or chemical, and this occurs with rapid kinetics. Allied to the prompt reversibility of this effect on removal of AGEs from the neutrophil milieu, it is proposed that AGEs may play a hitherto novel role as neutrophil 'co-agonists'. Thus alone, AGEs do not possess any ability to activate the neutrophil respiratory burst, but in the presence of AGEs, the stimulated neutrophil NADPH oxidase enzyme generates significantly enhanced ROS production by an average of two to three-fold. Importantly, AGE co-agonist activity provides a mechanism for augmenting NADPH oxidase activity that may in turn be triggered by a heterogeneous group of stimuli. That the NADPH oxidase enzyme is central to the basal production of ROS by neutrophils and the AGE-augmented production of ROS, is supported by the dose dependent inhibition of ROS output seen in the presence of DPI. This flavoprotein inhibitor is effectively regarded as an NADPH oxidase inhibitor, especially in neutrophils where NADPH oxidase is the dominant flavoprotein;[176] moreover there was a comparative lack of effect of the specific mitochondrial

flavoprotein inhibitor rotenone on the suppression of this ROS output. The stimulation of the NADPH oxidase enzyme by mechanical and chemical modes of stimulation and the augmentation of this process by AGEs may be mirrored pathophysiologically by the shear forces that leucocytes are subjected to in their passage through the vasculature[222] and by the encounters with bacterial peptides which may act as chemical stimulators of neutrophils. The temporal relationship of many acute ischaemic events to infective processes is well-established,[223] and inflammatory markers such as CRP are correlated with a predisposition to ischaemic events.[224] Therefore in the context of a surrounding mileu of AGEs and bacterial infection and the presence of mechanical shear stresses, the leucocyte in vivo can be seen as a potential perpetrator of vascular damage.

The dominant role of the NADPH oxidase enzyme in vascular oxidant stress is further emphasised by the fact that iso-enzymes of this complex are found in other vascular components such as the endothelium and vascular smooth muscle cells. The activity of vascular NADPH oxidases may be regulated by cytokines, hormones and mechanical forces that are also known to be involved in the pathogenesis of vascular disease such as angiotensin II, thrombin and platelet-derived growth factor.[225-229] Several studies have demonstrated a critical role for NADPH oxidase in angiotensin II-induced hypertension; angiotensin II stimulates superoxide generation in cultured rat vascular smooth muscle cells through an increase in NADPH oxidase activity and chronic infusion of angiotensin II into rats results in hypertension, increased vascular superoxide production and increased NADPH oxidase activity.[227] In the latter example, both

blood pressure and vascular reactivity are restored by the exogenous administration of SOD.[230] Meanwhile, in terms of mechanical forces, exposure of human umbilical endothelial cells to unidirectional laminar shear stress (5-20 dyne/cm^2) also results in a transient elevation in NADPH-dependent superoxide formation, whereas oscillatory shear stress caused a sustained increase in oxidase activity.[231] Therefore if the generation of ROS through NADPH oxidase activity is a common pathway by which a variety of atherogenic risk factors impose their deleterious effects on the vasculature, the influence of AGEs on this same activity merely follows a common motif.

Although a homogenous concentration of AGEs existed in the experimental media in vitro, it should be noted that in vivo AGE concentrations may not be uniform throughout the body. AGEs are often closely allied to the locations of atherosclerotic lesions,[2,47] although whether this is cause or effect has not been definitively answered; indeed both elements may be present. Notwithstanding this debate, in the vicinity of atherosclerotic plaques, AGE concentrations may be higher locally than in the circulation, resulting in locally enhanced neutrophil ROS production. This may also facilitate localised accelerated AGE formation, thus further perpetuating this cycle.[232] This non-homogenous distribution of AGEs in the body may also account for the apparent paradox between an increase in AGE-mediated neutrophil ROS generation and the observation that diabetic patients, especially those with poor metabolic control, appear to have a greater frequency of infective complications.[233] The reversibility of the AGE effect may lead to neutrophils being only transiently in an activated state at sites of heavy

AGE accumulation and reverting to a near normal state on relocation to other parts of the circulation or non-vascular compartments. Also hyperglycaemia per se is cytotoxic, impeding effective neutrophil functioning.[234] Thus neutrophil function under conditions of acute hyperglycaemia differs from that during times when metabolic control is closer to physiological parameters, but when the legacy of past metabolic indiscretions has led to accelerated AGE accumulation. Another factor which may rationalise the greater propensity for infection in diabetic patients with the presence of an augmented neutrophil respiratory burst lies in the ability of AGEs to ligate lactoferrin and lysozyme,[235] which are two important components of humoral immunity, involved in the opsonisation of pathogens.

The evidence from this study therefore adds further insight to possible pathogenic effects of AGEs in conditions such as diabetes and uraemia. Through their action as unique neutrophil co-agonists, it has been demonstrated that AGEs could play a key role in the induction of a state of increased oxidative stress by augmenting neutrophil ROS production and this may be in part responsible for the acceleration of vascular disease noted in these conditions.

4.2 The utility of chemiluminescence in the study of cellular ROS production

The possibility that AGEs may significantly contribute to the vascular oxidative burden through a direct effect on leucocyte ROS production provided the focus

for examination in this study. For ROS detection, a chemiluminescent assay was used in which the reaction of free radical species with a chemical probe results in the release of photons. The two chemiluminescent compounds deployed in the study were lucigenin and luminol, but in fact it is the univalently *reduced* form of lucigenin and the univalently *oxidised* form of luminol that react with radicals forming unstable intermediate compounds, an unstable dioxetane in the case of lucigenin and an endoperoxide in the case of luminol. The unstable intermediate compounds then decompose to electronically excited products with the release of photons as they subsequently fall to the ground state.[236]

Because of its sensitivity, lucigenin-derived chemiluminescence (LDCL) has frequently been used in the specific detection of free radical production by both in vitro enzymatic systems and in intact cells[237] and lucigenin has been shown to be especially specific for the detection of the superoxide radical.[238] However, the validity of lucigenin as a chemiluminescent probe for detecting biological superoxide has been questioned based on the possibility that lucigenin itself is able to act as a source of superoxide via the auto-oxidation of the lucigenin cation radical. If the lucigenin radical then reacts with molecular oxygen, superoxide will be generated and return the lucigenin radical back to its base state. Through such redox cycling, lucigenin might be able to repeatedly generate superoxide.[239] Weight has been lent to this concern from the observation that in several in vitro enzymatic systems that either do not produce superoxide or have a very limited ability to produce it, the use of lucigenin has resulted in the apparent detection of superoxide;[239] examples of such systems

include the combinations of glucose oxidase/glucose at pH 9.5, xanthine oxidase/NADH and endothelial nitric oxide synthase/NADPH. However despite these concerns, it has subsequently been shown that such redox-cycling does not occur at low concentrations of lucigenin (≤50μM) and there is no spontaneous generation of superoxide, even in the enzymatic systems already mentioned.[237]

Whereas lucigenin is a specific detector of superoxide, the luminescence of luminol can be elicited by a wide variety of oxidants, including superoxide, hydrogen peroxide, hypochlorous acid, the hydroxyl radical, singlet oxygen and nitric oxide.[240] In a slightly more complex reaction than occurs with lucigenin, it appears that nitric oxide (NO), produced by nitric oxide synthase (NOS) is required for the constitutive functioning of luminol-dependent chemiluminescence.[241] Thus inhibitors of NOS reduce luminol-dependent chemiluminescence, and the addition of the substrate L-arginine enhances it. Neither of these measures has any effect on triggering the absolute production of superoxide though. When phagocytic cells were incubated with LPS, superoxide was not released but NO levels increased. In this situation there was no gross elevation in luminol-dependent chemiluminescence and so it appears that luminol-dependent chemiluminescence requires the conjoint presence of superoxide and NO, perhaps taking place through the formation of peroxynitrite.[241]

Chemiluminescence was selected as the preferred mode of ROS detection due to its superior sensitivity when compared with other commonly deployed methods such as cytochrome c reduction. Moreover, such an assay was also advantageous because it allowed the observation of ROS generation in real time. Therefore in addition to being able to measure absolute quantities of ROS, assessments could also be made concerning its production kinetics. The only disadvantage with the chemiluminescent assays related to the effect of high protein levels in the medium on the suppression of photon detection. Such a light quenching effect by protein concentrations has been noted previously with respect to chemiluminescent reactions,[174] though at the concentrations of protein used in the study, such light quenching was minimal.

The varying predilections of chemiluminescent probes for detection of the varieties of free radicals was reflected in the current studies by the different sensitivities observed when the two probes were used in conjunction with different cellular substrates. Thus lucigenin was the preferred agent for superoxide detection in neutrophils whilst lymphoblast free radical generation was more appropriately detected with luminol; this may reflect differences in the ROS species produced by the two cell types. Unexpectedly, the results obtained with luminol in lymphoblasts did not mirror those found with lucigenin in neutrophils. However, with respect to luminol, it has been shown that this probe may cause inhibition of priming under certain conditions. Although AGEs do not function as neutrophil priming agents, the mechanism by which luminol appears to inhibit priming responses in neutrophils, probably involves alterations to

intracellular signalling processes and not an effect on the function or assembly of the oxidase subunits themselves because luminol is able to exert an inhibitory effect on whole cells but not on a cell-free NADPH oxidase system.[242] Therefore it is possible that such a degree of intracellular interference may affect other signalling processes, including the ones involved in the mediation of AGE co-agonist activity. Other possible reasons for the lack of an augmenting effect on stimulated lymphoblast ROS production by AGEs would include processes pertinent to the lymphoblasts themselves which will be discussed in the following section.

4.3 The relevance of examining neutrophils and lymphoblasts to vascular disease processes

As physiological ROS producers in antimicrobial defence, neutrophils were chosen for investigation because they may also contribute significantly to intravascular oxidant stress. When neutrophils have been examined from patients with obliterative atherosclerosis, they have been found to exhibit an enhanced respiratory burst and also a tendency to increased degranulation in response to stimulation with classical leucocyte-activating agents such as the bacterial cell wall peptide, fMLP, and calcium ionophores.[243] Evidence also suggests that even controlling for other factors, a higher blood leucocyte count and especially the granulocytic component, predicts a greater likelihood of future vascular events. In a similar vein, infection is often found preceding the development of ischaemic events.[244,245] Inflammation has been linked to all

stages of the development of vulnerable plaques, from the initial deposition of lipid to plaque rupture and its thrombotic complications. Evidence of leucocyte activation and degranulation is found in patients with unstable angina[137,246] and extensive monocyte and neutrophil infiltration is seen in fissured, thrombosed plaques in patients with acute coronary syndromes.[247,248] In vitro studies suggest numerous mechanisms through which leucocytes may affect the stability of plaques in acute coronary syndromes including the release of toxic chemicals such as ROS and proteolytic enzymes. Thus levels of the leucocyte enzyme myeloperoxidase have been shown to be elevated in persons with angiographically documented cardiovascular disease[249] and within culprit lesions prone to rupture.[250] Myeloperoxidase has been linked to the development of lipid-laden soft plaque,[251] the activation of protease cascades affecting the stability and thrombogenicity of plaques,[252] the production of cytotoxic and prothrombogenic oxidised lipids,[251] and the consumption of nitric oxide,[253] leading to vasoconstriction. Indeed, myeloperoxidase levels have been shown to predict the early risk of myocardial infarction and other major adverse cardiac events in the absence of prior myocardial necrosis.[254] However, as well as the secretion of myeloperoxidase, the activation of leucocytes prompts the concomitant generation of oxidants important in host defence and it is widely thought that the interaction of myeloperoxidase with ROS species, particularly peroxides, leads to the generation of the pathophysiologically important radical, hypochlorous acid. Quite apart from this, myeloperoxidase levels also reflect by surrogacy, the degree of neutrophil activation at any one point in time and therefore are likely to

reflect upon the level of ROS production. Neutrophils may thus be seen to be involved in the primary pathogenesis and progression of occlusive vascular disease[244,245] and they also have an acknowledged role in reperfusion damage following ischaemic events.[175]

Increased oxidative stress is also a recognised phenomenon in hypertensive animals[178] and seems to be more consistently present in subjects with a family history of hypertension[255] along with the observation that total leucocyte counts and neutrophil activity are often elevated to the higher end of the normal range in patients with hypertension.[256] The current studies provide confirmatory support for this pattern by demonstrating that transformed lymphocytes from otherwise matched hypertensive and normotensive individuals are able to maintain this phenotypic response in terms of ROS production, appropriate to the previous level of blood pressure. Such demonstration of phenotypic persistence in transformed lymphoblasts has also previously been shown with increased ion transporter activity and increased expression of NADPH oxidase subunits in lymphoblasts derived from hypertensive subjects. Therefore, even though lymphocytes may not contribute so greatly to atherogenic processes as neutrophils, their immortalisation to lymphoblasts provides a useful model for the investigation of phenotypic responses. Their use is additionally advantageous because they are devoid of any stimulatory influence imposed by a traumatic extraction process, such as that taking place with neutrophil isolation from whole blood, even though attempts were made to moderate for the latter by incubating the neutrophils at 37°C in a rotating incubator for a given period prior to use.

As vascular risk profiling is a multifactorial process involving well-recognised contributions from hypertension and diabetes, it is conceivable that some sort of complementary action or even synergy might take place between a hypertensive and diabetic phenotype. One explanation might lie in the increased shear stress that leucocytes are subject to in the hyperdynamic hypertensive circulation; the stimulatory effect of shear stress on neutrophil ROS production per se was demonstrated clearly in the current studies and was enhanced in the presence of AGE-albumin. Synergy may also take place in terms of ROS production. In this study, the surrogate for a diabetic phenotype was the presence of AGE-Albumin, since AGEs accumulate to excess in diabetes and have been demonstrated to be powerful mediators of a spectrum of pathologies, in particular vascular pathology; moreover, the potent augmenting effect of AGEs on ROS production in neutrophils has already been highlighted. The current study was unable to lend support to the hypothesis of synergistic ROS production in hypertensive, diabetic subjects, though it did not absolutely refute this possibility either. There was no detectable effect of AGEs on lymphoblast ROS production and although this may reflect reality, it may also have occurred for a number of technical and biological reasons. The lack of effect of AGEs on lymphoblast ROS production occurred over a wide range of AGE concentrations. The lower overall ROS detection in lymphoblasts subjected to the highest concentrations of AGE/Albumin (400μg/ml) was in keeping with previous findings in neutrophils and again probably reflected an artefactual response secondary to luminescence quenching in media with high protein concentrations. As well as lacking co-agonist activity on

lymphoblasts, AGEs were also unable to enhance lymphoblast ROS production by the mode of priming, even though it is known that lymphocytes are susceptible to the process of priming by certain chemical agents.[257]

The inability of AGEs to upregulate lymphoblast responses by mechanisms examined in the current study might be explained by a number of factors. It might be concluded that a signalling pathway is absent in lymphoblasts with respect to AGE-mediated responses. Thus although the Western blot analyses of lymphoblast lysates demonstrate the unequivocal presence of the RAGE receptor in these cell lines it is possible that the effects of AGEs on the respiratory burst are not mediated by RAGE, but rather by one of the other AGE-binding receptors which may be absent in lymphoblasts. Even given the prospect that the RAGE receptor is key to these processes in neutrophils, carriage of the RAGE receptor in lymphoblasts may be for a different purpose; the RAGE receptor may mediate one of numerous non-ROS-producing functions and any signalling systems downstream of the RAGE receptor in lymphoblasts may relate to one of these other processes rather than to stimulation of the NADPH oxidase system. Other well-known RAGE-mediated processes in cells include the production of various inflammatory cytokines, transducing responses from other inflammatory ligands such as the calgranulins[258] and assisting in inflammatory responses through other forms of stimulation. However, none of these roles were examined in this particular study and so a role for AGE-RAGE interactions in the mediation of such processes in lymphoblasts cannot be excluded. The possibility also exists that when the lymphocytes were immortalised, the transformation

process to lymphoblasts altered the intracellular machinery, thus rendering a previously effective mechanism inactive. Even assuming that intracellular machinery is present and remains unperturbed, experimental technique may be insufficient to demonstrate a response. Thus luminol was selected as the chemiluminescent agent for ROS production in lymphoblasts, because out of the chemiluminescent probes assessed for this purpose, only luminol was able to achieve satisfactory detection of ROS in this cell type; the differential sensitivity of the probes probably relates to differences in the ROS species produced by the different cell types. Unfortunately, with respect to luminol, it has been shown that this probe may inhibit neutrophil NADPH oxidase function under certain conditions through an as yet unidentified mechanism affecting the intracellular machinery;[242] this may have negated any priming effect of AGEs on lymphoblasts. Meriting consideration is the fact that the overall generation of ROS was also significantly less when comparing lymphoblasts with neutrophils, in keeping with their respective respiratory capacities, meaning that any small difference in ROS production between lymphoblast groups would also be harder to detect.

4.4 Mechanisms governing novel AGE-effects on neutrophil ROS generation.

Under certain pathological conditions, neutrophils exhibit a greater magnitude of inflammatory response to usual stimulation. This has been seen already with respect to many ischaemic vascular pathologies. It has also been recognised in

neutrophils derived from azotemic patients, which exist in a primed state characterised by an enhanced respiratory burst of stimulated but not resting neutrophils. In azotemia, it is assumed that this property may be secondary to a circulating factor that fails to undergo renal elimination and whose identity has yet to be elucidated. Transplantation but not haemodialysis is able to normalise such neutrophil oxidative burst activity in these azotemic patients, suggesting that high molecular weight circulating factors may be responsible. Candidates have included normal high molecular weight plasma proteins that have been non-enzymatically modified by the uraemic milieu, thus constituting glycoxidatively-modified proteins. Indeed, 1.5-6.0 kDa range proteins that have been accordingly modified to AGEs, have been isolated in the plasma of diabetic and non-diabetic patients with end stage renal disease. Although they are theoretically dialysable, the concentration of these AGEs is still appreciably increased in dialysis patients,[145] providing a basis for examining the potential of AGEs to prime neutrophil stimulatory responses. However, the current studies refute any role of AGEs in priming, but rather give evidence for a new co-agonist action of AGEs on the neutrophil respiratory burst and in particular the neutrophil NADPH oxidase enzyme.

As 'co-agonists', AGEs are distinct from priming agents which also enhance cellular function but require significant interaction time with cells to bring about their response.[150] The complementary effect on ROS production gained by combining AGEs with the previously characterised classical priming agents, PAF and TNFα, illustrates potential in vivo synergism, but also further distinguishes

the distinct pathways mediating AGE 'co-agonist' action from the mechanisms of neutrophil priming agents,[151] albeit with the common endpoint being the activation of the neutrophil NADPH oxidase. Any greater overlap in signalling mechanisms would be likely to reduce the chances of further upregulation by the secondary stimulus; this was previously demonstrated when primed neutrophils from azotemic patients were unable to gain further upregulation of their respiratory burst with subsequent TNFα exposure.

