MODULATION OF TISSUE FACTOR ACTIVITY EXPRESSED FROM A HUMAN MONOCYTIC CELL LINE

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By

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ABSTRACT
The contribution of oxidised low-density lipoprotein (oxLDL) in the pathogenesis of atherosclerosis is well established, as is the presence of oxLDL within atherosclerotic plaques. An important concomitant of atherosclerosis is the increased tendency for thrombosis in the region of the plaque. This may be explained by an interaction between oxLDL and the constituent cells of the plaque as in vitro studies have demonstrated tissue factor (the initiator of the coagulation cascade) is expressed from vascular cells following incubation with oxLDL. The exact mechanism by which LDL is oxidised in vivo has yet to be elucidated however, one candidate for the oxidising species is peroxynitrite, which forms via a reaction between nitric oxide and the superoxide anion (both released from macrophages during inflammatory periods). In addition to its ability to oxidise LDL, peroxynitrite has been shown to nitrate tyrosine to 3-nitrotyrosine, an effect that influences the biological activity of certain proteins.

In the present study, the effect of native LDL and LDL oxidatively modified by cupric ions and peroxynitrite were studied with respect to their ability to influence maximal cell surface tissue factor activity and to induce tissue activity expression in THP-1 macrophages. Native LDL inhibited maximal cell surface tissue factor activity and did not induce tissue factor expression in the macrophages. Conversely, LDL modified by both cupric ions and peroxynitrite enhanced maximal cell surface tissue factor activity and induced tissue factor activity expression, above basal levels. These results indicate peroxynitrite-oxidised LDL modulates the tendency for blood to coagulate contributing to the hypercoagulable-state associated with atherosclerosis.

The direct effect of peroxynitrite on tissue factor was investigated as 11 of its 12-tyrosine residues are in its active domain. 8 of these are exposed and thus susceptible to attack by peroxynitrite. Following exposure of tissue factor to peroxynitrite, there was an inhibition of procoagulant activity, accompanied by an increase in the nitrotyrosine content of the tissue factor, determined by ELISA. Furthermore, exposure of LPS to peroxynitrite was shown to diminish its ability to induce tissue factor expression in THP-1 monocytes.

These results may indicate a protective mechanism afforded by peroxynitrite under inflammatory conditions as an anticoagulant, to minimise the extent of the coagulation response, but also indicate procoagulant properties through oxidation of LDL.
“One does not need to be terrifically brainy to be a good scientist...Common sense one cannot do without, and one would be the better for owning some of the old-fashioned virtues...application, diligence, a sense of purpose, the power to concentrate, to persevere and not to be cast down by adversity...”

Peter Medawar
“Advice to a Young Scientist”
Dedicated to my father and the memory of my mother
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CHAPTER 1

MAIN INTRODUCTION
Atherosclerosis is a multifactorial, degenerative disease of the coronary and cerebral arteries that contributes towards the major cause of death and disability in Western style cultures, myocardial and cerebral infarction. The risk factors associated with developing the disease include smoking, obesity, hypercholesterolaemia, and pathological conditions such as diabetes and hypertension. The disease is characterised by a progressive thickening of the arterial intima resulting in localised narrowing of the lumen by complex structures, namely atherosclerotic plaques, which have a focal distribution, almost certainly governed by haemodynamic factors.

1.1 Pathogenesis of atherosclerosis

Atherosclerosis evolves over a number of years, beginning in childhood and progressing through middle age or later. The prolonged time-scale of the progression of this disease means it is not possible to follow its development closely, thus a great debate has arisen with regards to its pathogenesis. For many years it was widely believed that injury to the endothelium, caused by hypertension or hypercholesterolaemia, triggered the atherosclerotic lesion, but studies have shown that fatty streak lesions can and do develop under an intact endothelium. Circulating monocytes penetrate between endothelial cells, enter the intima and become loaded with lipoprotein-derived lipids. This raises the question what part does injury to the endothelium play? Moreover, the question arises how does the early lesion, the fatty streak, progress into the more complicated lesion – the fibrous plaque? The current concepts with regard to the underlying pathology of atherosclerosis include the 'endothelial damage' and the 'lipid infiltration' hypotheses, which are summarised in Fig. 1.2.

The earliest lesion in atherogenesis, the fatty streak, is characterised by an accumulation of cells loaded with lipoprotein-derived cholesteryl esters (foam cells) just beneath the endothelium. Most foam cells arise from circulating monocytes that
have taken up residence beneath the endothelium. Gerrity et al., (1979) showed that an atherogenic diet promoted an increase in adherence of monocytes to the endothelium and was followed by their penetration into the sub-endothelial space, where they became loaded with cholesteryl esters. The progression of fatty streak into a more advanced lesion involves additional, quite different processes, including release of growth factors and cell proliferation. The end result, the atherosclerotic plaque (Fig. 1.1), comprises of a core of extracellular lipids derived from plasma lipoproteins, a base of necrotic connective tissue and a cap of fibromuscular tissue, primarily composed of smooth muscle cells, covered by the endothelium (Stary et al., 1995).

There is evidence that LDL may become oxidised in vivo (Steinberg et al., 1989). Oxidised LDL (see section 1.3) is known to be highly cytotoxic towards cells and also may be responsible for damage to the endothelium. It may be that its uptake by macrophages serves a protective function in preventing endothelial damage. However, death of the macrophages in the underlying fatty streak, may cause release of their contents, exposing the overlying endothelial cells to the cytotoxic oxidised LDL. This has been documented as loss of endothelial cells by electron microscopy (Gerrity, 1981). At this point the sequence of events proposed by Ross in the original response to injury hypothesis (Ross & Harker, 1976) may come into effect. Adherence and aggregation of platelets to the damaged endothelium, results in the release of platelet-derived growth factor, and generation of additional growth factors, which promote the proliferation of smooth muscle cells, and culminate in a thickening of the atheroma. In this way, the key events in the lipid-infiltration hypothesis and the endothelial damage hypothesis can be brought together (Fig. 1.2).

Rupture of an atherosclerotic plaque triggers thrombosis by activation of the extrinsic pathway of blood coagulation (see section 1.5.1.2), culminating in the acute clinical events, myocardial and cerebral infarction. The shoulder areas of the plaque are most prone to rupture (Ross, 1986; Ross, 1993), which may be a consequence of the high occupancy of macrophage foam cells in these regions, which are known to release
proteolytic enzymes that exert their action, particularly on lipid-rich fibrous plaques (Falk, 1992). The risk of rupture is dependent upon plaque composition rather than size. Plaques rich in soft extracellular lipids are particularly vulnerable compared to plaques that are rich in collagen (Falk, 1992). Moreover, the thrombogenicity of atherosclerotic plaques can also be influenced by plaque composition. A report by Toschi et al., (1997) stated that plaques with a lipid-rich core acted as a more potent stimulus for thrombus formation, than fibrous, collagen-rich lesions, an effect that may arise because they are mechanically less stable.
Fibrosis
Lipid – Media: muscle and elastic tissue
Basic lesion is the patchy deposition of yellow lipid in plaques deep in the intima with overlying fibrous.

EARLY PROLIFERATIVE PHASE
- Pale yellow streaks slightly raised above surrounding intima

PROGRESSING PHASE
- Raised yellow plaques
- Reduction of lumen

LATE ULCERATIVE PHASE
- Raised irregular rough ulcerated plaques. Partially calcified with thrombus material on surface
- Lumen narrowed and distorted
- Branch vessel orifice narrowed and distorted

Fig. 1.1 The development of a mature atherosclerotic plaque

5 mm

Fig. 1.2 An atherosclerotic plaque
The plaque marked by the arrow has occluded most of the lumen of this blood vessel
High plasma LDL

LDL infiltration into intima

ox LDL + macrophages

Foam cells

Fatty streak

Endothelial injury

Adherence of blood platelets

Other growth factors

Release of PDGF

Cell proliferation

Advance lesion

Fig. 1.3 Postulated linkage between the 'lipid infiltration' and 'endothelial injury' hypotheses
The 'lipid infiltration' hypothesis (right column) may be sufficient to account for fatty streaks, and the 'endothelial injury' hypothesis (left column) may account for the progression of the fatty streak to more advanced lesions. Taken from Steinberg et al., (1989).
1.2 Lipoproteins

As lipids are relatively insoluble in an aqueous environment they are transported as large molecules complexed with apolipoproteins. *In vivo*, lipoproteins transport cholesterol to the liver for excretion and cholesterol and triglycerides from the liver to cells and tissues where they are utilised in membrane synthesis and steroid hormone production. In addition lipoproteins provide a supply of energy to adipose tissue and muscle, in the form of free fatty acids.

Lipoproteins are categorised on the basis of their relative density that arises from their lipid profile, which is greater as the density decreases. This enables their separation by density ultracentrifugation. Each class of lipoprotein is heterogeneous, containing a range of particles within a particular density range. There are five main classes of lipoproteins, namely chylomicrons, very low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). The lipoproteins differ with respect to their lipid content, density, and apolipoprotein moiety (Table 1.1).

The largest lipoprotein particles, chylomicrons, are predominantly composed of triglycerides, with a protein moiety, apolipoprotein B-48 (apo B-48). The role of chylomicrons is to transport triglycerides from the intestinal site to the systemic circulation, where the enzyme lipoprotein lipase (LPL), gradually hydrolyses the triglycerides, generating fatty acids, which are taken up by adipose tissue and muscle cells for further catabolism for energy, or resynthesised as triglyceride for storage. In structure and composition VLDL are similar to chylomicrons but are smaller, and contain less triglyceride, but more cholesterol, phospholipid and apolipoprotein B-100 (apo B-100). VLDL are mainly synthesised in the liver and their chief function is the transport of endogenously synthesised triglyceride. As with chylomicrons, triglycerides are removed from VLDL by LPL in the peripheral circulation. Loss of triglycerides results in lipoproteins with an intermediate density, IDL. Further hydrolysis by lipoprotein lipase converts IDL to LDL. LDL is the major cholesterol-
carrying particle in plasma. LDL has a half-life in circulation (1-2 days) compared to VLDL and IDL (1-2 h). LDL is removed from the circulation via a receptor-mediated process (see section 1.2.2). Nascent HDL are synthesised by the liver and gut as disk-shaped particles comprising protein, free cholesterol and phospholipid. In the circulation, the enzyme lecithin: cholesterol acyl transferase (LCAT) associates with the nascent HDL and esterifies the cholesterol, resulting in HDL becoming a mature spherical particle with a cholesterol ester core. HDL removes excess cholesterol from cell membranes. Such cholesterol is esterified by LCAT, and passes into the LDL core as cholesterol ester thus, HDL acts as a reverse pathway of cholesterol transport. HDL may markedly minimise the atherosclerotic process through its cholesterol removal from cells, resulting in smaller fatty streaks, fibrous plaques and less complex atherosclerotic lesions. Moreover HDL has also been shown to inhibit the process of LDL oxidation (Parthasarathy et al., 1990) (see section 1.3), which may consequently reduce foam cell formation.

1.2.1 Structure and composition of LDL

LDL is a spherical particle with a diameter of about 22nm and an average molecular weight of 2.5 million. LDL is composed of 20-24% phospholipid, 10% free cholesterol, 40-44% cholesteryl esters, 3-5% glycerides, and 21-26% protein (apo B-100). The neutral lipids (cholesteryl esters and triglycerides) form a hydrophobic core that is surrounded by a surface monolayer consisting of phospholipids and cholesterol (Fig. 1.4). Apo B-100 (mw 512 KDa) is a flexible protein that is embedded in the phospholipid monolayer (Esterbauer et al., 1990) with at least 50% exposure at the interface. It is the apo B moiety of LDL that is recognised by the LDL receptor (Section 1.2.2). The major antioxidant within LDL is α-tocopherol. Each LDL particle contains about 6 molecules of α-tocopherol. The other antioxidants present within the LDL particle include β-carotene, γ-carotene and lycopene, although these are present in much smaller quantities (Esterbauer et al., 1990).
1.2.2 The LDL receptor

The LDL receptor provides a highly regulated pathway for the clearance of LDL from the plasma compartment, with more than 70% of LDL particles removed via receptor mediated endocytosis. Although most tissues have some LDL receptors the highest concentration is found in the liver where LDL cholesterol is converted to bile acids for secretion into the duodenum. In addition, the adrenal gland and ovaries also have a rich population of receptors, due to the requirement for cholesterol in steroid hormone synthesis. When cells need cholesterol they synthesise LDL receptors, which are processed in the cells and assembled in clathrin coated pits of the cell membrane. The receptor recognises a specific domain of positive charges from lysine, arginine and histidine residues, within apo B-100, and binding occurs. The receptor-LDL complex is internalised by endocytosis and forms endocytotic vesicles. Fusion of several of these vesicles gives rise to an endosome. The acidic environment of the endosome causes the LDL to dissociate from the receptor, which is recycled to the membrane. The LDL is delivered to a lysosome, which contain degradative enzymes, resulting in the hydrolysis of apo B-100 to free amino acids and cholesterol esters to free cholesterol. Three different biochemical processes regulate the intracellular cholesterol level in the cell. The ability of the cell to synthesise its own cholesterol is reduced by switching off HMG CoA reductase (3-hydroxy-3-methyl-glutaryl co enzyme A reductase). The incoming LDL cholesterol promotes storage by the activation of acyl CoA cholesterol acyltransferase (ACAT), resulting in the formation of cholesterol esters, which are deposited, as storage droplets. Finally, and most significant, the LDL receptor becomes downregulated to prevent further cholesterol entry.

The physiological importance of the LDL receptor was revealed by Brown & Goldstein’s pioneering work in the field of familial hypercholesterolaemia (FH). Patients suffering FH have markedly elevated levels of LDL: 680 mg/dl in homozygotes, 300 mg/dl heterozygotes, compared with a desirable level of 175 mg/dl in normal individuals (Stryer, 1988). The high plasma LDL concentration in
these patients is due to a deficiency or absence of the receptor for LDL. Consequently, these patients develop atherosclerosis prematurely. However, their lesions are not qualitatively different from individuals with normal LDL receptor. The massive accumulation of LDL cholesterol in these patient’s developing lesions must occur by way of some pathway other than the LDL receptor, as macrophages could not endocytose LDL fast enough to become foam cells, partly because of receptor down-regulation. This observation led to the discovery of the scavenger receptor.

1.2.2.1 The scavenger receptor

LDL which have been chemically modified through acetylation are recognised by a different family of receptors to the normal LDL receptor, which are located on macrophages (Goldstein et al., 1979). The so-called scavenger receptors avidly bind and internalise modified LDL 3-10 times more rapidly than native LDL. The chemical modification alters the overall charge of the LDL making it more negative and no longer recognisable to the normal LDL receptor. Oxidatively modified LDL (see section 1.3) are also recognised by the scavenger receptor family. As the ε-amino groups of the apo B-100 lysine residues become masked by lipid oxidation products such as malonaldehyde (MDA), there is a decreased recognition of the LDL by the native receptor, which is accompanied by an increased recognition by the scavenger receptor (Haberland et al., 1984). Unlike the normal LDL receptor, the scavenger receptor is not under the control of intracellular cholesterol. This leads to an accumulation of lipids, particularly cholesterol and cholesterol esters within macrophages, which can not be compensated for by intracellular biosynthesis, resulting in foam cell formation.
<table>
<thead>
<tr>
<th></th>
<th>Density (g/ml)</th>
<th>Major Lipid</th>
<th>Main apolipoprotein</th>
<th>Diameter (nm)</th>
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<tr>
<td>Chylomicron</td>
<td>&lt;0.95</td>
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</tr>
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<td>Apo B-100/Apo E Apo C-I,II,III</td>
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<td>IDL</td>
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<td>Cholesterol and Triglyceride</td>
<td>Apo B-100/ Apo E</td>
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<tr>
<td>LDL</td>
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<td>Cholesterol</td>
<td>Apo B-100/ Apo E Apo C-III</td>
<td>22</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063-1.21</td>
<td>Cholesterol and phospholipid</td>
<td>Apo A-I, II, Apo C-I,III Apo D/Apo E</td>
<td>6.5-9.5</td>
</tr>
</tbody>
</table>

Table. 1.1 Characteristics and composition of the lipoproteins
Fig. 1.4 Schematic model of low density lipoprotein (LDL)
The expression of the scavenger receptor has been demonstrated on murine macrophages (Goldstein et al., 1979), human monocyte-macrophages (Fogelman et al., 1980) and the monocytic cell line, THP-1, following treatment with phorbol ester to induce differentiation (Hara et al., 1987; Via et al., 1989). In addition these receptors are found on Kupffer cells from rats and guinea pigs, tumour cell lines of the mouse (J774 and P338) as well as on endothelial cells. Macrophage scavenger receptors have been cloned and characterised in detail (Kodama et al., 1990; Matsumoto et al., 1990). This led to the identification of two isoforms, Type I and Type II, which share five identical domains, but differ in their C-terminal domains.

More recently other receptors have been identified that are also capable of binding modified lipoprotein, including CD36 (Nozaki et al., 1995) and LOX-1 (Yoshida et al., 1998). Structural differences have led to the grouping of such receptors into three classes, scavenger receptor (SR)- A, B and C. The original type I and II macrophage scavenger receptors constitute the current membership of the SR-A class. SR-A have been implicated in the receptor-mediated uptake of modified LDL and the transformation of macrophages to foam cells, with the expression of SR-A Type I receptors during monocyte-macrophage differentiation detected by Geng et al., (1994). This is also true of the LOX-1 receptor expression, which is not expressed in monocytes (THP-1 and human blood) but is present in mature macrophages (Yoshida et al., 1998).

The role of the macrophage scavenger receptor does not solely lie in removal of lipoproteins from the circulation. SR-A have also been associated with the removal of dead or apoptotic thymocytes (Platt et al., 1996; Terpstra et al., 1997). Scavenger receptors also have the ability to bind and internalise bacterial pathogens (Dunne et al., 1994) and may explain the presence of bacteria within atherosclerotic plaques (Jackson et al., 1997). A relationship between bacterial infection and coronary events has been indicated (Whincup et al., 1996), however the risk is greatly reduced when other factors are taken into consideration. A causal role for bacteria in the development of atherosclerotic lesions has yet to be established. However, the presence of bacterial endotoxin within the structure of the atherosclerotic plaque
may promote thrombus formation following plaque rupture through activation of
the extrinsic pathway of blood coagulation (see section 1.5.1.2) by tissue factor (see
section 1.5.1. and 1.6) expressed on the surface of the constituent cells of the plaque.

1.3 Oxidative modification of LDL

It has been known for a number of years that isolated lipoproteins, in particular
LDL, are very susceptible to oxidation during prolonged storage. The deterioration
of LDL is particularly obvious visually with the initial loss of yellow colour due to β-
carotene degradation, followed by the fragmentation and aggregation of apo B-100 by
cleavage of the polypeptide chain by lipid peroxyl radicals, resulting in a cloudy
appearance.

As approximately half of the lipid component of LDL constitutes polyunsaturated
fatty acids (PUFA), particularly linoleic and arachidonic acid, within the cholesterol
esters and phospholipids, it is highly susceptible to oxidative attack by free radicals.
Oxidation of LDL starts with subtraction of an allylic hydrogen atom from a
reactive methylene group of polyunsaturated fatty acid side-chains, by an initiating
radical X to generate an alkyl radical, L• (Fig. 1.5). The alkyl radical then takes up
oxygen to form the peroxyl radical LOO•, which then regenerates itself and leads to
the formation of lipid hydroperoxides, LOOH and a new lipid radical L• during the
propagation phase. Termination of this process is dependent upon removal of the
radicals associated with the propagation phase, either by the combination of two
peroxyl radicals to form non-radical products or by the presence of antioxidants, e.g.
α-tocopherol when donation of a hydrogen atom to the peroxyl radical results in a
relatively stable hydroperoxide.

Oxidation of LDL generates lipid oxidation products: - alkenals and alkanals such as
4-hydroxynoneal (4-HNE) (Esterbauer et al., 1987, 1988) and MDA, by scission of
the alkyl chains of fatty acids (Esterbauer et al., 1991); lysophosphatidylcholine from
the oxidation of phosphatidylcholine; oxysterols from the oxidation of cholesterol.
The formation of aldehydes leads to a secondary process, modification of the apolipoprotein by neutralisation of the \( \varepsilon \)-amino groups of lysine residues on the surface of the apo B-100 molecule (Steinberg 1989; Esterbauer, 1990). This results in an increase in the net negative charge and causes a change to its electrophoretic properties, which parallels the progressive decrease in lipoprotein affinity for the LDL receptor, as it becomes a ligand for the scavenger receptor (Haberland et al., 1984). It is unlikely that oxidation of LDL occurs within the plasma as it is protected by ascorbate (Frei et al., 1990), which prevents the initiation of lipid peroxidation by scavenging peroxy radicals in the aqueous phase of the plasma, before they can diffuse into plasma lipoproteins. Transferrin and caeruoplasmin provide antioxidant protection by sequestering transition metal ions, which prevents the stimulation of free radical reactions. Enzymatic antioxidants (superoxide dismutase, glutathione peroxidase, catalase, and glutathione reductase and glutathione transferase) are also present in the plasma, but only in low concentrations, thus their function remains unclear. It is proposed that oxidation of LDL occurs within the sub-endothelial space, where cellular components of the vessel wall may mediate oxidation (see section 1.3.2) and the quantity of antioxidants within LDL (see section 1.2.1) are only sufficient to protect from oxidation for a relatively short time.

1.3.1 Transition metal ion oxidation

A standard method of studying LDL oxidation \textit{in vitro} is by incubation with transition metal ions, in particular cupric ions, which catalyse the breakdown of lipid peroxides and thereby propagate further lipid oxidation. Indeed the oxidation of LDL by different cells types can be repeated by incubation with transition metal ions, with cupric ions being the most effective, but ferrous ions also initiating the process (Heinecke et al., 1987).
Fig. 1.5 The mechanism of polyunsaturated fatty acid oxidation initiated by free radicals
Based on time-dependent analyses the chronology of LDL oxidation by cupric ions can be divided into three consecutive time phases: lag phase, propagation phase and decomposition phase (Esterbauer et al., 1992). During the lag phase endogenous antioxidants (e.g. α-tocopherol and β-carotene) are consumed and only minimal lipid peroxidation of LDL occurs. The duration of the lag phase is determined by the endogenous antioxidant content of the LDL (Esterbauer et al., 1989) and when the antioxidants are depleted, the rate of lipid peroxidation rapidly accelerates giving rise to the propagation phase. The peroxide peak is reached when about 70 - 80% of the PUFA are oxidised, thereafter the lipid peroxide content of the LDL starts to decrease again because of decomposition reactions when hydroperoxides are converted to reactive aldehydes e.g. MDA and 4-HNE. Interactions between reactive aldehydes and apo B-100 give rise to the altered functional properties of oxidised LDL (Steinbrecher, 1987).

1.3.2 Cellular Oxidation

LDL cultured with endothelial cells are converted to a form that are recognised by the scavenger receptor and results in increased uptake of LDL into macrophages. In 1981, Henrickson et al., discovered that when LDL were incubated with endothelial cells for 24 h, the LDL became oxidatively modified. The modified LDL were no longer recognised by the normal LDL receptor, but were bound by the scavenger receptor located on macrophages, which internalised them. Subsequently other cell types were discovered that oxidised LDL, including monocytes, macrophages and smooth muscle cells (Heinecke et al., 1984; Leake & Rankin, 1990). The phenomenon of LDL oxidation using different cell types and media is summarised by Esterbauer, (1990).

It was noted that the oxidation of LDL only occurred when the cells were grown in specific medium, in particular Ham's F10, which contained significant concentrations of transition metal ions. Indeed, oxidation in such cell culture systems was almost completely inhibited by the addition of a metal ion chelator.
However, *in vivo*, metal ions are carefully sequestered, tightly bound to specific metal binding proteins and plasma proteins. Whether or not the concentration of free metal ions available *in vivo* under normal circumstances is ever high enough to mimic the system *in vitro* has yet to be elucidated. However, they may be present under pathological conditions as Lamb *et al.* (1995) reported the presence of catalytically active metal ions in tissue homogenates from atherosclerotic lesions.

In view of the uncertainty whether free metal ions oxidise LDL *in vivo*, alternative mechanisms for the cell catalysed oxidation of LDL have been proposed. Lipoxygenases are cytosolic enzymes, which are present in a number of cell types that have the ability to oxidise LDL. Various mechanisms have been proposed to explain this pro-oxidant effect. During the catalytic cycle of the enzyme free radicals are generated and these could diffuse into the LDL particle and initiate lipid peroxidation (Cathcart *et al.*, 1991). Another possibility is that lipoxygenase could act directly on the fatty acid side chains of the LDL, generating peroxyl (LOO) and alkoxyl (LO) radicals (Kalyanaraman *et al.*, 1990). However, studies by Sparrow *et al.*, (1992) and Jessup *et al.*, (1991) indicated that neither 5-lipoxygenase nor 15-lipoxygenase is essential for modification of LDL by cultured cells.

Monocytes and macrophages, as part of their biological function, generate large amounts of the superoxide anion and hydroxyl radicals, which are thought to be able to initiate lipid peroxidation (Steinbrecher *et al.*, 1988). The role of the superoxide anion in the transition metal ion oxidation of LDL was demonstrated by Heinecke *et al.*, (1986). Radicals of haem-containing proteins, usually associated with oxygen transport, for example myoglobin activated by hydrogen peroxide, generate ferryl myoglobin species, which have also been found to be potent agents in the oxidation of LDL (Dee *et al.*, 1991). Furthermore, nitric oxide (see section 1.7) has been shown to inhibit LDL oxidation in the presence of these species. However, in the presence of excess hydrogen peroxide, the action of nitric oxide is fundamentally different in that it appears to enhance the oxidation of LDL. Nitric oxide also acts as a pro-oxidant when it reacts with superoxide anions to generate the potent oxidising
species, peroxynitrite. The oxidation of LDL by peroxynitrite will be discussed in more detail in section 1.7.1 and in Chapter 4.

The modification of LDL, independent of lipid peroxidation, must also be taken into consideration. Activated macrophages secrete myeloperoxidase, which can generate a range of reactive species including hypochlorite and the tyrosyl radical. Hypochlorite was shown to modify the protein content of LDL without involving lipid peroxidation (Hazell et al., 1994) and there is evidence for the presence of hypochlorite modified proteins in human atherosclerotic lesions (Hazell et al., 1996). The generation of tyrosyl radicals by myeloperoxidase (Heinecke et al., 1993a) promotes the peroxidation of LDL lipids (Savenkova et al., 1994) and cross-links tyrosine residues into o-o' dityrosine (Heinecke et al., 1993b).

1.4 Oxidised LDL and atherosclerosis

As early as 1952, Glavind and co-workers noted that the chloroform extracts from atherosclerotic lesions contained lipid peroxides and that the peroxide content was positively correlated with the size of the atheroma. More recently, further evidence has emerged supporting the presence of oxidised LDL in vivo. Oxidised LDL has been detected in extracts of atherosclerotic lesions from both rabbits and humans (Ylä-Herttuala et al., 1989). Antibodies directed against oxidised LDL reacted with materials in atherosclerotic lesions of both rabbits and humans and autoantibodies against oxidised LDL have been detected in both rabbit and human plasma (Wu & Lefvert, 1995) implying the presence of either oxidised LDL itself or some very closely related antigen.

It has been demonstrated that the administration of antioxidants including Probucol, an anti-hypercholesterolaemic agent, (Carew et al., 1987), and the commercial antioxidant used by the food industry, butylated hydroxytoluene (BHT), (Bjorkhem et al., 1991) prevented the oxidative modification of LDL and significantly slowed the progression of experimental atherosclerosis in animal models. Several clinical studies
have indicated an inverse correlation between dietary intake of antioxidants and the risk of coronary artery disease (CAD) (Hertog et al., 1993; Rimm et al., 1993; Stampfer et al., 1993).