There are theoretically many potential pathways mediating AGE co-agonist activity. The key trigger is likely to involve the interaction of AGEs with a neutrophil trans-membrane receptor of which several candidates have been identified;[2] indeed the concentrations of AGEs used in the current studies bear similarity to those validated in previous receptor-binding studies.[259] Also, the presence of the RAGE receptor has been recently demonstrated in neutrophils[260] and confirmed in the current study. RAGE is a good candidate for the transduction of this particular AGE-effect as it is known to participate in inflammatory processes. Interestingly with respect to RAGE, it has a very short intracytoplasmic domain with no recognisable intrinsic enzyme activity and may function in tandem with an adaptor molecule.[261, 262] Attempts however at blocking RAGE signal transduction per se with an antibody to the cytoplasmic domain of the RAGE receptor, using electroporation to facilitate the interaction of the antibody with its intracellular target, were unfruitful. The antibody may have been denatured by the electroporation process, but even if it did remain functional, its attachment to the cytoplasmic domain of the RAGE receptor may not have been

sufficient to sterically hinder the binding of this domain to downstream signalling components. Future attempts to investigate this interaction would be merited utilising antibodies generated against the RAGE receptor's extracellular variable domain. Another approach could be the use of small interfering RNA (siRNA) to specifically reduce RAGE expression. Should even these measures be unable to prevent the AGE-augmented stimulated neutrophil respiratory burst, it does not necessarily point to a redundancy of the RAGE receptor in neutrophils, since receptor function may be cell-specific. This is exemplified by the different consequences of RAGE receptor ligation in vascular smooth muscle cells as opposed to neural cells.

Whilst a co-agonist effect on the neutrophil respiratory burst has been identified as a novel property of AGEs, there has been pre-existing evidence that arachidonic acid may effect similar responses from the neutrophil NADPH oxidase leading to an exploration of its role in mediating AGE-stimulated events. It has been subsequently demonstrated that arachidonic acid is central to this process, mimicking the co-agonist activity of AGEs. The details of this mechanism may only be speculated upon, but could reflect the ability of arachidonic acid to alter the structural conformation and functional efficiency of the $p47^{phox}$ subunit of NADPH oxidase[179] or an action on other intracellular components vital to the function of the oxidase.[180] The proportionally small increment in arachidonic acid generation induced by AGE-exposure that is required to facilitate a response is consistent with a catalytic function. If arachidonic acid is able to function through enhancing $p47^{phox}$ activity then this

may be an alternative mechanism to the increased $p47^{phox}$ membrane translocation that enables neutrophil NADPH oxidase priming in response to lipopolysaccharide,[152] but which was certainly not demonstrable in the context of AGE co-agonist action in the current studies.

A role for arachidonic acid metabolites in the signalling pathway of $fMLP^{263}$ is supported by noting that the highest doses of the cyclo-oxygenase and lipo-oxygenase inhibitors, Indomethacin and MK-886, induced a decline in ROS output from both control and experimental groups when the cells were stimulated with fMLP. However, the metabolites themselves were not influential in the action of AGEs on the NADPH oxidase as shown by the lack of effect of the aforementioned inhibitors on reducing AGE-augmented neutrophil responses. In neutrophils, it therefore appears that arachidonic acid production is increased by AGEs, a process in turn that is dependent upon $cPLA_2$ activation, manifested by phosphorylation and membrane-translocation of the latter on exposure to AGEs. As shown by measurements of intracellular calcium, both through fluorescence media and also through chelation experiments, alterations in intracellular calcium do not regulate this process; rather it is the constitutive presence of calcium that facilitates the mechanism. Indeed membrane-translocation may take place in the presence of calcium alone without phosphorylation, accounting for the significant presence of non-phosphorylated $cPLA_2$ in the other membrane fractions.[187]

There is a hint that redox-sensitive mechanisms may also play a role in transducing any action of AGEs on the neutrophil NADPH oxidase. Incubation of

neutrophils with N-acetylcysteine reduced the final ROS output, but more importantly abrogated the AGE-induced differential increase in ROS output. This superiority of N-acetylcysteine over other antioxidants may relate to its roles as a glutathione precursor and principal intracellular free radical scavenger. Reduced glutathione may inhibit an intracellular redox-sensitive pathway where trace quantities of free radicals mediate signalling[264] and the depletion of intracellular glutathione has been previously noted in AGE-exposed cells.[218] There may be a lesser capacity for N-acetylcysteine and other antioxidants to scavenge the larger extracellular ROS output of the respiratory burst. The current in vitro data only supports Tiron as an extracellular free radical scavenger of any significance. In fact, of the other anti-oxidants, acetylsalicylic acid and ascorbic acid, higher doses actually led to an increase in detected ROS. There remains the potential for any antioxidant, under given circumstances, to become a pro-oxidant[265] and such a phenomenon may be occurring at the higher doses of anti-oxidants in this case. With regards to ROS-disposing enzymes, both catalase and the SOD-mimetic (SOD-M) were able to reduce detected ROS production, but only if the enzymes remained in the reaction medium whilst the neutrophils were stimulated and ROS was being measured. Thus such enzymes have no real ability to alter signalling processes but merely catalyse the breakdown of any generated extracellular ROS in a dose-dependent manner. Judging from the ability of N-acetylcysteine to reduce arachidonic acid production and $cPLA_2$ translocation, this putative redox signalling is likely to occur upstream of $cPLA_2$ activation. Thus the relevance of the RAGE-receptor to the augmenting effects of AGEs in

neutrophils should not be excluded since RAGE is the only AGE-receptor so far demonstrated to convert an AGE signal into an intracellular redox signal.[112, 221] A summary of the proposed pathway mediating AGE-upregulation of the neutrophil respiratory burst is shown in Figure 4.41.

Figure 4.41

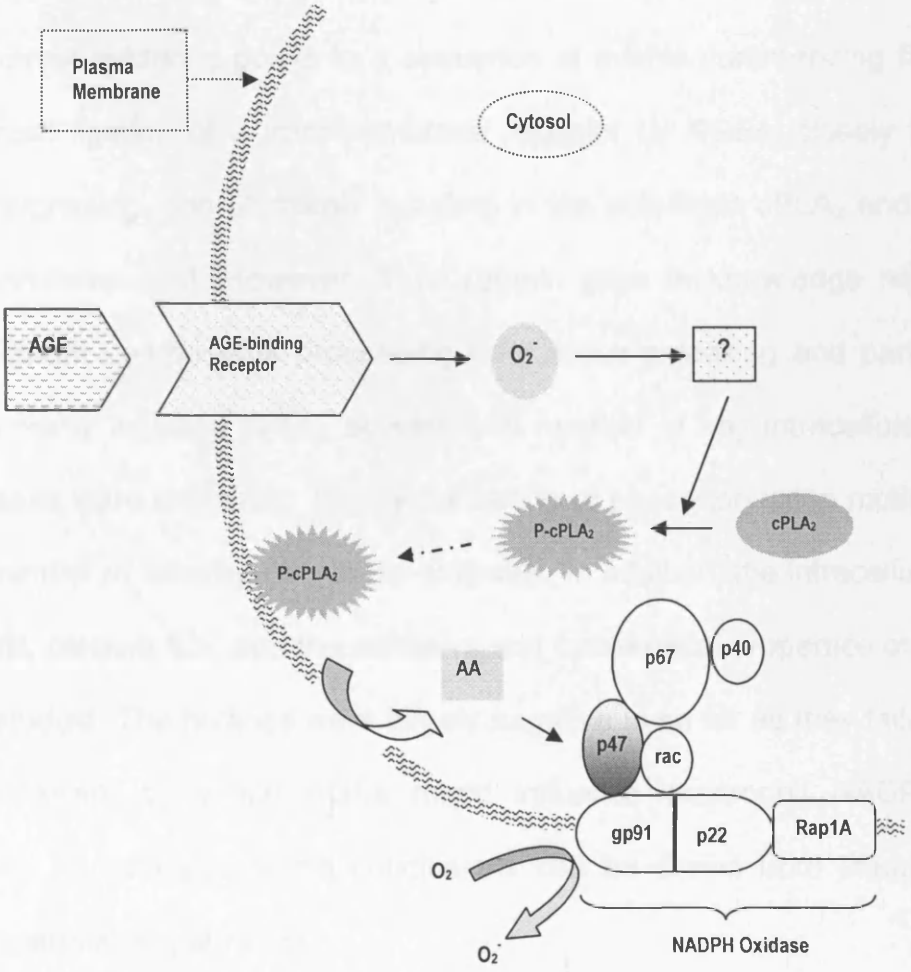


Figure 4.41 A putative pathway by which AGEs may enhance stimulatory burst activity of the neutrophil NADPH oxidase. Possible ligation of a receptor by AGEs generates intracellular superoxide (O_2^-) which through a redox-sensitive pathway involving one or more protein kinases, results in the phosphorylation, membrane translocation and subsequent activation of $cPLA_2$. The latter hydrolyses AA from membrane glycerophospholipid which then conformationally alters the $p47^{phox}$ subunit of the NADPH oxidase (subunits illustrated) to increase functional efficiency of the oxidase complex.

4.5 Unanswered questions regarding mediation of the AGE-augmented neutrophil respiratory burst

The current evidence points to a sequence of events commencing first with the presumed ligation of a transmembrane receptor by AGEs, closely followed by redox signalling, and ultimately resulting in the activation $cPLA_2$ and generation of arachidonic acid. However, there remain gaps in knowledge regarding the downstream components proceeding from redox signalling and particularly the mechanisms initiating $cPLA_2$ activation. A number of key intracellular signalling processes were examined, largely consisting of phosphorylation motifs mediated by a number of intracellular kinase enzymes. In addition, the intracellular traffic of proteins, calcium flux, and the adhesive and cytoskeletal properties of neutrophils were studied. The findings were largely negative in so far as they failed to reveal a mechanism by which AGEs might influence neutrophil NADPH oxidase function. Nonetheless, some conclusions can be drawn from interrogating the various signalling pathways:

Protein Kinase C (PKC):

PKC was examined as it is a known activator of the neutrophil NADPH oxidase enzyme, being directly activated by phorbol esters and leading to a stimulated respiratory burst. Moreover, inhibition of neutrophils with chelerythine or Ro-31-8220 reduced the magnitude of both the immediate peak and later prolonged phase of ROS production in response to fMLP stimulation.[266] The current data is concordant, with inhibition of PKC by the same two compounds resulting in a

lower overall ROS output from cells that had been stimulated either mechanically or with fMLP. There was no diminution in the augmenting effect on ROS output conferred by AGEs, except at high inhibitor doses which resulted in cellular toxicity.

Tyrosine Kinases:

Protein tyrosine kinases are similarly established in intracellular signalling processes and in particular are believed to be central to the mediation of priming responses in neutrophils.[156] Inhibition of tyrosine kinase activity with any of three inhibitors, genistein, tyrphostin and herbimycin, resulted in dose-dependent reductions in ROS output irrespective of the mode of cellular activation. The general tyrosine kinase inhibitors, genistein and tyrphostin, have previously been shown to reduce superoxide output from fMLP-stimulated neutrophils[267-269] whilst herbimycin, an inhibitor of src-family tyrosine kinases, is also known to diminish ROS output following NADPH oxidase activation by fMLP.[270] Src-family tyrosine kinases are a class of non-receptor tyrosine kinases that are classically expressed and activated in response to growth factor and mitogenic signals[271] and could consequently be pertinent to the effects of AGE-stimulation. The current and previous data underscore the importance of tyrosine kinases to cellular activation although their inhibition still does not impact upon AGE-augmenting mechanisms.

Phosphatidylinositol-3-kinase (PI3K):

Although hypotheses regarding tyrosine kinases have received the most attention with respect to neutrophil priming, the role of PI3K has also been recognised to be important in mediating certain priming responses, notably that of the cytokine TNF-α upon neutrophil ROS output.[149] Certainly phosphatidylinositol-3,4,5-triphosphate is rapidly produced upon exposure of neutrophils to fMLP and is proposed to act as a second messenger mediating actin polymerisation and respiratory burst activity.[154] The PI3K inhibitor, wortmannin, reduces phosphatidylinositol-3,4,5-triphosphate levels and causes a corresponding increase in levels of the precursor substrate phosphatidylinositol-bisphosphate.[154] The output of ROS appeared exquisitely sensitive to wortmannin, with the global impairment of neutrophil NADPH oxidase function implying a key role for PI3K in mediating NADPH oxidase responses; yet despite this, there was again little evidence of any interference with the NADPH oxidase-augmenting effect of AGEs.

Mitogen Activated Protein Kinases (MAPK):

The MAPK family are a group of cytoplasmic enzymes responsible for linking diverse cellular stimuli with cytoplasmic and nucleic regulatory molecules that dictate a whole range of responses, notably proliferation, differentiation and development.[195] MAPKs are subject to tyrosine phosphorylation possibly accounting for the interference with ROS production by the tyrosine kinase inhibitor genistein.[267, 268] Two MAPKs were examined in these studies, p44MAPK

and p38MAPK. Mechanistically, both directly activate MK2 (MAPK activated protein kinase 2) which subsequently mediates multiple neutrophil functions including ROS release, exocytosis, and chemotaxis.[272] Additionally, both MAPKs directly phosphorylate components of the NADPH oxidase, with p44MAPK and p38MAPK having predilections for $p47^{phox}$ and $p67^{phox}$ respectively; [273,274] as such, the MAPKs are attractive candidates for mediating upregulation of the NADPH oxidase.

In the current studies, though inhibition of both p44MAPK with PD98059 and p38MAPK with SB203580 inhibited ROS output from stimulated neutrophils to some degree, inhibition of p44MAPK had no impact on the AGE co-agonist component of stimulation. Also, the reductions in ROS output with p44MAPK inhibition were less than those achieved with p38MAPK inhibition. SB203580 exhibited a dose-dependent inhibition of ROS output, both for mechanical and fMLP stimulation, whereas a plateau effect existed for PD98059 - a wide range of concentrations caused a small and similar magnitude inhibition of the ROS response. This is in accordance with previous observations that p44MAPK inhibition is less effective at reducing ROS output in neutrophils, certainly in response to fMLP stimulation, than p38MAPK inhibition.[275] Moreover, inhibition of p38MAPK activation with increasing doses of SB203580 showed a trend towards a reduction in the AGE-augmenting effect on the neutrophil respiratory burst although two provisos should be noted; firstly there still remained a significant difference between the ROS output in AGE-stimulated versus control neutrophils, and secondly the absolute levels of ROS production at the highest

concentrations of SB203580 used were significantly reduced altogether, which may render any reduction of the AGE-augmenting effect more difficult to interpret. The lack of an increased p44MAPK moiety in cell extracts subjected to AGE-exposure is consistent with the inhibitor findings though it would have been interesting to compare the outcome with p38MAPK antibodies if these had been available. There is existing evidence though that p38MAPK itself may be phosphorylated and activated in neutrophils that have been primed by peritoneal dialysis effluent. [276] Since the latter has been postulated to contain AGEs which accumulate in uraemia, it is possible that p38MAPK might be activated preferentially following the presentation of AGEs to cells. However, the fact that such a pathway is likely to be central to other aspects of a cell's functioning mean that its inhibition results in diminution of cellular functioning in general and it is difficult to dissect out the effect on AGE stimulation specifically.

Phospholipase D (PLD):

The generation of phosphatidic acid from PLD activity has been shown to activate the NADPH oxidase in response to fMLP stimulation[277,278] and provides another potential pathway to superoxide release following agonist stimulation. The activation of PLD itself appears to occur via p44MAPK[277,278] but the relationship is more complex because in addition PLD also activates p38MAPK directly in neutrophil-like cell lines;[279] there thus appears to be cross-talk with the prominent MAPK family of enzymes. However, PLD per se certainly appears involved with ROS productivity in neutrophils to some degree as butanol

(compared to control iso-alcohol butan-2-ol) was able to impair general ROS production when cells where stimulated mechanically or with fMLP, though it did not affect the co-agonist effect of AGEs.

Calcium:

One of the other major second messengers in intracellular signalling is calcium flux, which like the tyrosine kinases, forms an essential component of many signalling cascades. In monocytes, intracellular calcium rises just before the respiratory burst.[280] This may facilitate the tyrosine phosphorylation of MAPKs, since EGTA, an extracellular calcium chelator, reduces the tyrosine phosphorylation of MAPK.[281] Interruption of calcium transients is also known to attenuate the upregulation of the neutrophil oxidative burst that occurs through priming mechanisms. This has been noted in the context of the priming agents PAF[282] and also F(ab) fragments generated against the L-selectin receptor. These F(ab) fragments exert a priming effect through cross-linking of the receptors, an effect which may be blocked by employment of the intracellular calcium chelator, BAPTA.[283] However, in the current studies, when intracellular calcium transients were examined directly through the fluorescence intensity of the calcium-fluorophore Fura-2, there was no significant difference in intracellular calcium concentrations following exposure of neutrophils to either AGE or HSA, and inhibition of calcium transients did not attenuate the co-agonist action of AGEs on neutrophil ROS production. Instead, a general reduction in ROS output was seen, compatible with the notion that calcium is an important intracellular

second messenger and also consistent with previous data demonstrating that depletion of intracellular calcium with BAPTA reduces fMLP-induced ROS production.[284] When extracellular calcium was chelated, the attenuation in ROS output following neutrophil stimulation was more marked following fMLP stimulation than following mechanical stimulation, although this finding appears to bear little practical significance. More important is the fact that interference with calcium made no impact on the augmented respiratory burst conferred by AGE co-activation.

Apart from the absolute reductions in ROS that were observed there was also a change in the kinetics of ROS production following calcium chelation. This was particularly noted with BAPTA, in the context of stimulation with fMLP. There was still a peak of ROS production, albeit greatly diminished and with a time lag before the rise in peak NADPH oxidase output. Previous findings demonstrating the superiority of BAPTA to EGTA in reducing fMLP-induced PLD activation[285] may explain this phenomenon, as the inhibition of PLD has already been shown to have a greater effect on the peak ROS burst following fMLP stimulation. These findings further highlight the complex interplay between different messenger and enzyme systems in agonist-activation of the NADPH oxidase.

Cytoskeletal and adhesive properties:

Much evidence points to a possible involvement of the cellular cytoskeleton in matters related to NADPH oxidase activity. In activated neutrophils, the superoxide-producing activity and portions of all the oxidase components are

found in the cortical cytoskeleton,[286] implicating cytoskeletal involvement in oxidase functioning. Interestingly, it has also been demonstrated that AGE-Albumin is able to increase intracellular f-actin assembly, albeit transiently. The latter effect followed a 5-10 second delay and could be abrogated by anti-RAGE monoclonal antibody.[260] The kinetics and time course of superoxide production vary greatly between neutrophils that are allowed to adhere to a surface compared to those remaining suspended in solution.[287] It was similarly observed in the current study that the interaction of neutrophils with their substrate is vital to the processes of degranulation and the respiratory burst. Thus it was serendipitously observed that the kinetics of ROS production in neutrophils depended upon the volume and type of vessel in which ROS production was analysed, even though both systems were analysed by chemiluminescence. A more gradual rise in ROS production was encountered when measurements took place in relatively large volume tubes, as opposed to the much smaller volumes of suspension contained in microplate wells. Only the kinetics of ROS production can be commented upon in this context, because the different calibration of the two luminometers makes a direct comparison of the absolute ROS production between the two systems difficult. When ROS production was observed for longer time periods with the tube luminometer, a similar peak and plateau pattern of ROS production emerged, although the time course for this was significantly longer than in the microplate luminometer.