It has long been recognised that cigarette smokers have a markedly increased risk of CAD. A study by Harats et al. (1990) reported that LDL isolated from the plasma of cigarette smokers following inhalation of 6 cigarettes was more susceptible to in vitro oxidative modification than LDL isolated from control plasma obtained prior to smoking. Treatment with ascorbate or α-tocopherol for 2 weeks prior to the acute experiment blocked the effects of cigarette smoking. These results may indicate a role for antioxidant therapy in the prevention of atherosclerosis (Esterbauer et al., 1992).

1.4.1 The biological properties of oxidised LDL

Oxidised LDL differs from native LDL in many ways. Compared to native LDL, there is a rapid uptake of oxidised LDL by macrophages, resulting in the formation of the foam cell (Gerrity, 1981). Oxidised LDL are known to play a role in recruitment and retention of human monocytes during atherogenesis (Quinn et al., 1987). Moreover individual LDL oxidation products, such as lysophosphatidylcholine, have also been shown to exert a chemoattractant effect (Quinn et al., 1988). Mildly oxidised LDL stimulates endothelial cells to release monocyte chemoattractant protein (MCP-1) (Cushing et al., 1990); granulocyte and macrophage growth factors (Rajavashisth et al., 1990) and promote adherence of monocytes to the endothelium (Berliner et al., 1990). Frostegard et al. (1990) reported that oxidised LDL induced the differentiation of monocytes to macrophages. Thus, oxidised LDL can play a role in the recruitment of monocytes to the lesion, promote their adherence to the endothelium and stimulate their differentiation into macrophages. Furthermore, oxidised LDL inhibits macrophage mobility and prevents their exit from the vessel wall back into the circulation. This could result in a vicious circle of monocyte recruitment to the lesion site combined with the retention of the product of their differentiation, macrophages.
Oxidised LDL is known to be cytotoxic towards a number of cell types including fibroblasts (Morel et al., 1983; Cathcart et al., 1985), endothelial and smooth muscle cells (Hessler et al., 1979). In 1995, Marchant et al. showed the cytotoxicity of oxidised LDL towards human monocyte derived macrophages could be reversed by antioxidants suggesting that cytotoxicity originates from the products of lipid oxidation. Indeed, the lipid oxidation products 4-hydroxynonenal (4-HNE) and lysophosphatidylcholine have both been shown to be cytotoxic (Esterbauer, 1993; Stary, 1985). The toxic effect of oxidised LDL towards various cell types is of particular importance during necrosis, in the later stages of lesion development.

As macrophages are able to bind and sequester oxidised LDL, then it is likely that they are subjected to severe oxidative stress. Therefore defence mechanisms are employed to prevent cell death. When macrophages were treated with oxidised LDL or 4HNE their intracellular glutathione content increased (Darley-Usmar et al., 1991). This increase in the level of glutathione is due to de novo synthesis in response to the oxidised LDL, as inhibition of glutathione synthesis resulted in cytotoxicity at a concentration that did not effect untreated cells (Gotoh et al., 1993).

Oxidised LDL have also been shown to stimulate smooth muscle cell proliferation (Görög, 1997) and induce platelet activation and aggregation (Bruckdorfer, 1989; Ardlie et al., 1989). Oxidised LDL also has direct effects on the vasomotor properties of blood vessels (Plane et al., 1992), as does lysophosphatidylcholine (Kugiyama, 1990). This may explain the impairment of vascular relaxation associated with hypercholesterolaemia and atherosclerosis. The influence of mildly oxidised LDL, on the haemostatic system, by acting as a local mediator in thrombosis promotion (Drake et al., 1991), will be discussed in more detail in Chapter 4.
Fig. 1.6 The effects of oxidised low density lipoprotein (LDL) on arterial cells
1.5 Haemostasis

The haemostatic mechanism is poised to maintain the closed, high-pressure circulatory system, following blood vessel injury. Essentially, three major phases are recognised; the constriction of the injured blood vessel, the adherence and aggregation of platelets at the site of injury to form a haemostatic plug, and finally, the coagulation phase leading to the formation of a clot. Each stage is not regarded as a separate entity and in fact all three are closely and intricately inter-linked. Binding to the damaged vessel causes platelets to release arachidonic acid, some of which is converted to thromboxane A$_2$, which attracts additional platelets to the site of injury and causes the damaged vessel to constrict. Thromboxane A$_2$ also causes the platelets to release adenosine diphosphate (ADP), which causes the platelets to flatten and expose receptors for fibrinogen on their surface. Activation of Hageman factor (factor XII) by contact with the damaged area of blood vessel, initiates the intrinsic blood coagulation pathway (see section 1.5.1.1) or the release of tissue factor (factor III), initiates the extrinsic blood coagulation pathway (see section 1.5.1.2).

Both pathways culminate in the conversion of prothrombin to thrombin, which then causes the polymerisation of fibrinogen to fibrin. Fibrin strands form a meshwork lattice that binds the edges of the injured vessel together and traps platelets, erythrocytes and leukocytes, resulting in a blood clot. Clots are not permanent structures, indeed they are designed to dissolve when the structural integrity of the damaged vessel is restored. Fibrinolysis involves the enzymatic breakdown of fibrin (and fibrinogen). The active blood fibrinolytic agent responsible is plasmin, which is derived from its inactive precursor plasminogen, which is activated by tissue plasminogen activator (tPA) and regulated by inhibitors such as plasminogen activator inhibitor (PAI-1) and Lp (a).
1.5.1 Blood coagulation

Blood coagulation is a defence system that maintains the integrity of the closed, high-pressure circulatory system despite blood vessel injury. The blood contains a system poised to become engaged instantaneously in clot formation the moment tissue injury occurs. A cascade of reactions converts zymogens to active proteases by the cleavage of peptide bonds. For each level of the cascade a non-enzymatic cofactor (Ca$^{2+}$) and phospholipid are required. The initiation of blood coagulation was previously divided into two pathways, the intrinsic and extrinsic. The intrinsic pathway being activated when blood comes into contact with anionic surfaces and relies on only factors ‘intrinsic’ to flowing blood. Meanwhile, the extrinsic pathway, is activated by a component ‘extrinsic’ to the blood, the membrane bound glycoprotein tissue factor.

1.5.1.1 Intrinsic pathway of blood coagulation

The intrinsic pathway of coagulation is initiated by the activation of Hageman factor, also termed factor XII by contact with a negatively charged surface (e.g. collagen) or a blood incompatible molecule (e.g. ADP). When a coagulation factor is activated it is denoted by ‘a’, for example XIIa. High molecular weight kininogen and prekallikrein are two proteins that facilitate this activation. XIIa activates factor XI, which, in turn, activates factor IX. IXa complexes with factor VIII. Factor X binds to the factor IXa-VIII complex and is activated. Xa forms a complex with factor V. Prothrombin binds to this complex and becomes converted to thrombin, which, in turn, cleaves fibrinogen to fibrin and results in clot formation (Fig. 1.7).
INTRINSIC PATHWAY

HMWK
XI → XIIa
Prekallikrein

XI → Xla
Ca^{2+}

IX → IXa

VIII → VIIIa
Ca^{2+}
PL

X → Xa

V → Va

Prothrombin → Thrombin

Fibrinogen → Fibrin

Cross-linked fibrin clot

EXTRINSIC PATHWAY

TF
Ca^{2+}
PL

VII → VIIa

TF/VIIa

Fig. 1.7 The coagulation cascade

HMWK - High molecular weight kininogen; TF - tissue factor; PL - phospholipid
Feedback pathway
The common names of the coagulation factors and their corresponding numeric classification are shown in Table 1.2.

1.5.1.2 Extrinsic pathway of coagulation

The activation of the extrinsic pathway of blood coagulation is associated with trauma or injury to the blood vessel (e.g. rupture of an atherosclerotic plaque or laceration to the vascular endothelium at the site of a wound), whereby tissue factor (see section 1.6) is exposed to circulating inactive coagulation factors. Tissue factor serves as a cofactor with factor VII to facilitate the activation of factor X. Alternatively factor VII can activate factor IX, which, in turn, can activate factor X. Once activated factor X proceeds to activate prothrombin to thrombin in a reaction requiring factor V. The thrombin converts fibrinogen to fibrin resulting in clot formation (Fig. 1.7). Full functional activity of tissue factor requires its insertion into a phospholipid bilayer containing phosphatidylserine (PS). Because both factor VIIa and the substrates of the tissue factor/VIIa complex contain glutamic acid rich domains, the charge dependent phospholipid surface interactions of both VIIa and substrates may regulate function.

The extrinsic pathway was believed to play a secondary role in coagulation, based on the lack of natural mutants and limited survival of tissue factor knockout mouse mutants. However, tissue factor has been shown to activate factor IX, which was earlier believed to be an intrinsic pathway component (Osterud, et al. 1977). A revised coagulation cascade has been devised in which two stages as opposed to two pathways are proposed. This is composed of an initiation stage via the tissue factor dependent pathway and an augmentation stage, which is handled by the components of the previous intrinsic pathway. The tissue factor pathway is ‘shut down’ soon after initiation by tissue factor pathway inhibitor – TFPI (see section 1.5.2.1), however, by this stage there are sufficient amounts of thrombin generated to activate the ‘intrinsic factors’ V, VIII and XI. This augmentation stage ensures a prolonged and large-scale activation of the coagulation cascade.
1.5.2 Regulation of coagulation

Following the activation of the coagulation cascade, in order for the formation of thrombin and fibrin to be regulated, powerful anticoagulant mechanisms are immediately initiated. These mechanisms include activation of a fibrin-mediated mechanism; activation of plasmin, a protease that destroys the polymerisation properties of the fibrin monomer and the inhibition of thrombin and factor Xa by protease inhibitors.

1.5.2.1 Tissue Factor Pathway Inhibitor (TFPI)

Following clot formation the cofactor activity of tissue factor is suppressed by the serine protease inhibitor, tissue factor pathway inhibitor (TFPI) (Broze et al., 1988). TFPI in its mature form contains 276 residues and has an acidic amino terminal, followed by 3 tandem domains with homology to Kunitz-type protease inhibitors and a basic carboxyl-terminal (Broze, 1992). Kunitz-type inhibitors act by imitating the substrate, but following binding of the enzyme the cleavage between amino acid residues at the active site of the inhibitor occurs very slowly or not at all. TFPI not only inhibits factor VIIa/tissue factor, but also produces a direct inhibition of factor Xa by binding at or near its serine active site (Broze et al., 1988). The proposed mechanism for the Xa-dependent inhibition of factor VIIa/tissue factor by TFPI involves the formation of a quaternary factor Xa-TFPI-factor VIIa/tissue factor complex (Broze et al., 1988). This inhibitory complex can result from the initial binding of factor Xa to TFPI with subsequent binding of the factor Xa-TFPI complex to factor VIIa/tissue factor, or alternatively, TFPI could bind to a pre-formed factor Xa-factor VIIa/tissue factor complex (Fig. 1.8).
Fig. 1.8 Proposed mechanism for the inhibition of the factor VIIa/tissue factor complex

Two alternative pathways are shown for the final quaternary complex. On the right, TFPI binds to Xa, the factor Xa-TFPI complex then binds to factor VIIa/tissue factor complex. On the left, TFPI binds to a pre-formed factor VIIa/tissue factor complex. Based on Broze (1992).
A small percentage of TFPI is free in the plasma and some believe that this is the biologically active form. However the majority of TFPI is carried in association with the plasma lipoproteins, LDL and HDL. TFPI is secreted predominantly by the liver cells and has been isolated from HepG2 cells, a human hepatoma cell line (Broze, 1987b). *In vitro*, TFPI can be induced in the monocytic cell line U937, by stimulation with phorbol ester or lipopolysaccharide (LPS) (Rana *et al.*, 1988). Furthermore, TFPI is secreted simultaneously with tissue factor in monocytes in response to LPS, with TFPI peaking later, indicating a mechanism for the localised control of blood coagulation (McGee *et al.*, 1994). However, this effect was not detectable in human peripheral blood monocytes (HPBM) treated with PMA and LPS (van der Logt *et al.*, 1994). Recent reports demonstrated TFPI expression in atherosclerotic lesions from the same cells that express tissue factor, namely macrophages, endothelial cells and smooth muscle cells (Lupu *et al.*, 1995).

TFPI may play a role outside that of its traditional part in the regulation of blood coagulation. TFPI was shown to prevent restenosis following tissue injury in an animal model of atherosclerosis. Kamimkubo *et al.* (1997), elucidated that this was due to TFPI exerting an antiproliferative effect on aortic smooth muscle cells. Moreover, TFPI was also shown to inhibit smooth muscle cell migration induced by tissue factor/Factor VIIa complex (Sato *et al.*, 1997), an important factor in atherogenesis.

**1.5.2.2 Apolipoprotein B-100**

Native LDL also possesses an anticoagulant effect distinct from that of TFPI, which is related principally to its apolipoprotein – apo B-100 (Section 1.3). Purified apo B was shown to inhibit tissue factor in the absence of previously defined tissue factor inhibitors (Ettelaie & Howell, 1992). This effect was previously demonstrated with apolipoprotein AII, a protein component of HDL (Carson, 1987). The inhibition of the extrinsic pathway by LDL occurs by a direct interaction of the protein component of tissue factor and apo B (Ettelaie *et al.*, 1996). Using spectroscopic
<table>
<thead>
<tr>
<th>Numeral</th>
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<tr>
<td>I</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>II</td>
<td>Prothrombin</td>
</tr>
<tr>
<td>III</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>IV</td>
<td>Calcium</td>
</tr>
<tr>
<td>V</td>
<td>Proaccelerin (Labile Factor)</td>
</tr>
<tr>
<td>VII</td>
<td>Proconvertin (Stable Factor)</td>
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<tr>
<td>VIII</td>
<td>Thromboplasminogen-Antihaemophilic Globulin (AHG)</td>
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<td>PlasmaThromboplastin Component (PTC)-Christmas Factor</td>
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<td>Hageman Factor</td>
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<tr>
<td>XIII</td>
<td>Fibrin Stabilizing Factor</td>
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</table>

Table 1.2 Numeric classification and common names of the coagulation factors
analysis and gel electrophoresis the formation of a complex between tissue factor and apo B-100 was demonstrated (Ettelaie et al., 1996). The complex formed by a two-stage process; the development of a hydrophobic environment during first 20 min, followed by interactions with the anionic amino acids, glutamate and aspartate, over a longer period of time (up to 60 min). This coincides with the progressive inhibition of tissue factor activity with apo B-100 that reaches a maximum after 60 minutes. Ettelaie et al., (1995) reported mild proteolytic degradation of apo B does not affect the inhibitory potential of this protein, indicating that that the inhibition may be resultant from one particular domain. Indeed, further studies in this field have led to the identification of the inhibitory domain within apo B-100, which is located in the LDL receptor-binding region. The receptor-binding region of apo B-100 has two distinct regions that are rich in the positively charged amino acids lysine and arginine (3121-3217 and 3300-3400). These residues interact with a peptide within tissue factor, containing the negatively charged amino acids glutamate and aspartate, that closely resembles a repeated domain within the LDL receptor protein (Ettelaie, et al., 1998).

1.5.3 The influence of lipoproteins on the haemostatic system

Lipoproteins may modulate the haemostatic balance of the vasculature by altering the expression and or function of thrombotic and fibrinolytic factors. Platelet aggregation studies in vitro have shown that hypercholesterolaemic patients are more sensitive to aggregatory agents than normocholesterolaemic subjects (Di Minno et al., 1986). Moreover, the authors reported that ADP induced fibrinogen binding to platelets is increased in a dose dependent manner by LDL, which results in faster aggregation and the formation of larger platelet aggregates. Conversely, HDL have been shown to inhibit platelet aggregation (Aviram & Brook, 1983), an effect that may be explained by apolipoprotein E enriched particles (Desai et al., 1989).

The expression of tissue factor, the initiating protein in the extrinsic pathway of blood coagulation, by endothelial cells and macrophages by minimally modified
LDL (Drake et al., 1991) and acetyl modified LDL (Colli et al., 1997) respectively, has been demonstrated. A further dimension to this work was described by Howell & Ettelaie (1989), who reported that in its native form LDL inhibited tissue factor activity. Upon oxidation of the LDL there was a reversal of the inhibitory effect, with a loss and even reversal of the inhibitory effect towards tissue factor. Oxidation of LDL resulted in alterations to the secondary structure of apoB, which were accompanied by the formation of protein-lipid, adducts on the ε-amino groups of basic amino acid residues. The region in which a major effect is seen is the receptor-binding region (Ettelaie et al., 1997). Moreover, the isolated apolipoprotein moiety of LDL, apo B-100, was found to exert the same effect (Howell & Ettelaie, 1990). The inhibition of tissue factor activity by HDL has also been demonstrated (Carson, 1981; Carson, 1987).

The influence of lipids and lipoproteins on soluble coagulation factors has also been demonstrated; factor VII activity has been shown to correlate with fasting concentrations of each of the major lipid and lipoprotein classes (Mitropoulos, et al., 1989) and following dietary fat ingestion there is a rapid rise in factor VII activity, the magnitude of which correlates with the composition of the fatty meal (Mitropoulos et al., 1994; Sanders et al., 1996). Moreover, elevated fibrinogen levels have been detected in patients with familial hypercholesterolaemia (Di Minno et al., 1986) and in population studies, fibrinogen was shown to be weakly, but positively associated with LDL cholesterol, triglycerides, Lp (a) and inversely associated with HDL cholesterol (Koenig et al., 1992; Cremer et al., 1994). Fibrinolytic capacity is dependent upon the balance between plasminogen activators and inhibitors, both of which are also influenced by lipids and lipoproteins. The suppression of tPA release from endothelial cells by LDL, Lp (a) and HDL was demonstrated by Levin et al. (1994). Furthermore, the dose dependent production of PAI-1 from endothelial cells in the presence of VLDL was shown (Stikorahm et al., 1990; Kaneko et al., 1994).
1.6 Tissue Factor

Tissue Factor (thromboplastin, factor III) is a transmembrane glycoprotein, which principally is known for its role in the initiation of the extrinsic pathway of blood coagulation (Section 1.5.1.2). Drake et al., (1989) reported the selective cytologic distribution of tissue factor in human tissues. Tissue factor activity is detectable in the brain, lung, kidney and placenta, particularly in the connective tissues encapsulating these organs. Moreover, tissue factor is expressed on the surface of adventitial cells surrounding blood vessels, though it is not detectable in the endothelium or peripheral blood cells. Thus, the tissue distribution of tissue factor can be thought of as constituting a haemostatic envelope around organs and blood vessels, ready to activate coagulation when vascular integrity is disrupted.

Tissue factor is most well known with regard to its role in the initiation of the extrinsic pathway of blood coagulation. Tissue factor synthesis can be induced in cells of the vasculature under inflammatory conditions through exposure to bacterial lipopolysaccharide (Colucci, et al., 1983; Robinson, et al., 1992). The intravascular coagulation disorders linked to septic shock, localised areas of coagulation - disseminated intravascular coagulation (DIC) can be explained by the aberrant expression of tissue factor (Thompson & Smith, 1989), as can the thrombotic complications associated with atherosclerosis (Taubman, et al. 1997) and with various types of cancer (Kakkar et al., 1995).

Recent data have given support for a role for tissue factor, outside that of coagulation, in cell proliferation as a response to vascular injury. The stimulation of quiescent human fibroblasts by serum-induced tissue factor mRNA plateaued within 1-2 h in the absence of de novo protein synthesis (Bloem et al., 1989). This led to the classification of the tissue factor gene as an ‘immediate early’ or ‘primary response’ gene. Furthermore, there is evidence that tissue factor is involved in tumour growth, via angiogenesis (Zhang et al., 1994), metastasis (Bromberg et al., 1995) and in

1.6.1 The structure of tissue factor

Tissue factor is synthesised as a 295 amino acid polypeptide including a 32-residue leader sequence, which is cleaved to produce the mature molecule. However, in as many as half of the molecules an additional two residues are removed resulting in a mature protein with a staggered amino terminus (Morrissey et al., 1987). Tissue factor consists of three domains: a short cytoplasmic domain of 19 residues (243-261), a single transmembrane domain of 23 residues (220-242) and an extracellular (receptor) domain of 219 residues (1-219). A single cysteine residue within the cytoplasmic domain is thioester bonded to stearate or palmitate, which may anchor it to the plasma membrane (Ruf & Edgington, 1994). Furthermore, cytoplasmic serine residues are phosphorylated by a protein kinase C dependent mechanism; however, this has yet to be assigned a functional role (Zioncheck et al., 1992). The extracellular domain of tissue factor contains two pairs of cysteines that are disulphide bonded. The importance of these disulphide linkages for factor VII binding was demonstrated by Rehemtulla et al. (1991). Three predicted N-linked glycosylation sites are available within the extracellular domain of human tissue factor for the assembly of carbohydrate moieties, although the glycosylation does not appear to be required for function (Paborsky et al., 1989). Recent studies indicate that the carbohydrate modification plays a role in the efficient presentation of cell surface tissue factor (Rickles et al., 1995).

Molecular cloning of tissue factor revealed three repeats of the uncommon tripeptide, tryptophan-lysine-serine, (WKS), previously hypothesised as a sequence motif that may be associated with structural loci that function to recognise serine proteases (Morrissey et al., 1987). The crystal structure of the extracellular domain of tissue factor, which interacts, with coagulation factor VII, to initiate the coagulation cascade, has been resolved to 2.0 Å (Banner et al., 1996). It was found to be
organised into two modules containing β-pleated sheets. The elucidation of the
structure of tissue factor, has revealed it to be similar to the cytokine receptor super-
family (Bazan, 1990), which are usually anchored through a single transmembrane
domain, followed by a cytoplasmic domain of varying length, and an extracellular
domain typically containing two β-strand folds.

1.6.2 Tissue factor and atherosclerosis

Under normal conditions, the cells of the vasculature do not express tissue factor. A
study of normal arterial and venous vessels by Wilcox et al. (1989), reported that
tissue factor mRNA and protein were absent from endothelial layers; however, tissue
factor was detectable in scattered cells in the tunica media and in the adventitia
surrounding these vessels. Circulating monocytes do not normally express tissue
factor, however, during episodes of inflammation, there is an induction of tissue
factor expression in these cells. It has been reported that hypercoagulability
associated with acute myocardial infarction and angina pectoris is induced by the
expression of tissue factor on circulating monocytes (Leatham, 1995). More recently,
Freeburn et al. (1998) reported that monocytes isolated from post myocardial
infarction patients had significantly higher procoagulant activity than age-matched
control patients did. A study by Misumi et al., (1998), on plasma tissue factor
antigen, showed that patients with unstable angina had higher plasma tissue factor
antigen levels than patients with stable angina

Tissue factor has been shown to contribute to the procoagulant activity of most
atherosclerotic lesions in the coronary artery (Marmur et al., 1996) and has been
detected in all stages of atherosclerotic lesions although it is more prominent in fatty
streaks and atheromatous plaques than in the early diffuse intimal thickenings
(Hatakeyama et al., 1997). The location of tissue factor within the atherosclerotic
lesion is also an important factor to take into consideration. In patients with
unstable angina, tissue factor was predominant in cellular areas containing
macrophages and smooth muscle cells, meanwhile in patients suffering with stable
angina, tissue factor was predominant in acellular areas (Moreno et al., 1996). In 1997 Ardissino et al., reported that it is the accumulation of tissue factor in an atherosclerotic plaque that plays a role in determining plaque thrombogenicity, as patients with unstable angina or who have suffered myocardial infarction had higher levels of tissue factor antigen and activity detectable in plaque regions than patients with stable angina.

1.7 Nitric oxide and peroxynitrite

Nitric oxide (NO) the gaseous free radical that is synthesised from L-arginine, is rapidly neutralised by haemoglobin and superoxide. Nitric oxide is synthesised in numerous mammalian cell types including endothelial cells, neutrophils (McCall et al., 1989) and fibroblasts (Ishii et al., 1991), by the nitric oxide synthase (NOS) family of isoenzymes. NOS converts the terminal guanidine group of L-arginine to generate nitric oxide and citrulline. The reaction requires nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor and uses molecular oxygen to oxidise the substrate.

The generation of nitric oxide by NOS is by either a constitutive or inducible pathway. The constitutive forms of NOS (neuronal and endothelial) are rapidly activated by receptor-mediated and flow-related stimulation of the endothelium and generate low rates of NO synthesis. Endothelial NOS, the isoform responsible for the nitric oxide released by agonist-stimulated vascular endothelial cells, results in the relaxation of vascular smooth muscle. The regulation and function of the inducible form of NOS (iNOS) is mainly associated with inflammatory and immune responses, generating large amounts of nitric oxide over a prolonged period, following activation by endotoxin and cytokines. iNOS is found in monocyte/macrophages, but, until recently it has been difficult to demonstrate the release of NO from human cells. However, Sharara et al., (1997) demonstrated the induction of NO synthesis by human cells following activation with α-interferon.
1.7.1 Physiological effects of nitric oxide

NO plays a significant role in the regulation of vascular tone, by promoting relaxation of vascular smooth muscle cells and inhibiting platelet aggregation (Moncada & Higgs, 1993). It acts as an important regulator of blood flow and perfusion pressure to isolated vascular beds in response to appropriate physiological stimuli. The involvement of a dysfunctional endothelium has been associated with pathologies such as atherosclerosis (Forstermann, *et al.*, 1988). Thrombosis is analogous with advanced atherosclerotic lesions and in part is controlled by nitric oxide dependent inhibition of platelet aggregation. However, in atherosclerotic lesions an excess production of superoxide may cause loss of the modulatory action of nitric oxide and at the same time yield peroxynitrite which is pro-aggregatory, thus promoting thrombus formation (Moro *et al.*, 1994). Oxidised LDL and lipid oxidation products have also been implicated in the impairment of vascular response associated with atherosclerosis and hypercholesterolaemia (see section 1.4). Nitric oxide, under certain conditions, has been demonstrated to play a role in the oxidative modification of LDL. In the presence of nitric oxide the ubiquinol content of LDL was found to be oxidised, but this was accompanied by only a small accumulation of lipid peroxides and there was no evidence that the integrity of apo B-100 had been altered (Jessup *et al.*, 1992). However, a study by Dee *et al.* (1991) reported that nitric oxide could act as both an antioxidant and a pro-oxidant of LDL, in the presence of myoglobin and hydrogen peroxide, depending upon the relative concentration of nitric oxide to hydrogen peroxide. Furthermore, nitric oxide promotes LDL oxidation when it reacts with superoxide, generating the potent oxidising species, peroxynitrite.

1.7.2 Peroxynitrite

Peroxynitrite forms from a reaction between nitric oxide and the superoxide anion (Fig. 1.9). The formation of peroxynitrite is dependent upon the relative concentrations of nitric oxide and superoxide present at any one time. Pathological
conditions can substantially upregulate the production of nitric oxide and superoxide released (Mugge et al., 1994), however a critical factor in peroxynitrite formation is the presence of superoxide dismutase, found in eukaryotic cells. Under normal physiological conditions, levels of superoxide will be kept low by the action of superoxide dismutase. Superoxide dismutase is present in micromolar concentrations and reacts with superoxide at $2 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$. As the concentration of nitric oxide required to cause vasorelaxation is only 5-10 nM, nitric oxide cannot compete with superoxide dismutase for superoxide; thus peroxynitrite does not form. However, if nitric oxide concentrations rise to micromolar levels, for example during periods of inflammation, it rapidly competes with superoxide dismutase for superoxide, due to its rapid rate constant ($6.7 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$), resulting in the formation of peroxynitrite. Peroxynitrite has a half-life under 1s in phosphate buffer at pH 7.4. This means under physiological conditions it is stable enough to diffuse away from the site of formation before reacting with target molecules. In its protonated form, peroxynitrous acid (ONOOH), it decomposes forming a species with the reactivity of the hydroxyl radical (Fig 1.9) (Beckman et al., 1990).

Evidence is emerging about the cellular formation of peroxynitrite, which has been detected in bovine aortic endothelial cells (Kooy & Royall, 1994) and rat macrophages (Ischiropoulos et al., 1992 b). However, the ability of human cells to form peroxynitrite has always been a matter of debate. In 1994 Carreras et al. found that stimulated human neutrophils released nitric oxide and superoxide at rates that favour the formation of peroxynitrite. More recently a study by Gagnon et al. (1998) discovered that peroxynitrite was formed by neutrophils, monocytes and lymphocytes in response to lipopolysaccharide.
NO\textsuperscript{−} + O\textsubscript{2}\textsuperscript{−} \rightarrow ONOO\textsuperscript{−}

ONOO\textsuperscript{−} + H\textsuperscript{+} \leftrightarrow ONOOH \rightarrow HO\textsuperscript{−} + NO\textsuperscript{2−} \rightarrow NO\textsuperscript{3−} + H\textsuperscript{+}

Fig. 1.9 The formation of peroxynitrite and its decomposition

Fig. 1.10 The nitration of tyrosine by peroxynitrite
1.7.2.1 Actions of peroxynitrite

Peroxynitrite has been shown to exert an effect on a variety of target molecules, through a number of different reactions. Peroxynitrite modifies proteins by nitration of specific amino acids, in particular the ortho positions of tyrosine residues to form nitrotyrosine (Ischiropoulos et al., 1992a) (Fig. 1.10). In addition phenylalanine may be nitrated (van der Vliet et al., 1994) and sulphydryl groups damaged (Radi et al., 1991b), which may, in turn, influence the biological activity of proteins. Protein modification, in particular tyrosine nitration, and its influence on the biological activity of proteins will be discussed further in Chapter 5.

Peroxynitrite has been shown to induce membrane lipid peroxidation, an effect that may explain its cytotoxic potential (Radi et al., 1991a). Hogg et al., (1993b) showed that α-tocopherol in human LDL could be oxidised by the simultaneous generation of superoxide and nitric oxide, whilst Graham et al., (1993) showed that LDL could be oxidised in vitro by peroxynitrite, to a form that was recognised by macrophage scavenger receptors, indicating modification to the apo B-100 moiety. The exact mechanism by which peroxynitrite oxidises LDL is as yet uncertain. It may be caused by the decomposition of peroxynitrite, which generates in addition to nitrogen dioxide, a hydroxyl radical - like oxidant, which has been shown to initiate lipid peroxidation (Hogg et al., 1992). Peroxynitrite may also contribute the modification of LDL by releasing copper from the caeruloplasmin, which would then promote lipid peroxidation (Swain et al., 1994). Oxidation of LDL by peroxynitrite has been shown to release a family of compounds called F₂ isoprostanes, through peroxidation of arachidonic acid (Moore et al. 1995). F₂ isoprostanes are known to exert potent biological activity, such as vasoconstriction. Thus, the oxidation of LDL by peroxynitrite may have a dual role in atherosclerosis, in the development of the atherosclerotic plaque and on vasomotor tone, through the release of oxidation products.
1.7.2.2 Peroxynitrite in vivo

The presence of nitrotyrosine is often considered as a specific footprint of peroxynitrite, although recent studies have suggested that tyrosine nitration may serve as an indicator of the presence of reactive nitrogen species (Halliwell, 1997). The presence of nitrotyrosine residues within atherosclerotic plaques was demonstrated by Beckman et al., (1994) using immunohistochemical techniques. However, nitrotyrosine residues were not detected in normal blood vessels (Beckman, 1994; Buttery et al., 1996), indicating a possible role for the involvement of peroxynitrite in the pathogenesis of atherosclerosis. Moreover, analysis of LDL isolated from human atherosclerotic lesions has revealed higher levels of nitrotyrosine compared with circulating LDL (Leeuwenburgh et al., 1997b). This appears to be convincing evidence for a role of peroxynitrite in atherosclerosis and other pathological conditions, including Alzheimer’s disease (Good et al., 1996), rheumatoid arthritis (Kaur & Halliwell, 1994), sepsis and viral myocarditis (Kooy et al., 1997), as tyrosine cannot be nitrated by nitric oxide alone (Ischiropoulos et al., 1992a; van der Vliet et al., 1994). However, there is also evidence for the presence of peroxynitrite in vivo, in the absence of any underlying pathology. Nitrated proteins have been detected in normal human plasma by ELISA (Khan et al., 1997), which may indicate low levels of peroxynitrite are being released from cells in vivo.
1.8 Aims of present work

- To establish that a human monocytic cell line could be used as a model system to study the induction of tissue factor activity in the macrophagic phenotype.

- To study the effect on cellular tissue factor activity of lipoproteins oxidised in the presence of cupric ions or by peroxynitrite.

- To determine the direct effects of peroxynitrite on isolated and cellular tissue factor.
CHAPTER 2

METHODS
CHAPTER 2: METHODS

2.1 Isolation of LDL

Low-density lipoprotein (LDL) was isolated, by discontinuous gradient ultracentrifugation, using a modified method based on Chung et al., (1980), to include a wash phase. The wash phase was incorporated in order to ensure purity of the LDL isolated.

Blood was extracted from healthy individuals, who gave informed consent, by venopuncture with a 19 gauge butterfly needle, into acid citrate dextrose (ACD – Appendix C), in the ratio of 4 parts blood to 1 part anticoagulant. The plasma fraction was isolated by centrifugation at 1500g for 20 min, in IEC Centra GP8R. The density of the plasma was adjusted by the addition of solid NaBr at 0.4428 g/ml and the plasma layered under 154 mM NaCl with 2 μM diethylenetriaminepenta-acetic acid (DTPA – Appendix C) (Fig 2.1. A). The sample was centrifuged at 200,000g for 2.5 h in a Beckman XL70 ultracentrifuge, using a 70 Ti fixed angle rotor. The resulting LDL band (B) was removed and layered between 1.151 and 1.063 SG density solutions (Appendix C) prior to a final centrifugation at 200,000g for 16 h (the wash phase). The LDL were then situated at the top of the tubes (C) and were removed from the centrifuge tubes using sterile 19-gauge needle and syringe. All centrifugation steps were performed at 4°C.

The LDL samples were then dialysed twice against Tris/NaCl buffer with 2 μM DTPA (Appendix C) to remove NaBr, whilst protecting the LDL against metal ion oxidation. The final dialysis was performed against Tris/NaCl buffer without DTPA to enable the LDL to be oxidatively modified at a later stage using copper ions. LDL were then passed through a 0.22 μm sterile filter and refrigerated at 4°C prior to use within 3-5 days.
Fig 2.1 A schematic representation of the isolation of LDL from human plasma by discontinuous density ultracentrifugation, based on the method by Chung et al., (1980)
2.2 Determination of LDL concentration

The concentration of isolated LDL was determined by the measurement of the protein moiety - apo B100, utilising the Bradford assay (Bradford, 1976). A standard curve was constructed for each assay, by the addition of 500 µl Bradford reagent (Appendix C) to 100 µl bovine serum albumin (BSA) standard (0-100 µg/ml). The absorbance was read at 585nm on a DU70 spectrophotometer. The concentration of apo B100 was calculated from the standard curve using straight-line regression constants.

2.3 Oxidative modification of LDL

2.3.1 Native LDL

Following the final dialysis, in the absence of DTPA, the freshly isolated LDL was classified as native LDL (nLDL). In order to protect against oxidation it was supplemented with 1 µM DTPA and stored at 4°C, protected from light, for no longer than 5 days.

2.3.2 Oxidised LDL

Following the final dialysis, in the absence of DTPA, the LDL was oxidised by different procedures in order to vary the extent of oxidation and nitration.

1. Air oxidation- minimally modified (mmLDL) – LDL was diluted to 1 mg protein/ml in phosphate buffered saline (PBS) and exposed to air at 37°C for 18 h. The sample was placed into a sterile glass vial and sealed with a rubber septum. This was pierced with a 19-gauge needle attached to a 0.22 µm sterile filter, thus allowing for gaseous exchange. Following the incubation the LDL were supplemented with 1 µM DTPA to prevent further oxidation.
2. Cupric ion oxidation - fully oxidised (oxLDL) - LDL were diluted to 1 mg protein/ml in PBS and put into a sterile glass vial. Cu$_2$SO$_4$.5H$_2$O was added at a final concentration up to 500 $\mu$M, and the LDL incubated at 37°C for 18 h. Following the incubation an excess of DTPA (up to 1 mM) was added to the LDL to halt the oxidation process by chelating the cupric ions. The LDL was dialysed twice against Tris/NaCl buffer, at 4°C, over 8 h, to remove the chelated cupric ions and then supplemented with 1 $\mu$M DTPA to prevent further oxidation.

3. Peroxynitrite oxidation (PNLDL) - LDL were diluted to 1 mg protein/ml in PBS supplemented with 2 $\mu$M DTPA (to prevent metal ion oxidation and to inhibit transition metal-dependent decomposition of lipid hydroperoxides). The LDL were exposed to 0 - 1 mM peroxynitrite at 37°C for 90 min. The LDL were then stored at 4°C overnight prior to use (Modified method based on Graham et al., 1993). This step was included so that the LDL had received a comparable length of incubation to air and cupric ion oxidised LDL. The LDL was dialysed twice against Tris/NaCl buffer, at 4°C, over 8 h, to compensate for the change in pH caused by peroxynitrite treatment and then supplemented with 1 $\mu$M DTPA to prevent further oxidation.

4. SIN-1 oxidation (SINLDL) - LDL were diluted to 1 mg protein/ml in PBS supplemented with 2 $\mu$M DTPA (as above) and exposed to 0 - 1 mM 3-morpholinosydnonimine-hydrochloride (SIN-1) at 37°C for 90 min. The LDL was then stored at 4°C overnight prior to use (Modified method based on Jessup et al., 1992). This step was included so that the LDL had received a comparable length of incubation to air and cupric ion oxidised LDL. The LDL was dialysed twice against Tris/NaCl buffer, at 4°C, over 8 h, to compensate for the change in pH caused by SIN-1 treatment and then supplemented with 1 $\mu$M DTPA to prevent further oxidation.
2.4 Determination of LDL oxidation

2.4.1 Lipid extraction

Lipid extracts were prepared using a standard extraction procedure (Bligh & Dyer, 1959). 376 µl chloroform/methanol (1:2 v/v) was added to 100 µl LDL (1 mg/ml) in PBS. The sample was vortexed and left to extract for 30 min. 125 µl chloroform and 125 µl PBS were then added, the sample was vortexed and then centrifuged at 210g for 10 min. This resulted in two clearly defined phases. 200 µl of the lower phase (chloroform) was removed and dried under N$_2$ and then resuspended according to the assay.

2.4.2 Peroxide determination

An iodometric method had been used previously (El-Saadani, et al., 1989), to measure the lipid peroxide content of lipoprotein samples. However, this proved unsuitable when determining the lipid peroxide content of lipoprotein samples oxidised by peroxynitrite, because addition of the Chod-iodide reagent to dilute peroxynitrite generated a positive reading for lipid peroxides.

2.4.2.1 FOX assay

The ferrous oxidation/xylenol orange (FOX) assay (Jiang, Z-Y. et al., 1992) measures the oxidation of Fe$^{2+}$ to Fe$^{3+}$ at low pH by lipid hydroperoxides. The Fe$^{3+}$ then reacts with the ferric complexing dye, xylenol orange, resulting in the conversion of the acidified yellow of the xylenol orange to a blue colour. This assay was used to measure lipid peroxides in all lipid extracts of lipoproteins (Section 2.4.1). A standard curve was constructed using 50 µl cumene hydroperoxide (0 - 200 µM), dissolved in ethanol, 150 µl FOX reagent was added (Appendix C), and the absorbance read at 620nm in an Anthos Labtec HT2 plate reader. The peroxide
content of ethanol extracts of samples was calculated from the standard curve using straight-line regression constants.

2.4.3 Thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive substances (TBARS) assay provides a measurement of malondialdehyde (MDA), a breakdown product of arachidonic acid that is produced in relatively constant proportions to the peroxidation process (Esterbauer et al., 1990). A standard curve was constructed using 625 μl 1,1,3,3-tetraethoxypropane (TEP) (0 - 20 μM) to which 750 μl, 0.67% 2-thiobarbituric acid (Appendix C), was added. Samples were mixed and heated at 95-100°C for 15-30 min and the absorbance read at 532nm on a DU70 spectrophotometer. The TBARS content of samples was determined from the standard curve using straight-line regression constants.

2.4.4 Agarose gel electrophoresis

The principle of electrophoresis is based on the fact that lipoproteins, when placed in an electric field will migrate towards one of the electric poles (Noble, 1968). Oxidation of LDL results in the neutralisation of ε-amino groups of lysine residues of apo B-100, conferring upon it a net increase in electronegative charge which can be detected by agarose gel electrophoresis (Steinbrecher, 1987) and measured by the migration distance to the anode. The relative electrophoretic mobility (REM) is defined as the ratio of migration distance of oxidised LDL to native LDL. Agarose gel electrophoresis was undertaken using the Sebia Hydragel Lipo and Lp (a) kit.

2.4.4.1 Reagent preparation for agarose gel electrophoresis

Tris barbital buffer concentrate was diluted to 1l with ultra pure water to give a final solution containing: Tris 7.20 g/l, Barbital 1.84 g/l, Sodium barbital 10.30 g/l,
Sodium azide 0.10 g/l. The reconstituted buffer was stored at 4°C for up to 4 weeks in a closed container.

Sudan black stain was prepared by mixing with a magnetic stirrer the solutions in the following order: - 160 ml pure ethanol, 2 ml Sudan black concentrated solution, 140 ml ultra pure water. The stain was prepared and used within one day.

Destain - a 45% ethanol solution was prepared with ultrapure water and stored in a closed container at room temperature.

2.4.4.2 Electrophoresis procedure

Agarose gels were blotted to remove excess moisture and sample template positioned on the gel. 2 µl sample (LDL 1 mg protein/ml) was placed on the template wells and allowed to diffuse into the gel (approx. 10 min). The sample template was removed and the gel placed upside down into the electrophoresis tank, containing 150 ml Tris barbital buffer per compartment. Following electrophoresis for 90 min at 50 volts, the gel was removed from the tank and dried in hot air. The dried (and cooled) gel was immersed in the staining solution for exactly 15 min and destained for exactly 5 min in 45% ethanol. The gel was washed in a bath of distilled water and dried in hot air.

2.5 The human monocytic cell line - THP-1

The human myelomonocytic cell line THP-1 was originally cultured from the blood of a one-year old infant with acute monocytic leukaemia (Tsuchiya et al., 1980). This cell line represents human monocytes with respect to criteria such as its morphology, secretory products, ability to phagocytose and expression of membrane antigens. (Tsuchiya et al., 1980; Auwerx, 1991). These cells are blocked at a certain step of the differentiation process. They can, however, be artificially induced to differentiate into a macrophage-like form by treatment with phorbol ester (Tsuchiya et al., 1982). A more detailed account of this process is covered in Chapter 3.
2.5.1 Culture of THP-1 monocytes

THP-1 monocytes were routinely cultured in RPMI 1640 medium supplemented with 10% heat inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 20 μM mercaptoethanol, 100 units/100 μg/ml penicillin/streptomycin (Medium A), and incubated in a humidified atmosphere of 95% air and 5% CO₂. At this stage, the culture was a homogeneous population of round cells which grew in suspension, sometimes forming loose clumps.

2.5.2 Determination of cell number and viability

Cell number was determined using Fast-Read counting chamber, consisting of a 5 x 2 matrix of small (3 x 3) grids. The cell number within the small grids was counted. As the dimension of an individual 3 x 3 grid is 0.33 mm x 0.33 mm and the depth is 0.1 mm; the volume of each 3 x 3 grid is $10^{-4}$ ml (0.1 μl).

Thus the cell number can be derived from:

$\frac{\text{Total number of cells}}{\text{No. of grids counted}} \times 10^4 = \text{No. cells/ml}$

Cell viability was determined by the addition of Trypan blue in the ratio of 1:1 to cell suspension. Viable cells exclude Trypan blue and non-viable cells are stained blue. The percentage viability can be derived from:

$\frac{\text{No. viable cells}}{\text{total cell No.}} \times 100 = \% \text{ viability}$

Cells were routinely maintained between $2 \times 10^5$ - $9 \times 10^5$ cells /ml and at this concentration retained a viability greater than 98%.
Fig. 2.2 The growth characteristics of THP-1 monocytes
Cells were counted using a Fast Read counting chamber and viability determined by Trypan Blue dye exclusion. Results represent the mean ± SEM of 4 independent experiments.
The growth curve for THP-1 monocytes initiated at a concentration of $2.5 \times 10^5$ cells/ml is shown in Fig 2.2. The cells initially enter a 24 h lag phase, followed by a logarithmic phase of growth where the cell doubling time is 48 h. The cells enter a plateau phase at a concentration of $8.5 \times 10^5$ cells/ml (6 days). At this stage in order for continuing proliferation to occur, the cells were passaged in medium A.

2.6 Measurement of procoagulant activity

The amount of tissue factor activity in a sample can be determined by the one-stage prothrombin time assay (Fig. 2.3). The one-stage prothrombin time assay relies upon the initiation of the extrinsic pathway of coagulation (see section 1.5.1.2). Tissue factor, in the presence of calcium and phospholipid, acts as a cofactor for factor VII, to facilitate the activation of factor X and subsequent coagulation factors, which culminates in the formation of a fibrin clot. An unknown quantity of tissue factor activity in a sample can be determined through examination of the time taken to produce a clot. With increasing concentration of tissue factor, present within a sample, less time is taken for clot formation.

2.6.1 One stage prothrombin time assay

The one-stage prothrombin time assay was performed on a Cascade - M - coagulometer (Helena Laboratories). 100 $\mu$l CaCl$_2$ was pre-incubated at 37°C for 1 min prior to the addition of 100 $\mu$l of sample with unknown quantity of tissue factor (e.g. recombinant tissue factor or cell suspension), and a further 1 min incubation at 37°C. Clotting was initiated by the addition of 100 $\mu$l reconstituted plasma, containing clotting factors in their inactive form, thus initiating the extrinsic pathway of coagulation through the formation of the tissue factor- factor VIIa complex. Clot formation was detected via a change in optical density of the sample, and a reading of clotting time supplied.
Fig. 2.3 A diagrammatic representation of the one-stage prothrombin time assay

100 μl CaCl₂ is pre-incubated at 37°C for 1 min, prior to the addition of 100 μl standard or 100 μl sample with an unknown quantity of tissue factor activity. This mixture is incubated for 1 min at 37°C. Coagulation is initiated by the addition of 100 μl plasma. The time taken for clot formation is measured and tissue factor activity quantified by reference to a standard curve constructed using recombinant tissue factor.
From the time taken for clot formation to occur, tissue factor activity was quantified by reference to recombinant tissue factor standard, which at a concentration of 1000 units/ml clotted plasma in 14 sec. A standard curve was constructed using recombinant tissue factor diluted with distilled water, and expressed as log tissue factor concentration (units/ml) versus log clotting time (seconds). The concentration of tissue factor was calculated using straight-line regression constants.

Changes in procoagulant activity, for example the influence of modified forms of lipoproteins on tissue factor activity (see Chapter 4) were determined by a comparison of the sample in question against a control sample in which the lipoprotein had been replaced with an equal volume of PBS. As before procoagulant activity was determined by time taken to produce a clot and through reference to a standard curve. The change in procoagulant activity was then determined by the equation:

\[
\frac{\text{Initial activity} - \text{Residual activity} \times 100}{\text{Initial activity}}
\]

Whereby a negative value indicates an enhancement of procoagulant activity and a positive value indicates an inhibition of procoagulant activity.

2.7 Flow Cytometry

2.7.1 Principles of Flow Cytometry

Flow cytometry enables us to count and size particles, and to quantify the physical and biological properties of a large number of individual cells within a few minutes. It is the measurement of cells in a flow system delivered in single file, by the process of hydrodynamic focusing, past a point of measurement, the light source, normally a laser (Fig 2.4). Interaction of the cells with the light source may scatter the light and/or result in the excitation of bound fluorescent molecules present on the surface.
Fig. 2.4 A flow chamber on a flow cytometer
Samples are injected into the centre of a stream of liquid, the sheath fluid, which hydrodynamically focuses the sample stream, delivering the cells to the laser, the point of detection, in single file.
or interior of the cells. The light collected by a series of lenses is passed to a photomultiplier tube, which converts optical signals to electrical pulses, which are proportional to the amount of scattered or fluorescent light. Analysis of a homogeneous population of cells can be achieved by placing a 'gate' around the cells. This ensures that any cells outside of this region are excluded from the analysis.

2.7.2 Preparation of cells for Flow Cytometry measurement of tissue factor antigen expression

Cells were resuspended in 1% BSA/PBS (w/v) at 10⁶ cells/ml. 5 μl Fluorescein isothiocyanate (FITC) conjugated anti-tissue factor antibody or FITC conjugated mouse IgG antibody (negative control) was added and incubated over ice in the dark for 30 min. The cells were then washed in 1% BSA/PBS and resuspended in 1% BSA/PBS (w/v) prior to fluorescence analysis on a Coulter EPIC MCL flow cytometer. Forward angle and side scatter parameters were collected logarithmically and the cells were gated to exclude debris and microparticles. Fluorescence histograms of these gated cells were used to assess tissue factor antigen expression. This was calculated by subtracting the negative control fluorescence histogram from the tissue factor fluorescence histogram.

2.8 Preparation of peroxynitrite

Peroxynitrite was prepared by mixing equal volumes of NaNO₂ and HNO₃ through a T-junction and passing the solution into NaOH (Fig 2.5), as described previously (Beckman, 1990). Excess H₂O₂ was removed by filtering the solution over a MnO₂ column. Decomposed peroxynitrite was prepared by passing the NaNO₂/HNO₃ into H₂O resulting in a mixture of nitrate and nitrite. The pH was then adjusted by addition of NaOH. The concentration of peroxynitrite was determined by measuring its absorption at 302nm using the extinction coefficient (ε) of 1670 M⁻¹ cm⁻¹. (Fig 2.6)
Fig. 2.5 The preparation of peroxynitrite
Equal volumes of NaNO₂ and a mixture of HNO₃, H₂O₂ and H₂O are passed through a T-junction into NaOH, over ice, as described by Beckman, 1990.
Fig. 2.6 The \( n\nu \) absorption spectrum of peroxynitrite
The concentration of peroxynitrite can be determined by use of the extinction
coefficient (\( \epsilon \)) of 1670 M\(^{-1}\) cm\(^{-1}\) at 302nm.
2.9 Measurement of nitrotyrosine residues

Immuno-reactive nitrotyrosine residues in samples were quantified by competitive ELISA (Khan et al., 1997). Fatty acid free BSA at 2 mg/ml in 50 mM PBS, was nitrated with peroxynitrite over a 1 h period, with three aliquots of peroxynitrite added to 10 ml of the BSA solution (final concentration of peroxynitrite – 30 mM). The nitrated BSA was partly purified by chromatography.

Spectroscopic analysis of an alkaline (pH 10, 50 mM carbonate buffer) solution of the nitrated BSA was used to determine nitrotyrosine content. A scan was performed over the range 350 - 500nm and the optical density at 438nm noted. The molar ratio of nitrotyrosine to BSA was 4.7:1. The nitrated BSA (NT-BSA) was frozen at -70°C. This batch of NT-BSA was used throughout the series of experiments.

96 well plates were prepared for the assay by addition of NT-BSA, diluted to 5 µg/ml in 50 mM carbonate buffer, incubated at room temperature for 5 h, followed by an overnight incubation at 4°C. Unbound NT-BSA was washed away by three washes with PBS. Non-specific protein binding sites on each well were blocked by the addition of dilution buffer (0.05% Tween 20 (v/v) in 10 mM PBS containing ovalbumin 0.5% (w/v)), for 1 h at 37°C. The wells were then washed four times with wash buffer (PBS + 0.05% Tween 20).

A standard curve (0.005 – 100 µg/ml) was constructed using NT-BSA (100 µl) as the competing antigen. 100 µl polyclonal rabbit antibody (IgG fraction) to nitrated keyhole limpet hemocyanin was added. The plate was incubated for 2 h at 37°C and washed four times with wash buffer. 100 µl polyclonal antibody to rabbit IgG was added and the incubated further for 1 h at 37°C and then washed four times as before. 100 µl avidin/biotinylated horseradish peroxidase complex, prepared 60 min before use, was added. After the final incubation (1 h) the plate was washed four times as before. 100 µl of colour development solution, comprising solvent of
Fig. 2.7 A representative nitrotyrosine standard curve
A standard curve was constructed between (0.005 – 100 μg/ml) and the optical density read at 490nm on a MRX plate reader
phosphate/citrate buffer and perborate and a tablet of orthophenylene diamine as the chromogen, was added.

The plate was left for full colour development to take place and the reaction stopped by the addition of 50 μl 4M H₂SO₄. Optical density of each well at 490nm was determined using a computer-linked MRX (Dynex Technologies, UK) plate reader. A standard curve of Abs₄₉₀ versus log (n) μg/ml NT-BSA was constructed (Fig. 2.7). Competition assays were performed by adding appropriate samples instead of NT-BSA and inhibition of the antibody binding determined from the standard curve.

2.10 Quantification of bacterial lipopolysaccharide activity

Lipopolysaccharide activity was measured using the QCL 1000 chromogenic assay from Biowhittaker. The Limulus Amebocyte Lysate (LAL) assay is used to detect endotoxin associated with Gram-negative bacteria.

2.10.1 Principle of QCL 1000 chromogenic LAL method

\[
\text{Proenzyme} \xrightarrow{\text{Endotoxin}} \text{Enzyme} \\
\text{Substrate} + \text{H}_2\text{O} \xrightarrow{\text{Enzyme}} \text{Peptide} + \text{pNA}
\]

Gram-negative bacterial endotoxin catalyses the activation of a proenzyme in LAL. The initial rate of activation is determined by the concentration of endotoxin present. The activated enzyme catalyses the splitting of p-nitroaniline (pNA) from a colourless substrate. The pNA released is measured spectrophotometrically at 405nm. The correlation between the absorbance and the endotoxin concentration is linear in the 0.1 - 1 EU/ml range (EU- endotoxin unit, 1 EU = 0.1 ng/ml).
2.10.2 QCL 1000 chromogenic LAL test procedure

50 µl of standard, sample or water (control) was mixed with LAL (50 µl), in a 96 well plate and incubated at 37°C for 10 min, 100 µl of substrate solution added and the mixture incubated at 37°C for a further 6 min. The reaction was stopped by the addition of 25% v/v acetic acid (100 µl). In the presence of endotoxin a yellow colour developed. The absorbance of the sample was determined at 405nm on an Anthos Labtech HT2 plate reader. Absorbance is directly proportional to amount of endotoxin present, thus the concentration of endotoxin was calculated from the standard curve - 0.1 - 1 EU/ml (Fig. 2.8).