That the rate of ROS production is also dependent upon the rate of neutrophil interaction with the plastic substrate was further demonstrated by experiments in

which irradiated microplates that had been rendered negatively charged and hence hydrophilic, were able to retard ROS production from neutrophils, presumably secondary to a general repulsion of neutrophil interaction by this hydrophilic surface. Nevertheless, even though the ROS production from neutrophils suspended in AGEs was significantly reduced, it still exceeded the output from neutrophils suspended in albumin by a similar proportion as when the experiments were performed on normal microplates. It was therefore unsurprising that AGEs were also unable to enhance the adhesion of neutrophils to the plastic media when examined in 6-plate wells. A robust technique was used to determine neutrophil adhesion, utilising the fluorescence of lysed, adherent cells that had been pre-loaded with the fluorescent indicator BCECF. This technique was preferred in order to rule out potential bias caused by direct visualisation of cells on high-powered field. From these results, it can be concluded that although degranulation and production of oxygen radicals by neutrophils is largely dependent upon adherence to a surface following stimulation, such an adhesion process is certainly not employed by AGEs as a mechanism for enhancing ROS productivity in stimulated neutrophils.

Re-organisation of the cytoskeleton is one process that takes place when neutrophils change from a mobile to adherent state, and though this aspect of cytoskeletal activity evidently does not alter in response to AGE-stimulation, there are other aspects of cytoskeleton functioning that merit investigation in response to AGEs. With reference to the NADPH oxidase, it is conceivable that the actin cytoskeleton facilitates the intracellular traffic of enzymes and oxidase

components. Attempts were made to block the action of Rho-Kinase, an important regulator of the actin cytoskeleton by the use of its specific inhibitor Y-27632. This inhibitor retards the transendothelial migration of neutrophils and along with such decrease in physical motility, markers of migrational activity such as myosin light chain phosphorylation, actin polymerisation and myosin-II filament formation are reduced.[288] More pertinent to the functioning of the NADPH oxidase, Y-27632 has also been shown to reduce phorbol ester-dependent superoxide output in a dose-dependent manner.[289] In the current studies however, Y-27632 did not greatly inhibit ROS output following neutrophil stimulation, either mechanically or with fMLP, let alone retard the AGE-enhancing effect on stimulated NADPH oxidase output. In fact at lower doses there was an apparent facilitation of ROS output. This did not achieve significance for the cells stimulated mechanically, but following fMLP-stimulation there was a significant increase in ROS output in the presence of very low dose Y-27632 (100nM) from both the AGE-activated and control neutrophils. Such an enhancing effect required pre-incubation with Y-27632 and was additive to the co-agonist effect of AGEs. Y-27632 therefore appears to possess properties compatible with a neutrophil priming agent, which is an unexpected incidental finding and of no direct relevance to the current studies.

In conclusion, a wide range of intracellular signalling systems were examined in order to further detail the mechanism of AGE-augmentation of the neutrophil respiratory burst. A number of factors, ranging from biological to technical, may

account for the findings that the vast majority of these systems were inconsequential to this process:

a) The signalling pathways studied were either those that have been classically described in the traditional agonist-activation of neutrophils or at least those which have been implicated in priming processes. However, AGE co-agonism may involve novel, as yet unidentified pathways of activation.

b) The road to activation is unlikely to be discrete and could involve multiple pathways that are interconnected. Thus although PLD activates p38MAPK[279] the activation of PLD itself might involve p44MAPK.[278] Therefore the inhibition of one pathway may cause cross-over inhibition of other systems, having far reaching effects on cell function. As such, the use of inhibitors may not always elucidate pathways of action and this is heightened by the fact that some kinases have multiple isoenzymes, each with a slightly different specificity of action and different susceptibilities to the various inhibitors, further rendering the use of inhibitors difficult to interpret.

c) Further adding to complexity, multiple parallel pathways may exist that are capable of regulating cell activation. Blocking one pathway may simply result in compensation by alternative systems, so that no net effect is noted. Such parallel functioning has been seen previously with MAPK and SAPK. Inhibition of the two together results in an additive reduction in ROS output from phagocytes.[272] Alternative evidence for the plurality of pathways is

demonstrated in the context of the rho-GTPase, *rac2*. In murine neutrophils, *rac2* is important for phosphorylation of p38- and p44MAPK, with reduced phosphorylation of these moieties in homozygous null *rac* mutants. The latter also manifest greatly reduced superoxide production. However, inhibition of either p38- or p44MAPK results in only a modest reduction of superoxide production, implying that overall *rac2* may have an important role in regulating NADPH oxidase activation but that its effects may be exerted through multiple downstream pathways some of which are independent of MAPKs.[290]

Such a proposal for multiple intracellular signalling mechanisms is not novel but has also been implicated in priming phenomena. In the latter it has been proposed that calcium-activated systems may run parallel with tyrosine kinase mediated systems leading to a 'crosstalk' model for priming. Cellular agonists may trigger calcium signalling and activation of serine/threonine kinases such as PKC or the calmodulin-dependent kinases, leading in turn to the activation of MAPK or another serine kinase. The parallel pathway might run from the activation of tyrosine kinases through to MAPK/serine kinase activation and the two might converge upon $p47^{phox}$. The presence of up to eight different serine residues on $p47^{phox}$ and their differing susceptibility to various kinases makes this an attractive hypothesis.[150]

d) The potential for inter-species variation and specificity of the different kinases may also play a role in the different effects observed between the current study and previous work which may have been undertaken on a number of

non-primate species and differing leucocyte sub-types. A species-dependence has been demonstrated for p38MAPK in mediating priming responses to TNF-α, PAF, GM-CSF and the subsequent activation by phorbol esters or fMLP.[291] Even with conserved signalling motifs such as calcium, variation exists, so that in bovine neutrophils, BAPTA is a much more potent inhibitor of ROS output following phorbol ester stimulation compared to EGTA, a situation which is reversed in human phagocytes.[292]

4.6 Future therapeutic strategies

Elucidating the pathways leading from the interaction of AGEs with neutrophils to the excess production of ROS upon cell stimulation may allow for pharmacological intervention and manipulation of the components of this mechanism with the aim of therapeutically reducing oxidative stress in the clinical scenarios associated with excess AGE accumulation such as diabetes mellitus, ageing and renal failure.

The current information has led to the identification of two potential targets for therapeutic intervention, namely the inhibition of $cPLA_2$ and the prevention of intracellular redox signalling that is triggered by AGE-stimulation. In vitro these have been shown to attenuate the upregulation of stimulated NADPH oxidase activity that is seen with exposure to AGEs. Whilst there may be potential in exploring the effects of inhibiting SAPK activity, further work is required to determine the strength of association between MAP Kinases and the AGE-augmented neutrophil respiratory burst.

Of the two $cPLA_2$ inhibitors used in the current study, mepacrine is already licensed for use in the treatment of protozoal infections but has too unfavourable a side-effect profile to be used on a long-term basis which is what would be required in the context of reducing AGE-induced oxidative stress. Its side effect profile includes potentially serious neurological sequelae, hepatitis and bone marrow failure.[293] Its use in the elderly is also cautioned[293] and this group is one of the main targets of therapies against AGE-mediated signalling processes. The other $cPLA_2$ inhibitor, MAFP, is also likely to have significant toxicity and has yet to be evaluated in human studies.

The greatest cause for optimism surrounds the goal of modifying the intracellular redox state, which appears highly relevant to transduction of the AGE signals per se. This has been exemplified by previous analyses of RAGE-receptor ligation and the subsequent activation of MAP Kinases[112] but also in the current study where the intracellular glutathione precursor N-acetylcysteine was able to diminish the amplification of stimulated ROS output induced by AGEs. Oxidant stress therefore has a two-fold contribution to the pathogenesis of vascular disease, both through previously mentioned effects on plaque formation/rupture and alteration of vascular tone and secondly through intracellular signalling, the latter of which demands only trace levels for effect. The expectation has been that by reducing oxidative stress, principally by the administration of exogenous antioxidants, adverse cardiovascular outcomes might be prevented. However, trials on this subject have provided mixed outcomes. The prospective,

randomised and double-blinded CHAOS study[294] was a secondary prevention study looking at cardiovascular outcomes in patients administered with relatively high doses of α-tocopherol. There was a large 80% reduction in subsequent rates of non-fatal myocardial infarction, but this result has not been replicated in other studies, notably the only other large secondary prevention study (GISSI) [295] and a major primary prevention study (HOPE).[296] It may be that the effects are more marked in secondary as opposed to primary prevention due to ambient oxidative stress levels and the dose of α-tocopherol was also higher in the CHAOS than in the GISSI trial. Meanwhile, the SPACE trial,[297] conducted in patients with end-stage renal failure, found α-tocopherol to be beneficial in opposing adverse cardiovascular outcomes. The uraemic patients included in this trial have consequently very high levels of oxidative stress and possess high cardiovascular risk, factors which may be important to the positive outcome of the study.

The lack of a consistent benefit with anti-oxidant therapy in the clinical trials may thus be partly due to inadequate plasma levels of anti-oxidants in some studies. Nonetheless, the current data, which included a variable dose range of free radical scavengers, also did not demonstrate any attenuation of AGE-augmented neutrophil ROS production. It may be that in attempting to attenuate final cellular ROS output, the levels of ROS generated exceed the potential for scavenging by traditional doses of antioxidants. The alternative therefore lies in preventing the final ROS production from the NADPH oxidase in the first place. Redox signalling appears to be one mechanism contributing to this process and is amenable to

intervention by the use of an intracellular scavenger; it is for this reason that N-acetylcysteine is probably more effective in vitro than the other scavengers in reducing the AGE-induced amplification of ROS generation.

Longer term therapeutic strategies might focus on the retardation of AGE accumulation per se. Apart from enhancing neutrophil oxidative stress AGEs have been seen to evoke other unfavourable vascular responses. A Glycation model for ageing has already been proposed[298] and its central tenet may also apply to diabetic and uraemic processes. Caloric restriction, a proven method of life-prolongation in lower species, has a number of attractive explanations for its effect, with one aspect concerning the reduction in glycation processes that occur in such animals. Further work is required to explore the effect of reducing AGE accumulation, either through caloric restriction or through novel AGE crosslink-breakers, on the reduction in processes such as oxidant stress and vascular ischaemic outcomes. There is also scope for exploring the role of N-acetylcysteine in reducing vascular events in individuals with diabetes, uraemia or in an older population per se. It is a relatively well tolerated agent, and though judging from the in-vitro data it will require relatively high oral dosing, it certainly has the potential for benefit in the high risk vascular patient.

In summary, the current studies have led to a further understanding of mechanisms by which the accumulation of AGEs in certain clinical states may contribute to abnormal vascular processes, predominantly through the co-agonist stimulation of neutrophil NADPH oxidase, a process which can be measured

directly and in real time by chemiluminescent techniques. Future hope for ameliorating this process appears to best lie with the attenuation of intracellular oxidative signalling that is contingent upon the interaction of AGEs with neutrophils. It would remain to be seen from clinical trials however, whether such therapy actually impacted upon cardiovascular outcomes in subjects prone to high AGE accumulation such as diabetic and uraemic individuals and the older population in general.

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APPENDIX

Publications arising from this thesis:

Augmentation of the Neutrophil Respiratory Burst through the Action of Advanced Glycation Endproducts – A Potential Contributor to Vascular Oxidant Stress.
Richard K.M. Wong, Andrew I. Pettit, Joan E. Davies, Leong L. Ng. **Diabetes** Sep.2002; 51(9):2846-53

Advanced glycation endproducts stimulate an enhanced neutrophil respiratory burst mediated through the activation of cytosolic phospholipase A2 and generation of arachidonic acid.
Richard K.M. Wong, Andrew I. Pettit, Paulene A. Quinn et al. **Circulation** Oct 2003; 108(15):1858-64

Increased free radical production in hypertension due to increased expression of the NADPH oxidase subunit $p22^{phox}$ in lymphoblast cell lines.
Andrew I. Pettit, Richard K.M Wong, Virginia Lee, et al. **Journal of Hypertension** Apr.2002; 20 (4):677-83

Augmentation of the Neutrophil Respiratory Burst Through the Action of Advanced Glycation End Products

A Potential Contributor to Vascular Oxidant Stress

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An accelerated accumulation of advanced glycation end products (AGEs) occurs in diabetes secondary to the increased glycemic burden. In this study, we investigated the contribution of AGEs to intravascular oxidant stress by examining their action on the neutrophil burst of reactive oxygen species (ROS); this may be a significant donor to the overall vascular redox status and to vasculopathy. AGEs exerted a dose-dependent enhancement on the neutrophil respiratory burst in response to a secondary mechanical stimulus (up to 265 ± 42%, $P = 0.022$) or chemical stimulation with formyl-methylleucylphenylalanine 100 nmol/l (up to 218 ± 19%, $P < 0.001$), although they possessed no ability to augment the neutrophil respiratory burst alone. This phenomenon was both immediate and reversible and depended on the simultaneous presence of AGEs with the additional stimulus. It appeared to work through an upregulation of the neutrophil NADPH oxidase, the enzyme responsible for ROS generation, as seen by a diphenyleneiodonium-dependent suppression of basal and augmented ROS output. Moreover, this action of AGEs was found to be complementary to that of neutrophil priming agents, also known to upregulate neutrophil ROS production, implying the presence of distinct intracellular transduction pathways mediating the effect of these two classes of agents. *Diabetes* 51:2846–2853, 2002

Advanced glycation end products (AGEs) are irreversible adducts resulting from the nonenzymatic reaction of reducing sugars with the amino groups of proteins. Their formation is partly dependent on prevailing sugar and protein concentrations, accounting for their increased accumulation in patients with diabetes, in whom they have been implicated as mediators of a spectrum of pathologies (1,2). This may relate to their ability to covalently cross-link proteins (3) causing structural changes to tissues, but there has also been realization that AGEs are able to effect a host of direct cellular responses through interaction with cellular receptors recognizing AGE ligands, of which the receptor for AGE (RAGE) is the most well characterized (2). Induced responses may include cytokine induction, adhesion molecule expression, smooth muscle and fibroblast proliferation, and chemoattraction of inflammatory cells, which may influence vascular tissue remodeling (1,2).

Another key to the progression of much AGE-related pathology may be via induction of oxidative stress, reflecting an excess production of highly reactive oxygen species (ROS), including free radicals and peroxides, compared with countering antioxidant defenses. Indeed, oxidative stress has been reported in the context of diabetes (4), whereas RAGE ligation by AGEs has been shown to deplete intracellular antioxidant defenses (5), and extraneous addition of antioxidant compounds may ameliorate the downstream effects of AGE/RAGE interaction (6,7).

Free radical production is an important component of an organism's defense against microbial invasion, but inappropriate production may have detrimental sequelae, resulting in molecular and tissue damage (8,9). Additionally, ROS have been recognized to possess messenger roles themselves within certain biological pathways (10). Their function within such pathways may again result in deleterious cellular responses when overactivated. Through such mechanisms, AGEs and oxidative stress may contribute to a range of pathological phenomena, which may be especially pertinent to the pathogenesis of macrovascular disease.

The possibility of directly visualizing cellular ROS production resulting from AGE exposure in real time was investigated in this study. Neutrophils are one of the main producers of ROS within the vascular compartment, and their main pathway to ROS production involves the NADPH oxidase. This enzyme acts through the one-electron reduction of molecular oxygen to form superoxide ($O_2^{\cdot-}$) (11), which in turn undergoes dismutation by superoxide dismutase (12), thus generating hydrogen peroxide as well. In the current study, we have investigated the effect of AGEs on neutrophil ROS generation in the

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AGE, advanced glycation end product; DPI, diphenyleneiodonium; fMLP, formyl-methylleucylphenylalanine; HBSS, Hank's balanced salt solution; HSA, human serum albumin; MM-albumin, minimally modified albumin, PAF, platelet activating factor; RAGE, receptor for AGE; RLU, relative light unit; ROS, reactive oxygen species; TC199, tissue culture media 199; TNF-α, tumor necrosis factor-α.

context of a potential contribution to the vascular oxidative burden.

RESEARCH DESIGN AND METHODS

Materials. Bis-*N*-methylacridinium nitrate (Lucigenin), diphenyleneiodonium (DPI), formyl-methylleucylphenylalanine (fMLP), glucose, platelet activating factor (PAF), percoll, tissue culture media 199 (TC199), and tumor necrosis factor-α (TNFα human recombinant) were all purchased from Sigma-Aldrich, and 20% endotoxin-free human serum albumin (HSA) was obtained from BioProducts Laboratory.

Preparation of AGE-albumin. AGE-albumin and minimally modified albumin (MM-albumin) were prepared by preincubation of endotoxin-free HSA (20%) with 1 or 30 mmol/l glucose, respectively, at 37° for 12 weeks in 100 mmol/l phosphate (pH 7.4). At the end of the incubation, AGE-albumin/MM-albumin were dialysed against PBS for 24 h and 0.9% sodium chloride for 12 h. Nonglycated HSA was used as a negative control.

Neutrophil isolation. Neutrophils were prepared according to the method of Baron et al. (13). After informed consent was received, 20 ml fresh citrated blood was obtained from healthy adult volunteers, added to Dextran solution (300 mg in 5 ml Hank's balanced salt solution [HBSS] containing 140 mmol/l NaCl, 4 mmol/l $NaHCO_3$, 0.3 mmol/l Na_2HPO_4 [anhydrous], 5 mmol/l KCl, 0.4 mmol/l KH_2PO_4 [anhydrous], 5.5 mmol/l glucose, 0.4 mmol/l $MgSO_4$, and 4.7 mmol/l H_2O), and allowed to sediment for 30 min. Plasma (containing platelets and leukocytes) was removed and centrifuged for 10 min at 482*g*. The cell pellet was vortexed with dilute HBSS for 10 s to lyse contaminating erythrocytes, and then osmolarity was restored by the addition of excess HBSS. A mixed leukocyte pellet was then obtained by further centrifugation at 482*g* for 10 min and layered onto a prepared continuous density gradient of Percoll (65% Percoll, 10% 10 × TC199, 1.6% 1 mol/l HEPES, 0.4% 5 mol/l NaCl, and 23% sterile water, pH 7.4, and centrifuged at 21,982*g* for 15 min at 4°). The neutrophil band was recovered, washed in TC199, and incubated at 37° for 20 min before use. The viability of neutrophils isolated by this method was 95%, as determined by the trypan blue dye exclusion test.

Detection of ROS. ROS detection was based on the chemiluminescent technique described by Liu et al. (14), with chemiluminescence recorded on an EG&G Berthold microplate luminometer LB96V. All experiments were performed at 37°. Lucigenin (50 μmol/l) was made up in a balanced salt solution containing 140 mmol/l NaCl, 15 mmol/l HEPES, 5 mmol/l KCl, 5 mmol/l glucose, 1.8 mmol/l $CaCl_2.2H_2$ 0, and 0.8 mmol/l $MgSO_4.7H_2O$ (pH 7.4). All experiments were performed in triplicate with 10^6 cells per well.

Neutrophil stimulation. Mechanical stimulation of neutrophils was by multiple passages through a standard 1-ml pipette tip. The number and intensity of passages were equal for each experiment, although it was found that with more than one passage, the same amplitude of neutrophil ROS production was induced whatever the actual number or intensity of passages (data not shown). Such a plateau effect of shear stress on neutrophil activation parameters has been previously observed (15) when neutrophils were subject to a certain range of shear stresses. The chemical stimulus used in this study was fMLP (100 nmol/l), a bacterial cell-wall peptide that activates neutrophils via specific G-protein–coupled cell-surface receptors (16).