2.11 Statistical analyses

All experiments were performed at least three times. Averages were expressed as mean ± SEM. Statistical significance was analysed by paired Student’s t-test. Values of P < 0.05 were considered statistically significant.
Fig. 2.8 A representative Limulus amoebocyte lysate assay standard curve
A standard curve was constructed between 0.1 – 1 endotoxin units (EU)/ml and the absorbance read on a DU 70 spectrophotometer at 405nm ($R^2 = 0.9977$).
CHAPTER 3

INDUCTION OF MATURATION OF THP-1 MONOCYTES AND CORRESPONDING EXPRESSION OF TISSUE FACTOR ACTIVITY
CHAPTER 3: INDUCTION OF MATURATION OF THP-1 MONOCYTES AND CORRESPONDING EXPRESSION OF TISSUE FACTOR ACTIVITY

3.1 Introduction

Studies of monocyte derived macrophages are often impeded by the limited availability and difficulties in purification of monocytes from whole blood, and in any case there is still the need to transform the monocytes into macrophages. The immortalised human leukaemia cells lines (e.g. U937, THP-1 and MONO MAC 6) have phenotypes corresponding to the early stages of the monocyte differentiation pathway, with the added advantage of existing as a homogeneous population (Åbrink et al., 1994). The additional advantages of using such cell lines rather than freshly isolated human monocytes, include the viability of the cells and their unlimited growth potential, which provides a continuous and well-defined source of material. However, the use of such cell lines must be exercised with some caution, as they do not necessarily possess the same characteristics of the cell type from which they were derived. The monocytic cell line THP-1, originally isolated from an infant suffering from acute monocytic leukaemia, exhibits a phenotype more similar to native monocyte-derived macrophages than the other human myeloid cell lines with regard to criteria such as: 1) morphological characteristics, 2) expression of membrane antigens and receptors, 3) transient expression of proto-oncogenes, 4) production of secretory proteins. (Tsuchiya et al., 1980; Auwerx, 1991).

Under the influence of chemoattractants and growth regulatory molecules released from different cell types monocytes adhere to and migrate into the endothelium (Ross, 1993). Following penetration into the subendothelial space the monocytes undergo differentiation. Monocytes can be induced to differentiate by stimulatory agents, including phorbol esters (Matikainen et al., 1994), gamma interferon (IFN-γ) (Vey et al., 1992), Vitamin D3 (Rigby et al., 1984; Vey et al., 1992) and retinoic acid (Matikainen et al., 1994). The study of parameters such as morphological characteristics and the expression of surface antigens can be used to chart the process
of the differentiation of monocytes into macrophages. One such marker CD14, is associated with mature monocytes (Åbrink et al., 1994). It has been demonstrated that treatment of monocytes with IFN-γ and retinoic acid, to induce differentiation, is accompanied by an increase in CD14 expression (Rigby et al., 1984; Vey et al., 1992).

Lo et al., (1995) found that adhesion of monocytes to cytokine activated endothelial cells, an event that is associated with the early stages in the pathogenesis of atherosclerosis, was also accompanied by an increase in tissue factor activity expression. Spontaneous differentiation of peripheral blood monocytes into macrophages has been demonstrated to be accompanied by the transient expression of tissue factor (van den Eijnden et al., 1997). The transient expression of tissue factor during monocyte-macrophage differentiation can be replicated in vitro, in peripheral blood monocytes (Lyberg & Prydz, 1981); U937 cells (Rana, 1988); and HL60 cells (Bach & Moldow, 1997) stimulated with phorbol esters, including phorbol-12-myristate-13 acetate (PMA) and 12-O-tetradecanoylphorbol-13-acetate (TPA). The mechanism by which tissue factor synthesis is activated by phorbol esters is not yet fully understood, although activation of protein kinase C (PKC) is known to be involved, as pre-incubation of monocytes with PKC inhibitors have been shown to block PMA induced tissue factor activity (Ternisien et al., 1993). PKC acts as a receptor for PMA, which substitutes for the normal substrate, diacyl glycerol, in activating PKC. As analogues of diacyl glycerols, phorbol esters may act to cause perturbations in the phospholipid bilayer of the membrane (Dawson et al., 1984), which would influence tissue factor activity. Membrane reorganisation involves the exposure of negatively charged lipids, in particular phosphatidylserine, which is normally sequestered in the inner leaflet of the plasma membrane, to the outer surface of the membrane bilayer, which enhances procoagulant activity by favouring the assembly of the tissue factor/factor VII complex (Bach et al., 1986).

The lack of thrombotic events in the circulation can be explained by the fact that vascular cells including monocytes and endothelial cells either lack or express very
little tissue factor activity. However, on exposure to a variety of stimuli including P-selectin (Celi et al., 1994); monocyte chemotactant protein (MCP) (Schecter et al., 1997), and bacterial lipopolysaccharide (LPS) (van der Logt, 1994; Colucci, M., 1983), tissue factor is expressed by monocytes. Serum has also been reported to cause a transient increase in tissue factor mRNA in quiescent murine fibroblasts, human fibroblasts, human epithelial cells and rat vascular smooth muscle cells, via the action of serum growth factors (Mackman, 1995). Bloem et al. (1989) reported a transient increase in tissue factor mRNA, followed by an increase in tissue factor activity and antigen in quiescent human fibroblasts, following stimulation with serum. This finding was also replicated in human epithelial cells (HeLa), when tissue factor mRNA and activity were induced by serum and PMA (Cui et al., 1994). However, unlike stimulation with phorbol esters, PKC is not involved in the serum induction of tissue factor mRNA (Cui, 1994).

Pathological activation of the coagulation cascade by aberrant expression of tissue factor on the surface of monocytes or endothelial cells has been implicated in life-threatening conditions including, the thromboembolic disorders associated with cancer (Kakkar et al., 1995) and atherosclerosis (Taubman et al., 1997). Indeed, monocytes isolated from post-myocardial infarction patients were shown to have elevated tissue factor-like activity (Freeburn et al., 1998), which could act to increase the risk of subsequent ischaemic episodes. Tissue factor is also implicated in the complications associated with Gram-negative infections. Monocytes isolated from the blood of patients with meningococcal infection were shown to have an increased procoagulant activity, compared with monocytes from normal volunteers (Osterud & Flaegstad, 1983), which may explain disseminated intravascular coagulation (DIC), a complication associated with sepsis. DIC is thought to arise from a shift in the vascular endothelium from an anticoagulant to a procoagulant state, by the induction of tissue factor. A role for tissue factor in DIC has been demonstrated by (Creasey et al., 1993), who found that tissue factor pathway inhibitor reduced mortality in baboons, with E. coli induced septic shock.
Via the LPS/LBP/CD14 pathway, LPS induces tissue factor activity expression from monocytes, promoting coagulopathy (Steinemann et al., 1994). The lipid A moiety of LPS (commonly known as endotoxin), associates with LPS binding protein (LBP), a 60 kDa serum protein. The LPS-LBP complex interacts with the cell surface receptor CD14, a 55 kDa glycoprotein expressed on cells of the monocyte-macrophage lineage, that is commonly associated with maturation. Steinemann et al., (1994) reported that monocytes cultured in the presence of serum were induced to express tissue factor antigen at LPS concentrations 100 times lower than monocytes cultured in serum-free medium. The use of an anti-LBP antibody indicated that this effect was dependent on the presence of LBP in serum. Furthermore, the requirement for CD14 was demonstrated by the inhibition of LPS/LBP induction of tissue factor antigen in the presence of an anti-CD14 monoclonal antibody. However, the presence of LBP or serum does not appear to be necessary to elicit a procoagulant response to LPS in macrophages. A study by Jungi et al., (1996) demonstrated that monocyte-derived macrophages respond to LPS, in a similar fashion, with respect to an up-regulation of procoagulant activity, in the presence or absence of serum, suggesting that LBP is not required for the activation of macrophages. However, both serum dependent and independent stimulation of macrophages still required the presence of CD14. Thus, during monocyte-macrophage differentiation macrophages may acquire a functional substitute for LBP, enabling the macrophages to recognise low concentrations of LPS.

The induction of LPS-induced tissue factor activity in human monocytes is mediated by PKC activation (Ternisien et al., 1993), shown by the inhibition of LPS-induced tissue factor activity in human monocytes, by PKC inhibitors. Tissue factor induction in peripheral blood monocytes by LPS appears to be regulated at the transcriptional level, by the accumulation of tissue factor mRNA (Gregory et al., 1989). Studies using the monocytic cell line THP-1, indicate that tissue factor induction by LPS is regulated at both transcriptional and posttranscriptional levels (Brand et al., 1991). The authors reported that in addition to transcriptional control,
previously detected by Gregory et al., (1989) it appears that LPS also exerts a stabilising effect on the tissue factor mRNA during the accumulation period.

The differentiation of monocytes into macrophages is accompanied by the capacity of the macrophages to take up oxidised LDL in an un-regulated fashion by the scavenger receptor, leading to the formation of the foam cell. Foam cells found in the atherosclerotic plaque have been shown to express high levels of tissue factor mRNA and tissue factor protein (Wilcox et al., 1989). Furthermore it has been demonstrated that tissue factor is over-expressed in macrophages deposited in the extracellular matrix of atherosclerotic intimas (Kato et al. 1996). These findings indicate that at some point during differentiation, macrophages and foam cells start to express tissue factor constitutively. Alternatively it may be that in the atherosclerotic lesion macrophages are induced to express tissue factor through an endogenous stimulus. One such stimulus may lie in the presence of oxidised LDL within the plaque, as studies by Brand et al. (1990) have shown an induction of tissue factor mRNA in the monocytic cell line, THP-1 following incubation with oxidised LDL.

The aim for this section of work was to develop a model of monocyte-macrophage differentiation, in which the macrophages remained viable and the expression of tissue factor associated with differentiation had subsided, so that the effects of native and oxidatively modified LDL on macrophage tissue factor expression could be studied independently.
3.2 Results

3.2.1 An investigation into viability and tissue factor activity expressed on the surface of THP-1 monocytes over a 7-day period.

Cell surface tissue factor activity and viability in a routine culture of THP-1 monocytes, over a 7 day period, was measured by the one-stage prothrombin time assay (see section 2.6.1) and Trypan Blue dye exclusion (see section 2.5.2). THP-1 monocytes were passaged in Medium A (see section 2.5.1) at an initial concentration of $5 \times 10^5$ cells/ml. The high starting concentration was necessary so that small culture volumes would contain sufficient cells for the assays used, in particular the one stage prothrombin time assay, which requires $10^7$ cells/ml to achieve detectable clotting times on the coagulometer. The cell surface tissue factor activity and viability of the cells over a 7-day period is shown in Fig. 3.1. During the first 5 days the viability and cell surface tissue factor activity remained relatively constant. After 5 days there was a drop in viability, most likely to be the result of the cells reaching their optimal concentration required for sustained growth and the depletion of culture medium nutrients. The accompanying increase in cell surface tissue factor activity is likely to be the consequence of cell death, as increased procoagulant activity has been associated with apoptotic endothelial cells (Greeno et al., 1996; Bobeli et al., 1997).

3.2.2 An investigation into the influence of serum on tissue factor activity expression from THP-1 monocytes

As serum is known to induce tissue factor gene expression in epithelial cells (Cui et al., 1994) and fibroblasts (Bloem et al., 1989) studies were performed in order to determine the effect of serum on tissue factor activity expressed on the surface of THP-1 monocytes during routine culture. Parallel cultures were set up using
Fig. 3.1 The association between viability of THP-1 monocytes and cell surface tissue factor activity
THP-1 monocytes were passaged on day 0 as described in section 2.5.1. Cell surface tissue factor activity was measured by the one-stage prothrombin time assay and results expressed as tissue factor activity units / 10^6 cells and represented by the bar graph. The viability of the cells was determined by Trypan Blue dye exclusion and represented by the linear graph. Both measurements were determined up to 7 days after passage. The results shown are one typical experiment, which was performed at least 3 times. *P < 0.05; **P < 0.01; + P < 0.001
Fig. 3.2 Comparison of serum versus serum free medium on basal cell surface tissue factor activity 24 h post passage
The cell surface tissue factor activity of THP-1 monocytes that had been routinely passaged 24 h previously in medium ± serum, was determined by the one-stage prothrombin time assay. Results represent the mean ± SEM of 6 independent experiments, P < 0.001
medium containing serum (Medium A) or medium in which the serum had been replaced with the serum-free supplement, Nutridoma HU (Medium B – see Appendix C). Cells were seeded at an initial concentration of 5 x 10⁵ cells/ml. The cells were left for 24 h to re-enter logarithmic phase and the cell surface tissue factor activity determined by the one-stage prothrombin time assay.

The basal level of tissue factor activity in cells passaged with serum (1.4 - 2.3 tissue factor activity units/10⁶ cells – average 1.8) was at least 2 fold higher than cells that had been passaged in serum-free medium (0.41 - 0.56 tissue factor activity units/10⁶ cells – average 0.49) (Fig. 3.2). This indicated that passaging the cells in medium containing serum promoted a higher level of induction of tissue factor expression on the surface of THP-1 monocytes than passaging the cells in medium containing a serum-free substitute, resulting in a higher basal level of cell surface tissue factor activity.

As the overall aim for this section of work was to develop a model of monocyte-macrophage differentiation with low basal levels of cell surface tissue factor activity, it was apparent that it would be necessary to omit serum from the culture medium to achieve this. Based on this fact, a study was set up to determine the effects of a long-term culture of THP-1 monocytes using serum-free conditions.

3.2.3 A study into the effects of long-term culture using serum-free medium

The cells were routinely cultured in Medium B in which the 10% serum had been replaced with 1% Nutridoma, a serum-free supplement. Although the cell surface tissue factor activity was lower than cells routinely cultured in medium containing serum (Fig. 3.2), after a period of about 2 weeks in these culture conditions the cells became very ‘clumpy’, forming strands of cells that could be visualized without the microscope. When the cells were viewed under the light microscope, they no longer
Fig 3.3 Morphology of THP-1 monocytes in the absence and presence of PMA.
Photographs show THP-1 monocytes in the absence of PMA (A) and in the presence of 100 nM PMA following a 24 h incubation (B). Both photographs were taken on a Leitz Epivert inverted microscope, magnification 320x.
looked healthy and smooth. Instead their surface appeared to be granular. Moreover, there was more cell debris present in cells cultured in the serum free medium when compared to the cells cultured in the presence of serum. Thus, the continuous use of serum-free medium for the long-term culture of the cells could not be applied.

To overcome this problem the cells were routinely cultured in the presence of serum. For experimentation purposes, the cells were re-passaged into serum-free medium 24 h prior to the experiment, allowing for the cells to re-enter logarithmic phase. Seeding into serum free medium prior to experimentation resulted in viable cells that had lower basal levels of tissue factor than cells that remained in serum, with the added advantage that this medium could also be used in later studies looking at the effects of lipoproteins on the induction of tissue factor expression in macrophages.

3.2.4 An investigation into the differentiation of THP-1 monocytes into macrophages using PMA

In order to differentiate THP-1 monocytes to macrophages, cells in logarithmic phase, (round and growing in suspension, with a doubling time of 24 h), were passaged at a density of 5 x 10⁵ cells /ml into Medium B (section 2.5.1), in which the serum had been replaced by the serum free supplement Nutridoma HU. The cells were not used for experimentation until they had re-entered logarithmic phase, 24 h later.

PMA was made up as a stock solution of 1 mM in dimethyl sulfoxide (DMSO) and diluted in PBS to give a 10 µM working solution which was added directly to the cells in Medium B at concentrations between 0.1-1000 nM (0.00001-0.1% DMSO).

The differentiation process of THP-1 monocytes to macrophages by PMA was studied. Cells were incubated with PMA (0.1-1000 nM) for periods up to 48 h at 37°C and examined by light microscopy for signs of differentiation. Within 1 h of the addition of PMA, the cells incubated with 10 nM-1000 nM PMA had started to adhere to the tissue culture plate. Adherence increased over the 24 h incubatory period and was
accompanied by a cessation of proliferation and a change in morphology, with the cells adopting an irregular flattened and amoeboid-like morphology, characteristic of macrophages (Fig 3.3). This change in morphology was not observed in the cultures incubated with 0.1 nM-1 nM PMA, although they were adherent. Following 48 h incubation with 100 nM-1000 nM PMA the cells appeared granular and were dissociating from the culture dishes indicating cell death was occurring. Following 48 h incubation the cells that had received the lower concentrations of PMA (0.1-10 nM) remained adherent, but only cultures in 10 nM PMA exhibited a macrophage-like appearance. Addition of DMSO at the same dilution required for obtaining the given concentration of PMA did not cause adherence or differentiation of the cells.

3.2.5 An investigation into the induction of tissue factor expression from THP-1 monocyte-derived macrophages by PMA

In all of the following studies, cell surface tissue factor activity was measured, as opposed to tissue factor activity in cell homogenates as the measurement of surface activity provides a more physiological model of the biologically effective response of monocytic tissue factor expression following exposure to stimulatory agents or during differentiation. However, the fact that cell surface tissue factor activity is only a small fraction of total tissue factor activity induced following exposure to stimulus, is an important fact to take into consideration, when studying such a model.

Tissue factor was confirmed as the source of the procoagulant activity in all of the experiments described, as coagulation did not proceed when normal plasma was replaced with factor VII deficient plasma. The culture medium, additives (serum, glutamine, mercaptoethanol and penicillin-streptomycin) and PBS all tested negative for tissue factor activity by the one-stage prothrombin time assay.

In order to determine the optimum dose of PMA required to induce the maximal expression of tissue factor activity and to study the kinetics of this expression in THP-1 monocytes, the cells were seeded into Medium B, 24 h prior to experimentation. The cells were incubated with PMA at 37°C. Following incubation, the supernatant was decanted into a centrifuge tube. The adherent cells
Fig. 3.4.1 Dose-response of cell surface tissue factor activity on THP-1 monocytes following 4 h incubation with PMA

Cells were incubated with the specified concentration of PMA for 4 h. Following the incubation the cell surface tissue factor activity was determined by the one-stage prothrombin time assay and results expressed as tissue factor activity units/10^6 cells. Results represent the mean ± SEM of 4 independent experiments. *P<0.05; **P<0.02; ***P<0.01.
were washed with PBS in order to remove excess calcium ions. Versene, a commercially available solution of EDTA, was added to the culture vessel to promote dissociation of the cells. This treatment was favoured over mechanical removal, so as to preserve the integrity of the cells. The supernatants were pooled and the cells pelleted by centrifugation at 210g for 10 min at room temperature, in an IEC Centra GP8R centrifuge. The cells were washed with PBS and then resuspended in PBS at 10^7/ml.

The cell surface tissue factor activity was determined by the one-stage prothrombin time assay (Section 2.6.1) and results expressed as tissue factor activity units per million cells (tissue factor activity units/10^6 cells).

3.2.5.1 The dose-response of tissue factor activity expressed on the surface of THP-1 monocytes incubated with PMA

THP-1 monocytes were incubated with PMA (0 -1 μM) for 4 h. The dose-dependent expression of tissue factor activity from the monocytes following incubation with PMA is shown (Fig. 3.4.1). Note the basal level of tissue factor activity even in the unstimulated cells (0.5 tissue factor activity units /10^6 cells). This may be a consequence of continuous culture of the cells in the presence of serum, prior to the experiment, which was essential to maintain healthy suspension cultures (see section 3.2.3). However, from earlier studies this was the lowest basal level of cell surface tissue factor activity that was achievable with this cell type. A significant increase in tissue factor activity after the 4 h incubation, was attainable with even the lowest concentration of PMA used (1 nM) and the tissue factor activity expressed followed a dose-dependent pattern, with maximal activity at 1 μM PMA, giving rise to a 4.2 fold increase of tissue factor activity, when compared to un-stimulated cells.
3.2.5.2 The temporal profile of tissue factor activity expressed on the surface of THP-1 monocytes incubated with PMA

From the results in the section of 3.2.4, the highest concentration that could be used to induce differentiation over a 48 h period, without visible cell death, was 10 nM. This concentration also resulted in significant expression of tissue factor activity following a 4 h incubation (see section 3.2.5.1). Thus 10 nM PMA was used to investigate the kinetics of the expression of cell surface tissue factor activity, over a 48 h incubation. THP-1 monocytes were seeded into Medium B 24 h prior to experimentation and the PMA added at time 0 h. At each of the specified time points, flasks were removed from the incubator, the adherent cells removed, and the cells resuspended in PBS as previously described (see section 3.2.5). The cell surface tissue factor activity was determined by the one-stage prothrombin time assay. The temporal profile of tissue factor activity expressed on the surface of THP-1 monocyte-macrophages during 48 h incubation with 10 nM PMA is shown (Fig. 3.4.2). A 2-fold increase in cell surface tissue factor activity was detected with only a 2 h incubation, whilst, maximal activity was detected following a 24 h incubation and resulted in a 6-fold increase in cell surface tissue factor activity. This returned to basal levels by 48 h. Throughout the 48 h incubation, the cells were adherent and did not appear granular, in agreement with the results in section 3.4.1. However uptake of Trypan Blue, indicated that the viability of the cells was being compromised by the 48 h incubation with PMA, with only 70% viability at 48 h compared with 93% viability at 24 h, and 98% viability of control cells (Fig. 3.5.1). This posed the problem that, although the cells were in a macrophagic-form with basal levels of cell surface tissue factor activity, they were not viable and could not be used for further experimentation.
Fig. 3.4.2 Temporal profile of tissue factor activity on the surface of THP-1 monocytes incubated with PMA over a 48 h period
Cells were incubated with 10 nM PMA for up to 48 h. PMA was added at time = 0 and at the specified time points tissue factor activity was determined by the one-stage prothrombin time assay and expressed as tissue factor activity units/10⁶ cells. Results represent the mean ± SEM of 4 independent experiments. *P < 0.05; **P < 0.001; +P < 0.0002; ++P < 0.0001
3.2.5.3 Viable macrophages and basal levels of tissue factor

It was concluded that prolonged incubation with PMA even at low concentrations was toxic to the cells. The toxicity is more pronounced in this model of monocyte-macrophage differentiation, than in the original experiments on phorbol ester induction of differentiation of THP-1 monocytes, performed by Tsuchiya et al. (1982), due to the absence of serum in the culture medium used in this study. From the results in section 3.2.4 it was clear that PMA exerted an effect after only 1 h into the incubation period, and that the induction of tissue factor expression was occurring as early as 2 h (section 3.2.5.2). This led to the question whether a prolonged incubation was absolutely necessary to induce differentiation and the transient tissue factor expression?

The continuous PMA incubation was replaced with shorter incubation periods of 1, 2, 4 and 8 h. Following incubation for the specified duration, the cells were then washed 3 times with PBS and resuspended at the same density in Medium B, to complete the remainder of the 48 h incubation. For example, cells that had been incubated for 2 h with PMA, were washed and resuspended in fresh serum-free medium prior to a further 46 h incubation in the absence of PMA. In parallel, cultures were set up where the PMA incubation was continuous. After a total incubation of 48 h, the viability of the cells was then determined by Trypan Blue dye exclusion. The results were encouraging as even the cells incubated for the shortest duration could not be visually distinguished, when viewed under the microscope, from cells incubated in parallel without the PMA being washed out. Moreover, the 1 and 2 h incubation period cells retained viability above 95%, compared with the cells incubated continuously for 48 h with PMA, which only achieved 70% viability (Fig. 3.5.2).
Fig 3.5.1 The viability of THP-1 monocytes during a 48 h incubation with PMA THP-1 monocytes were incubated with 10 nM PMA for up to 48 h, following which the viability was determined by Trypan Blue dye exclusion. Results represent the mean ± SEM of 4 independent experiments. *P < 0.05; **P < 0.01
Fig. 3.5.2 The effect of duration of PMA treatment on the viability of THP-1 monocyte-derived macrophages
The cells were incubated with PMA for the time indicated and viability determined by Trypan Blue dye exclusion, following a complete incubation of 48 h. Results represent the mean ± SEM of 4 independent experiments. *P < 0.05; ***P < 0.01.
Fig. 3.5.3 A comparison between the temporal profile over a 48 h period of tissue factor activity expressed from THP-1 monocytes incubated with 10 nM PMA for a total of 2 and 48 h respectively.

THP-1 monocytes were incubated with 10 nM PMA for up to 48 h and the kinetics of tissue factor activity expressed as measured by the one-stage prothrombin time assay. Cultures were either exposed to continuous 48 h incubation with PMA or to a shorter incubation of 2 h duration, followed by a wash, and a further incubation of 46 h, which has been shown to preserve the viability of the cells. Results represent the mean ± SEM of 4 independent experiments.
For further experiments the 2 h incubation period was used. When the kinetic profile of cell surface tissue factor activity was repeated using this ‘washout method’ with 2 h incubation, it was comparable to that of cells which had received the ‘continuous’ PMA treatment (Fig. 3.5.3), again returning to basal levels after 48 h. However the levels of tissue factor activity were lower. This could have been caused by one of two things. Firstly, the washing process following the short PMA treatment caused the cell surface tissue factor to be sheared off, or secondly, less tissue factor activity was expressed originally as the result of the reduced PMA stimulation through less activation of protein kinase C. When the former explanation was tested it was found that some of the tissue factor activity on the surface of THP-1 monocytes was lost as the result of repeated washes. Nevertheless, the procedure provided a possible model in which the cells were viable after exposure to PMA, had basal tissue factor activity and were suitable to study the effects of plasma lipoproteins on the expression of tissue factor activity from monocyte-derived macrophages.

3.2.6 Induction of tissue factor antigen by PMA- measured by flow cytometry

In order to determine whether the tissue factor activity expressed on the surface of the cells during PMA treatment was accompanied by an increase in cell surface tissue factor antigen, the cells were seeded into Medium B, 24 h prior to the experiment. The cells were incubated with 10 nM PMA for up to 48 h. At the specified time-points, the cells were removed from the culture vessel and resuspended in PBS as described in section 3.2.5. The cells were then stained for tissue factor antigen and tissue factor antigen was measured by flow cytometry, Fig. 3.6, as described in section 2.7.2.

The kinetic profile of tissue factor antigen expression in THP-1 monocytes incubated with 10 nM PMA for up to 48 h is shown in Fig. 3.7A. Tissue factor antigen levels dropped during the first 8 h of incubation, but rapidly rose to give maximal expression at 18 h. Following this, the antigen levels dropped to below baseline levels at 24 h and remained relatively constant thereafter. These results are not consistent with the expression of cell surface tissue factor activity, reported in section 3.2.5.2, when
Fig. 3.6 Histogram display of flow cytometric analysis of the measurement of tissue factor antigen on the surface of THP-1 monocytes following incubation with PMA. Histogram A represents tissue factor antigen on the surface of THP-1 monocytes in the absence of PMA. Histogram B represents tissue factor antigen on the surface of THP-1 monocytes following an 18 h incubation with 10 nM PMA. Forward and side scatter parameters were collected logarithmically and the cells gated to exclude debris and microparticles (gate c). Fluorescence histograms of the gated cells (a & b) were used to assess tissue factor antigen.
Fig. 3.7 The temporal profile of tissue factor antigen expression on THP-1 monocytes following stimulation with PMA
THP-1 monocytes were incubated with 10 nM PMA for up to 48 h and the expression of tissue factor antigen measured by flow cytometry. PMA was added at time = 0. Diagram A represents cells that had been incubated with PMA for a total of 48 h. Diagram B represents cells in which the PMA had been washed out of the system after a 2 h incubation as described in section 3.2.5.3 followed by a further 46 h incubation in the absence of PMA. Results represent the mean ± SEM of 4 independent experiments. *P < 0.05
maximal tissue factor activity was detected following 24 h incubation with PMA. Thus, even though tissue factor antigen is expressed on the surface of the monocytes, it does not necessarily mean that maximal tissue factor activity is present. The kinetic profile of cells, which had received continuous PMA treatment, was comparable to that of cells that had only received the 2 h incubation (Fig 3.7 B).

3.2.7 Secondary stimulation of THP-1 monocyte-derived macrophages with PMA to induce tissue factor activity expression

The earlier studies established a method for differentiating the monocytes into a macrophagic form, with accompanying basal levels of cell surface tissue factor activity, but whether these macrophages would respond to a further stimulus needed to be elucidated. THP-1 monocytes in their macrophagic form were re-stimulated with 10 nM PMA for up to 24 h. This time-scale was chosen based on the results in section 3.2.5.2 in which maximal cell surface tissue factor activity was detected following 24 h incubation with 10 nM PMA.

The expression of surface tissue factor activity, following a secondary stimulation with PMA is shown in Fig. 3.8. Tissue factor activity on the surface of the macrophages was significant after only 4 h, and reached maximal levels following an 8 h incubation, however, the increase in activity was approximately 2-fold less than was seen with the initial stimulation. After 8 h the levels of tissue factor activity decreased, returning to basal levels by 24 h. The earlier maximal activity response, compared to the results in section 3.2.5.2 could be due to differences in the expression of tissue factor between monocytes and macrophages, or, as the tissue factor expression was already primed by the initial stimulation with PMA used in the differentiation of the cells, a shorter incubation was sufficient to induce maximal tissue factor activity expression.
3.2.8 A study of cell differentiation and tissue factor activity expression in THP-1 monocytes by LPS

From the literature it is known that LPS is capable of inducing the expression of tissue factor in peripheral blood monocytes (van der Logt et al., 1994) and the monocytic cell line U937 (Rana et al., 1988). In order to ascertain if this response was reproducible in THP-1 monocytes and if this method could be used to induce the differentiation of these cells, THP-1 monocytes were incubated with LPS. Cells were passaged at 5 x 10^5 cells/ml into Medium A (containing 5% serum – see section 2.5.1) 24 h prior to experimentation, in order to study the expression of tissue factor activity by LPS. This medium was used as opposed to the Medium B used in previous experiments, as there is an augmentation in the response to LPS when serum is present in the medium due to the presence of lipopolysaccharide binding protein (Steinemann et al., 1994). Incubation of THP-1 monocytes with LPS did not promote adherence to the culture vessel. Thus, following incubation, the cells were pelleted by centrifugation at 210g, for 10 min, at room temperature in IEC Centra GP8R. The cells were washed once in PBS and resuspended to 10^7 /ml in PBS and cell surface tissue factor activity determined by the one-stage prothrombin time assay (Section 2.6.1). Results were expressed as tissue factor activity units/ 10^6 cells.

3.2.8.1 The dose- response of tissue factor activity expressed on the surface of THP-1 monocytes incubated with LPS

The dose response of tissue factor expression THP-1 monocytes, incubated with LPS (E. coli 026:B6) (0-10 µg/ml) for 4 h, is shown in Fig. 3.9.1. The greatest increase in tissue factor activity was seen with 0.1 µg/ml with a 2.3 fold increase in activity compared to control cells (no LPS). The highest concentrations tested (1 µg/ml and 10 µg/ml), caused a 1.73 fold and 1.66 fold increase in tissue factor activity, respectively. 10 µg/ml was chosen for further experiments, as this concentration resulted in a significant expression of tissue factor activity, and did not compromise the viability of the cells. Moreover, the lowest concentration of LPS that was used in such studies in
Fig. 3.8 The effect of a second exposure to PMA on the induction of tissue factor activity from THP-1 monocyte-derived macrophages

THP-1 monocytes that had previously been treated with 10 nM PMA for 2 h followed by a further 46 h incubation in order to induce differentiation and basal levels of cell surface tissue factor activity were then re-stimulated with a second dose of 10 nM PMA for up to 24 h and cell surface tissue factor activity measured. Results represent the mean ± SEM of 3 independent experiments. *P<0.002; **P<0.001.
the literature, was at least 1 μg/ml (van der Logt et al., 1994; Robinson et al., 1992). In all the experiments the viability of the cells, determined by Trypan Blue dye exclusion, remained greater than 95%.

3.2.8.2 The temporal profile of tissue factor activity expressed on the surface of THP-1 monocytes incubated with LPS

The temporal profile of the induction of tissue factor activity by LPS was established by incubating THP-1 monocytes with 10 μg/ml LPS (E. coli 026:B6), for up to 48 h (Fig. 3.9.2). Maximal tissue factor activity was expressed during 16 h incubation; 8 h earlier than the maximal activity induced by PMA treatment. After this period tissue factor levels started to decline, as before, until they reached baseline levels at 48 h. The earlier peak in tissue factor activity detected on the surface of the monocytes using this treatment compared to incubation with PMA may simply be an effect of the different stimulatory agents.
Fig. 3.9.1 Dose response of tissue factor activity on the surface of THP-1 monocytes incubated with LPS
Cells were incubated with LPS (E. coli 026:B6) for 4 h. Cell surface tissue factor activity was determined by the one-stage prothrombin time assay. Results represent the mean ± SEM of 3 independent experiments. **P<0.02; ***P<0.01
Fig 3.9.2 Temporal profile of tissue factor activity on the surface of THP-1 monocytes incubated with LPS
Cells were incubated with 10 μg/ml LPS (E. coli 026:B6) for a 48 h period. LPS was added at time = 0. At the specified time points cell surface tissue factor activity was determined by the one-stage prothrombin time assay. Results represent the mean ± SEM of 3 independent experiments. *P < 0.05; **P < 0.01
3.3 Discussion

The present chapter reports the preparatory work to develop a suitable model of monocyte-macrophage differentiation, which culminated in viable macrophages and a return to basal levels of tissue factor activity and antigen. The overall aim of this work was then to use the macrophages in the study of expression of tissue factor activity by native and oxidatively modified LDL (Chapter 4).

The THP-1 monocytic cell line was chosen in this study as it behaves more like native peripheral blood monocytes than any of the other myelomonocytic cell lines available, however a drawback in using this particular cell line is its high basal tissue factor activity level compared with peripheral blood monocytes. Tsuchiya et al. (1982) reported that maturation was accompanied by adherence to the culture vessel and cessation of cellular proliferation. In agreement with the results presented in section 3.2.4, the authors reported that the cells started to adhere to the culture vessel within one hour of the initiation of phorbol ester treatment, following which, they proceeded to spread over the culture vessel. However, unlike the present study, the authors reported that the adherent cells could not be removed from the culture vessel with either EDTA or trypsin treatment. A commercially available solution of EDTA (Versene) was used here, which did not cause any problems in the dissociation of the cells from the culture vessel. The difference between the two studies may be a consequence of the use of continuously cultured monocytes in the present study, compared with Tsuchiya and co-workers (1982) who used the original type of THP-1 that had been cryopreserved for 24 months prior to the study.

In agreement with the results in section 3.2.4, the authors also reported detachment of the cells from the culture vessel following 48 h incubation with phorbol ester, indicating that cell death was taking place. This occurred even though they cultured their cells in the presence of serum, which may reduce the effective concentration of PMA reaching the cells due to serum components binding the PMA. Cell death appears to be a problem in the use of phorbol esters in order to induce
differentiation. In the present study a method was developed to overcome the toxicity, associated with the use of PMA, but in serum free conditions, so that the possibility of PMA binding to serum and reducing the effective dose reaching the cells could be eliminated.

A more physiological way of inducing differentiation of monocytes into macrophages may lie in oxidised LDL, which was been shown to promote differentiation of U937 monocytes (Frostegard et al., 1990). Additionally, 1,25-dihydroxy vitamin D3 (calcitrol) has been shown to induce differentiation in U937 monocytes (Rigby et al., 1984). However, 1,25-dihydroxy vitamin D3 has been shown to only promote partial differentiation in THP-1 monocytes. This may be the result of the completely different signal transduction pathway used by vitamin D3 analogues (steroid receptor), to the pathway used by phorbol esters and LDL (protein kinase C) (Auwerx, 1981).

Tissue factor is constitutively expressed in vivo, in many cell types that are not normally in contact with flowing blood, such as epithelial cells lining the body cavities (Drake et al., 1989). However, in two cell types, monocyte/macrophages and endothelial cells, tissue factor synthesis can be induced by a variety of stimuli including cytokines (Schorer et al., 1986; Terry & Callahan, 1996), monocyte chemoattractant protein (Schechter et al., 1997), and bacterial lipopolysaccharide (LPS) (Colucci, M., 1983; van der Logt, 1994). Increase of tissue factor activity may also arise following induction by various conditions, including exposure of cells to membrane perturbing agents such as hydrogen peroxide (Schorer et al., 1985). Increased procoagulant activity on the surface of cells may be the result of changes in the amount of tissue factor available on the surface of the cell as a consequence of increased synthesis of tissue factor or by exposure of functional tissue factor that is existing in an encrypted form. The exact mechanism of encryption is not known, however, one current hypothesis is that increases in cell surface procoagulant activity is related to a redistribution of membrane lipids, including the availability of anionic phospholipids in proximity to tissue factor (Nemerson, 1968; Bach et al., 1986).
Alternatively, it has been shown that cell surface tissue factor activity increases following exposure to proteolytic enzymes. Mulder et al., (1996 a & b) reported that, in smooth muscle cells, tissue factor was constitutively expressed within or near microinvaginations of the plasma membrane. These microinvaginations were recognised as caveolae. This encrypted tissue factor could be activated by processes that alter the structure or phospholipid content of the membrane such as detergent treatment (Carson, 1996). A further dimension to studies into the localisation of tissue factor was revealed by Schecter et al. (1997) in a study using smooth muscle cells. The authors reported that tissue factor activity was partitioned into three pools, an active cell surface form that has transient exposure following induction, a latent cell surface form and an intracellular pool. The limited availability of the active form may be an important adaptive mechanism for limiting the procoagulant potential of the vessel wall, whilst the presence of caveolae-associated tissue factor may function as a latent pool of procoagulant activity, which can rapidly be activated at sites where the integrity of the vessel wall is compromised.

The dose-response effect of PMA on the expression of tissue factor activity in THP-1 monocytes was studied (section 3.2.5.1). The results are in agreement with the work of Lyberg & Prydz (1981) who reported a concentration-dependent increase in phorbol ester induction of tissue factor activity in peripheral blood monocytes which was maximal with 1 μM phorbol ester. However, the extent to which tissue factor activity increased differs, with an approximate 15-fold increase in tissue factor activity above basal levels detected by Lyberg & Prydz, compared with a 6-fold increase above basal levels detected in the present study. Several important factors must be taken into consideration when comparing these results. In the present study, tissue factor activity was measured after 4 h incubation, whilst Lyberg & Prydz looked at an increase of tissue factor activity following 14 h incubation. In addition, the present study measured tissue factor activity on the surface of intact cells, compared to Lyberg & Prydz who measured tissue factor activity in cell homogenates. Thus, they would have measured not only the cell surface tissue factor activity, but also intracellular tissue factor activity. Lyberg & Prydz (1981) reported
an increase of tissue factor antigen accompanied the increase in tissue factor activity, but was not seen when the inhibitors of tissue factor synthesis (cycloheximide and actinomycin D) were studied, indicating that induction of tissue factor activity involves de novo synthesis of tissue factor, as opposed to an intracellular accumulation of tissue factor or enhanced expression.

The study of the temporal profile of tissue factor activity expression on the surface of the cells following incubation with PMA (section 3.2.5.2), showed that the maximal expression of tissue factor activity was seen following a 24 h incubation, after which a decline was observed. Lyberg & Prydz (1981) showed the maximal response in peripheral blood monocytes occurred at about 18 h and, in U937, monocytes maximal tissue factor activity has been observed at about 12 h (Rana et al., 1988). The difference between these results could be due to: 1) differences between cell lines; 2) the alternative methods used to measure tissue factor activity, for example measurement of procoagulant activity; measurement of factor Xa formation, or 3) whether tissue factor activity was measured in whole cells or lysates. Despite the discrepancies between the time-point of the expression of maximal tissue factor, all the results are in agreement with respect to the decline in tissue factor activity following maximal expression. One explanation for this decline in activity is that tissue factor is being shed from the surface of the monocytes during the incubation. When the medium that the cells had been cultured in during incubation with PMA was tested for procoagulant activity, it was positive. However, this required the volume of the conditioned medium to be reduced by centrifugation at 2000g. When the remaining medium was viewed under the light microscope there was no evidence for the presence of cells. However, due to the reduction in volume of the conditioned medium and the length of the clotting times detectable in the one-stage prothrombin time assay, the procoagulant activity in terms of tissue factor activity units could not be quantified without using much larger volumes of cells, seeded at a higher density, than was used in these studies. Bona & Rickles (1987) studied vesiculation in HL60 monocytes. They showed maximal plasma membrane tissue factor expression 12-18 h after stimulation with phorbol esters; as this declined there
was an increase in vesicular tissue factor, which reached maximal levels between 24 - 36 h. Monocyte vesiculation may be implicated in the dissemination of membrane associated procoagulant activity following exposure to LPS (Satta et al., 1994).

An alternative explanation for the decrease in tissue factor activity with prolonged stimulation with phorbol esters may be the down regulation of PKC. Fournier & Murray (1987) described the down regulation of PKC by a single application of phorbol ester to mouse skin. This effect has also been detected in peripheral blood monocytes (Brozna & Carson, 1988), where phorbol esters were reported to cause a decrease in monocyte-associated tissue factor expression. A study by van der Logt and colleagues (1994) also reported that long-term exposure of cells to PMA down-regulated PKC activity. After prolonged exposure to PMA, human peripheral blood monocytes contained only low levels of tissue factor mRNA. These authors reported that a secondary stimulation with PMA or LPS failed to induce tissue factor mRNA expression, indicating that the cells were refractory to stimulation. In this present study, when THP-1 macrophages were re-stimulated with PMA (section 3.2.7) there was a 5-fold increase in the cell surface tissue factor activity. This may be a consequence of the short stimulation with PMA that the THP-1 macrophages had received, thus avoiding the down-regulation of PKC. However, when the macrophages in this study were incubated with 10 µg/ml LPS, they were refractory to induction of tissue factor activity, even though the cells were transferred to medium containing serum. The findings of Jungi et al. (1996) showed that monocyte - derived macrophages responded to LPS both in the absence and presence of serum. Further investigation with regards to a double stimulation with LPS and stimulation with LPS followed by PMA may give some explanation to this result.

The use of LPS to promote maturation of monocytes failed in the criteria used in this study to define differentiation, that is, adherence of cells to the culture vessel, the cessation of proliferation and the accompanying change in morphology. Thus, LPS treated cells were excluded from further experimentation on macrophages.
However, exposure to LPS did induce the expression of tissue factor activity in the monocytes. When the monocytes were incubated with LPS for different time intervals, tissue factor activity was maximal at 18 h and decreased to basal levels at 48 h. Other researchers have reported maximal tissue factor activity at 9 h, decreasing to basal levels at 26 h, in human peripheral blood monocytes (van der Logt et al., 1994). Possible explanations for this discrepancy were discussed previously, however, vesiculation must also be taken into consideration. The time-dependent down-regulation of tissue factor, following peak tissue factor activity, is thought to be caused by PKC. PKC inhibitors have been shown to prolong LPS-induced tissue factor expression in monocytes (Brozna et al., 1994). In view of this and the work of Ternisien (1993), who showed that tissue factor induction in human monocytes is dependent upon PKC activation, PKC can be assigned a role, both in the induction and down-regulation of tissue factor activity.

In this study, a model for monocyte-macrophage differentiation was developed. This model provides viable macrophages, which express basal levels of tissue factor and remain responsive to secondary stimulation. In addition, LPS was used to induce tissue factor activity in monocytes. However, unlike treatment with PMA, the induction was not accompanied by a cessation of proliferation, nor was there a change in morphology indicating differentiation. Hence, monocytes treated with LPS could not be described as macrophages or used in further studies requiring macrophages.
CHAPTER 4

EFFECT OF NATIVE LDL AND MODIFIED LDL ON TISSUE FACTOR ACTIVITY AND EXPRESSION
Disruption of atherosclerotic plaque and subsequent thrombus formation lead to acute coronary events and the progression of atherosclerotic disease. The link between elevated plasma lipoproteins has long been recognised with respect to plaque development and the pathogenesis of atherosclerosis. It has been proposed that the progression of atherosclerosis is not only associated with an increased plasma concentration of LDL, but the oxidative state of LDL (Steinberg, 1993). Carew et al., (1987) demonstrated that Probucol, an agent originally used to lower plasma cholesterol levels, exhibited an antioxidant effect, which slowed the progression of the development of atherosclerotic lesions in an experimental model of the disease. In addition, other factors must also be taken into account when considering the development of ischaemic heart disease. The studies of Meade and colleagues, reported that the coagulation factors fibrinogen and activated factor VII were better predictors for the development of ischaemic heart disease in middle aged men, than cholesterol (Meade et al., 1980; Mitropoulos et al., 1989).

Modified forms of LDL have been detected in healthy, normolipidaemic humans (Avogaro et al., 1988) and within atherosclerotic plaques of both rabbits and humans (Yla-Herttuala et al., 1989; Carpenter et al., 1995) indicating peroxidation of LDL in vivo. The process of oxidation of LDL in vivo is not fully understood, particularly the origin of the oxidising species. Mass spectrometric analysis of protein oxidation products isolated from atherosclerotic tissue implicate a role for the tyrosyl radical (Leeuwenburgh et al., 1997a) and hypochlorous acid (Hazen et al., 1996). A study by Smith et al., (1992) detected iron and copper ions in gruel samples from advance atherosclerotic lesions, whilst Lamb et al., (1995) reported that extracts from human atherosclerotic lesions were capable of catalysing the oxidation of LDL by macrophages, a reaction that requires the presence of transition metal ions. The
presence of catalytically active metal ions in advanced atherosclerotic lesions may give an explanation to why LDL is oxidised in those parts of the body, however, what remains unexplained is how the metal ions become available in the first instance as they are normally sequestered by binding proteins. One explanation may come from results presented by Swain et al., (1994) who reported peroxynitrite is extremely effective in releasing copper from caeruloplasmin, even when the protein is in its normal plasma environment.

The modification of LDL in vitro can be achieved by the initiation of peroxidation within the lipid phase of the LDL particle. In vitro modification has been observed by incubation with cupric ions and a number of cell types including endothelial cells, smooth muscle cells and macrophages (Esterbauer et al., 1990). However, this has been shown to be dependent upon the presence of exogenous transition metal ions in the medium (Heinecke et al., 1984; Leake et al., 1990). As yet the physiological process that contributes to the oxidative process in vivo has not been elucidated. A number of mechanisms have been proposed, including the seeding of lipid peroxides in the LDL particle by 15-lipoxygenase (Cathcart et al., 1991), however other authors have provided conflicting evidence to these mechanisms (Jessup et al., 1991). The seeding of peroxides into the LDL particle requires the presence of agents, such as transition metal ions, to promote the breakdown of peroxide. An alternative mechanism lies in the direct abstraction of a hydrogen atom from an unsaturated fatty acid. This would also lead to lipid peroxidation within the LDL particle without the requirement for the initial seeding peroxide and the presence of transition metal ions. The initiation of this reaction requires the formation of a highly reactive free radical oxidant, such as the hydroxyl radical.

Peroxynitrite is an oxidising species, which arises from a reaction between nitric oxide and the superoxide anion. Pathological conditions can substantially up-regulate the production of nitric oxide and superoxide from macrophages and neutrophils, both of which are released from vascular cells under inflammatory conditions. Peroxynitrite has been shown to generate an oxidant with similar
reactivity to the hydroxyl radical (Beckman et al., 1990), and has also been shown to initiate membrane lipid peroxidation (Radi et al., 1991a). Hogg et al., (1993a) reported peroxynitrite is capable of oxidising LDL to a potentially atherogenic form and that exposure of LDL to peroxynitrite increased the susceptibility of LDL to transition metal ion oxidation. In addition to oxidation by peroxynitrite, LDL has been shown to be oxidised with a combination of nitric oxide and superoxide, generated from SIN-1 (Darley-Usmar et al., 1992; Hogg et al., 1993b; Thomas et al., 1998).

The oxidative modification of LDL is accompanied by the generation of a wide-range of lipid oxidation products including hydroperoxides and aldehydes and the modification of the apolipoprotein (see section 1.3). Lipoproteins and modified lipoproteins have been demonstrated to act on cells of the vasculature (see section 1.4) and to modulate haemostasis by altering the expression and or function of thrombotic and fibrinolytic factors. Oxidation of LDL by peroxynitrite generates F2-isoprotanes, a family of prostaglandins formed from peroxidation of arachidonyl-containing phospholipids (Moore et al., 1995). The presence of F2-isoprotanes promotes vasoconstriction, which may contribute to the vascular pathobiology associated with atherosclerosis. Endothelium derived relaxation has been shown to be impaired in the vessels of patients with atherosclerosis (Försterman et al., 1988) and in hypercholesterolaemic animal models. Furthermore, formation of peroxynitrite has been shown to attenuate nitric oxide vasodilatation (White et al., 1994). Thus, peroxynitrite can be implicated in the development of atherosclerosis, not only by acting as a potent mediator in the oxidation of lipoproteins, but also by influencing vasomotor tone.

Normally, cells that are in direct contact with the blood do not constitutively express tissue factor. However, endothelial cells, monocytes and monocyte-derived macrophages have the ability to express tissue factor following induction by phorbol esters, lipopolysaccharide or cytokines (Lyberg & Prydz, 1981; Colucci, 1983; Schorer et al., 1986). Spontaneous differentiation of peripheral blood monocytes into
macrophages has been shown to be accompanied by a transient expression of tissue factor (van den Eijnden et al., 1997). Tissue factor has been detected on the surface of monocyte-derived foam cells (Landers et al., 1994), whilst a study by Colli et al., (1999) found that the transformation of monocyte-derived macrophages into foam cells was accompanied by an increase in their thrombogenicity. Tissue factor has been implicated in the thrombogenicity of atherosclerotic plaque following rupture (Wilcox et al., 1989; Toschi et al., 1997).

Elevated levels of tissue factor on circulating monocytes has been detected in coronary disease (Leatham et al., 1995) and in post myocardial infarction patients (Freeburn et al., 1998), which may explain the hypercoagulability associated with these conditions. The heightened levels of tissue factor in patients with coronary disease has been shown to be accompanied by elevated levels of TFPI (Falciani et al., 1998; Soejima et al., 1999). TFPI provides physiological inhibition of tissue factor initiated coagulation by binding to factor Xa and TF-VIIa complex (see section 1.5.2.1). Caplice et al., (1998) demonstrated the presence of TFPI within atherosclerotic plaques, where it attenuates tissue factor activity. A study by Badimon et al. (1999), reported that plaque thrombogenicity was reduced by TFPI, which may indicate a novel therapeutic strategy in the prevention of acute intravascular thrombosis following plaque disruption.

Lipoproteins modulate haemostasis by altering the expression and or function of thrombotic and fibrinolytic factors. Aupeix et al., (1996) found that oxidised cholesterol compounds (oxysterols) were capable of enhancing tissue factor activity in THP-1 monocytes. However, this was not caused by an increased expression of tissue factor, rather an induction of phosphatidylserine exposure, on the outer leaflet of the plasma membrane, which is known to enhance tissue factor activity by promoting the formation of the tissue factor /factor VIIa complex (Bach, 1986). Schuff-Werner et al. (1989) reported that chemically modified LDL (acetyl - and malonaldehyde-LDL), but not native LDL, induced tissue factor in human monocyte-macrophages. Weis (1991) replicated these results in endothelial cells,
using oxidatively modified LDL. Others have found that VLDL was more effective in enhancing tissue factor expression in endothelial cells (Kaneko et al., 1994), and peripheral blood mononuclear cells, an effect that was found to be dependent upon the presence of T cells and suppressed by LDL (Schwartz et al., 1981: Levy et al., 1981).

Drake and his colleagues (1991) studying the influence of minimally modified (mildly oxidised) LDL on human endothelial cells reported an induction of tissue factor mRNA and procoagulant activity in human endothelial cells. Brand and colleagues (1990) showed an induction of tissue factor mRNA and activity in THP-1 monocytes incubated with oxidised LDL, however, a study using adherent monocytes found that LPS-free oxidised LDL failed to induce tissue factor expression in the monocytes, but significantly enhanced tissue factor induced by LPS (Brand et al., 1994). The ability of oxidatively modified LDL to induce the expression of tissue factor from vascular cells is subject to controversy because early studies in this field did not take into consideration the possibility of LPS contamination of LDL during the isolation process, which also is capable of inducing tissue factor expression in vascular cells (Colucci et al., 1983; Rana et al., 1988; Robinson et al., 1992). LPS is the major glycolipid molecule present in the cell wall of all Gram-negative bacteria. LPS is composed of repeating units of oligosaccharide covalently attached to a core oligosaccharide-lipid moiety (Fig.4.1). It is the lipid moiety (lipid A) of LPS, known as endotoxin, that is responsible for many of the activities associated with LPS, including mitogenicity and cytokine activation (Preston et al., 1996).

A further dimension to the influence of modified lipoproteins on the induction of tissue factor activity from cells of the vasculature lies in the interaction of LDL, in particular the apoB-100 moiety with tissue factor. Earlier work carried out in this laboratory has shown that native LDL inhibits isolated tissue factor activity in vitro. However, oxidation of the LDL removes this inhibitory action and acts to augment its activity (Howell & Ettelaie, 1990). Further studies have shown that these contrasting influences of LDL and oxidised LDL on the procoagulant activity of
tissue factor arise from a direct interaction of apo B-100 with the protein moiety of tissue factor (apoprotein III) (Ettelaie & Howell, 1995), and is distinct from the effect of the established inhibitor of tissue factor, TFPI (Ettelaie et al., 1999). TFPI is carried on the surface of lipoproteins. As with inhibition of tissue factor activity by apo B-100, the inhibitory potential of TFPI is also compromised by LDL oxidation (Lesnik et al., 1995). The inhibitory action of TFPI is well documented (Broze, 1995). However, at physiological concentration, TFPI is not sufficient to control procoagulant activity (Rao & Rapaport, 1987). Thus, the presence of other inhibitors of tissue factor may be of physiological relevance, in order to provide sufficient inhibition of the extrinsic pathway of blood coagulation, following blood vessel damage or plaque rupture.

The aim of this study was to investigate whether LDL oxidised by a variety of agents, including cupric ions and peroxynitrite, could induce tissue factor activity and antigen expression in monocyte-derived macrophages. Moreover, effects of native and the oxidatively modified forms of LDL were investigated to determine their influence on recombinant and cell surface tissue factor activity.
Fig. 4.1 The structure of LPS (taken from Preston et al., 1996)
4.2 Results

4.2.1 An investigation into the oxidative modification of LDL using a variety of oxidising agents

LDL was isolated as described in section 2.1. LDL was diluted to 1 mg protein/ml with PBS and oxidised using various oxidising agents; including air, cupric ions, peroxynitrite and SIN-1 (see section 2.3). The conditions required to achieve optimal oxidation were determined by analysis of lipid peroxides and TBARS and by measurement of protein modification by agarose gel electrophoresis, as described in section 2.4.

4.2.1.1 Lipid peroxide, TBARS and nitrotyrosine content of native and oxidatively modified LDL

The lipid peroxides, TBARS and nitrotyrosine content of the different preparations of native and oxidatively modified LDL is shown in Table 4.1. As the LDL became progressively more oxidised there was an increase in its lipid peroxide and TBARS content. Oxidation using cupric ions resulted in the most dramatic change in lipid peroxide, and consequently TBARS content in the samples. Oxidation of LDL by peroxynitrite and SIN-1 resulted in a more gentle oxidation, with the lipid peroxide and TBARS content resembling those detected in minimally modified (air oxidised) samples. Decomposed peroxynitrite did not increase the lipid peroxide or TBARS content of the LDL.