Coating of microplate wells. Microlite-2 wells (Dynex) were filled with 100 μl PBS containing 500 μg AGE-albumin/albumin, covered, and left overnight on a plate shaker. They were then washed three times with PBS and left to dry before use.

Data analysis. Chemiluminescent values are presented as relative light units (RLUs) per minute and means ± SE. For the analysis of total ROS production (RLU), we calculated the area under the curve (cut off at 6 min). We used two-tailed *t* tests for comparisons of two groups and one-way ANOVA with Tukey's post hoc analysis for between-group analyses involving more than two groups (SPSS).

RESULTS

AGE-albumin cooperates with a secondary stimulus resulting in enhancement of the neutrophil respiratory burst. The direct effect of AGEs on neutrophil ROS production was assessed through the addition of AGE-albumin or albumin control (50–800 μg/ml) to 5 × 10^5 neutrophils, which had been allowed to settle onto microplate wells. The subsequent integrated ROS production over 15 min was measured and compared with baseline ROS production (also over 15 min), as measured by Lucigenin-enhanced chemiluminescence. Although addition of both AGE-albumin and albumin resulted in slight increases in neutrophil ROS production over basal levels, there was no significant difference between them (comparing the two groups over the given dose range, P = 0.774).

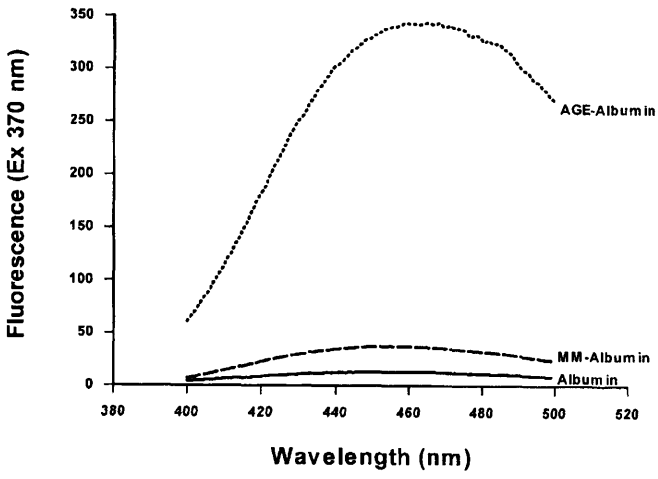

FIG. 1. Fluorescence spectra of AGE-albumin, MM-albumin, and albumin. The excitation wavelength is 370 nm.

However, neutrophils subjected to a secondary stimulus (mechanical or chemical) in the presence of AGE-albumin responded with a respiratory burst that was significantly enhanced compared with activation in the presence of control albumin, with the magnitude of enhancement dependent on the AGE concentration. Both mechanical and fMLP stimulation resulted in a two-phase neutrophil ROS burst, a primary peak, and a secondary trough phase, with AGE-albumin able to enhance both of these aspects. The intensity of the fluorescence spectra of the albumin and AGE-modified albumin samples used in this study give some quantitative idea to the relative degrees of AGE modification of these entities (Fig. 1). MM-albumin was able to augment neutrophil ROS release with much reduced gain compared with AGE-albumin (Fig. 2*A* and *B*). At a concentration of 400 μg/ml (the optimum dose for effect of MM-albumin), AGE-albumin and MM-albumin enhanced peak ROS production in response to mechanical stimulation by an average of 362% (P < 0.001) and 157% (P = 0.011), respectively, and total ROS production by an average of 282% (P < 0.001) and 169% (P = 0.009), respectively (P < 0.001 between groups in both cases) (Fig. 2*C* and *D*). With fMLP stimulation, AGE-albumin and MM-albumin increased peak ROS production by an average of 177% (P < 0.001) and 119% (P = 0.021), respectively, but only AGE-albumin enhanced total ROS production (by an average of 145%, P < 0.001).

Even within each class of AGE modification there existed a dose-response relationship. For AGE-albumin, optimum responses were achieved at concentrations of AGE-albumin ≥200 μg/ml for both mechanical and fMLP stimulation (Fig. 3*A* and *B*) (for both forms of stimulation, P < 0.001 between groups). At progressively higher doses of AGE-albumin, this relative difference was maintained, although the absolute chemiluminescent intensity from the samples diminished, probably due to impairment of light transmission by higher protein concentrations (17).

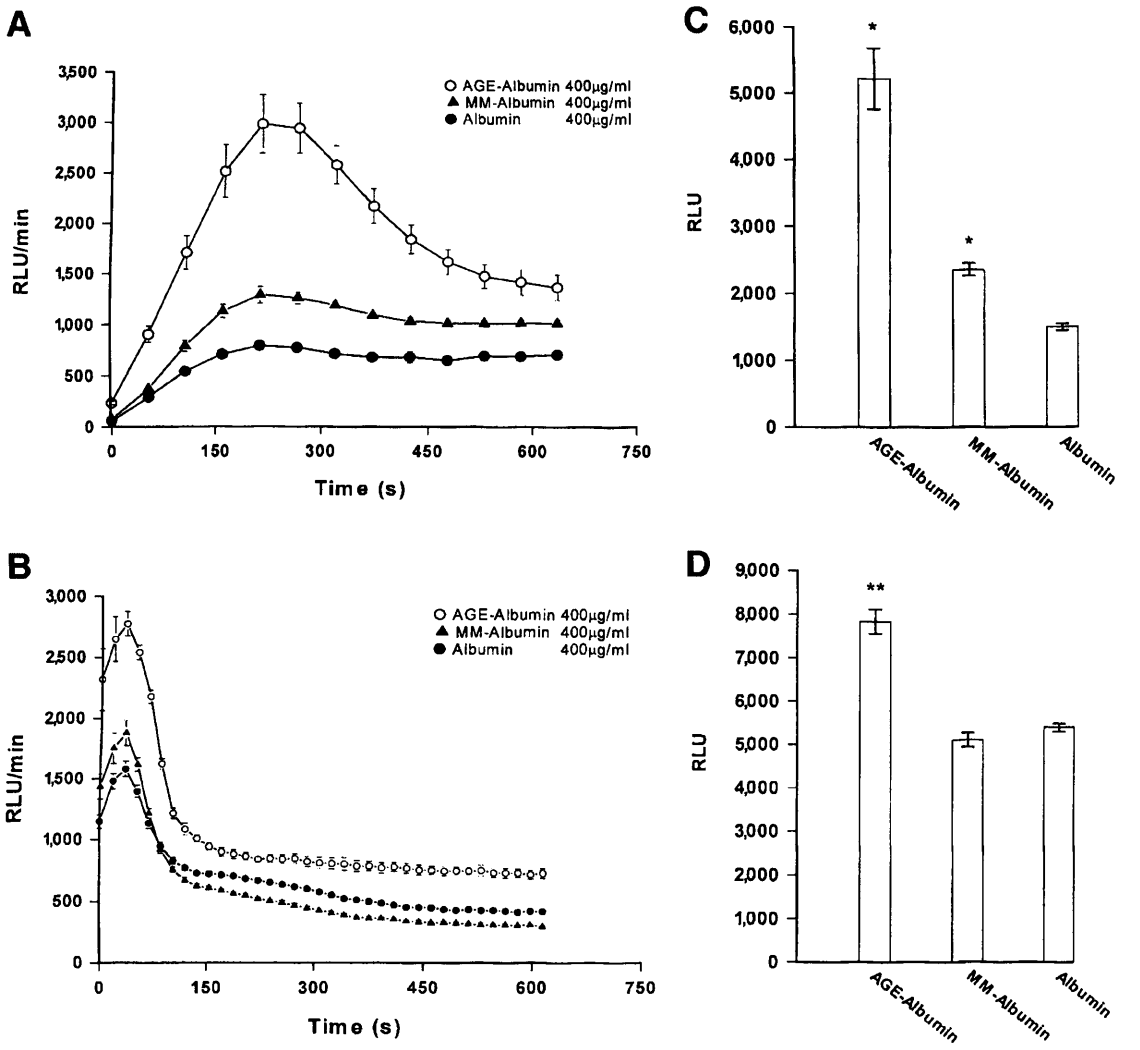

FIG. 2. Acute neutrophil ROS production as detected by lucigenin-enhanced chemiluminescence in the presence of AGE-albumin, MM-albumin, or albumin, all at 400 µg/ml. *A*: Response to a mechanical stimulus ($P < 0.001$ between groups by ANOVA). *B*: Response to stimulation with fMLP ($P < 0.001$ between groups by ANOVA). *C*: Total ROS production after mechanical stimulation ($P < 0.001$ between groups by ANOVA). For comparisons with control, *$P < 0.001$ and **$P = 0.009$ (post hoc Tukey's analysis). *D*: Total ROS production after stimulation with fMLP ($P < 0.001$ between groups by ANOVA). For comparisons with control, **$P < 0.001$ (post hoc Tukey's analysis between groups). Results are representative of multiple experiments and each result represents the mean of three separate aliquots of 10^5 cells recorded simultaneously. Where error bars are not seen, SE is less than the size of the symbol.

The dose response for MM-albumin was much less marked, although still significant for both forms of stimulation (Fig. 3*C* and *D*) (for both, $P < 0.001$ between groups). There was little response below a concentration of 400 µg/ml of albumin, but at higher concentrations (800 µg/ml and above) it was also not possible to detect any small differences in ROS production due to the increasing chemiluminescent quenching caused by the high protein load (Fig. 3*C* and *D*). There appeared to be no requirement for significant preincubation of neutrophils with AGE-albumin to elicit this response; immediate suspension of cells in AGE-albumin before stimulation was as effective as preincubation with AGE-albumin for longer periods (data not shown).

We also investigated the effect of temporally reversing the two processes of AGE-albumin addition and mechanical stimulation. When neutrophils had been mechanically stimulated first, before the subsequent addition of AGE-albumin or control, there was no differential enhancement of ROS generation (total or peak) in the cells exposed to AGE-albumin (Fig. 4*A*) ($P = 0.603$ comparing total ROS production). Likewise, mechanically stimulating neutrophils and then releasing them onto AGE-albumin or albumin-coated microplate wells also did not result in any major difference in neutrophil ROS production (total or peak) (Fig. 4*B*) ($P = 0.319$ comparing total ROS production). Therefore, there appears to be a prerequisite for the presence of AGEs in the immediate neutrophil milieu, immediately before or simultaneously with the mechanical stimulus so that augmentation of the neutrophil ROS burst may take place.

It is well recognized that the cellular enzyme NADPH oxidase is responsible for phagocytic ROS production (12), and it could be inferred that any increase in neutrophil ROS output is attributable to increased NADPH oxidase activity. As confirmation, neutrophils were

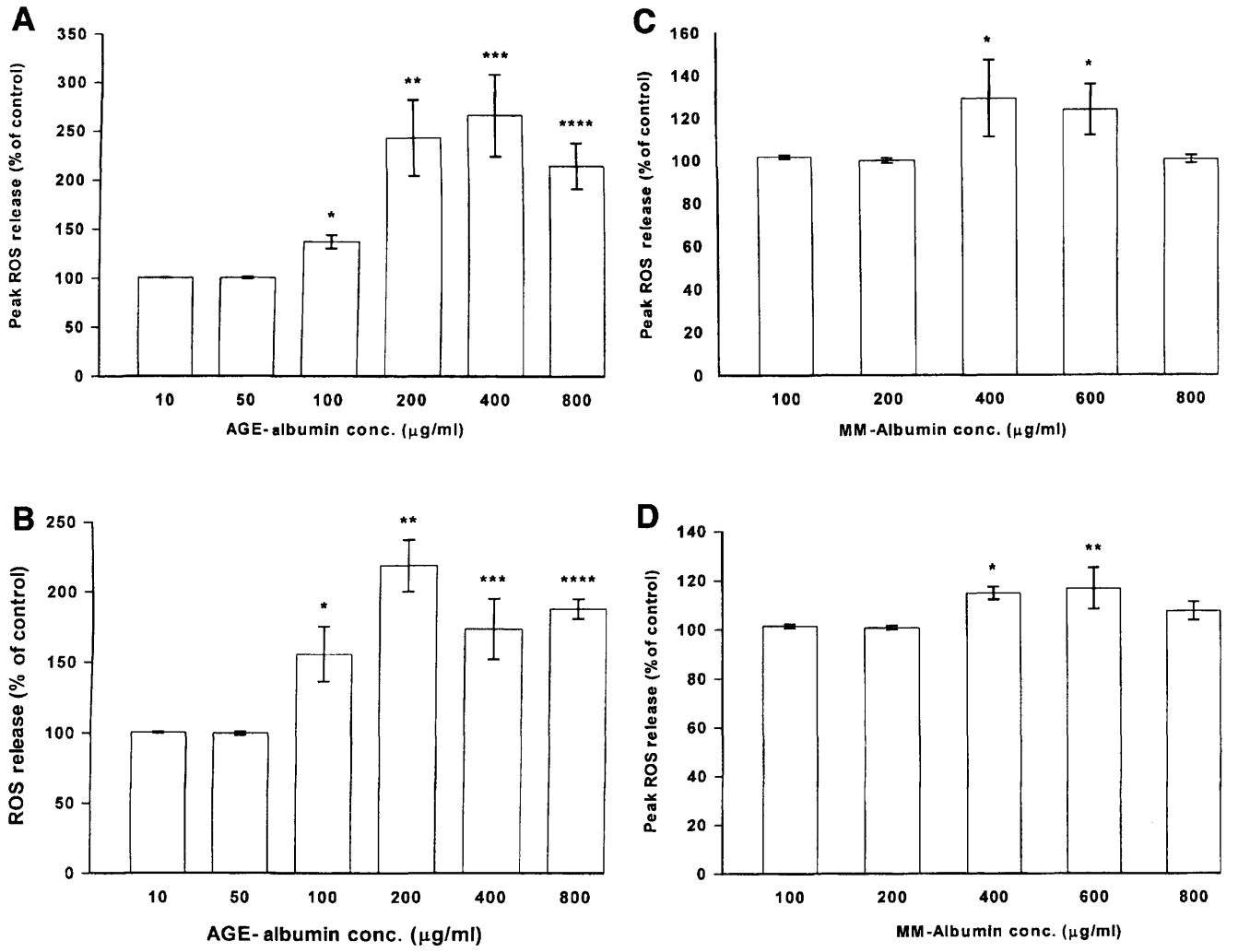

FIG. 3. The dose response of AGE-modified albumin on its relative enhancement of the peak neutrophil ROS release over control. *A*: Peak ROS release after mechanical stimulation in the presence of varying doses of AGE-albumin. $P < 0.001$ by ANOVA between groups. With post hoc Tukey's analysis comparing with control, *$P = 0.047$, **$P = 0.013$, ***$P = 0.022$, and ****$P = 0.002$. *B*: Peak ROS release after stimulation with fMLP in the presence of varying concentrations of AGE-albumin. $P < 0.001$ by ANOVA between groups. With post hoc Tukey's analysis comparing with control, *$P = 0.03$, **$P < 0.001$, ***$P = 0.003$, and ****$P = 0.032$. *C*: Peak ROS release after mechanical stimulation in the presence of varying doses of MM-albumin. $P < 0.001$ by ANOVA between groups, with post hoc Tukey's analysis comparing with control, *$P < 0.001$. *D*: Peak ROS release after stimulation with fMLP in the presence of varying concentrations of MM-albumin. $P < 0.001$ by ANOVA between groups. With post hoc Tukey's analysis comparing with control, *$P = 0.009$ and **$P < 0.001$. The results for each dose are the mean results from 10 different sets of neutrophils.

preincubated with the flavoprotein inhibitor DPI (18) for 30 min and at various concentrations before the addition of AGE-albumin/albumin (200 μg/ml) and application of a mechanical or chemical stimulus, all in the continuing presence of DPI. The result was a dose-dependent abrogation of ROS release, albeit maintaining the relative differential production of ROS between experimental and control groups (Fig. 5*A* and *B*) (for both, $P < 0.001$ between groups). In contrast, the specific mitochondrial flavoprotein inhibitor rotenone (100 nmol/l, 10 μmol/l) had little effect on neutrophil ROS output in this situation (data not shown). Therefore, it would appear that the action of AGEs on neutrophils requires a functioning NADPH oxidase and that the most likely mechanism of action involves an upregulation of the activity of the NADPH oxidase.

The effect of AGE-albumin on the neutrophil respiratory burst is a reversible process. We examined the reversibility of the AGE effect on neutrophils as an extension of the notion that AGEs must be present simultaneously with another stimulus to generate enhanced neutrophil ROS production. Neutrophils were incubated in AGE-albumin or control albumin (both 200 μg/ml) for a period of 30 min before being washed and then activated immediately with either mechanical or chemical (fMLP) stimuli. With both stimuli, the differential enhancement of ROS production previously induced by AGE-albumin was abrogated ($P = 0.71$ and $P = 0.12$, respectively, for total ROS production; data not shown). It is therefore apparent that the effect of AGE on neutrophils is a reversible phenomenon with no imprinting of any previous interaction on cell memory and response.

The effect of AGE-albumin is complementary to the effect of neutrophil priming agents in enhancing neutrophil respiratory burst capacity. A variety of

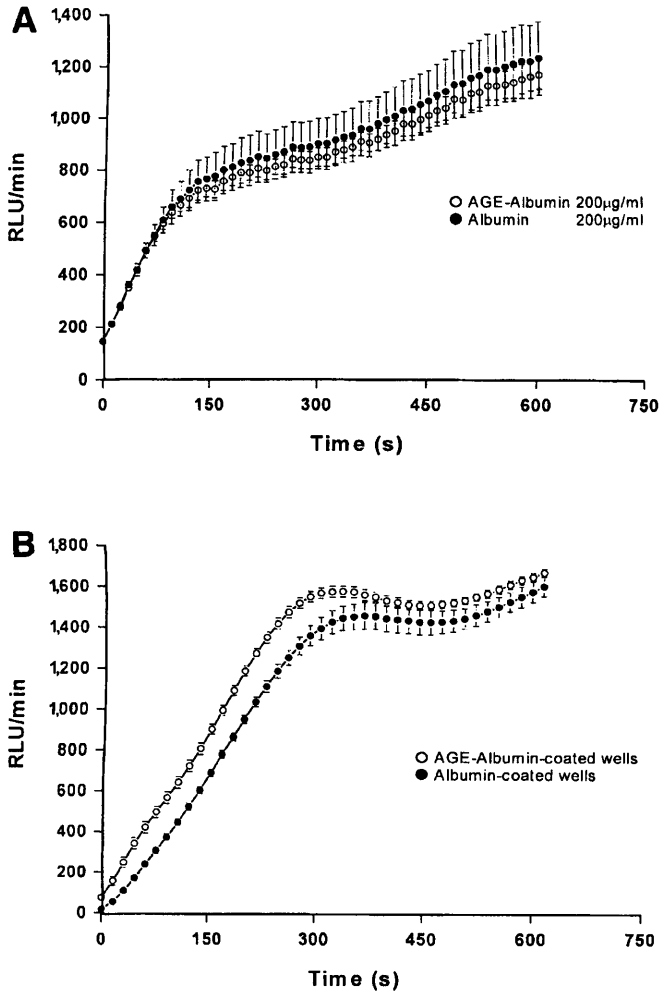

FIG. 4. Acute neutrophil ROS production detected by lucigenin-enhanced chemiluminescence when subjected to a reversal in the order of AGE-albumin addition and mechanical stimulation. *A*: Neutrophils were subjected to a mechanical stimulus first, before subsequent addition of AGE-albumin or albumin. *B*: Neutrophils were mechanically stimulated and then released onto microplate wells coated with AGE-albumin or albumin. Results are representative of multiple experiments, and each result is the mean of three aliquots of 10^5 cells recorded simultaneously. Where error bars are not seen, SE is less than the size of the symbol.

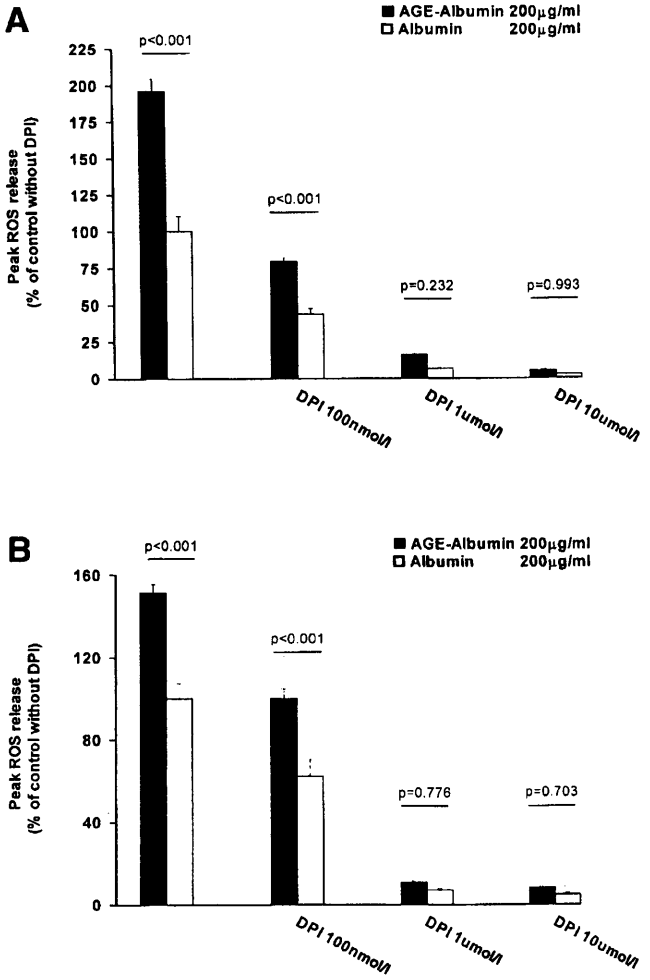

FIG. 5. Peak neutrophil ROS release with exposure to AGE-albumin or control albumin under conditions of increasing DPI concentration. *A*: ROS release after mechanical stimulation ($P < 0.001$ between groups by ANOVA). *B*: ROS release after stimulation with fMLP ($P < 0.001$ between groups by ANOVA). Peak ROS release is expressed as a percentage of peak ROS release with albumin control and without addition of DPI.

other agents have also been documented to possess the capacity for increasing neutrophil ROS production. One such group of agents, collectively termed "neutrophil priming agents" (19) and including two well-studied agents, TNF-α and PAF, differs from AGE-albumin in requiring significant preincubation time with cells in order to achieve an effect. In investigating the possibility of shared cellular upregulation mechanisms between AGEs and priming agents, we studied the effects of combined exposure to these agents. Neutrophils were preincubated with TNF-α, PAF, or control vehicle for the required priming time (30 min) with the subsequent addition of AGE-albumin or albumin (both at 200 μg/ml) and further stimulation of the cells either mechanically or by fMLP (Fig. 6*A–D*). Both TNF-α and PAF appeared to exert even more potent augmentation effects on the neutrophil respiratory burst than AGE-albumin alone, and the effects appeared to be complementary when AGE-albumin was combined with them. For mechanical stimulation, there was an ~10-fold increase in the absolute values of total and peak ROS production in both AGE-albumin and albumin groups when TNF-α was added (data not shown), generally maintaining the differential increase in ROS production between the AGE-albumin and albumin groups at 150–200%, whether assessed by peak or total ROS production (Fig. 6*A*). With a combination of TNF-α priming and fMLP stimulation, there was an ~6-fold increase in peak ROS and an 11-fold increase in total ROS production (data not shown), with a maintenance of the differential increase in ROS production (peak or total) between 150 and 160% (Fig. 6*B*).

PAF priming led to a lesser increase in ROS production than TNF-α. Allied to mechanical stimulation, there was an approximate twofold increase in peak ROS and a 1.5-fold increase in total ROS production (data not shown), maintaining a 200–230% differential increase (in peak or total ROS production) in AGE-albumin compared with albumin

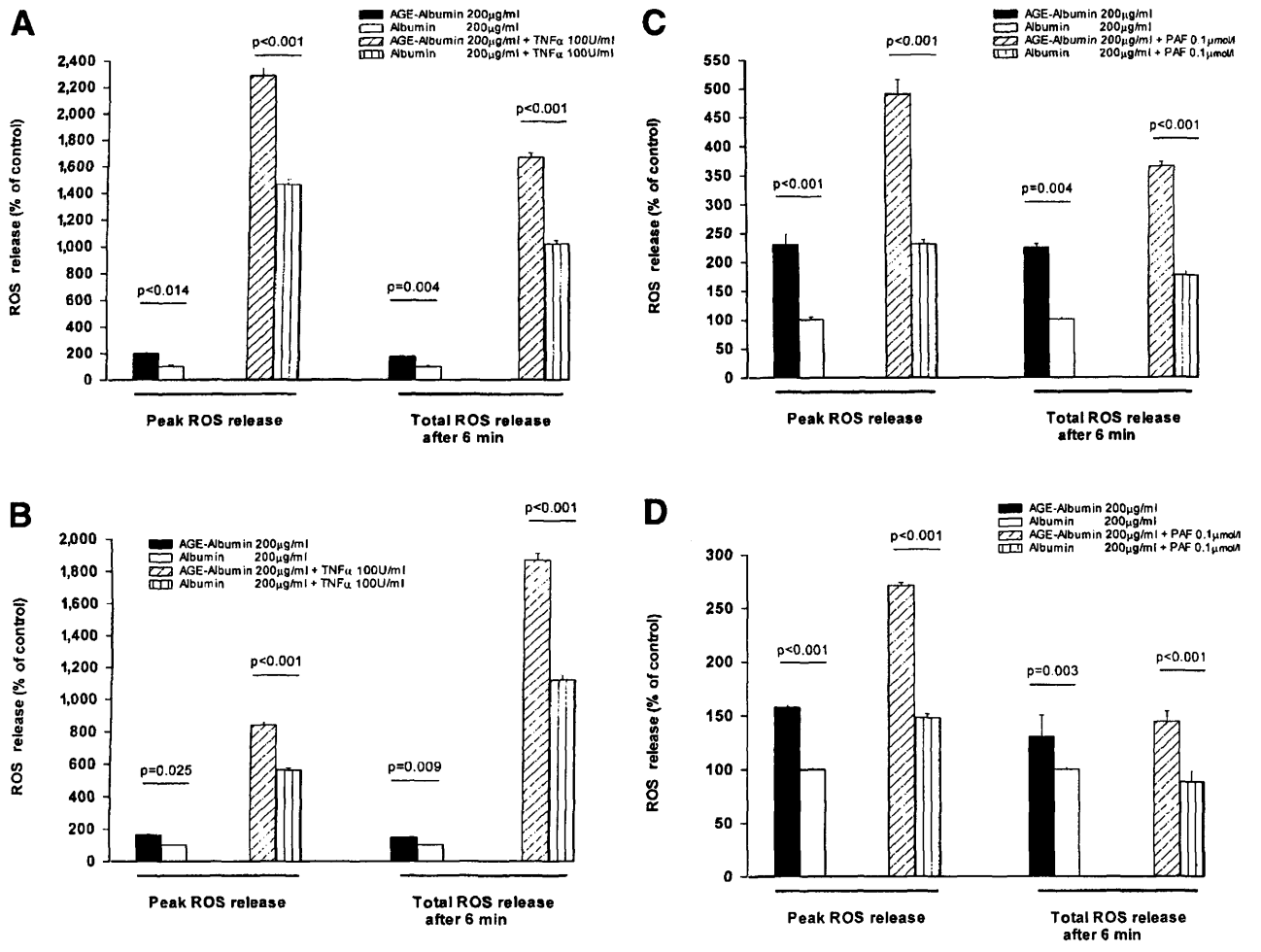

FIG. 6. AGE-albumin complements both TNF-α and PAF to further augment neutrophil ROS production. Neutrophils were preincubated with TNF-α (100 units/ml), PAF (0.1 µmol/l), or DMSO vehicle for 30 min before addition of AGE-albumin or albumin (both at 200 µg/ml). They were then subjected to a mechanical stimulus or activation by fMLP in the continuing presence of TNF-α (*A* and *B*, respectively) or PAF (*C* and *D*, respectively). Each recording is the mean of three separate aliquots of 10^5 cells measured simultaneously, and the results are representative of multiple experiments. For *A–D*, $P < 0.001$ between groups by ANOVA.

groups (Fig. 6*C*). With fMLP stimulation, there was only a 1.5-fold increase in peak ROS production but no real change in total ROS production, principally caused by lower secondary trough phase ROS production in the PAF groups, negating the effect of the higher peak ROS values in these groups (data not shown). There was nonetheless still a differential increase in peak and total ROS production to the level of 130–180% in the AGE-albumin compared with albumin groups (Fig. 6*D*).

DISCUSSION

Premature macrovascular disease is a prevalent accompanying pathology in diabetes (20) and in renal failure (21), with the increased rate of accumulation of AGEs in these conditions recognized as a potential contributor to this pathology (22). Oxidative stress may play a vasculopathic role (23) by neutralization of the natural vasodilator nitric oxide (NO) (8) and augmentation of plaque development through LDL oxidation, rendering it more susceptible to uptake by scavenger macrophages (24). The possibility that AGEs may significantly contribute to this vascular oxidative burden has been examined in this study. For ROS detection, we used a chemiluminescent assay in which the reaction of free radical species with a chemical probe (Lucigenin) generates light. The application of this technique to the biological substrates involved in this study has previously been validated (15,25). Moreover, such an assay was pertinent to this situation because of its great sensitivity and ability to allow observation of ROS production in real time.

A state of increased oxidative stress has been reported previously upon ligation of RAGE by AGEs (5–7,26). Most of this evidence has been indirect inference, through observing the activation of intracellular pathways normally regulated by oxidant stress or through the abrogation of AGE/RAGE responses by antioxidants (6,7). There has also been one report of the direct detection of hydrogen peroxide release when AGEs were allowed to interact with the RAGE receptor on endothelial cells. Although peroxide release was a direct response in this case, it occurred as a slow, integrated accumulation measured over a period of 60 min (26).

In the current study, further evidence for AGE-induced oxidant stress has been outlined. As physiological ROS

producers in antimicrobial defense, neutrophils were chosen for investigation because they may also contribute significantly to intravascular oxidant stress. Their role in reperfusion damage after ischemic events is already acknowledged (12). Evidence also suggests that even controlling for other factors, a higher blood leukocyte count and especially the granulocytic component, predicts a greater likelihood of future vascular events. Infection is similarly often found preceding the development of ischemic events (27,28). In comparing neutrophils from healthy individuals with those from patients with stable and unstable coronary artery disease, there exists a gradation of progressive neutrophil activation that may provide a pathophysiological milieu for the progression of stable to unstable coronary artery disease. Activated neutrophils release toxic chemicals including ROS and proteolytic enzymes that damage the endothelium and basement membrane. ROS release also causes lipid oxidation and initiates platelet activation/aggregation; neutrophils may thus be involved in the primary pathogenesis and progression of occlusive vascular disease (27,28).

In this study there was no evidence for a direct, rapid induction of oxidant stress upon the exposure of neutrophils to AGE-albumin. However, the presence of AGEs allowed for a dose-dependent enhancement of the ROS burst imparted by a secondary stimulus, whether it was mechanical or chemical, occurring with rapid kinetics. Allied to the prompt reversibility of this effect on removal of AGEs from the neutrophil milieu, we conclude that AGEs may play an as yet undescribed role as neutrophil "coagonists." As coagonists, AGEs are distinct from priming agents, which also enhance cellular function but require significant interaction time with cells to bring about their response (19). There is in vivo relevance to the coagonist actions of AGEs, because leukocyte passage through the vasculature generates enough shear stress to potentially act as a secondary mechanical agonist (29). In vivo chemical agonists, on the other hand, may be provided by pathogen exposure (fMLP is a bacterial derivative [16]), and some acute vascular events have already been correlated with infective episodes (30).

The complementary effect on ROS production gained by combining AGEs with the previously characterized priming agents PAF and TNF-α illustrates potential in vivo synergism and also further distinguishes AGE coagonist action from that of neutrophil priming agents. Complementarity implies that there are distinct intracellular pathways governing upregulation of the neutrophil response (31), of which the common end point is the activation of the neutrophil NADPH oxidase. That the NADPH oxidase enzyme is central to both the basal production of ROS by neutrophils and the AGE-augmented production of ROS is suggested by the dose-dependent inhibition of ROS output with DPI. This flavoprotein inhibitor is effectively regarded as an NADPH oxidase inhibitor, especially in neutrophils where NADPH oxidase is the dominant flavoprotein (18); moreover, there was a comparative lack of effect of the specific mitochondrial flavoprotein inhibitor rotenone on suppressing this ROS output.

Many candidate pathways exist for mediation of the effect of AGEs on the neutrophil respiratory burst. The key trigger probably involves the interaction of AGEs with a neutrophil transmembrane receptor of which several candidates have been identified (2). Indeed, the presence of RAGE has been recently demonstrated in neutrophils (32). Whatever the subsequent pathway of signal transduction, its end result provides for a mechanism that is able to augment NADPH oxidase activity triggered by a heterogeneous group of stimuli.

The AGE dosages in this study bear similarity to those validated in previous receptor-binding studies (33). Relating these to actual pathological levels in diabetic patients, our unpublished data shows that AGE-albumin doses in this study were ~10-fold greater than the concentration in serum from diabetic patients with macrovascular complications, as assessed by AGE fluorescence. The latter was already threefold greater than serum from healthy control subjects. This difference between in vitro and in vivo intensities does not necessarily render our experimental dosages irrelevant. In vitro pharmacological effects generally occur at greater dosages than are required in vivo, and the difference here is barely one order of magnitude. Additionally, AGE concentrations in vivo are not uniform throughout the body. AGEs are often closely allied to the locations of atherosclerotic lesions (2,34), although whether this is cause or effect has not been definitively answered; indeed both elements may be present. Nevertheless, in the vicinity of atherosclerotic plaques, AGEs may have greater local compared with circulating concentrations resulting in locally enhanced neutrophil ROS production. This may also facilitate localized accelerated AGE formation, thus further perpetuating this cycle (35).

This nonhomogenous distribution of AGEs in the body may account for the apparent paradox between an increase in AGE-mediated neutrophil ROS generation and the observation that diabetic patients, especially those with poor metabolic control, appear to have a greater frequency of infective complications (36). The reversibility of the AGE effect may lead to neutrophils being only transiently in an activated state at sites of heavy AGE accumulation and reverting to a nearer normal state on relocation to other parts of the circulation or nonvascular compartments. Also, hyperglycemia per se is cytotoxic, impeding effective neutrophil functioning (37). Thus, neutrophil function under conditions of acute hyperglycemia differs from that during times when metabolic control is closer to physiological parameters but when the legacy of past metabolic indiscretions has led to accelerated AGE accumulation.

The evidence from this study therefore adds further insight to possible pathogenic effects of AGEs in conditions such as diabetes and uremia. Through their action as unique neutrophil coagonists, it has been demonstrated that AGEs could play a key role in the induction of a state of increased oxidative stress through augmentation of neutrophil ROS production, and this may be in part responsible for the acceleration of vascular disease noted in these conditions.

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Advanced Glycation End Products Stimulate an Enhanced Neutrophil Respiratory Burst Mediated Through the Activation of Cytosolic Phospholipase A_2 and Generation of Arachidonic Acid

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Background—Advanced glycation end products (AGEs) enhance NADPH oxidase, and hence respiratory burst activity, of stimulated neutrophils. They are thus potentially vasculopathic, especially in diabetes, uremia, and aging, in which AGEs classically accumulate. We investigated the underlying mechanisms.

Methods and Results—Neutrophils prelabeled with [^{3}H]arachidonic acid display increased [^{3}H]arachidonate release on exposure to AGE-albumin over exposure to albumin alone (by 151±16%, $P<0.01$). Arachidonic acid (AA) itself seems to mediate the AGE-augmented neutrophil respiratory burst (ascertained by chemiluminescence). Inhibitors of the cyclooxygenase pathway (indomethacin) and lipoxygenase pathway (MK-886) do not impair this AGE effect, excluding a contribution from AA metabolites. Cytosolic phospholipase A_2 (cPLA$_2$) controls AA generation. Its inhibition by methyl arachidonyl fluorophosphonate abrogates the AGE-enhanced activated neutrophil respiratory burst, and it is demonstrably stimulated in AGE-exposed neutrophils, as evidenced by isoform gel-shift and an increasingly membrane-translocated state in Western blots of neutrophil subfractions. Inhibition of other PLA$_2$ isoforms, secretory PLA$_2$ and calcium-independent PLA$_2$, by manoalide and haloenol-lactone suicide substrate, respectively, does not affect this effect of AGEs relative to inhibitor-treated controls. The thiol antioxidant NAC reduces activation of cPLA$_2$ (assessed by isoform gel-shift and membrane translocation), production of AA in AGE-albumin–exposed neutrophils (H^3 release reduced to 104±17%, $P=0.94$ compared with albumin-exposed neutrophils), and the AGE-augmented neutrophil respiratory burst.