The nitrotyrosine content of the oxidatively modified LDL was determined using a competitive ELISA (see section 2.9). The content of nitrotyrosine residues in native LDL was classified as the basal level and was 1.7 nmol/mg apoB.
<table>
<thead>
<tr>
<th>LDL preparation</th>
<th>Lipid peroxides nmol/mg apoB</th>
<th>TBARS nmol/mg apoB</th>
<th>Nitrotyrosine nmol/mg apoB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native (n=10)</td>
<td>10.1 - 20.1 (18.0 ± 2.3)</td>
<td>0.04 - 2.0 (0.9 ± 0.3)</td>
<td>0.91 - 4.11 (1.4 ± 0.7)</td>
</tr>
<tr>
<td>Air (n=12)</td>
<td>14.3 - 68.4 (34.3 ± 4.2)</td>
<td>0.3 - 7.9 (2.3 ± 0.9)</td>
<td>2.1 - 4.2 (3.2 ± 0.6)</td>
</tr>
<tr>
<td>CuSO₄ (5 μM) (n=4)</td>
<td>53.5 - 89.0 (72.5 ± 9.1)</td>
<td>2.5 - 6.6 (4.7 ± 0.8)</td>
<td>Not tested</td>
</tr>
<tr>
<td>CuSO₄ (100 μM) (n=8)</td>
<td>64.4 - 224.1 (156.5 ± 18.6)</td>
<td>25.0 - 78.0 (53.3 ± 8.2)</td>
<td>4.0 - 5.7 (4.8 ± 0.4)</td>
</tr>
<tr>
<td>SIN-1 (1 mM)(n=8)</td>
<td>16.7 - 169.0 (76.8 ± 22.5)</td>
<td>1.0 - 9.5 (4.2 ± 1.2)</td>
<td>5.0 - 16.5 (10.9 ± 2.4)</td>
</tr>
<tr>
<td>Peroxynitrite (1mM)(n = 8)</td>
<td>18.3 - 86.2 (41.4 ± 8.9)</td>
<td>1.4 - 8.4 (4.3 ± 1.0)</td>
<td>561 -1165 (862 ± 175)</td>
</tr>
<tr>
<td>Decomposed Peroxynitrite (n = 8)</td>
<td>11.5 - 38.9 (18.4 ± 7.7)</td>
<td>0.06 - 4.8 (1.5 ± 0.7)</td>
<td>1.3-2.0 (1.7 ± 0.2)</td>
</tr>
</tbody>
</table>

Table 4.1: Measurement of lipoprotein oxidation and tyrosine nitration in different preparations of LDL

The extent of oxidation in all LDL preparations was characterised by the concentrations of TBARS and lipid peroxides (Lpo). The results are expressed as nmol/mg apoB/ml and represent the range of values obtained. Values in brackets are the mean ± SEM and n = the number of independent experiments on individual LDL preparations. Significant difference compared to native LDL * P < 0.05; ** P < 0.01, as determined by Wilcoxon rank sum test.
There was an approximate two-fold increase in the basal level when the LDL was air oxidised (3.2 nmol/mg apoB), and an approximate four fold increase in LDL oxidised with cupric ions (4.8 nmol/mg apoB). LDL oxidised with SIN-1 resulted in an average nitrotyrosine content of 8.4 nmol/mg apoB, however, the greatest increase in nitrotyrosine residues was seen following oxidation with 1 mM peroxynitrite (942.4 nmol/mg apoB). This result was not replicated when decomposed peroxynitrite was used, where the nitrotyrosine content was the same as basal levels. Obviously, the increase in nitrotyrosine detected in LDL oxidised by cupric ions and air is likely to be artefactual, as nitration would only occur with peroxynitrite and SIN-1 oxidation.

4.2.1.2 Detection of lipoprotein oxidative modification by electrophoretic mobility

4.2.1.2.1 Cupric ion oxidation

The effect of oxidising LDL (1 mg protein/ml) with up to 500 μM CuSO₄ for 18 h, at 37°C and analysis by agarose gel electrophoresis is shown in Fig. 4.2.1. There was little detectable change in electrophoretic mobility in LDL incubated with up to 50 μM cupric ions (Lanes 3-6), when compared to native LDL (Lane 2). Oxidation with 100 μM cupric ions (Lane 7) resulted in a shift in mobility and a widening of the band, indicating modification of the apo B-100. However, following the oxidation of the LDL with 500 μM cupric ions (Lane 8), the band on the gel was extremely diffuse, indicating fragmentation of apo B-100 had occurred, due to the extent of oxidation. From these results, it was concluded that the highest concentration of cupric ions that could be used in the oxidation of LDL in order to promote maximal change in electrophoretic mobility, without causing fragmentation of the apo B moiety, was 100 μM. This concentration was used in all subsequent experiments.
Fig. 4.2.1 Agarose gel showing the electrophoretic changes of apo B-100 treated with increasing concentrations of CuSO₄ (1-500 μM)

Native LDL (1 mg protein/ml) was incubated with the indicated concentration of CuSO₄ for 18 h at 37°C. Each sample (2 μl) was applied to the gel and subjected to electrophoresis (Section 2.4.2). Lane 2: nLDL; Lanes 3 - 8: 1, 5, 10, 50, 100, 500 μM CuSO₄ oxidised LDL. Results shown are from one experiment that was representative of 3 independent experiments.
4.2.1.2.2 Peroxynitrite and SIN-1 oxidation

LDL (1 mg protein/ml) was oxidised with up to 1 mM peroxynitrite and decomposed peroxynitrite, in PBS supplemented with DTPA, (in order to eliminate transition metal ion oxidation and prevent the decomposition of lipid hydroperoxides by transition metal ions), for 90 min at 37°C and was followed by 16 h incubation at 4°C, in order to replicate the time-scale of cupric ion oxidation. As peroxynitrite decomposes rapidly at physiological pH, by the end of the initial incubation it is unlikely that any of the active form remained, hence the incubation was transferred to 4°C.

The same conditions were applied when using SIN-1 to maintain comparability between the different methods of oxidation. Oxidation of LDL with up to 1 mM peroxynitrite and 1 mM SIN-1 and analysis by agarose gel electrophoresis is shown in Fig. 4.2.2. Native LDL remained at the origin (Lanes 1 & 4) and there was no change in mobility following oxidation with peroxynitrite at 200 and 400 μM (Lanes 5 & 6 respectively). With 600 μM peroxynitrite and above (Lanes 7, 8 & 9) a change in mobility could be detected, which was greatest when the LDL was oxidised with 1 mM peroxynitrite (Lane 3 - Lane 9). When the LDL was treated with 1 mM decomposed peroxynitrite there was no change in electrophoretic mobility (Lane 10), indicating the absence of oxidative modification of apo B-100.

Oxidation of LDL with 1 mM SIN-1 (Fig. 4.2.2 - Lane 2) caused less mobility change than LDL oxidised with 1 mM peroxynitrite (Lane 3). The change in mobility of the LDL oxidised by peroxynitrite and SIN-1 may not be solely due to masking of lysine residues within apo B-100 by aldehydic products of oxidation. Another factor that must be taken into consideration when these oxidising agents are used, is their ability to nitrate tyrosine residues, which may, in turn affect the overall charge of the LDL particle. Indeed, measurement of the nitrotyrosine content of all forms of modified LDL showed that extensive nitration occurred when the LDL was oxidised with peroxynitrite. Although oxidation with SIN-1 resulted in some nitration of the LDL
it was not comparable to levels of nitration achieved following peroxynitrite oxidation (see section 4.2.2 and Table 4.1).

1 mM peroxynitrite and 1 mM SIN-1 was used in all subsequent experiments as they promoted the maximal change in electrophoretic mobility.

4.2.1.2.3 Comparison of changes in electrophoretic mobility following oxidative modification of LDL by different oxidising agents

Direct comparisons were made of LDL oxidation using all the oxidising conditions by agarose gel electrophoresis (Fig. 4.2.3). Whilst native LDL remained at the origin (Lanes 1 & 2) the greatest change in electrophoretic mobility was detected in the LDL that had been oxidised by incubation with 100 μM cupric ions for 18 h (fully oxidised - Lanes 5 & 6). In comparison, when LDL was oxidised with 1 mM SIN-1 (Lanes 7 & 8) there was less change in mobility, an effect also noted by Jessup et al., (1992). Indeed the modest increase in mobility was comparable to LDL that had been oxidised by air (minimally modified – lanes 3 & 4) for 18 h (see section 2.3). Oxidation with 1 mM peroxynitrite (Lanes 9 & 10) resulted in a change in mobility in the region between that achieved with cupric ions and SIN-1.
Fig. 4.2.2 Agarose gel showing the electrophoretic changes of apo B-100 treated with increasing concentrations of peroxynitrite (200 - 1000 μM) and SIN-1 (1000 μM)

Native LDL (1 mg protein/ml) was incubated with the indicated concentration of peroxynitrite and SIN-1 for 90 min at 37°C. The samples were stored overnight at 4°C prior to application to the gel and electrophoresis completed (Section 2.4.4.2). Lane 1: nLDL; Lane 2: 1000 μM SIN-1; Lane 3: 1000 μM peroxynitrite; Lane 4: nLDL; Lanes 5-9: 200, 400, 600, 800, 1000 μM peroxynitrite; Lane 10: 1000 μM decomposed peroxynitrite. Results shown are from one experiment that was representative of 3 independent experiments.
Fig. 4.2.3 Agarose gel showing the electrophoretic changes of apo B-100 treated with a variety of oxidants

Native LDL (1 mg protein/ml) was incubated with the oxidants indicated, all under the optimal conditions to give maximal electrophoretic mobility. Lanes 1 & 2: nLDL; Lanes 3 & 4: mmLDL; Lanes 5 & 6: CuSO4 oxidised LDL; Lanes 7 & 8: SIN -1 oxidised LDL; Lanes 9 & 10 peroxynitrite oxidised LDL. Results shown are from one experiment that was representative of 3 independent experiments.
4.2.2 Prevention of LPS contamination of LDL samples

A problem that can arise in the study of lipoprotein induction of tissue factor expression in vascular cells is LPS contamination of the LDL, as LPS also promotes the induction of tissue factor in vascular cells. Every precaution was taken during the isolation and modification of LDL used in this project to prevent such contamination. All glassware used was rinsed with a 1% solution of the endotoxin-cleaning agent, E-Toxaclean (prepared with pyrogen free water) and baked at 200 °C for a minimum of 4 h, prior to use. All reagents were prepared with pyrogen free water. Finally, samples were tested using the Limulus amoebocyte lysate assay (see section 2.10).

All LDL used in the experiments were tested for LPS and found to have < 10 pg/ml endotoxin, and subsequently were termed ‘LPS-free LDL’. From the results in Chapter 3, a study of the dose-dependent induction of tissue factor activity expression from THP-1 monocytes (Fig. 3.9.1) indicated that the minimum concentration of LPS that caused a significant increase in tissue factor activity following a 4 h incubation was 0.1 μg/ml. Thus, the low levels of LPS detectable in the LDL preparations, by the Limulus amoebocyte assay, would not influence the expression of tissue factor activity in further experiments designed to investigate the influence of modified LDL on the induction of tissue factor in monocyte-macrophages, performed over a 4 h period.

4.2.3 Investigation into the effect of LDL oxidatively modified by different procedures on recombinant tissue factor activity

In this study the effect of native and oxidatively modified forms of LDL (air, cupric ion, peroxynitrite and SIN-1) on the procoagulant activity of tissue factor were investigated using commercially available recombinant tissue factor, which was reconstituted to give a stock solution of 1000 U/ml.
Recombinant tissue factor (150 μl -10U/ml) was incubated with native and the oxidatively modified forms of LDL (150 μl - 0.2 mg protein/ml or 1 mg/ml, diluted in PBS), to give a final activity of recombinant tissue factor of 5U/ml and a concentration of LDL of 0.1 mg protein/ml and 0.5 mg protein/ml respectively. The highest concentration used in these experiments was 0.5 mg protein/ml, as opposed to the more physiological concentration of 1 mg protein/ml. This produced the same effect in terms of influencing tissue factor activity as the more physiological concentration, but meant that less LDL needed to be isolated in order to perform the experiments. Samples were incubated for 90 min at 37° C. This time-scale was chosen, based on the observations of Ettelaie et al., (1999), who found that plasma TFPI caused maximal inhibition of tissue factor activity following a 15 min incubation. Thus, by incubating for 90 min, we could be sure that any effects exerted by TFPI, early in the incubation, had diminished. The tissue factor activity was determined by the one-stage prothrombin time assay and the percentage inhibition calculated against initial values of 5U/ml tissue factor incubated with PBS for 90 min at 37° C (see section 2.6.1).

The effect of native and oxidatively modified lipoproteins on recombinant tissue factor activity is shown in Fig. 4.3.1. At 0.5 mg protein/ml, native LDL inhibited tissue factor activity (Mean ± SEM 41% ± 13; P<0.05). When LDL was oxidised this anticoagulant effect was lost and values exceeded that of control values to exhibit procoagulant activities. There was little difference between the LDL that had been oxidatively modified with a variety of agents, with the mean enhancement of tissue factor activity ranging between 38% for air oxidised LDL to 43% with LDL oxidised with SIN-1. However, only LDL oxidised with peroxynitrite caused an enhancement of tissue factor activity that was statistically significant (Mean ± SEM 39% ± 10; P<0.02).

When the concentration of LDL used in the experiment was reduced to 0.1 mg protein/ml statistical significance was not reached with any of the samples tested. In
Fig. 4.3.1 The effect of LDL oxidised by different procedures on recombinant tissue factor activity

Recombinant tissue factor (final concentration 5U) was incubated with the LDL at concentrations of A.) 0.5 mg/ml and B.) 0.1 mg/ml, at 37°C, for 90 min. Tissue factor activity was determined by the one-stage prothrombin time assay and compared to control sample of recombinant tissue factor incubated with PBS.

KEY: n LDL - native LDL; mm LDL - air oxidised LDL; ox LDL - copper oxidised LDL; PN LDL - peroxynitrite oxidised LDL; SIN LDL - SIN-1 oxidised LDL. Data are shown as an inhibition or enhancement of control values and represent the mean ± SEM of 3 independent experiments, samples assayed in triplicate. Statistics represent comparison of oxidised LDL versus native LDL*P < 0.05, **P < 0.01.
all the experiments LDL that had been treated with decomposed peroxynitrite behaved in the same way as native LDL (not shown).

4.2.4 Investigation of the effect of LDL oxidatively modified by different procedures on optimal tissue factor activity on the surface of THP-1 monocytes

Following the results in 4.2.3, where the effects of native and oxidatively modified LDL on recombinant tissue factor activity was determined, the following study set out to establish whether this effect could be replicated on the cell surface tissue factor activity. In order to do this tissue factor activity had to be induced on the surface of THP-1 monocytes. The results presented in section 3.2.5.2, the study of the temporal profile of tissue factor activity expressed on the surface of THP-1 monocyte-macrophages, showed maximal tissue factor activity was detectable following 24 h incubation of the cells with 10 nM PMA. As the cells in this experiment were required to have maximal cell surface tissue factor activity but not necessarily required to be in the macrophagic form, the PMA washout method developed in section 3.2.5.3 was not used here. With continuous 10 nM PMA incubation for 24 h the cells were expressing maximal surface tissue factor activity, with viability 93%, therefore ideal for these experiments.

Following the 24 h incubation with PMA, adherent cells were lifted from the culture plate by treatment with the Versene (EDTA solution). The cells were washed with PBS and resuspended in PBS to $10^7$ /ml. A cell suspension (150 µl) was incubated with an equal volume of LDL (0.2 mg protein/ml or 1 mg/ml, diluted in PBS) to give final concentrations of LDL 0.1 mg protein/ml and 0.5 mg protein/ml respectively. Samples were incubated for 90 min at 37 °C. The tissue factor activity was determined by the one-stage prothrombin time assay and the percentage inhibition calculated against initial values, (150 µl cell suspension incubated with 150 µl PBS).
The influence of oxidatively modified forms of LDL on tissue factor activity on the surface of THP-1 monocyte-macrophages, induced by PMA, is shown in Fig. 4.3.2. Incubation of the cells with native LDL (0.5 mg protein/ml) resulted in an inhibition of cell surface tissue factor procoagulant activity (Mean ± SEM 22% ± 5; P<0.02). As with the effect of oxidised LDL on recombinant tissue factor, when the LDL was oxidised its anticoagulant properties were reversed and it became procoagulant, with values ranging from 34% enhancement with LDL oxidised with 5 µM cupric ions to 64% enhancement with LDL oxidised with SIN-1. Only air oxidised LDL reached statistical significance (35% ± 8; P<0.01). The exception to the procoagulant effect of oxidatively modified LDL was LDL that had been oxidatively modified using 100 µM cupric ions, which appeared to inhibit tissue factor activity, but this was not a significant effect.

Native LDL at 0.1 mg protein/ml caused an inhibition of tissue factor activity (12% ± 3; P<0.05), whilst all forms of oxidised LDL enhanced tissue factor activity, although to a lesser extent than the values seen with 0.5 mg protein/ml, with a range of values from 3% with peroxynitrite modified LDL to 21% enhancement with LDL oxidised with peroxynitrite. Statistical significance was only reached with air oxidised LDL (12% ± 4; P<0.02). In all the experiments LDL that had been treated with decomposed peroxynitrite behaved in the same way as native LDL (not shown).
Fig. 4.3.2 The effect of LDL oxidised by different procedures on the optimal cell surface tissue factor activity on THP-1 monocyte-macrophages

THP-1 monocytes were stimulated with PMA for 24 h (Section 3.2.5.3), to express maxima tissue factor activity. Cells were resuspended in PBS at $10^7/\text{ml}$ and incubated with the LD at A.) 0.5 mg/ml or B.) 0.1 mg/ml, for 90 min, at 37°C. Tissue factor activity was determined by the one-stage prothrombin time assay and values compared to control cells, incubated with PBS.

KEY: n LDL - native LDL; mm LDL - air oxidised LDL; ox LDL - copper oxidised LDL; PN LDL - peroxynitrite oxidised LDL; SIN LDL - SIN-1 oxidised LDL.

Data are shown as an inhibition or enhancement of control sample tissue factor activity and represent mean ± SEM of at least 3 independent experiments, samples assayed in triplicate. Statistics represent comparison of oxidised LDL versus native LDL.*$P < 0.05$. 

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4.2.5 Induction of tissue factor activity expression from basal levels in THP-1 monocyte-derived macrophages by oxidatively modified LDL

The work of Drake and colleagues (1981) showed that minimally modified LDL promoted the induction of tissue factor mRNA and tissue factor activity in endothelial cells. This study set out to determine whether LDL, oxidatively modified by different agents including cupric ions and peroxynitrite, could induce the expression of surface tissue factor activity and antigen in THP-1 derived macrophages.

The experiments performed in section 3.2.5.3, provided a method for differentiating THP-1 monocytes into viable macrophages in which the cells had basal levels of cell surface tissue factor and thus could be used to investigate tissue factor activity induction by lipoproteins. Briefly, THP-1 monocytes were incubated with 10 nM PMA for 2 h. The cells were then washed free of the PMA with PBS and resuspended at the same density in fresh medium. The cells were then incubated for a further 46 h, by which time they were in a macrophage-form, adherent to the culture vessel and had basal levels of cell surface tissue factor activity.

The cells were incubated with LDL (0 - 100 µg/ml) for 4 h at 37 °C. The concentration of LDL used in this set of experiments was restricted to 0 - 100 µg/ml, primarily to prevent cytotoxicity, especially when the oxidatively modified forms of LDL were being studied. Work by Wilson, 1995 (PhD thesis) indicated that incubation of THP-1 monocyte-derived macrophages with oxidised LDL above 75 µg/ml compromised the viability of the cells although the experiments were performed over a 24 h period. In this study, effects of LDL up to 100 µg/ml were studied, but with a shorter incubation period. The shorter incubation period also meant that further oxidative modification of the LDL was unlikely. Furthermore, from the results described in section 3.2.7, when the monocyte-derived macrophages were re-stimulated with PMA, a 4 h incubation was sufficient to cause a significant increase in the expression of cell surface tissue factor activity. Thus, it was not
unreasonable to expect that this would also be true with respect to induction of tissue factor activity by oxidatively modified LDL.

Following the incubation the adherent cells were removed from the culture vessel by treatment with Versene, washed with PBS and resuspended to $10^7/\text{ml}$ in PBS. The viability was determined by Trypan Blue dye exclusion. A sample of cells was removed and tissue factor activity determined by the one-stage prothrombin time assay. The percentage inhibition/enhancement was calculated against the initial activity, of cells that had been incubated with LPS-free PBS. The remaining cells were placed on ice and prepared for tissue factor antigen detection by flow cytometry (section 2.7.2).

Incubation of the THP-1 monocyte derived macrophages with the native and oxidatively modified LDL ($0 - 100 \, \mu\text{g} \, \text{protein/ml}$) for $4 \, \text{h}$ did not compromise the viability of the cells as determined by Trypan Blue dye exclusion, which remained at $95\%$ or above. In native and minimally modified LDL a bell – shaped dose-response was detected, with maximal effect at $60 \, \mu\text{g} \, \text{protein/ml}$ (Fig. 4.4.1). A comparison of the effects of native and oxidatively modified LDL at $60 \, \mu\text{g/ml}$, on the induction of tissue factor activity in THP-1 monocyte-derived macrophages is shown in Fig. 4.4.2. Both native and air oxidised LDL exhibited less tissue factor activity than that of control cells, indicating an absence of tissue factor activity induction or that basal level of tissue factor activity had been inhibited. Air oxidised LDL resulted in statistically significant inhibition of tissue factor activity (Mean ± SEM $38\% ± 4$; $P<0.01$). LDL oxidised with cupric ions, peroxynitrite and SIN-1 all acted to increase tissue factor activity above that of control cells, which indicated an induction of tissue factor activity or an enhancement of basal levels of tissue factor activity. Copper oxidised LDL resulted in statistically significant enhancement of tissue factor activity ($37\% ± 7$; $P<0.05$). In all experiments LDL that had been treated with decomposed peroxynitrite behaved in the same was as native LDL (not shown).
Fig. 4.4.1 The influence of LDL and modified forms of LDL on induction of tissue factor activity in THP-1 monocyte-macrophages
THP-1 macrophages with basal levels of tissue factor were incubated with native and oxidatively modified forms of LDL at the specified concentrations for 4 h at 37°C. Following incubation, the procoagulant activity on the surface of the macrophages was determined by the one-stage prothrombin time assay. Results represent the % inhibition (negative values) or % enhancement (positive values) of procoagulant activity, compared with macrophages incubated with PBS (LPS free) for the same duration. Results represent mean ± SEM of 3 independent experiments. Statistics represent comparison of oxidised LDL versus native LDL*P < 0.05; +P < 0.005.
Fig 4.4.2 The influence of LDL and modified forms of LDL on the induction of tissue factor activity in THP-1 macrophages

THP-1 monocyte-derived macrophages with basal levels of tissue factor activity were incubated with LDL and modified forms of LDL (60 μg/ml) for 4 h. Procoagulant activity was then determined by the one-stage prothrombin time assay. Results represent the % inhibition (negative values) or % enhancement (positive values) of procoagulant activity, compared with macrophages incubated with PBS (LPS free) for the same duration. Results shown are the mean ± SEM of 3 independent experiments. Statistics represent comparison of oxidised LDL versus native LDL + P < 0.005.
Table 4.2 Tissue factor antigen levels on the surface of THP-1 monocyte-derived macrophages following 4h incubation with different forms of modified LDL. THP-1 monocytes were differentiated into a macrophage -like form by treatment with PMA (section 3.2.3). The cells were then incubated with the LDL (0-100μg/ml) for 4 h at 37°C, prior to staining for tissue factor antigen and analysis by flow cytometry. Results represent a percentage increase (positive values) or percentage decrease (negative values) of tissue factor antigen in cells incubated with LDL compared to cells incubated with PBS. The results represent the mean ± SEM of 3 independent experiments. †P < 0.05

<table>
<thead>
<tr>
<th></th>
<th>Native</th>
<th>Air</th>
<th>Copper</th>
<th>Peroxynitrite</th>
<th>SIN-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 μg/ml</td>
<td>11.3 ± 6.4</td>
<td>-15 ± 12.0</td>
<td>-5.7 ± 13</td>
<td>3.7 ± 6.2</td>
<td>7.7 ± 9.8</td>
</tr>
<tr>
<td>40 μg/ml</td>
<td>-6.7 ± 17.4</td>
<td>3.3 ± 2.0</td>
<td>-3.7 ± 18.4</td>
<td>5.7 ± 3.2</td>
<td>-2.3 ±14.3</td>
</tr>
<tr>
<td>60 μg/ml</td>
<td>-9.7 ± 11.4</td>
<td>-4.0 ± 6.2</td>
<td>19.7 ± 4.7†</td>
<td>-3.7 ± 7.3</td>
<td>11.7± 9.4</td>
</tr>
<tr>
<td>80 μg/ml</td>
<td>4.3 ± 9.9</td>
<td>-3.0 ± 4.6</td>
<td>8.3 ± 5.2</td>
<td>-0.3 ± 7.2</td>
<td>21.0±16.2</td>
</tr>
<tr>
<td>100 μg/ml</td>
<td>-11 ± 5.5</td>
<td>0.3 ± 9.2</td>
<td>-1.0 ± 6.0</td>
<td>4.0 ± 7.2</td>
<td>12.7± 9.4</td>
</tr>
</tbody>
</table>
There was no obvious induction in tissue factor antigen levels (Table 4.2) on the surface of THP-1 monocyte-derived macrophages by any of the LDL tested. The only LDL that caused a significant increase in tissue factor antigen (Mean ± SEM 19.7% ± 4.7; P < 0.05) was the copper oxidised, but this was only seen at one concentration – 60 μg/ml.

4.2.6 Investigation of the inhibitory potential of serum on recombinant tissue factor activity

The results discussed in section 4.2.4.1 & 4.2.4.2 showed native LDL inhibited both recombinant tissue factor activity and cell surface tissue factor activity respectively. This inhibition has been demonstrated to be an effect of apo B-100 interacting with a specific domain of tissue factor (Ettelaie et al., 1995; Ettelaie et al., 1998). However, this is by no means the only inhibitor of tissue factor. The apolipoprotein of HDL – apo A-II, has been demonstrated to inhibit tissue factor activity (Carson, 1987). In addition, tissue factor pathway inhibitor (see section 1.5.2.1) circulates in the plasma, bound to LDL. In order to determine whether the inhibitory effects of TFPI and apo B-100 could be identified in the presence of serum, human venous blood was collected from five volunteers who gave informed consent. The blood was collected in the absence of anticoagulant and allowed to clot at room temperature for 2 h and the sera decanted, pooled and centrifuged on a MSE bench centrifuge to remove any cells. The supernatant was removed and used within two days. 2.85 ml recombinant tissue factor (5U tissue factor activity /ml final concentration) was incubated with 150 μl serum at 37°C. 100 μl was removed immediately and tissue factor activity measured by the one-stage prothrombin time assay. Subsequently, 100 μl samples were removed at intervals up to 90 min and assayed. The percentage inhibition was calculated against the initial activity. In addition, similar samples of serum were pre-incubated at 37 °C for 60 min with either anti-human apo B-100 antibodies (73 μM final concentration) or anti-human TFPI antibodies (7.5 μM final concentration) and examined as above.
Fig. 4.5 The inhibition of recombinant tissue factor by serum
The biphasic inhibition of recombinant tissue factor activity by serum, and the influence of pre-incubating serum with anti TFPI antibodies and anti apolipoprotein B antibodies, for 60 min at 37°C, prior to the incubation with tissue factor are shown. Results shown are one representative experiment that was performed at least 3 times.
Following incubation of tissue factor with serum, it was found that the initial rise in inhibition plateaued after 30 min to a maximum of 50%. This was followed by a later increase in inhibition, levelling at 70%, indicating the presence of at least two separate tissue factor inhibitors in serum with distinct kinetics. Pre-incubation of serum with anti-TFPI antibodies greatly reduced the rate of the early phase of inhibition. In comparison, pre-incubation with anti-apolipoprotein B-100 diminished the late wave of inhibition, without affecting the initial inhibitory potential of serum (Fig. 4.5)

4.2.7 An investigation into the effect of exposing LPS to peroxynitrite

Lipopolysaccharide is a complex structure composed of repeated oligosaccharide units and the lipid A moiety. As the lipid A moiety of LPS is saturated it is unlikely that peroxynitrite would promote lipid oxidation in this molecule. However, the complex carbohydrate moiety would still remain a target for modification by peroxynitrite, based on previous studies (Pryor & Squadrito, 1995), thus, the effect of peroxynitrite on LPS was investigated.