Conclusions—AGE augmentation of the activated neutrophil respiratory burst requires AA generation, through which neutrophil NADPH oxidase may be upregulated, enhancing reactive oxygen species output. AA is generated by cPLA$_2$, which may be stimulated through an AGE-activated redox-sensitive pathway. ***(Circulation.* 2003;108:1858-1864.)**

Key Words: glycosylation end products, advanced ■ phospholipases ■ arachidonic acid ■ free radicals ■ NADPH oxidase

The accumulation of advanced glycation end products (AGEs) resulting from the nonenzymatic reaction of amino acids with aldose sugars, is implicated in pathological conditions, particularly the vascular disease encountered in diabetes mellitus, uremia, and aging.[1-3] This may be mediated by oxidative stress[4,5]; the aforementioned states are associated with increased oxidative stress.[6-8] The role of oxidative stress in the pathophysiology of vascular disease is already established.[9] One mechanism by which AGEs may induce oxidant stress is by enhancing free radical generation from neutrophils. AGEs act as neutrophil coagonists, having no direct, discernible effect on neutrophil function, yet augmenting the respiratory burst induced by a secondary stimulus.[10] This is dependent on neutrophil NADPH oxidase, a multicomponent enzyme that generates superoxide.[11]

The mechanism governing this AGE effect has not been characterized, although its initiation is probably receptor-mediated. Receptors capable of binding AGEs have been described, of which the receptor for AGEs is the best characterized,[12] with a common feature of its ligation being the activation of redox-sensitive pathways.[13] Also of particular pertinence regarding the coagonist action of AGEs on the NADPH oxidase are similar reports ascribing a facilitatory action on NADPH oxidase to arachidonic acid (AA), an unsaturated fatty acid produced by phospholipase A_2 (PLA$_2$) enzymes.[14,15]

In seeking to understand how AGEs may contribute to vascular pathology, we investigated the costimulatory actions of AGEs on the neutrophil NADPH oxidase with respect to generation of reactive oxygen species (ROS). We found that

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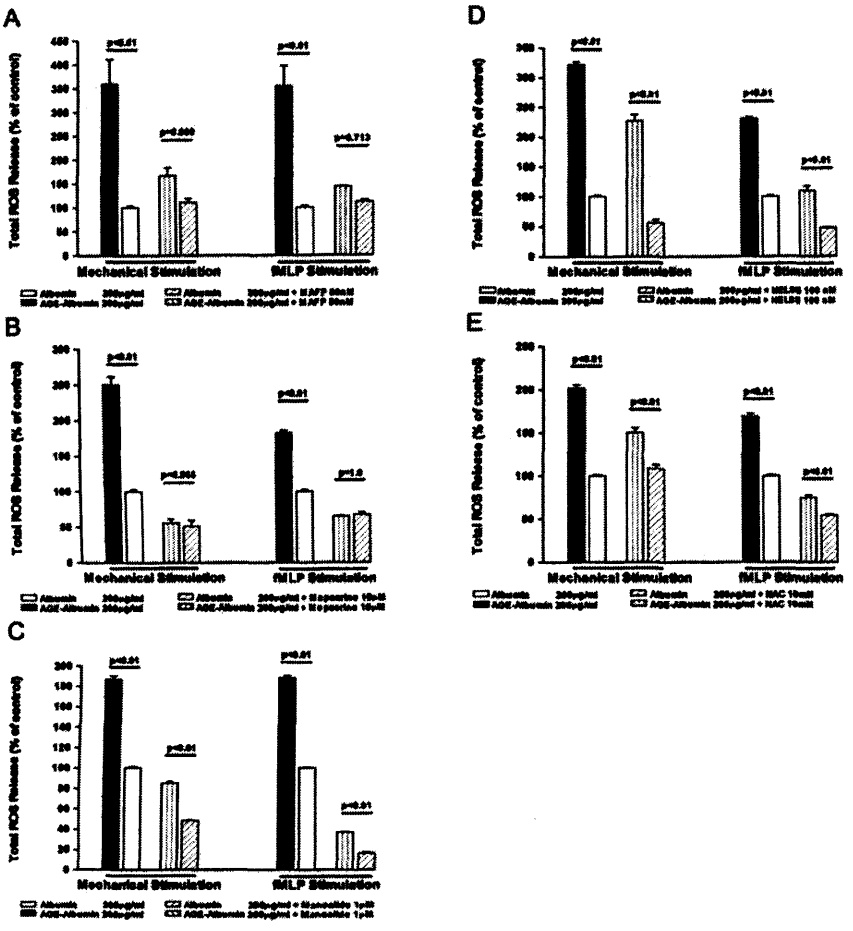

Figure 1. Total neutrophil ROS production (over a period of 6 minutes), detected by lucigenin ECL, in presence of AGE-albumin/albumin (200 μg/mL) and in response to mechanical or chemical (fMLP) stimuli. ROS release is expressed as a percentage of output from control neutrophils. Effect of preincubation with various compounds on ROS production from neutrophils exposed to AGE-albumin/albumin: A, MAFP 50 nmol/L ($P<0.01$ between groups by ANOVA). B, Mepacrine 10 μmol/L ($P<0.01$ between groups by ANOVA). C, HELSS 100 nmol/L ($P<0.01$ between groups by ANOVA). D, Manoalide 1 μmol/L ($P<0.001$ between groups by ANOVA). E, NAC 10 mmol/L ($P<0.01$ between groups by ANOVA). Results are representative of multiple experiments, each result representing mean of 3 separate aliquots of 10^5 cells recorded simultaneously.

AGE-induced generation of AA, produced by cytosolic PLA_2 ($cPLA_2$), is the basis of this phenomenon and may be regulated by a redox-sensitive mechanism.

Methods

Materials

Bis-*N*-methylacridinium nitrate (lucigenin), fMLP, glucose, indomethacin, *N*-acetylcysteine (NAC), Percoll and tissue culture medium 199 (TC199) were all from Sigma-Aldrich. Haloenol-lactone suicide substrate (HELSS), manoalide, and methyl arachidonyl fluorophosphonate (MAFP) were from Biomol. MK-886 was from Calbiochem, 20% endotoxin-free human serum albumin (HSA) was from BioProducts Laboratory, and [^{3}H]arachidonic acid (^{3}H-AA) was obtained from Amersham.

Preparation of AGE-Albumin

AGE-albumin was prepared by preincubation of endotoxin-free HSA (20%) with 1 mol/L glucose at 37°C for 12 weeks in 100 mmol/L phosphate (pH 7.4). Control albumin was produced by incubation of the same HSA preparation under identical conditions but without the glucose. After incubation, HSA preparations were dialyzed against PBS and 0.9% sodium chloride for 24 hours before sterilization by filtration.

Neutrophil Isolation and Fractionation

Fresh citrated blood was obtained from healthy adult volunteers after they had given informed consent. Neutrophils were separated by dextran sedimentation and purification on a Percoll gradient.[10] Membrane and cytosol fractions were prepared according to the method of Levy and Malech.[16] Briefly, neutrophils were centrifuged down, resuspended in relaxation buffer (in mmol/L: 10 KCl, 3 NaCl, 3.5 $MgCl_2$, 1.25 EGTA, 10 HEPES, pH 7.4, containing 1 mmol/L PMSF, 100 μmol/L leupeptin, 10 μg/mL aprotinin, 1 mmol/L Na_3VO_4, and 25 mmol/L NaF), snap-frozen in liquid nitrogen, defrosted, and sonicated 3 times for 15 seconds on ice. The postnuclear supernatant was centrifuged at 150 000*g* for 60 minutes (Beckman Optima Max Ultracentrifuge) to obtain a cell membrane pellet and cytosol supernatant. Membrane pellets were resuspended in the above relaxation buffer.

Neutrophil Stimulation and Inhibition

Mechanical stimulation of neutrophils was by multiple passages through a standard 1-mL pipette tip.[10] Chemical stimulation was in the form of fMLP 100 nmol/L.[17]

When we investigated the effects of various enzyme inhibitors on the AGE-augmented neutrophil ROS burst, neutrophils were preincubated with the respective inhibitors, after which neutrophils were spun down and resuspended in inhibitor-free buffer (containing AGE-albumin/HSA) before stimulation.

Detection of Reactive Oxygen Species

ROS detection was based on lucigenin enhanced chemiluminescence (ECL)[18] recorded on an EG&G Berthold microplate luminometer LB96V. All experiments were performed at 37°C. Lucigenin 50 μmol/L was made up in a balanced salt solution containing (in mmol/L) 140 NaCl, 15 HEPES, 5 KCl, 5 glucose, 1.8 $CaCl_2 \cdot 2H_2O$, and 0.8 $MgSO_4 \cdot 7H_2O$ (pH 7.4). All experiments were performed in triplicate with 10^5 cells per well.

Western Blotting

Cytosol and membrane fractions (100 μg protein) were resolved on 10% (wt/vol) SDS-polyacrylamide gels. The resolved proteins were

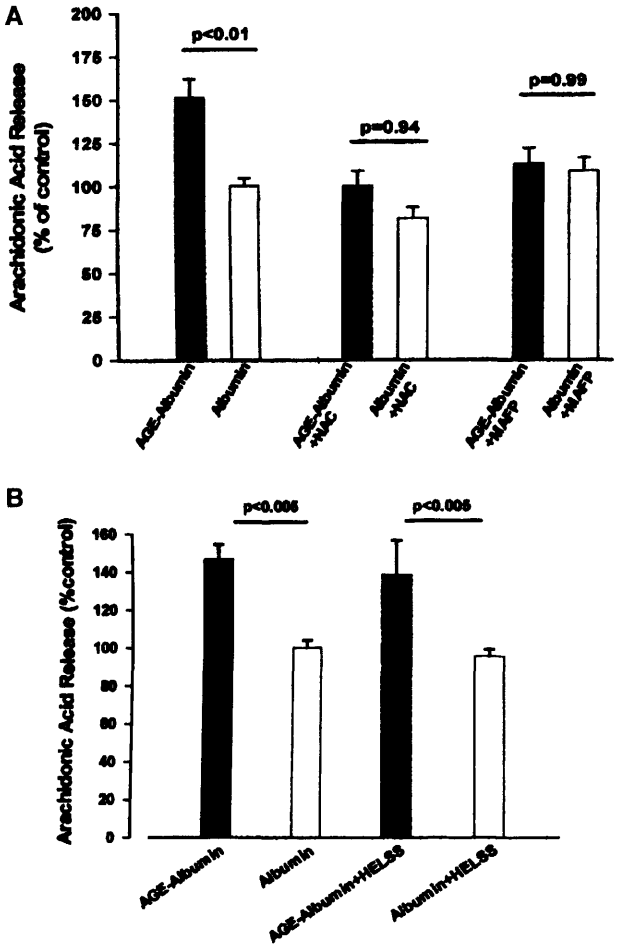

Figure 2. A, Neutrophil AA release over a period of 30 minutes after addition of AGE-albumin (black bars) or albumin (white bars), both at 200 μg/mL and with previous incubation in NAC or MAFP. Results are expressed as percentage increase in AA release over control (albumin group) and are mean of 5 experiments. Analysis was by 1-way ANOVA with Tukey's test ($P<0.01$ between groups). AA released in control HSA group was 2962±136 dpm/10^6 cells in 30 minutes. B, Neutrophil AA release over a period of 30 minutes after addition of AGE-albumin or albumin (both at 200 μg/mL) with and without pretreatment with HELSS (100 nmol/L). Results are mean of 8 experiments. AA released in control HSA group was 2051±81 dpm/10^6 cells in 30 minutes.

blotted to nitrocellulose, which was then blocked in 2.5% BSA in PBS with Tween. The blots were incubated for 1 hour with a 1:500 dilution of mouse monoclonal $cPLA_2$ antibody (Santa Cruz). After extensive washes, the blot was incubated for 1 hour more in a 1:2000 dilution of peroxidase-conjugated anti-mouse antibody (Sigma). Detection was by ECL (Amersham).

Detection of AA Production

Labeling of neutrophils with [^{3}H]-AA and the release of radiolabeled AA were performed according to the method of DiPersio et al.[19] Briefly, the cells were incubated with 2.5 μCi/mL [^{3}H]-AA for 45 minutes at 37°C. Those cells requiring further treatment with MAFP, NAC, or HELSS were incubated for a further 60 minutes in TC199 with 1% BSA. Cells were then washed 3 times in TC199 and incubated for 30 minutes with AGE-albumin or albumin at 200 μg/mL in TC199. After this final incubation, cells were centrifuged, and an aliquot of the supernatant was counted by liquid scintigraphy.

AA Binding by AGE-Albumin

To examine whether binding of AA was different between AGE-albumin and control HSA, we incubated and equilibrated 1 μCi/mL [^{3}H]-AA for 6 hours at 37°C with both AGE-albumin and control HSA and then measured the free fraction of [^{3}H]-AA by ultrafiltering the solutions on 30-kD cutoff Microcon filter devices (Millipore). The ultrafiltrates were counted by liquid scintigraphy.

Data Analysis

Chemiluminescence values are presented as relative light units per minute (RLU/min) (means with SEMs). For analysis of total ROS production (RLU), we calculated the area under the curve (cutoff at 6 minutes). Comparisons were with 2-tailed *t* tests or 1-way ANOVA with Tukey's post hoc analysis as appropriate (SPSS Inc).

Results

AGE augmentation of the stimulated neutrophil respiratory burst is abolished by inhibitors of $cPLA_2$ and by NAC. We previously demonstrated the ability of AGEs to augment the activated neutrophil respiratory burst.[10] Here, we investigated the effects of manipulating both AA production and intracellular redox status on this particular property of AGEs. AA is hydrolyzed from membrane glycerophospholipids by PLA_2,[20] of which different isoforms exist. As shown previously, the respiratory burst was augmented in the presence of AGE-albumin compared with control albumin, after mechanical or chemical (fMLP) stimulation (Figure 1, A–E).[11] This augmented response was abolished by a 30-minute preincubation of neutrophils with the minimum effective dose of either of 2 $cPLA_2$ inhibitors, MAFP 50 nmol/L[21] and mepacrine 10 μmol/L[22] (Figure 1, A and B) and was significantly attenuated by a 60-minute preincubation of neutrophils with the intracellular glutathione precursor NAC 10 mmol/L (Figure 1E). For the latter, AGE-augmented ROS output diminished from 201% to 138% ($P<0.01$) with mechanical stimulation and from 169% to 138% ($P<0.01$) with fMLP stimulation. However, a 60-minute preincubation with other direct free-radical scavengers (ascorbic acid, acetylsalicylic acid, and tiron) produced negligible effects (data not shown), and inhibition of other PLA_2 isoforms (secretory PLA_2 [$sPLA_2$] by manoalide 1 μmol/L[23] and calcium-independent PLA_2 by HELSS 100 nmol/L,[24] both being maximal effective doses)

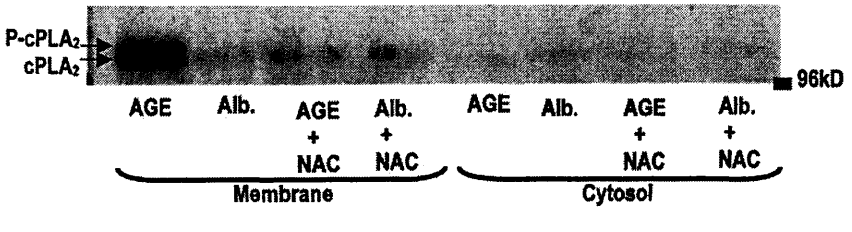

Figure 3. Qualitative assessment of $cPLA_2$ in subcellular neutrophil fractions. Neutrophils exposed to AGE-albumin (AGE) and albumin (Alb) with or without NAC were fractionated into membrane and cytosol fractions. $cPLA_2$ was detected by Western blotting. P-$cPLA_2$ represents phosphorylated $cPLA_2$, which has reduced electrophoretic mobility and is gel-shifted. A 96-kD marker is shown. Immunoblot shown is representative of 5 experiments.

Quantitative Comparison of $cPLA_2$ (Nonphosphorylated) in Different Neutrophil Fractions After Exposure to AGE-Albumin/Albumin

	Cytosol*			Membrane*		
	AGE-Albumin	Albumin	*P*	AGE-Albumin	Albumin	*P*
$cPLA_2$	0.53 (0.02)	0.75 (0.05)	0.018	2.53 (0.17)	0.51 (0.06)	0.024
$cPLA_2$ (+NAC)	0.56 (0.06)	0.72 (0.02)	0.086	1.01 (0.05)	0.89 (0.10)	0.762

The phosphorylated $cPLA_2$ band is seen only in the membrane fraction of neutrophils exposed to AGE-albumin. Mean (SEM) and *P* values (by Tukey's test) are quoted for 5 experiments.
*For both the cytosolic and membrane fractions, $P<0.01$ between groups by ANOVA.

did not result in significant reduction of the AGE-augmented neutrophil respiratory burst compared with inhibitor-treated controls (Figure 1, C and D). Higher concentrations of HELSS damaged cells, reducing their viability (data not shown).

AGE-Exposed Neutrophils Exhibit Enhanced Liberation of AA, Which Is Abrogated by Inhibitors of $cPLA_2$ and NAC

To confirm that AGE coagonist activity on neutrophil NADPH oxidase is mediated through AA, we assessed AA production. Neutrophils prelabeled with ^{3}H-AA were incubated for 30 minutes in AGE-albumin or albumin (200 μg/mL). AGE-albumin–exposed neutrophils exhibited a higher rate of AA release than albumin-exposed neutrophils (151±16%, $P<0.01$) [Figure 2A]. This enhancement was abrogated by preincubation of neutrophils for 30 minutes with a minimum effective dose (50 nmol/L) of MAFP, an inhibitor of $cPLA_2$, before AGE-albumin/albumin exposure (Figure 2A), confirming the role of $cPLA_2$ in AA synthesis in this context. Because the interaction of AGEs with some cellular receptors has been noted to activate redox-sensitive pathways,[13] we also sought to establish whether redox-sensitive pathways might govern the AGE-induced increase in neutrophil AA production. We manipulated intracellular levels of reduced glutathione using a 60-minute preincubation of neutrophils with NAC, a precursor of glutathione. Subsequent exposure of neutrophils to AGE-albumin did not result in any significant increase in AA production over control cells (104±17%, $P=0.94$) (Figure 2A). In another series of experiments, we confirmed that the enhancing effect of AGE-albumin on neutrophil AA release was not affected by pretreatment with the calcium-independent PLA_2 antagonist HELSS (100 nmol/L) (Figure 2B).

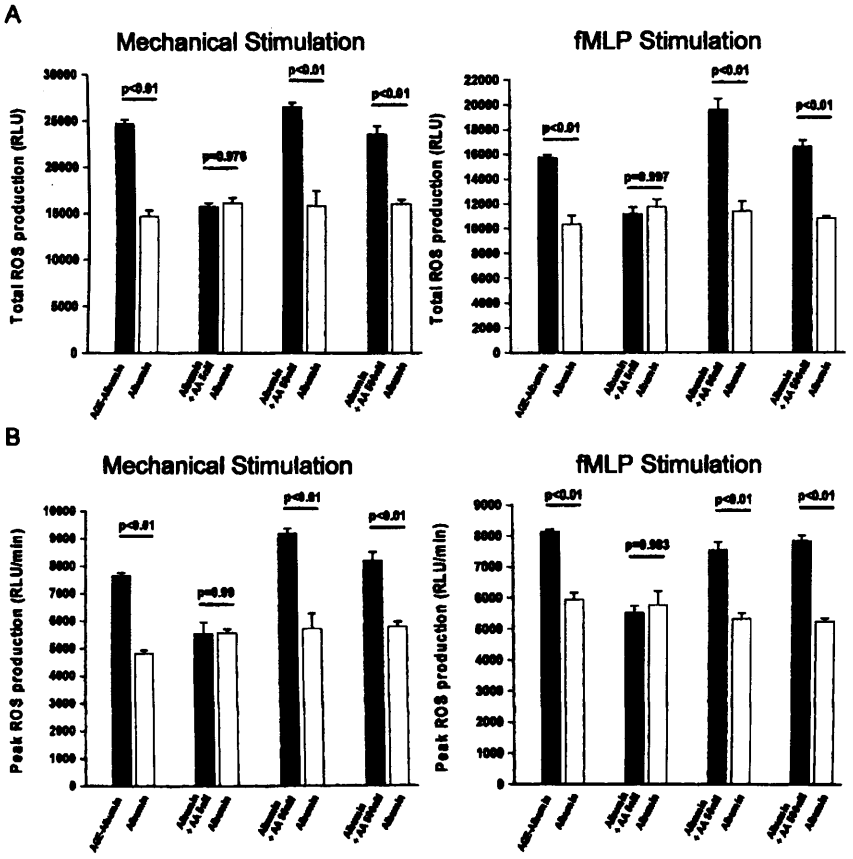

Figure 4. Acute neutrophil ROS production detected by lucigenin ECL in presence of AGE-albumin/albumin (200 μg/mL) and/or varying doses of AA and in response to mechanical or chemical (fMLP) stimuli. A, Total neutrophil ROS production (over a period of 6 minutes) after stimulation in presence of AGE-albumin (200 μg/mL), albumin (200 μg/mL), or albumin (200 μg/mL) plus varying doses of AA ($P<0.01$ between groups by ANOVA). B, Peak neutrophil ROS production after stimulation in presence of AGE-albumin (200 μg/mL), albumin (200 μg/mL), or albumin (200 μg/mL) plus various doses of AA ($P<0.01$ between groups by ANOVA). Each recording is mean of 3 separate aliquots of 10^5 cells measured simultaneously, and results are representative of multiple experiments. Total ROS production was measured in RLU and peak ROS production in RLU/min.

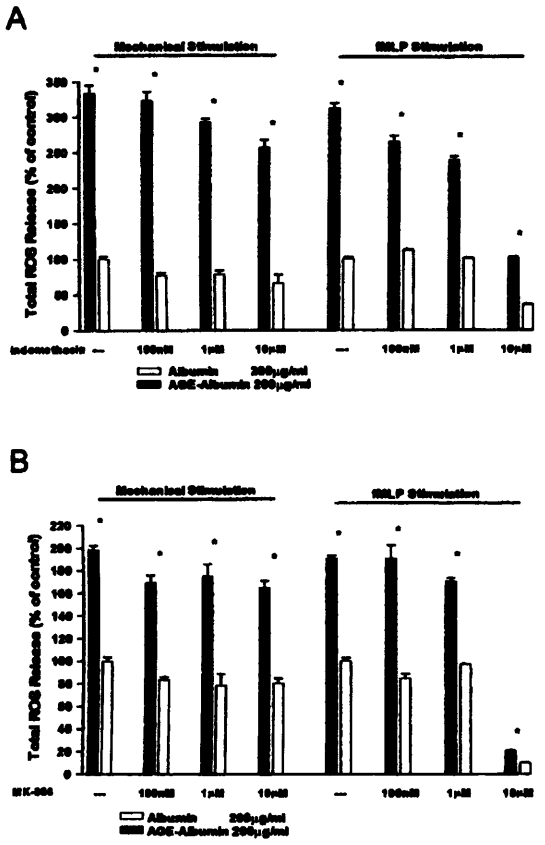

Figure 5. Acute neutrophil ROS production detected by lucigenin ECL in presence of AGE-albumin/albumin (200 μg/mL) in response to mechanical or fMLP stimulation and/or varying doses of indomethacin (A; $P<0.01$ between groups by ANOVA) or MK-886 (B; $P<0.01$ between groups by ANOVA). Each recording is mean of 3 separate aliquots of 10^5 cells measured simultaneously. Results are representative of multiple experiments. *$P<0.01$ by post hoc Tukey's analysis of ANOVA.

The differences in AA release may have been a result of altered binding or trapping of AA by AGE-albumin compared with control HSA. We therefore measured the AA counts in ultrafiltrates of buffer containing either AGE-albumin or control HSA with 1 μCi/mL [^{3}H]-AA. In 5 experiments, 200 μg/mL AGE-albumin bound 79.3±0.12% of the available [^{3}H]-AA, compared with 200 μg/mL HSA, which bound 81.0±0.13% of the AA ($P<0.001$). This small difference, although significant, would have had minimal effect on the trapping of AA released from the cells and would not have accounted for the increased AA release in AGE-albumin–treated cells, because AGE-albumin bound less of the labeled AA.

AGEs Mediate Activation of cPLA$_2$

Activation of the enzyme cPLA$_2$ requires its phosphorylation and translocation from the cytosol to the plasma membrane.[25] After transient exposure to AGE-albumin/albumin followed by resuspension in relaxation buffer, neutrophils were rapidly fractionated into membrane and cytosolic components. Immunoblots revealed increased cPLA$_2$ in the plasma membrane fractions, with a corresponding decrease in the cytosolic fractions, in AGE-albumin–exposed compared with albumin-exposed neutrophils (Figure 3 and the Table). In addition, the presence of a phosphorylated cPLA$_2$ moiety in the membrane fraction derived from cells exposed to AGE-albumin was revealed through reduced electrophoretic mobility or gel-shift (Figure 3). Such AGE-induced cPLA$_2$ phosphorylation and increased membrane translocation was abolished by a 60-minute preincubation of neutrophils with 10 mmol/L NAC (Figure 3 and the Table).

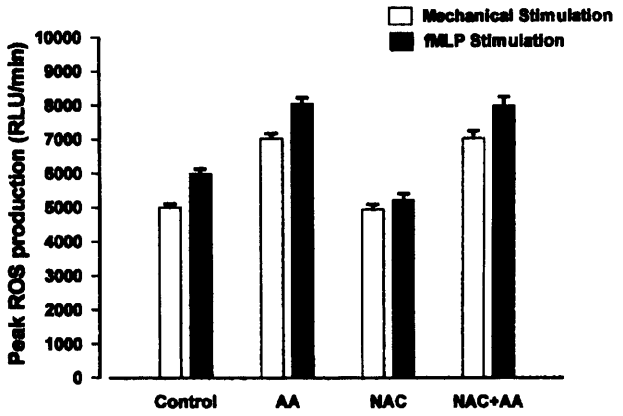

Figure 6. Effect of NAC pretreatment (10 mmol/L) on AA (50 nmol/L) enhancement of mechanical and fMLP-stimulated ROS output from neutrophils in presence of 200 μg/mL AGE-albumin or albumin (n=8 experiments).

AA Mimics AGE-Induced Augmentation of the Stimulated Neutrophil Respiratory Burst

Because cPLA$_2$ inhibitors nullified AGE-induced AA release and AGE-enhanced ROS release from stimulated neutrophils, we investigated ROS production after direct addition of AA to neutrophils. Neutrophil ROS release (both total and peak) in response to stimuli was enhanced in the presence of AA ≥50 nmol/L (average enhancement of total ROS production by 157% with mechanical stimulus and 163% with fMLP stimulus), and the magnitude of this enhancement was similar to that induced by AGE-albumin itself (average enhancement of total ROS production by 168% with mechanical stimulus and 152% with fMLP stimulus) (Figure 4, A and B).

AA and Not One of Its Metabolites Is Responsible for Augmenting the Stimulated Neutrophil Respiratory Burst

To determine whether the facilitatory role of AA in NADPH oxidase activation might be mediated through its downstream metabolites, we assessed the effects of specific inhibitors of the cyclooxygenase and lipoxygenase pathways on AGE-augmented neutrophil ROS production. The cyclooxygenase inhibitor indomethacin[23] and the lipoxygenase inhibitor MK-886[26] did not attenuate the differential enhancement of ROS production caused by AGE-albumin in response to mechanical/chemical stimuli (Figure 5, A and B).

NAC Does Not Affect the AA-Enhanced Response of Neutrophils to Mechanical or Chemical Stimuli

The direct effect of AA on ROS production of neutrophils with both mechanical and chemical stimuli was tested in the

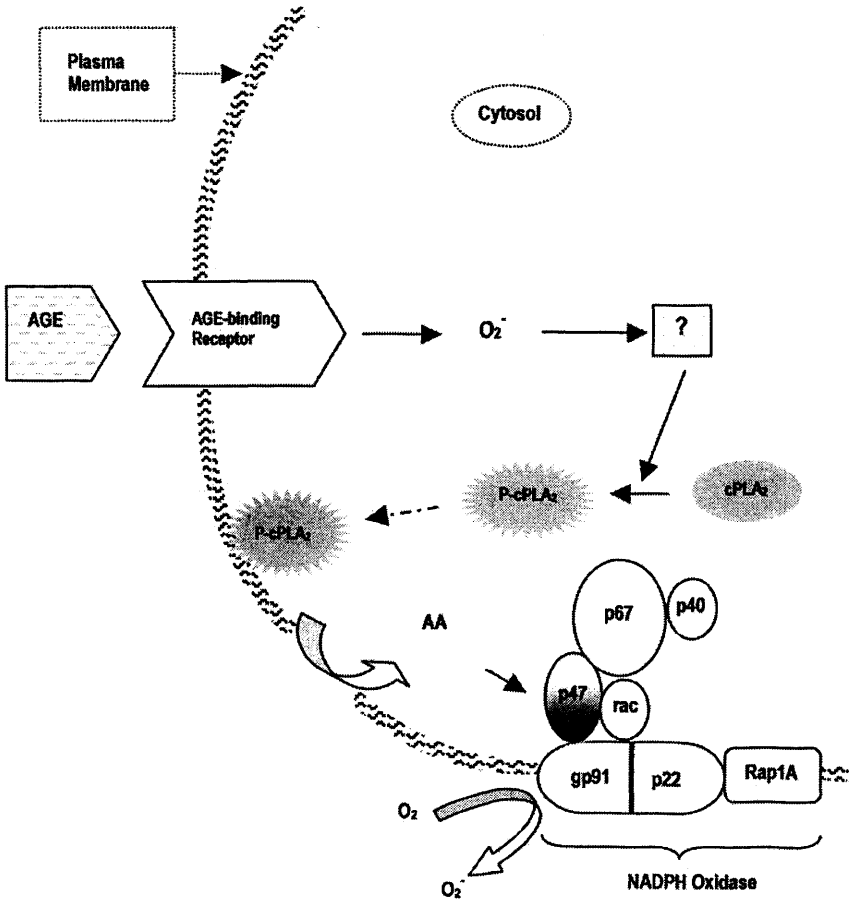

Figure 7. A putative pathway by which AGEs may enhance stimulatory burst activity of neutrophil NADPH oxidase. Possible ligation of a receptor by AGEs generates intracellular superoxide (O_2^-), which through a redox-sensitive pathway involving 1 or more protein kinases results in phosphorylation, membrane translocation, and subsequent activation of $cPLA_2$. $cPLA_2$ hydrolyzes AA from membrane glycerophospholipid, which then conformationally alters p47 subunit of NADPH oxidase (subunits illustrated) to increase functional efficiency of oxidase complex.

absence and presence of NAC pretreatment (10 mmol/L). NAC pretreatment had no effect on the AA enhancement of ROS production with either stimulus ($P<0.001$ by Tukey's test for both stimuli between control and AA-treated cells in the absence or presence of NAC, n=8 experiments, Figure 6).

Discussion

Evidence from this study suggests how AGEs might enhance neutrophil NADPH oxidase activity and contribute to the increased vascular oxidant stress and cardiovascular disease in diabetic, uremic, and elderly patients. AA seems central to this process, mimicking the coagonist activity of AGEs. This may reflect its ability to alter the structural conformation and functional efficiency of the p47 subunit of NADPH oxidase[14] or action on other intracellular components vital to the function of the oxidase.[15] The proportionally small increment in AA release induced by AGE exposure is consistent with a catalytic function.

AGE-induced AA production seems to be dependent on $cPLA_2$ activation, which becomes phosphorylated and membrane-translocated on exposure of neutrophils to AGEs. Calcium facilitates this process; indeed, membrane translocation may take place in the presence of calcium alone without phosphorylation, explaining the significant presence of nonphosphorylated $cPLA_2$ in the other membrane fractions.[25] In HELSS-treated neutrophils, AGE-albumin–stimulated AA release was unaffected, making it unlikely that calcium-independent PLA_2 is involved in these effects. In addition, others have documented that calcium-independent PLA_2 is not involved in the fMLP stimulation of neutrophil AA release.[27,28] In contrast, $cPLA_2$ has a major role in AA release from fMLP-stimulated neutrophils,[28] and this is associated with translocation of $cPLA_2$ to membranes and a retarded electrophoretic mobility consistent with enhanced phosphorylation.[28]

There is a hint that redox-sensitive mechanisms may also play a role in transducing the effect of AGE on neutrophil NADPH oxidase, but few clinical trials have demonstrated a reduction in vascular events from antioxidant supplementation.[29] This may be partly a result of inadequate antioxidant plasma levels, but our unpublished data on free radical scavengers showed no attenuation of AGE-augmented neutrophil ROS production. The exception was NAC, which reduced the final ROS output but more specifically abrogated the AGE-induced differential increase in ROS output. This superiority of NAC may relate to its role as a glutathione precursor, the principal intracellular free radical scavenger, because glutathione depletion occurs in AGE-exposed cells.[5] Reduced glutathione may inhibit intracellular redox-sensitive pathways in which trace quantities of free radicals mediate signaling.[30] From the ability of NAC to reduce AA production and $cPLA_2$ translocation, this putative redox signaling is likely to occur upstream of $cPLA_2$ activation. Activation of redox signaling by AGEs has been already described in the

context of ligation of receptor for AGE by AGEs,[13,31] and it remains likely that a receptor-mediated event is the initiating focus in the cascade of events described above (Figure 7).

Further work is needed to determine the role for receptor ligation in the initiation of events and to delineate mechanisms by which redox signaling results in $cPLA_2$ activation, although many candidate kinases exist.[32] Unraveling such intracellular pathways may widen the number of potential therapeutic options when targeting vascular oxidant stress and disease.

Acknowledgments

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Increased free radical production in hypertension due to increased expression of the NADPH oxidase subunit p22^{phox} in lymphoblast cell lines

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Objectives **To confirm increased production of reactive oxygen species (ROS) in hypertension, to demonstrate the source of ROS and to analyse NADPH oxidase subcomponent expression in hypertension.**

Design **A lymphoblast model was used, as this has previously been used in the study of hypertension and of NADPH oxidase. Chemiluminescence (CL) was chosen to assay ROS production, as it is simple and sensitive.**

Methods **Lymphocytes from 12 hypertensive patients (HT), and 12 age- and sex-matched normotensive (NT) subjects, were immortalized. Luminol, isoluminol and Cypridina luciferin analogue (CLA) CL were used to assay ROS production. NADPH oxidase subunits were measured by Western Blot analysis.**

Results **Stimulation with 50 μmol/l arachidonic acid (AA) resulted in increased ROS production in HT cell lines with luminol, CLA and isoluminol CL. Stimulation with 500 nmol/l 12-*O*-tetradecanoylphorbol-13-acetate (TPA) produced a detectable increase in HT ROS production with luminol and with CLA, whereas there was no significant difference with isoluminol. The ROS production was abolished by diphenyleneiodonium chloride (DPI) but not by rotenone, indicating that a non-mitochondrial flavoprotein such as NADPH oxidase is the source of ROS. Analysis of NADPH oxidase subcomponents revealed an increase in p22^{phox} in HT subjects.**

Conclusions **We have shown there is increased ROS production in lymphoblasts derived from hypertensive subjects, probably originating from NADPH oxidase. As the ROS production persists in transformed cells, this suggests a genetic predisposition to increased ROS production. Increased expression of p22^{phox} in HT lymphoblasts may account for some of the increased ROS.**

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Keywords: hypertension, NADPH oxidase, superoxide, hydrogen peroxide, free radicals, reactive oxygen species

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Introduction

A number of animal models show a relationship between reactive oxygen species (ROS) and hypertension (HT) [1–4]. Studies of human neutrophils and monocytes have produced mixed results, some showing an increase in ROS production in HT [5,6], and some showing no difference [7,8]. One group suggested that ROS was only significant in the context of a family history of HT [9]. Some of these differences could have arisen from different environmental influences that could have affected ROS production in cells freshly isolated from patients and subjects.

ROS may be involved in the pathophysiology of HT by reacting with endothelial-derived nitric oxide (NO) [10], thus producing endothelial dysfunction [11] and increased blood pressure [12]. The ROS production may be the link between HT and premature development of atherosclerosis. There are a number of reasons why ROS production could be linked to atherosclerosis. ROS have been shown to initiate endothelial cell apoptosis [13], to oxidize low-density lipoprotein making it more harmful to the vessel wall [14], to cause smooth muscle hyperplasia via activation of various kinases [15,16], to damage DNA [17], to activate matrix metalloproteinases [18], to recruit macrophages and neutrophils to the endothelium via intercellular adhesion molecule-1 (ICAM-1) [19] and to trigger platelet aggregation [20,21].

A number of enzymes produce ROS, but NADPH oxidase has been implicated as the major source in HT

[4,11]. In activated phagocytic cells there is a general assumption in the literature that NADPH oxidase is the source of ROS. NADPH oxidase (for a review see [22]) is a multicomponent enzyme that has a membrane portion collectively known as cytochrome b_{558}, which consists of $gp91^{phox}$, $p22^{phox}$, two haems and a flavin-adenine dinucleotide (FAD) subunit. The membrane portion of the enzyme is the catalytic core, but is inactive until it is united with the cytosolic components, which are $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$. The cytosolic components are closely associated with a G protein, Rac2, that is thought to facilitate translocation of the cytosolic components to the membrane. In the resting state there is little ROS production, but once stimulated the cytosolic components translocate to the plasma membrane, resulting in production of superoxide from the substrates NADPH, hydrogen ions and oxygen. The cells within the vascular system that contain NADPH oxidase are all leucocytes, vascular smooth muscle cells and fibroblasts.

It has been shown that $p22^{phox}$ mRNA is elevated in spontaneously hypertensive rats (SHRs) [23] and can be induced in rats with a ligated renal artery (an experimental model for angiotensin II induced HT) [24]. The rise in $p22^{phox}$ mRNA is associated with an increased ROS production.

As previous investigations have indicated that hypertensive phenotypes could persist in Epstein–Barr virus immortalized lymphoblasts [25], and that such cells also express components of NADPH oxidase [26], these cells could be used as models for investigating the production of ROS free from the influences of environmental factors present *in vivo*. The aim of the present research was to measure basal and stimulated ROS production in stable normotensive (NT) and hypertensive (HT) lymphoblast cell lines using Cypridina luciferin analogue (CLA), luminol and isoluminol chemiluminescence (CL), to demonstrate that NADPH oxidase is responsible for the increased ROS production in HT using inhibitors, and to examine NADPH oxidase subunit expression in NT and HT cell lines using Western blot analysis.

Materials and methods

Chemicals

Arachidonic acid (AA), diphenyleneiodonium chloride (DPI), fetal calf serum, Histopaque 1077, horseradish peroxidase type II (HRP), luminol, RPMI 1640, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and tissue culture medium 199 were all purchased from Sigma (Poole, Dorset, UK). Isoluminol was purchased from Fluka (Gillingham, Dorset, UK); 2-methyl-6-phenyl-3,7dihydro-imidazo[1,2-a]pyrazin-3-one (CLA) from Tokyo Kasei Kogyo Co. (Tokyo, Japan); and Rac2 polyclonal antibody was supplied by Santa Cruz Biotechnology Inc (Santa Cruz, California, USA). Anti-rabbit IgG conjugated to horseradish peroxidase was supplied by Amersham Life Science (Little Chalfont, Bucks, UK).

Tissue culture

Lymphoblast cultures were established as described previously [25]. We selected a study group of 12 HT patients and 12 age- and sex-matched NT subjects.