LPS (10 ng/ml - 1 Endotoxin unit) used to generate the maximal concentration in the Limulus amoebocyte lysate assay standard curve (Section 2.10) was exposed to peroxynitrite (0 - 3 mM) and decomposed peroxynitrite (300 µM) and tested in the assay in order for its activity to be determined. LPS activity was then quantified by the Limulus amoebocyte lysate assay and compared to samples that had not been exposed to peroxynitrite. The dose-dependent abolition of LPS activity following exposure to peroxynitrite (0 - 3 mM) is shown in Fig 4.6.1. A significant inhibition of LPS activity was detectable even with 30 µM peroxynitrite, which increased to 98% inhibition of activity, with 3 mM peroxynitrite. However, exposure of LPS to decomposed peroxynitrite (300 µM) did not cause an inhibition of activity.
To determine whether this effect of peroxynitrite could be replicated when LPS was present as a contaminant of a lipoprotein preparation, LDL that had previously been classified as 'LPS-free' was spiked with a known quantity of LPS (20 ng/ml - 2 Endotoxin units). The LDL was then exposed to peroxynitrite (0 - 3 mM) and decomposed peroxynitrite (300 μM). The results of this study are shown in Fig. 4.6.2. Incorporation of LPS into 'LPS-free LDL' and subsequent exposure to peroxynitrite produced a similar result to the effect of peroxynitrite on isolated LPS. However, in this investigation, only 3 mM peroxynitrite resulted in a significant inhibition of activity and the lowest concentration of peroxynitrite only caused a negligible effect on LPS activity (Fig. 4.6.2)
Fig. 4.6.1 The influence of peroxynitrite on LPS activity
LPS (10 ng/ml) was exposed to peroxynitrite (0-3 mM) and decomposed peroxynitrite (300 μM). LPS activity was then quantified by the Limulus amoebocyte lysate assay. The percentage inhibition of LPS activity was calculated against LPS, which had been treated with PBS. Results represent the mean ± SEM of 3 independent experiments. *P< 0.05; + P< 0.001.
Fig. 4.6.2 The effect of exposing LPS within LDL to peroxynitrite, on LPS activity
LDL was spiked with a known quantity of LPS prior to exposure to peroxynitrite (0-3 mM). LPS activity was then quantified by the Limulus amoebocyte lysate assay in all samples. The percentage inhibition of LPS activity in LDL exposed to peroxynitrite was calculated against LDL not exposed to peroxynitrite. Results represent the mean ± SEM of 3 experiments. *P<0.02.
4.3 Discussion

It is widely accepted that the formation of foam cells in vivo arises due to the presence of oxidatively modified LDL. The source of the oxidatively modified LDL has yet to be elucidated, however, a study by Graham et al., (1993) reported that peroxynitrite modified LDL is capable of being recognised by macrophage scavenger receptors and therefore may be the source of oxidised lipids within atherosclerotic lesions demonstrated by Yla Herttuala et al., (1989). Lipid laden macrophages within atherosclerotic plaque have been shown to express tissue factor (Landers et al., 1994), which may activate the extrinsic pathway of coagulation following plaque rupture, resulting in the formation of a thrombus and acute coronary syndromes. The aim of this investigation was to compare the influence of LDL, in its native form with LDL oxidised by a variety of agents, including cupric ions and peroxynitrite, on monocytic cell surface tissue factor. Furthermore, the native and oxidatively modified forms of LDL were compared with respect to their ability to induce the expression of tissue factor activity in the precursor of the foam cell, the monocyte-derived macrophage.

LDL were oxidised using air, cupric ions peroxynitrite and SIN-1. From the results presented in sections 4.2.1.1 and 4.2.1.2, the highest lipid peroxide and TBARS content was detected in LDL oxidised by cupric ions. LDL oxidised with a bolus dose of peroxynitrite had lipid peroxide and TBARS content only slightly higher than LDL that had been subjected to the more gentle air oxidation (mm LDL). This is likely to be the result of the rapid decomposition of peroxynitrite when introduced to physiological pH, limiting the extent of oxidation. Indeed, LDL incubated with the same concentration of SIN-1, which generates peroxynitrite through the slow simultaneous release of superoxide and nitric oxide, contained higher levels of lipid peroxides and TBARS, even though the duration of incubation was identical to that of peroxynitrite. However, when the electrophoretic mobility of LDL oxidised with peroxynitrite and SIN-1 were compared, LDL oxidised by peroxynitrite exhibited the greatest change in mobility. Unlike the results presented
by Hogg et al., 1992, who used 800 μM peroxynitrite to oxidise the LDL, the increase in electrophoretic mobility was less than that achieved when the LDL was incubated with 100 μM cupric ions. The discrepancy between these results is likely to be a consequence of the concentration of LDL used in the experiments; Hogg et al., (1992) used 0.2 mg protein/ml, compared with 1 mg protein/ml used in this study. Thus, if the concentration of peroxynitrite used to oxidise the LDL was increased, it is likely that the extent of oxidation would be comparable to LDL oxidised with cupric ions.

Hogg et al. (1992) reported that LDL incubated first with peroxynitrite and then with copper, had a shorter lag period than LDL that had not been pre-incubated with peroxynitrite. The incubation of LDL with peroxynitrite was also accompanied by a depletion of the vitamin E content of the LDL, usually associated with oxidation of the lipid component of LDL. However, the fact that peroxynitrite modified LDL in the presence of DTPA indicates that this process is not dependent upon transition metal ions. Swain et al., (1994) found that peroxynitrite promotes the release of copper from the caeruloplasmin. These results may explain the presence of catalytically active copper ions in advanced atherosclerotic lesions. Thus, peroxynitrite may have a dual role in the oxidation of LDL, primarily by directly modifying LDL, and secondly by releasing copper from the caeruloplasmin, which could then promote further oxidation.

A major product of the action of peroxynitrite on proteins is the nitration at the ortho position of tyrosine residues. This reaction occurs spontaneously, but is catalysed by superoxide dismutase (Ischiropoulos et al., 1992a). Nitrotyrosine has been detected in atherosclerotic lesions of the coronary arteries and observed in the early sub-intimal fatty streaks (Beckman et al., 1994a). A study by Buttery et al. (1996) demonstrated that nitrotyrosine was not present in normal blood vessels. However, within atherosclerotic lesions, nitrotyrosine was detected in macrophages, foam cells and smooth muscle cells. Leeuwenburgh et al., (1997) demonstrated that LDL isolated from atherosclerotic lesions had 90-fold higher levels of nitrotyrosine than the plasma of healthy subjects, indicating the presence of reactive nitrogen
intermediates in the artery wall, promoting in vivo oxidation of LDL. In the present study, oxidation of LDL with peroxynitrite resulted in a 615-fold increase in nitrotyrosine when compared with native LDL. This may be a consequence of the concentration of peroxynitrite used to oxidise the LDL. The concentration of 1 mM is rather high and it is questionable as to whether this could arise physiologically, although only a small fraction of peroxynitrite survives the dilution in buffer at neutral pH. Moreover, in this study, peroxynitrite was delivered as a bolus dose, another unlikely scenario in vivo. When LDL was oxidised with peroxynitrite generated from SIN-1, there was only a 7-fold increase in nitrotyrosine, which may indicate that the slow generation of peroxynitrite was not sufficient to promote extensive nitration, as found in vivo, in previous studies, or that only small amounts of peroxynitrite are formed and survive at neutral pH.

The effect of native and oxidatively modified LDL on recombinant tissue factor activity is shown in Fig. 4.3.1. As in earlier studies, performed on isolated tissue factor when the effect of native and oxidatively modified LDL were studied (Howell & Ettelaie, 1990), native LDL inhibited the procoagulant activity of recombinant tissue factor. Moreover, in agreement with the work of Howell & Ettelaie (1990), when the LDL became oxidised, there was a reversal in its action towards tissue factor, resulting in an enhancement of procoagulant activity. There was little difference between the different forms of 0.5 mg/ml oxidatively modified LDL with respect to degree of enhancement of procoagulant activity, despite differences in the lipid peroxides, unlike the results reported Ettelaie et al. (1999), who showed that the degree of inhibition or enhancement of tissue factor activity was proportional to the lipid peroxide content. The results of LDL oxidatively modified by peroxynitrite and SIN-1, behaving in the same way as LDL modified by cupric ions, are novel to this study. These results are particularly encouraging when taking into consideration the evidence that oxidation of LDL by peroxynitrite occurs in vivo (Leeuwenburgh et al., 1997b), as it may explain the source of the stimulus that promotes thrombogenicity in cells of the atherosclerotic plaque.
The contrasting influences of native and oxidatively modified LDL arise from a direct interaction between the protein moiety of LDL, apolipoprotein B-100 and the protein moiety of tissue factor (apoprotein III). Ettelaie et al., (1998) reported the presence of a peptide within tissue factor that closely resembles the repeated domain within the LDL receptor protein that is responsible for binding apo B-100. The binding of these proteins requires positively charged residues (lysine) and tryptophan within apo B-100 and negatively charged residues within tissue factor (glutamate and aspartate). Interaction between these proteins mask essential amino acids that are essential for binding and activation of coagulation factor VII and inhibition of tissue factor activity arises. The ability of apo B-100 to influence tissue factor activity has been shown to be dependent upon the whole lipoprotein complex and optimal inhibitory effect is achieved with a particular lipid composition and/or size (Ettelaie & Howell, 1992). Upon oxidation of LDL, the secondary structure of apo B-100 undergoes alterations with the greatest region affected being the receptor-binding region. These alterations are accompanied by the formation of protein-lipid adducts on the ε-amino groups of the basic amino acid residues (Ettelaie et al., 1997), resulting in a reversal of the inhibition and therefore augmentation of tissue factor activity (Ettelaie et al., 1995). Ettelaie et al. (1995) studying the influence of oxidation of the other classes of lipoproteins on tissue factor activity, found that the influence of HDL and lipoprotein (a) was unaltered by oxidation, both inhibiting tissue factor activity. LDL, which normally acted as an inhibitor of tissue factor activity, acted to enhance tissue factor activity, whereas the enhancing effect of VLDL was diminished.

When the influence of LDL and its modified forms were studied with respect to their effects on maximal cell surface tissue factor activity (Fig. 4.3.2) the results showed the same pattern as those seen with recombinant tissue factor, the exception being LDL oxidised with 100 μM cupric ions, which appeared to inhibit tissue factor activity. This may be due to the extent of oxidation of the samples, because if the apo B-100 moiety had been severely modified by the oxidation, then it would not exert any effect on LDL. Thus, the effect seen may be entirely due to oxidised lipids, an effect previously studied by Ettelaie et al. (1995), who reported that un-treated LDL lipids
had no effect on tissue factor activity, unlike LDL lipids oxidised by cupric ions or lipoxygenase which inhibited tissue factor activity. Thus, the oxidative state of LDL can alter the endogenous activity of tissue factor, through a direct action on tissue factor and therefore factor VII activity.

The oxidative state of LDL is also an important determinant in the induction of tissue factor by cells of the vasculature. The work of Brand et al., (1990) found that minimally modified LDL induced the expression of tissue factor activity in THP-1 monocytes, which reached a maximum at 6 h. These results were replicated in endothelial cells by Drake et al. (1991) who observed maximal tissue factor activity was detected between 4 and 6 h. In both of these studies, maximal induction was achieved with 40 μg/ml LDL. In the present study, minimally modified LDL did not increase tissue factor activity in the monocyte-derived macrophages following 4 h incubation. In fact it behaved in a similar way to native LDL, appearing to decrease tissue factor activity, an effect which was maximal at 60 μg protein/ml. As the lipid peroxides and TBARS content of the minimally modified LDL used in this set of experiments indicated that the LDL had been oxidised, there are two explanations for this result. Firstly, when the other researchers were testing minimally modified LDL, the longer duration of their incubations compared to the present study (24 h versus 4 h) promoted further oxidation of the LDL. Secondly, the differentiation of the monocyte-macrophages was accompanied by the expression of tissue factor activity, which was maximal at 24 h (see section 3.2.5.2). In section 3.2.7 the monocyte-macrophages were shown to be responsive to re-stimulation with PMA, which promoted maximal expression of tissue factor activity at 8 h. Re-stimulation with a different stimulus, LDL in this instance, may require a longer period of stimulation, to promote the induction of tissue factor activity above that of basal levels. The apparent decrease in tissue factor activity in monocyte-macrophages incubated with mm LDL may therefore be attributed to an inhibition of basal tissue factor activity on the surface of the macrophages with the longer period of incubation used in this experiment, when compared with the effects of LDL on
maximal cell surface tissue factor activity presented in section 4.2.4 (4 h versus 90 min).

Incubation of THP-1 macrophages with copper oxidised LDL resulted in an increase in cell surface tissue factor activity, which was also shown, to a lesser extent, in LDL oxidised with peroxynitrite and SIN-1. The maximal increase was detected when the concentration of LDL was at 60 μg protein/ml. Conversely, Brand et al., (1994) reported that 'LPS-free' LDL, oxidised with cupric ions did not increase tissue factor activity in human adherent monocytes following a 12 h incubation. However, the authors reported that copper ion oxidised LDL significantly enhanced tissue factor activity that had been induced by LPS.

In the present study, the increase of tissue factor activity on the surface of the monocyte-derived macrophages incubated with oxidatively modified LDL was not accompanied by an increase in cell surface tissue factor antigen levels. This result is in agreement with Petit et al., (1999), who reported that peripheral blood monocyte-derived macrophages incubated with oxidised LDL, had increased tissue factor activity, but no change in tissue factor antigen. An explanation for this may be found in the results in section 3.2.6. The temporal profile of tissue factor activity and antigen expression induced by PMA did not follow the same time-course, indicating that exposure of antigen was not an indicator of functional activity.

Penn et al., (1999) reported that LDL increased levels of tissue factor cell surface protein, but was not accompanied by an increase in cell surface procoagulant activity. However, in the presence of hydrogen peroxide, procoagulant activity was increased. The authors suggested that this was the result of a novel 2-step pathway for increasing tissue factor on the surface of cells (a small percentage of total cellular tissue factor) in which lipoproteins promote the induction of latent protein, which is then activated by oxidants. This may explain the findings of van den Eijnden et al., (1999) who reported that monocyte-derived macrophages incubated with modified lipoproteins did not lead to tissue factor expression, and concluded that the induction
of tissue factor expression in foam cells in the atherosclerotic lesion is triggered by additional components other than lipoproteins.

The presence of an inhibitor of tissue factor within plasma and serum is by no means a recent observation. The inhibition of tissue factor by serum was studied extensively by the early investigators (Loeb et al., 1922; Lanchantin & Ware, 1953). Such studies led to the discovery that the inhibition of tissue factor by serum was calcium dependent, reversible and exhaustible. From the present experiments where tissue factor was incubated with serum, it could be deduced that there are at least two forms of tissue factor inhibitors acting at different rates. Moreover, by using antibodies specific to apo B-100 and TFPI, it was shown that the first inhibitory peak was caused by the action of TFPI, whereas apo B-100 had a slower, more consistent effect. However, the action of other inhibitors within plasma, e.g. HDL (apolipoprotein A-II) (Carson, 1987), cannot be ruled out. Studies by Ettelaie et al., (1999) using isolated TFPI and apoB-100, showed that plasma TFPI had a transient inhibitory effect, which caused maximal inhibition of about 70%, after around 15 min, which is in agreement with the first inhibitory peak in the serum study. Conversely, inhibition by apo B-100, occurred over a longer period of time, but caused almost total inhibition of the tissue factor procoagulant activity at 120 min, accounting for the second inhibitory peak in the serum study. Upon oxidation of LDL the inhibitory potential of TFPI is lost (Lesnik et al., 1995). This coupled with the procoagulant influence of oxidised LDL on tissue factor (Howell & Ettelaie, 1990) may result in a severely compromised control of haemostasis, especially in conditions associated with tissue factor mediated coagulation, such as potentially thrombotic episodes associated with coronary heart disease.

The ability of macrophage scavenger receptors to bind and internalise bacteria (Dunne et al., 1994) may provide a source of entry for LPS into the atherosclerotic plaque, and induce tissue factor expression in the constituent cells. Furthermore, LPS has been shown to promote peroxynitrite formation in rat aorta (Szabo et al., 1995) and in human leukocytes (Gagnon et al., 1998). The results presented in this
study demonstrated that peroxynitrite inhibited the activity of both free LPS and LPS within LDL, which may ensure that LDL is free of LPS, whilst at the same time it provides a method of LDL-oxidation. The diminution of LPS activity by peroxynitrite may represent a localised control of LPS activity in areas of inflammation and indicate a positive role for peroxynitrite \textit{in vivo}, by limiting LPS induced tissue factor expression.

Peroxynitrite modification of LDL can have similar effects to those of other oxidised forms of LDL, in terms of its ability to enhance existing cell surface tissue factor activity and promote the induction of tissue factor activity from basal levels, in vascular cells. Therefore, peroxynitrite may be an important oxidant in atherosclerotic plaque, which produces effects consistent with the cellular changes seen in atherosclerosis, particularly in relation to the coagulation pathway.
CHAPTER 5

THE INFLUENCE OF NITRATION BY PEROXYNITRITE ON TISSUE FACTOR ACTIVITY
CHAPTER 5: THE INFLUENCE OF NITRATION BY PEROXYNITRITE ON TISSUE FACTOR ACTIVITY

5.1 Introduction

Nitric oxide is produced by endothelium, macrophages, neutrophils, and neurons from the NADPH-dependent oxidative deamination of L-arginine (Moncada & Higgs, 1993). Nitric oxide reacts rapidly with superoxide at a rate of at least $3.7 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ to form peroxynitrite (Pryor & Squadrito, 1995) (see section 1.7.2, Fig. 1.8). Peroxynitrite has a pKa of 6.8 (Radi et al., 1991a), hence less than 20% of it will be protonated at pH 7.4. The protonated form of peroxynitrite, peroxynitrous acid, is very unstable with a half-life of less than 1 sec, and decomposes to form various products, including potent oxidants with the reactivity of nitric oxide and the hydroxyl radical (Beckman et al., 1990). Peroxynitrite is an agent with a diverse chemistry that is capable of oxidation and modification of proteins, DNA (Szabo & Oshima, 1997) and lipids (Radi et al., 1991a). In addition peroxynitrite has been shown to act as a powerful bactericidal agent (Zhu et al., 1992; Hurst & Lymar, 1997).

Peroxynitrite has been shown to be relevant to a number of human pathologies, including atherosclerosis, inflammation (Boughton-Smith et al., 1993; Kaur & Halliwell, 1994) and neurodegenerative disease (Beckman et al., 1993 a & b). With regards to atherosclerosis the actions of peroxynitrite are diverse. Peroxynitrite may contribute to the development of the atherosclerotic lesion through the oxidation of the constituent lipids of LDL; a study by Graham et al., (1993) reported that macrophage scavenger receptors recognised LDL oxidised with peroxynitrite. Thrombosis associated with advanced atherosclerotic lesions is controlled in part by nitric oxide dependent inhibition of platelet aggregation (Moncada & Higgs, 1993). However, in atherosclerotic lesions an excess production of superoxide (Mugge et al., 1994) may cause loss of the modulatory actions of nitric oxide and at the same time yield peroxynitrite, which is pro-aggregatory and so promote thrombus formation.
Furthermore, peroxynitrite has been shown to contribute to defective vasorelaxation in atherosclerotic tissue (White et al., 1994) and impair the actions of several vasodilators on the coronary circulation (Villa et al., 1994).

Studies in vitro have demonstrated that stimulated human neutrophils release nitric oxide and superoxide at rates that favour the formation of peroxynitrite (Carreras et al., 1994). There is evidence that nitric oxide and peroxynitrite can be formed by bovine aortic endothelial cells (Kooy & Royall, 1994), rat macrophages (Ischiropoulos, et al., 1992b) and more recently by human monocytes (Sharara et al., 1997), following stimulation with α - interferon. A study by Gagnon et al. (1998) reported that human leukocytes produced high amounts of peroxynitrite in response to LPS, which may explain the source of the elevated plasma peroxynitrite levels observed in endotoxic shock (Szabo et al., 1995).

Peroxynitrite can nitrate phenylalanine (van der Vliet et al., 1994), oxidise (Kato et al., 1997) and nitrate tryptophan (Alvarez et al., 1996) and oxidise sulphydryl groups at a rate 1000 - fold higher that that of hydrogen peroxide (Radi et al., 1991b). However, one of the best known effects of peroxynitrite is its ability to nitrate free or protein-associated tyrosines, in particular at the ortho position to generate nitrotyrosine, by a reaction that is catalysed by superoxide dismutase (Ischiropoulos et al., 1992a). The presence of nitrotyrosine in vivo strongly suggests the formation of peroxynitrite, as tyrosine is nitrated by peroxynitrite but not by nitric oxide itself (Ischiropoulos et al., 1992a; Van der Vliet et al., 1994). However, nitration may also arise as the result of myeloperoxidase activity in neutrophils, since as well as producing hypochlorite, which may chlorinate tyrosine, this enzyme can nitrate proteins in the presence of nitrite (Eiserich et al., 1998).

Nitrotyrosine residues have been detected in both early fatty streaks and late atherosclerotic lesions of coronary arteries (Beckman et al., 1994a), nitrotyrosine is not detectable in normal blood vessels (Buttery et al., 1996), indicating that peroxynitrite or another oxidant derived from nitric oxide is produced and may play
a part in the evolution of the lesion. Nitrotyrosine has been associated with human pathologies including Alzheimer's disease (Good et al., 1996), AIDS dementia (Boven et al., 1999), rheumatoid arthritis (Kaur et al., 1994) and in myocardial inflammation (Kooy et al., 1997). Nitrotyrosine and its metabolites were detected in healthy human urine (Ohshima et al., 1990) and in healthy human plasma proteins (Khan et al., 1997), implying the exposure of these proteins to continuous low levels of oxidative stress, in the absence of any pathology.

The nitration of tyrosine in vitro can be achieved chemically and more selectively if performed at high pH with tetranitromethane (Chacko, 1985). HDL (Chacko et al., 1985), thrombin (Lundblad et al., 1988) and tissue plasminogen activator (Ploug et al., 1995) are all influenced in their activities by nitration with tetranitromethane. Moreover, nitration of tyrosine by peroxynitrite has been shown to influence the activity of surfactant protein (Haddad et al., 1993), inhibit cytochrome P450 (Janij et al., 1987) and inhibit protein phosphorylation (Martin et al, 1990). Proteins in which tyrosine has a key function in their biological activity are particularly sensitive to these changes. Ischiropoulos et al., (1992a) noted the inactivation of manganese and iron superoxide dismutase following tyrosine nitration. This has been studied further by Yamakura et al., (1998), who found that the inactivation of manganese superoxide dismutase was due to the exclusive nitration of tyrosine 34 of the enzyme. Nitration of active site tyrosine within human manganese superoxide dismutase has been demonstrated to occur in the inflammatory diseases, including chronic organ rejection and arthritis (MacMillan – Crow & Thompson, 1999).

Tissue factor is a glycosylated membrane spanning protein, best known as the initiator of the extrinsic pathway of blood coagulation following trauma to the blood vessel or exposure of cells of the vasculature to endotoxin (Colucci et al., 1983; Ternisien et al., 1993) during Gram-negative infection. The extracellular domain of tissue factor, the active domain, consists of 219 amino acids. Inspection of the primary sequence of tissue factor reveals there are 12 tyrosine residues in the structure of tissue factor, 11 of which are found in the extracellular domain. Based
on the structural studies of Ruf et al., (1994), 8 are exposed and therefore susceptible to attack by peroxynitrite. The others are buried within this peptide. The intracellular domain of tissue factor has no tyrosine residues, but does contain the only free thiol group. The relevant domains of tissue factor, with respect to factor VII binding and overall coagulation, have been investigated using site directed mutagenesis by alanine substitution (Ruf et al., 1994). The authors reported that mutation of Tyr 71 and Tyr 157 (both in the extracellular domain) affected overall coagulation, particularly the substitution of Tyr 71, which resulted in a 98% reduction in activity.

In view of the structure of the extracellular domain of tissue factor, particularly its tyrosine content, and from previous literature, indicating that nitration of tyrosine residues within proteins can influence their biological activity, the direct effects of peroxynitrite on the structure of tissue factor were investigated. Furthermore, the functional activity of this protein, its ability to induce coagulation, was tested following exposure to peroxynitrite. The functional activity study was extended to monocytes in which cell surface tissue factor activity had been induced by phorbol ester stimulation.

Furthermore, following the results presented in Chapter 4, which showed a reduction of LPS activity following exposure to peroxynitrite, as measured by the Limulus amoebocyte lysate assay, the present study was extended to investigate the effect of exposing bacterial endotoxin (LPS) to peroxynitrite and determining the effect, in terms of the ability of LPS to induce tissue factor activity in human monocytes.
5.2 Results

5.2.1 Spectrophotometric analysis of individual amino acid exposed to peroxynitrite

Prior to investigating the effect of peroxynitrite on tissue factor itself, the effects of peroxynitrite on the constituent amino acids found in the extracellular domain of tissue factor was studied. L-isomer amino acids were prepared in water to give a stock solution of 0.03 M. The pH was adjusted accordingly to enhance solubility. Individual amino acids were diluted to 300 µM and the pH adjusted to pH 7, prior to scanning on a Beckman DU-70 spectrophotometer, in the visible and \( \text{uv} \) regions, against a blank of water. Individual amino acids were then exposed to 300 µM peroxynitrite or 300 µM decomposed peroxynitrite, rapidly vortexed adjusted to pH 7. Individual amino acids exposed to peroxynitrite or decomposed peroxynitrite were scanned, as before, against a blank of water.