The lymphoblasts were maintained in RPMI 1640 growth medium containing glutamine, penicillin, streptomycin and 10% fetal calf serum, at a cell density of less than 10^6cells/ml. The cells were incubated at 37°C in an atmosphere of 5% $CO_2$and O_2. Aliquots were taken on the day of experiment and cells were recovered by centrifugation (1400 rpm for 10 min). The cells were spun through Histopaque 1077 at 1400 rpm for 10 min to isolate the viable cells and washed in TC199 before being suspended in 1–5 ml of TC199. The cells were stored at 37°C prior to the experiments. The cell number was estimated using a Beckman Coulter counter (Beckman, High Wycombe, Bucks, UK).

Chemiluminescence

ROS estimation was based on the technique described by Liu *et al.* [27]. The detection of ROS was enhanced by using CLA [28,29], luminol and isoluminol [30] CL, and recorded using an EG & G Berthold microplate luminometer LB96V (Bad Wildbad, Germany). All experiments were performed at 37°C. The luminescent probes were made up in a balanced salt solution containing: NaCl, 140 mmol/l; HEPES, 15 mmol/l; KCl, 5 mmol/l; glucose, 5 mmol/l; $CaCl_2.2H_2O$, 1.8 mmol/l; and $MgSO_4.7H_2O$, 0.8 mmol/l. The final concentrations were: luminol, 5 μmol/l; isoluminol, 50 μmol/l; and CLA, 1 μmol/l. The solutions of luminol and isoluminol contained 6units HRP/ml. The experiments were performed in triplicate, with 10^5 cells per well.

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*-transferase fusion protein was produced in *Escherichia coli* transfected with the plasmid (obtained from Professor D. Roos, the University of Amsterdam). The fusion protein was purified on a glutathione–Sepharose slurry column and eluted with free 10 mmol/l glutathione buffered by 50 mmol/l Tris to pH 8.0. $p47^{phox}$ 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.* [31]. The $gp91^{phox}$ was a thioredoxin-fusion protein obtained from Dr David Lambeth (Emory University). Antisera to the recombi-

nant phox components were raised in rabbits by monthly subcutaneous injections of 500 μg of the various proteins. The antibodies were purified by protein A columns and eluted with 100 mmol/l glycine, pH 2.5. The specificity of the $p47^{phox}$ and $p67^{phox}$ antibodies was determined by performing two Western blots for each subunit, one probed with the antibody alone (for concentration see below) and the other probed with a combination of the antibody and the antigen (for both $p67^{phox}$ and $p47^{phox}$ 10 μg/ml). The band of interest was taken as that of which the intensity was decreased in the antigen-blocked blot. 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 a cytosolic fraction and a membrane fraction. The membrane and cytosol fractions were separated as described by Abo and Pick [32]. The cell suspensions were sonicated three times for 10 s, the homogenate was spun at 300 *g* and the post-nuclear fraction was retained. The post-nuclear fraction was separated into cytosol (supernatant) and membranes (pellet) by centrifugation at 50 000 *g* for 1 h. The $p22^{phox}$ and $gp91^{phox}$ bands were enriched in the membrane fractions but absent in the cytosolic fractions.

SDS-polyacrylamide gel electrophoresis

The technique was based on that described by Laemmli [33]: 50–500 μg protein extracts from the lymphoblast cell lines were resolved on 7.5–15% SDS–polyacrylamide gels. The resolved proteins were Western blotted on to nitrocellulose, and probed with the polyclonal antibodies in phosphate-buffered saline (PBS) Tween with 5% Marvel, for 1 hour at room temperature. The concentration of the antibodies was: $p22^{phox}$, 10 μg/ml with 5% horse serum; $p47^{phox}$, 1 μg/ml; $p67^{phox}$, 5 μg/ml; $gp91^{phox}$, 10 μg/ml; and Rac2, 0.2 μg/ml. The primary antibody was labelled with anti-rabbit IgG conjugated to horseradish peroxidase diluted 1 in 1500 in PBS Tween and 5% Marvel, for 1 h at room temperature. The protein bands were detected using enhanced chemiluminescence kits from Amersham, and band intensities analysed on a BioRad densitometer (BioRad, Hemel Hempstead, Herts, UK).

Statistics

Each cell line underwent CL studies on three 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. The Western blot results are presented as arbitrary densitometry units, and represent the mean and SEM of the standardized results from at least three blots. Two-tailed values < 0.05 were considered significant. The two-sample *t*-test, Mann–Whitney test and Pearson's correlation coefficient were calculated using Minitab (Minitab Inc., Pennsylvania, USA). For the variation in ROS production found, the experiments have greater than 90% power to detect a 50% difference, at $P < 0.05$.

Results

The NT and HT groups were well matched for age and gender (Table 1). 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 mmHg in the HT subjects ($P < 0.01$).

Basal ROS production with luminol was NT 14.5 ± 1.4 versus HT 12.9 ± 2.6 (not significant; results expressed as the mean RLU ± SEM). The equality in basal ROS production was also reproduced with CLA and isoluminol (results not shown). Typical luminol-enhanced CL traces are shown for both AA stimulation (Fig. 1A) and TPA stimulation (Fig. 1B) with the same NT and HT cell lines in A and B. The CLA and isoluminol CL of AA- and TPA-stimulated cell lines showed similar kinetics to luminol. For stimulation with 50 μmol/l AA, the cell lines produced a rapid peak of ROS production between 45 and 90 seconds, and then declined to baseline. The peak was 679.3 ± 31.6 RLU for the NT cell line and 2718 ± 65.6 RLU for the HT cell line. The maximum rate of ROS production with TPA (500 nmol/l) plateaued much later, between 5 and 30 minutes. The maxima for these particular cell lines were NT 745.7 ± 12.3 RLU and HT 1258.7 ± 17.8 RLU. The AA- or TPA-stimulated ROS production was abolished with 20 μmol/l DPI (an inhibitor of flavoenzymes), but 10 μmol/l rotenone (a mitochondrial oxidase inhibitor) had no effect on luminol-enhanced CL, with either AA- or TPA-stimulated cells.

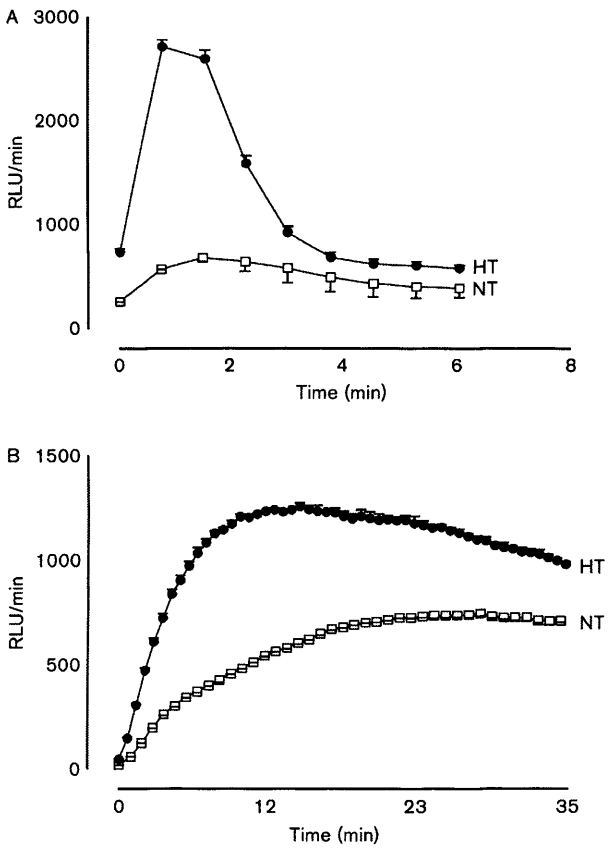
As the stimulated CL results were not normally distributed, we used a log transformation to normalize the data, which were then compared using a two-sample *t*-test; all results are expressed as mean ± SEM log RLUs. The raw data were also compared using the Mann–Whitney test (data not shown), which also revealed statistically significant results. We found that stimulation of the cell lines resulted in a significantly higher ROS production in HT, compared to NT cell lines (Fig. 2) when assayed

Table 1 Baseline characteristics of the study population

	NT	HT	*P*
AGE (years)	51.8 ± 4.1	59.5 ± 3.8	
SBP (mmHg)	115 ± 4	149 ± 5	<0.001
DBP (mmHg)	77 ± 2	91 ± 2	<0.001
n	12	12	
Males	5	5	
Family history of hypertension	0	6	

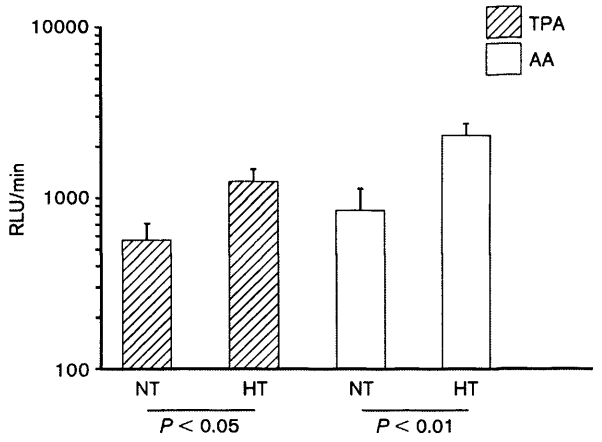
NT, normotensive; HT, hypertensive; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Fig. 1



Luminol-enhanced chemiluminescence of arachidonic acid (50 μmol/l) stimulated (A) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (500 nmol/l) 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. HT, hypertensive; NT, normotensive; RLU/min, relative light units produced per minute.

Fig. 2



Peak luminol-enhanced chemiluminescence, stimulated with 50 μmol/l arachidonic acid and 500 nmol/l 12-O-tetradecanoylphorbol-13-acetate (TPA). The results represent the mean of 12 NT and 12 HT cell lines. The results for the individual cell lines also represented the mean of the three separate recordings on different days. NT, normotensive; HT, hypertensive; RLU/min, relative light units produced per minute.

with luminol-enhanced CL and stimulated with 50 μmol/l AA (NT 2.60 ± 0.18 versus HT 3.26 ± 0.12; $P < 0.01$) and 500 nmol/l TPA (NT 2.51 ± 0.17 versus HT 3.01 ± 0.08; $P < 0.05$). These findings were also reproduced when assaying ROS production with CLA, whereas isoluminol-enhanced CL only showed a significant difference between NT and HT subjects when stimulating with AA (results not shown).

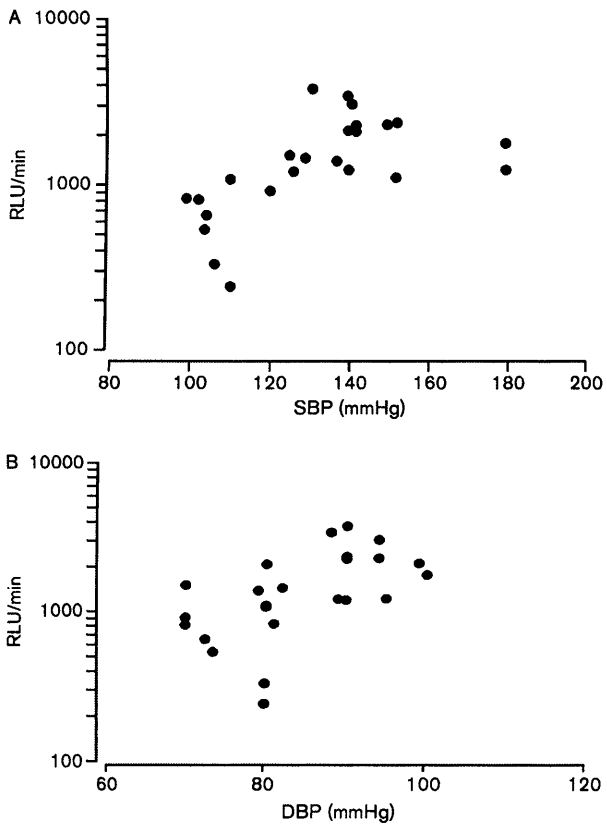
AA-stimulated ROS production assayed with luminol-enhanced CL correlated with DBP ($r = 0.53$, $P < 0.01$) and SBP ($r = 0.46$, $P < 0.05$) (Fig. 3). TPA-stimulated ROS production, measured with luminol-enhanced CL, correlated with SBP ($r = 0.61$, $P < 0.01$) but the correlation of TPA-stimulated ROS production with DBP failed to reach significance ($r = 0.35$, $P = 0.089$). The AA- and TPA-stimulated ROS production assayed by CLA-enhanced CL also correlated significantly with both SBP and DBP (results not shown).

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. We found that $p22^{phox}$ was overexpressed in HT versus the NT cell lines. A representative Western blot is shown in Figure 4. Using standardized results from four 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 was 0.15–1.06 ($P = 0.01$, as calculated by the Mann–Whitney test). We could detect no significant difference between the cell lines in $p47^{phox}$ (NT 1.0 ± 0.06 versus HT 1.0 ± 0.11), $p67^{phox}$ (NT 1.03 ± 0.33 versus HT 1.14 ± 0.28), $gp91^{phox}$ (median NT 1.04 versus median HT 1.14; 95% CI for difference, −0.49 to 0.13) and Rac2 (NT 1.13 ± 0.15 versus HT 0.88 ± 0.12) (Fig. 5). The results for all the phox subunits are the average of three standardized Western blots.

Discussion

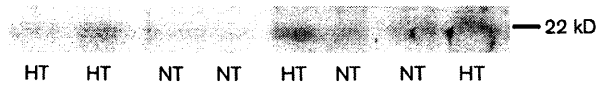
Using luminol- and CLA-enhanced CL for detection of ROS and two stimulants, we have demonstrated increased ROS production in HT compared to NT human lymphoblast cell lines. Isoluminol results were inconsistent. We chose to use isoluminol as well as luminol because some authors have shown that luminol attenuates immunoglobulin-primed ROS production in neutrophils [30]. The isoluminol probably could detect

Fig. 3



Luminol-enhanced chemiluminescence of 50 μmol/l arachidonic acid-stimulated lymphoblasts (12 normotensives and 12 hypertensives) plotted versus SBP (A) and DBP (B). Each point represents the mean of three values recorded on separate days. For SBP $r = 0.46$ ($P < 0.05$) and for DBP $r = 0.53$ ($P < 0.01$). RLU/min, relative light units produced per minute; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Fig. 4



A representative Western blot for $p22^{phox}$; 100 μg of the protein extract was loaded in each lane. Densitometric analysis revealed increased $p22^{phox}$ 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.

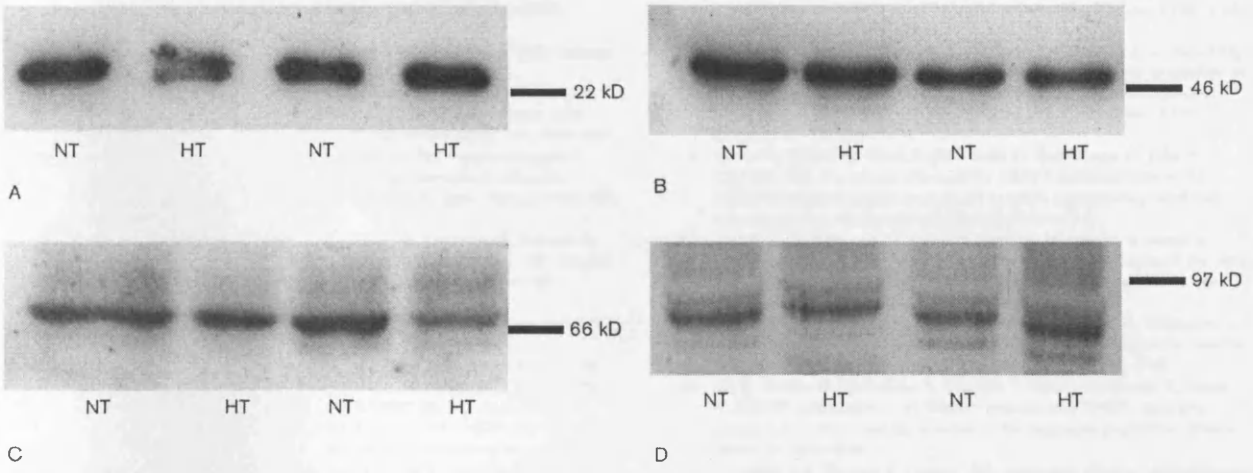
no difference in our lymphoblast model because isoluminol only measures extracellular ROS production. TPA is thought to stimulate NADPH oxidase to produce ROS indirectly by activation of protein kinase C [34] and AA directly activates NADPH oxidase [35]. DPI abolished ROS production whereas rotenone had no effect on ROS production. The use of two agonists, as well as demonstrating that the results are reproducible, show that direct activation of NADPH oxidase with AA also results in the excess production of ROS in the HT cell lines, suggesting that at least some of the increased production is due to a difference in NADPH oxidase activity rather than a pathway activating NADPH oxidase. DPI inhibits flavoproteins and has been shown to inhibit ROS production from unstimulated macrophage mitochondria [36]. 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 results add to the growing body of evidence in favour of an association between ROS production and hypertension. Previous work with neutrophils has been inconclusive, in that some groups have found an increase in ROS production in HT compared to NT subjects [5,6], while others have been unable to reproduce these results [7,8]. 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 ROS production is approximately 20–100 times less than in neutrophils [26,37]. As the increased ROS production of HT cells persists in transformed cells cultured *in vitro*, this suggests that the phenotype of increased ROS production is genetically predetermined.

The reason for the association of stimulated ROS production with HT is not clear, it may mean that the increased $p22^{phox}$ predisposes to HT but requires another trigger to unmask the phenotype. A possible trigger for increased ROS production is angiotensin II [11,24]. The increased stimulated ROS production may also have implications in the development of atheroma. Atherosclerosis is thought by many to be an inflammatory process [38]; 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 [39]; increased ROS production could mediate the decreased endothelial function. Stimulated ROS production may also be the link that associates chronic infections with endothelial dysfunction [40], and with the increased incidence of ischaemic heart disease.

In animal models of hypertension, two groups have shown increased production of $p22^{phox}$mRNA in hypertension [23,24]. We have shown a small but significantly increased expression of $p22^{phox}$in lymphoblasts

Fig. 5



Representative Western blots for Rac2 (A), $p47^{phox}$ (B), $p67^{phox}$ (C) and $gp91^{phox}$(D). Densitometric analysis of 12 NT and 12 HT subjects revealed no significant difference between NT and HT subjects for Rac2, $p47^{phox}$, $p67^{phox}$ and $gp91^{phox}$. HT, hypertensive; NT, normotensive.

from humans with hypertension. The presence of $p22^{phox}$ is known to be critical for ROS production in autosomal recessive chronic granulomatous disease [41] and in experimental models where $p22^{phox}$ is depleted [24]. $p22^{phox}$ interacts with an SH3 domain of $p47^{phox}$, allowing electron transfer to occur and therefore the reduction of oxygen [42]. The amount of $p22^{phox}$ may be a rate-determining step in the production of ROS. The small increase in $p22^{phox}$ is probably insufficient alone to account for all the variance in ROS production, but to formally assess this in such a small sample would be invalid. Other possible causes of increased ROS production are polymorphisms of $p22^{phox}$; the C242T polymorphism has been associated with increased ROS production [43] and may be associated with ischaemic heart disease [44,45] and stroke [46]. NADPH oxidase can be primed to produce increased ROS. Priming describes a complex process, often initiated by cytokines or infections, to enhance stimulated ROS production [47,48]. Increased levels of superoxide dismutase, catalase and glutathione can also negate ROS production.

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 overexpression of the $p22^{phox}$ subunit.

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