The \( \text{uv} \) absorption spectrum of L-tyrosine at neutral pH shows two major peaks in the region 230 and 290nm. However, these were unaffected by exposure to peroxynitrite (not shown). The visible region spectrum of 300 µM L-tyrosine, prior to and following, exposure to 300 µM peroxynitrite and decomposed peroxynitrite is shown in Fig. 5.1.1. L-tyrosine itself showed very little absorbance in the visible region (Trace a). Addition of 300 µM decomposed peroxynitrite to L-tyrosine showed a shoulder of a peak at 350nm (Trace b). This peak is likely to be a contaminant of peroxynitrite as it was also present when the absorption spectrum of decomposed peroxynitrite in water was measured (Fig. 5.1.2). Addition of 300 µM peroxynitrite to L-tyrosine (Trace c) also showed shoulder of a peak at 350nm; however, an additional peak was present in the region of 430nm, reminiscent of the spectrum of nitrotyrosine (Fig. 5.1.3)

Phenylalanine itself showed very little absorbance in the visible region (Trace a)(Fig.5.1.4). Addition of 300 µM decomposed peroxynitrite to phenylalanine
Fig. 5.1.1 Spectrophotometric analysis of L-tyrosine exposed to peroxynitrite
300 μM solution of tyrosine (Trace a) was exposed to 300 μM decomposed peroxynitrite (Trace b) and 300 μM peroxynitrite (Trace c), and the pH adjusted to 7. The visible region absorption spectrum was measured on a Beckman DU70 spectrophotometer, against a blank of water. Results shown are one representative experiment, which was performed at least 3 times.
Fig. 5.1.2 The absorption spectrum of 300 μM decomposed peroxynitrite in water
Peroxynitrite was diluted in water to give a final concentration of 300 μM and the pH adjusted to 7. The visible region absorption spectrum was measured on a Beckman DU70 spectrophotometer, against a blank of water. Results shown are one representative experiment, which was performed at least 3 times.
Fig. 5.1.3 The absorption spectrum of 3-nitrotyrosine
A 300 μM solution of 3-nitrotyrosine was prepared with water. The pH was adjusted to 7 and the solution examined spectrophotometrically on a Beckman DU70 spectrophotometer, against a blank of water. The results shown are one representative experiment, which had been performed at least 3 times.
Fig. 5.1.4 Spectrophotometric analysis of phenylalanine exposed to peroxynitrite
300 μM solution of phenylalanine (Trace a) was exposed to 300 μM decomposed
peroxynitrite (Trace b) and 300 μM peroxynitrite (Trace c) and the pH adjusted to 7.
The visible region absorption spectrum was measured on a Beckman DU70
spectrophotometer, against a blank of water. Results shown are one representative
experiment, which was performed at least 3 times.
resulted in a shoulder of a peak at 350nm (Trace b). Addition of 300 μM peroxynitrite to phenylalanine (Trace c) also showed the peak at 350nm; however, an additional peak was present in the region of 405 nm (Fig. 5.1.4).

The \( \text{uv} \) absorption spectrum of L-tryptophan at neutral pH shows two major peaks in the region 230 and 278nm. However, these were unaffected by exposure to peroxynitrite (not shown). The visible region spectrum of 300 μM L-tryptophan, prior to and following, exposure to 300 μM peroxynitrite and decomposed peroxynitrite is shown in Fig. 5.1.5.

L-tryptophan itself showed very little absorbance in the visible region (Trace a). As with L-tyrosine, addition of 300 μM decomposed peroxynitrite to L-tyrosine resulted in a shoulder of a peak at 350nm (Trace b), which increased in size following addition of 300 μM peroxynitrite to L-tryptophan (Trace c).

The fluorescence spectrum of 300 μM L-tryptophan was measured with an emission wavelength of 340nm (Kato et al., 1997) and scanning from 320-410nm on a Perkin Elmer MPF44B fluorimeter following exposure to peroxynitrite and decomposed peroxynitrite (0 - 3 mM). The fluorescence spectrum of 300 μM L-tryptophan, is shown in Fig. 5.1.6 (Trace a). Following exposure of tryptophan to 3 mM peroxynitrite the tryptophan fluorescence was largely eliminated (Trace c). The same concentration of decomposed peroxynitrite (Trace b) reduced the fluorescence intensity of tryptophan but not to the same extent as active peroxynitrite. In order to eliminate the possibility that the effect was not the result of \( \text{H}_2\text{O}_2 \), which may be present from the synthesis of the peroxynitrite, assuming no steps were taken to remove it by filtering through a manganese oxide column, the experiment was repeated in the presence of \( \text{H}_2\text{O}_2 \) at concentrations which were maximum (0 - 12 mM). There was no reduction in fluorescence, thus excluding the effect of \( \text{H}_2\text{O}_2 \) in the oxidation of tryptophan (not shown – see the effect of hydrogen peroxide on tissue factor fluorescence – Fig. 5.2.3).
Fig. 5.1.5 Spectrophotometric analysis of L-tryptophan exposed to peroxynitrite
300 μM solution of tryptophan was prepared with water (Trace a), was exposed to
300 μM decomposed peroxynitrite (Trace b) and 300 μM peroxynitrite (Trace c) and
the pH adjusted to 7. The absorption spectrum was measured on a Beckman DU70
spectrophotometer, against a blank of water. Results shown are one representative
experiment, which was performed at least 3 times.
Fig. 5.1.6 The oxidation of tryptophan by peroxynitrite

The oxidation of tryptophan residues, measured by detection of emission at 340nm on a Perkin Elmer MPF-44B fluorimeter is shown. Trace a represents 300 μM tryptophan prepared with water and the pH adjusted to 7. Traces b and c are the emission detected following exposure to 300 μM decomposed and 300 μM peroxynitrite respectively and the pH adjusted to 7. The results shown are one representative experiment, which had been performed at least 3 times.
5.2.2 Spectrophotometric analysis of recombinant tissue factor exposed to peroxynitrite

The \textit{uv} and visible spectra of recombinant tissue factor (100 \( \mu \text{g/ml} \)) exposed to peroxynitrite and decomposed peroxynitrite (0 - 3 mM) were scanned on a Beckman DU70 spectrophotometer against a blank of \( \text{H}_2\text{O} \). The \textit{uv} absorption spectrum of recombinant tissue factor shows two major peaks at 230 and 278 nm, which are reminiscent of those of tryptophan, but may include peaks of tyrosine at neutral \( \text{pH} \). The absorption spectrum did not change significantly on addition of peroxynitrite up to 3 mM (not shown). The absorption spectrum in the visible region of recombinant tissue factor prior to and following exposure to 3 mM peroxynitrite and decomposed peroxynitrite is shown in Fig. 5.2.1. In the visible region, recombinant tissue factor did not show strong absorbance (Trace a) but on addition of 3 mM peroxynitrite a peak appeared at 350 nm with a smaller peak at 410-440 nm region (Trace b), the latter corresponding to the visible absorption spectrum of nitrotyrosine (Fig. 5.1.3). Addition of 3 mM decomposed peroxynitrite to recombinant tissue factor (Trace c) led to the appearance of the peak at 350 nm which can be attributed to the presence of peroxynitrite as the same peak was detectable when decomposed peroxynitrite was added to water (Fig. 5.1.2). Moreover, the absence of a peak at 410 - 440 nm indicated that nitration had not occurred. Lower concentrations of peroxynitrite (30 - 300 \( \mu \text{M} \)) showed little change in absorption at any of these wavelengths (not shown) indicating that at the concentration of recombinant tissue factor used a high concentration of peroxynitrite was required to achieve nitration. At the lower concentrations of peroxynitrite tested, nitration may have occurred, but not to the extent that could be detected by this method.

The fluorescence spectrum of recombinant tissue factor following exposure to peroxynitrite and decomposed peroxynitrite (0 - 3 mM) was measured with an emission wavelength of 340 nm and scanning from 320-410 nm on a Perkin Elmer MPF44B fluorimeter. The fluorometric spectrum of recombinant tissue factor suggest that tryptophan was modified to a very limited extent by the addition of 300
μM peroxynitrite (Fig 5.2.2), but this was largely eliminated by the addition of 3 mM peroxynitrite. Decomposed peroxynitrite decreased fluorescence slightly, but less than active peroxynitrite. In order to eliminate the possibility that the effect was not the result of \( \text{H}_2\text{O}_2 \), which may be present from the synthesis of the peroxynitrite, the experiment was repeated in the presence of \( \text{H}_2\text{O}_2 \), assuming steps were not taken to remove it during the synthesis of peroxynitrite, by filtering through a manganese oxide column. There was little reduction in fluorescence, with either 300 μM or 3 mM \( \text{H}_2\text{O}_2 \), compared with that seen when peroxynitrite was used, thus excluding the likelihood of an artefact due to the oxidation of tryptophan by \( \text{H}_2\text{O}_2 \) (Fig. 5.2.3).
Fig. 5.2.1 Spectrophotometric analysis of recombinant tissue factor exposed to peroxynitrite
Recombinant tissue factor, prepared with water (Trace a) was exposed to 3 mM peroxynitrite (Trace b) or 3 mM decomposed peroxynitrite (Trace c) and the pH adjusted to 7. The visible absorption spectrum was measured on a Beckman DU70 spectrophotometer, against a blank of water. Results shown are one representative experiment, which was performed at least 3 times
Fig. 5.2.2 The oxidation of tryptophan residues within recombinant tissue factor following exposure to peroxynitrite

The tryptophan residues within recombinant tissue factor, measured by detection of emission at 340nm on a Perkin Elmer MPF-44B fluorimeter is shown (Trace a). Traces b and c show the emission detected following the exposure of recombinant tissue factor to 300 μM decomposed and 300 μM peroxynitrite respectively and traces d and e are the result of exposure to 3 mM decomposed and 3 mM peroxynitrite respectively. The results shown are one representative experiment, which had been performed at least 3 times.
Fig. 5.2.3 The influence of hydrogen peroxide on the fluorescence of tryptophan residues within recombinant tissue factor

The tryptophan residues within recombinant tissue factor, measured by detection of emission at 340nm on a Perkin Elmer MPF-44B is shown (Trace a). Traces b and c show the emission detected following the exposure of recombinant tissue factor to 300 µM and 3 mM hydrogen peroxide respectively. The results shown are one representative experiment, which had been performed at least 3 times.
5.2.3 Influence of peroxynitrite on recombinant tissue factor

Recombinant tissue factor (100 μg/ml) was exposed to peroxynitrite or decomposed peroxynitrite at concentrations between 0 - 3 mM diluted from a stock solution into PBS (pH 7.4) and added within the space of 5 s by rapid vortexing. All measurements were carried out within 10 min of peroxynitrite being added to recombinant tissue factor.

5.2.3.1 Measurement of lipid peroxidation

Recombinant tissue factor is dispersed in a small amount of lipid, which is necessary for its biological activity. It is therefore possible that any effects of peroxynitrite may be due indirectly to oxidation of lipids and modification of proteins. In order to determine whether any lipid peroxidation occurred in recombinant tissue factor following exposure to peroxynitrite, the lipid peroxide content of samples were estimated by the FOX assay (Section 2.4.2.1). There was not a significant increase in the oxidation of the lipid associated with the recombinant tissue factor. Recombinant tissue factor prior to peroxynitrite exposure contained mean lipid peroxide concentration 4.4 μM (range 3.6 - 5.3). Following exposure to 3 mM peroxynitrite, the maximal concentration used in these experiments the mean lipid peroxide concentration was 4.3 μM (range 2.7 - 5.9).

5.2.3.2 Measurement of nitrotyrosine residues

Immuno-reactive nitrotyrosine residues in recombinant tissue factor (100 μg/ml) following exposure to various concentrations of peroxynitrite were quantified by competitive ELISA (Section 2.9) as the spectrophotometric determinations were insufficiently sensitive for quantification of the extent of nitration, when low concentrations of peroxynitrite were used (300 μM and 30 μM). However, the spectroscopy studies did indicate that modification of tyrosine and tryptophan might occur with addition of peroxynitrite.
Fig. 5.3 The nitrotyrosine content of recombinant tissue factor following exposure to peroxynitrite and decomposed peroxynitrite
Recombinant tissue factor (100 $\mu$g/ml) was exposed to peroxynitrite (0 - 3 mM) and decomposed peroxynitrite (300 $\mu$M) diluted from stock solution with PBS. Control samples were recombinant tissue factor and PBS. Competitive ELISA quantified nitrotyrosine residues. The results represent the mean SEM of 3 independent experiments. * 0.05 < P; **0.02 < P.
The sensitive competitive ELISA procedure for nitrotyrosine (Khan et al., 1997) was used, as a convenient procedure for detection of protein modification by peroxynitrite at low concentrations. The effect of exposing recombinant tissue factor to peroxynitrite and the subsequent nitrotyrosine content of the samples is shown in Fig. 5.3. Exposure of recombinant tissue factor to 30μM peroxynitrite did not increase the nitrotyrosine content significantly above background levels. At 300 μM, although only low levels of nitration were detected, the increase over background levels was significant. This is likely to be the consequence of a high proportion of the nitrating species being lost during mixing, based on the short half-life of peroxynitrite at physiological pH. Exposure of recombinant tissue factor to 3 mM peroxynitrite resulted in a substantial increase in nitrotyrosine content of the samples. Decomposed peroxynitrite did not cause nitration of tyrosine residues in recombinant tissue factor, compared to controls where only buffer was used. This result may suggest that only the most exposed tyrosine residues in the extracellular domain of tissue factor are modified by the peroxynitrite. Furthermore, the nitration of key tyrosine residues, e.g. tyrosine 71, important to the procoagulant activity (Ruf et al., 1994) may be all that is required to inhibit coagulation. Therefore, additional tyrosine nitration may prove to be superfluous.

5.2.3.3 Measurement of procoagulant activity

Following exposure of 100 μg/ml recombinant tissue factor to peroxynitrite or decomposed peroxynitrite at concentrations between 0 - 3 mM, the samples were diluted (final concentration 2 μg/ml protein). Dilution to this concentration ensured that the clotting time achieved would be in the range of the standard curve in which a small change in clotting time represents a significant change in the procoagulant activity. The procoagulant activity of the samples was determined by the one-stage prothrombin time assay (Section 2.6.1), and compared to control samples, which had been treated with PBS. The degree of inhibition of procoagulant activity was calculated. All the assays were completed within 10 min after addition of peroxynitrite to the recombinant tissue factor.
The exposure of recombinant tissue factor (100 μg protein/ml) to 0-3 mM peroxynitrite resulted in a concentration dependent inhibition of procoagulant activity compared to that of the control sample, almost complete inhibition was attained at 3 mM peroxynitrite and significant inhibition at 30 μM and above (Fig. 5.4). Exposure to decomposed peroxynitrite resulted in a small enhancement in procoagulant activity (Fig. 5.4). Nitrate or nitrite, or indeed a mixture of the two did not cause the enhancement in tissue factor activity detected with decomposed peroxynitrite, when these were tested separately. Nor was it an effect of residual H₂O₂ assuming no steps were taken to remove this during the synthesis of peroxynitrite.

5.2.4 Influence of peroxynitrite on cell surface tissue factor activity

From the results in section 5.2.3.3 where peroxynitrite caused an inhibition of recombinant tissue factor activity, this study set out to investigate whether this could be replicated on the surface of cells, which had been induced to express tissue factor. Preliminary studies showed that the addition of peroxynitrite at concentrations above 100 μM resulted in cell lysis, thus, experiments using concentrations higher than this were not used in cellular studies.

THP-1 monocytes were incubated with 10 nM PMA for 24 h in order to induce the expression of cell surface tissue factor activity (3.2.5.2). Adherent cells were lifted from the culture plate by the treatment with Versene. The cells were washed once with PBS and resuspended in PBS to 10⁷ cells/ml and were exposed to 0 - 100 μM peroxynitrite and decomposed peroxynitrite, which had been diluted from a stock solution into PBS. The procoagulant activity of the samples was determined by the one-stage prothrombin time assay and percentage inhibition of procoagulant activity calculated (Section 2.6.1). The inhibition of procoagulant activity of cell surface tissue factor, following exposure to peroxynitrite is shown in Fig. 5.5. Inhibition was attained at concentrations as low as 10 μM peroxynitrite and an increase in inhibition accompanied higher concentrations of peroxynitrite.
Fig. 5.4 The inhibition of recombinant tissue factor activity by peroxynitrite treatment
Recombinant tissue factor was exposed to peroxynitrite and decomposed peroxynitrite or PBS (control). Tissue factor activity was determined by the one-stage prothrombin time assay and results expressed as an inhibition of activity compared to control samples. Results represent the mean ± SEM of 3 independent experiments. *0.05<P; **0.02<P; ***0.01<P.
Fig. 5.5 The inhibition of cell surface tissue factor activity by peroxynitrite
Tissue factor activity was expressed on the surface of THP-1 monocytes following treatment with PMA. The cells were then exposed with up to 100 μM peroxynitrite and the tissue factor activity measured by the one-stage prothrombin time assay. Results represent the mean ± SEM of 3 independent experiments. **0.02<P; ***0.01<P
5.2.5 Measurement of total nitrotyrosine residues in THP-1 monocytes following peroxynitrite exposure

As it is not possible to identify tissue factor antigen easily for a separate assay of tissue factor nitration, the ELISA was employed to measure nitrotyrosine residues in THP-1 monocytes following exposure to peroxynitrite. THP-1 monocytes growing under standard conditions (see section 2.5.1) were removed from the culture medium, washed with PBS and resuspended in PBS to 10⁷ cells/ml.

The cells were then exposed to peroxynitrite or decomposed peroxynitrite (0 - 100 μM), which had been diluted from stock solution into PBS. Higher concentrations of peroxynitrite could not be used in this experiment as above this concentration cell lysis occurred. Following addition of peroxynitrite and vortexing for 5s, 500 μl cells were removed within 10 s of the addition. This time point was classified as t = 0 h. The remainder of the cell suspension was incubated at 37°C for up to 2 h. The sample removed at t = 0 h was exposed to three bursts of ultrasound, over a 1 min period, while cooled with ice. The membrane and cytosolic fractions were separated by centrifugation (210g, 5 min). The membrane fraction was then washed three times with PBS and solubilised in 1% CHAPS buffer (Hjelmeland, 1980). Nitrotyrosine residues were quantified by competitive ELISA (section 2.9). In separate experiments it was shown that CHAPS buffer did not interfere with the ELISA.

The nitration of the cytosolic fraction of the monocytes is shown in Fig. 5.6.1. The only apparent increase in nitrotyrosine levels was obtained following exposure to 100 μM peroxynitrite and at all concentrations tested, decomposed peroxynitrite did not promote nitration. The lack of nitration with the lower concentrations of peroxynitrite tested, may be a consequence of the decomposition of peroxynitrite before it reached the cytosol.
Fig. 5.6.1 The nitration of THP-1 monocyte cytosolic proteins by treatment with peroxynitrite

THP-1 monocytes were resuspended in PBS and exposed to peroxynitrite and decomposed peroxynitrite (0-100 μM), both of which had been previously diluted from stock solution into PBS. A sample was removed immediately (t = 0 h) and whilst the remaining cells were placed at 37°C for up to 2 h. The cells were sonicated and the membrane component separated from the cytosol. The membrane was washed and resuspended in 1% CHAPS and nitrotyrosine content quantified by ELISA. Results represent mean ± SEM of 4 independent experiments. +Statistical significance is related to the increase in nitrotyrosine residues, compared to control sample, following exposure to peroxynitrite at specified time +P<0.01; ++P<0.001. * Statistical significance related to the decrease in nitrotyrosine residues over a period of time *P<0.02; **P<0.001.
Fig. 5.6.2 The nitration of THP-1 monocyte membrane proteins by treatment with peroxynitrite
THP-1 monocytes were resuspended in PBS and exposed to peroxynitrite and decomposed peroxynitrite (0-100 μM), both of which had been previously diluted from stock solution into PBS. A sample was removed immediately (t = 0 h) and whilst the remaining cells were placed at 37°C for up to 2 h. The cells were sonicated and the membrane component separated from the cytosol. The membrane was washed and resuspended in 1% CHAPS and nitrotyrosine content quantified by ELISA. Results represent mean ± SEM of 4 independent experiments.
In all the cytosolic fractions there was a decrease in nitrotyrosine levels over the 2 h incubation, with the most significant effect detected in the samples that had been nitrated with 100 μM peroxynitrite: -1 h following nitration there was a 38% reduction (P< 0.02), in nitrotyrosine residues detected whilst at 2 h following exposure to peroxynitrite, there was 40% reduction (P<0.001) in nitrotyrosine residues detected. However, a decrease in the nitrotyrosine content of the membrane fractions was not apparent. This may be the result of nitrated membrane proteins being more stable than nitrated cytosolic proteins or less accessible to enzymes that may remove them (discussed in section 5.3).

The nitration of the membrane fraction of the monocytes, following exposure to peroxynitrite, is shown in Fig. 5.6.2. The basal level of nitrotyrosine in the control membranes was at least 4 - fold greater than basal levels of cytosolic nitrotyrosine (0.8 μmol/mg protein, compared with 0.2 μmol/ mg protein). However, there was no obvious increase in membrane nitrotyrosine levels following exposure to peroxynitrite with any of the concentrations tested. The higher basal levels of nitrotyrosine detected in the membrane fraction and the apparent lack of nitration may indicate that all of the available tyrosine residues are already nitrated.

5.2.6 The influence of peroxynitrite on the ability of LPS to induce tissue factor expression in THP-1 monocytes

Lipopolysaccharide induces iNOS to generate nitric oxide which, in the presence of superoxide, promotes the formation of peroxynitrite. Furthermore, stimulation of leukocytes by LPS has been shown to generate peroxynitrite (Gagnon et al., 1998). The results presented in section 4.2.7 showed that peroxynitrite treatment of isolated LPS or LDL samples contaminated with LPS, abolished LPS activity, as determined by the Limulus amoebocyte lysate assay. This led to the question whether the exposure of LPS to peroxynitrite could influence the biological response exerted by LPS during periods of inflammation, that is, induction of tissue factor from cells of the vasculature.
Lipopolysaccharide (E. Coli 026:B6) was prepared as a 1 mg/ml stock solution in PBS as directed by the suppliers. LPS (1 mg/ml) was exposed to peroxynitrite (0-3 mM) and decomposed peroxynitrite (300 μM), which had previously been diluted in PBS. Native or peroxynitrite modified LPS was diluted further in PBS, to 10 μg/ml and added to cells that had been cultured as described previously (3.2.8), to induce tissue factor activity. The cells were incubated for 16 h, following which they were expressing maximal cell surface tissue factor activity, as determined in section 3.2.8.2. The cells were then washed once with PBS and resuspended to 10⁷/ml in PBS prior to tissue factor activity measurement by the one-stage prothrombin time assay. Values were compared to cells that had been incubated with PBS treated LPS (control) and expressed as the percentage inhibition of control LPS tissue factor expression.

Bacterial LPS that had been exposed to peroxynitrite prior to its addition to THP-1 monocytes evoked a lower cell surface tissue factor activity than the control LPS that had not received any treatment. At lower concentrations of peroxynitrite (30 and 300 μM) the inhibition of LPS activity did not reach statistical significance. Exposure of LPS to 3 mM peroxynitrite caused a 38% inhibition of tissue factor activity expressed when compared to cells incubated with un-treated LPS (Fig. 5.7). This value is significantly lower than the results discussed in Chapter 4 (section 4.2.7), when the effect of exposing LPS carried on LDL, to peroxynitrite was studied. In section 4.2.7 the exposure of LPS to 3 mM peroxynitrite resulted in almost complete inhibition of LPS activity. However, the concentration of LPS tested was only 20 ng/ ml, compared to the concentration of 1 mg/ml used in the present set of experiments. Thus, the difference in the degree of inhibition caused by peroxynitrite is likely to dependent upon the relative concentrations of LPS and peroxynitrite used.
Fig. 5.7 The effect of pre-treating LPS (E. coli 026:B6) with peroxynitrite on its ability to induce tissue factor activity in THP-1 monocytes

THP-1 monocytes were incubated with LPS (10 µg/ml) for 16 h that had been pre-treated with the specified concentration of peroxynitrite and decomposed peroxynitrite. The results represent the mean ± SEM of 3 independent experiments with values calculated from a comparison of treated LPS to untreated LPS. *** P<0.01
5.3 Discussion

The aim of this section of work was to determine the influence of peroxynitrite on the biological activity of tissue factor, in view of the fact that there are 11 tyrosine residues within the active domain of this protein, some of which have been shown to play an important role in its procoagulant activity e.g. tyrosine 71 (Ruf et al., 1994). Furthermore, nitration of key tyrosine residues within other proteins have been shown to influence their biological activity. Nitration of tyrosine residues within proteins have been associated with the inactivation of manganese superoxide dismutase (Ischiropoulos et al., 1992a) and prostacyclin synthase (Zou et al., 1997), the inactivation of the lipid aggregatory activity of surfactant protein (Haddad et al., 1993) and the inhibition of protein tyrosine kinase activity (Gow et al., 1996).

The nitration of proteins has been associated extensively with pathological conditions related to inflammation such as atherosclerosis and arthritis (Beckman et al., 1993a; Buttery et al., 1996; Kaur & Halliwell, 1994). However, the fact that the nitrated proteins are present in normal plasma and other biological fluids (Khan et al., 1997; Khan et al., 1998; Ohshima et al., 1990), strongly suggests that low levels of peroxynitrite may be released from cells in vivo or that nitration is arising from enzymic processes, including from prostaglandin H synthase (Gunther et al., 1997) or myeloperoxidase (Eiserich et al., 1998).

A study by van der Vliet et al., (1994) noted that treatment of tyrosine with peroxynitrite was accompanied by an increase in absorption at 425nm, indicative of the formation of 3-nitrotyrosine (Radi et al., 1991). The results in the present study are in agreement with this as the spectrophotometric analysis of L-tyrosine exposed to peroxynitrite revealed a peak in the region of 430nm, although this was not detected with decomposed peroxynitrite treatment (Fig. 5.1.1). Spectrophotometric analysis of the nitration of phenylalanine, revealed an increased absorption at 405nm, which was not present with decomposed peroxynitrite treatment, an effect also noted by Van der Vliet at al., (1994). The modification of tryptophan, not detectable...
with spectrophotometric analysis, was suggested by the spectrofluorometric data, at least at higher concentrations of peroxynitrite. These results are in agreement with the work of Kato et al., (1997), who found the intensity of fluorescence derived from tryptophan residues decreased with increasing peroxynitrite concentration. The authors also reported that decomposed peroxynitrite had no effect on tryptophan fluorescence, an effect also observed in the present study. In the current study, the concentrations of peroxynitrite used (0.03 - 3 mM) may appear to be high. However, the short half-life of this species at physiological pH results in much lower effective concentrations. For example, it has been calculated that 100 mM peroxynitrite is only equivalent to a steady state concentration of 2.8 mM for 1 min (Radi et al, 1991a).

The study of the influence of peroxynitrite on tissue factor showed the nitration of tyrosine residues was associated with the reduction in the procoagulant activity of tissue factor. At lower concentrations of peroxynitrite there was some inhibition of procoagulant activity with only very modest increases to the nitration of tyrosine. This may be because only certain key tyrosine residues are important to the procoagulant activity e.g. tyrosine 71 (Ruf et al., 1994) and that the additional nitrations are superfluous. The observed effects on tissue factor may not be exclusively related to nitration of tyrosine. There are no free thiols in the extracellular domain of tissue factor but there are 4 tryptophan residues in the extracellular domain, which may contribute to the interactions of tissue factor with Factor VII as part of the WKS motif - repeated 3 times in tissue factor (Ruf et al., 1994). Nitration of tryptophan residues, through exposure to peroxynitrite (Alvarez et al., 1996) may lead to the partial loss of the procoagulant activity of tissue factor. There are 10 phenylalanine residues within the extracellular domain of tissue factor, phenylalanine 19 & 140 seen to be required for optimal tissue factor activity. Free nitrophenylalanine competes with nitrated bovine serum albumin to a lesser extent than free 3-nitrotyrosine in the ELISA which in turn is less than protein bound nitrotyrosine (Khan et al., 1998). A contribution from nitrated phenylalanine residues in the tissue factor to coagulation activity cannot be completely excluded.
and deserves further investigation. Peroxynitrite oxidises lipids and sugars (Pryor & Squadrito, 1995) but the lipids associated with recombinant tissue factor were not oxidised when recombinant tissue factor was exposed briefly to peroxynitrite. As there are no carbohydrate residues in recombinant tissue factor, this was not relevant to the impairment of procoagulant activity, but it may influence the glycosylated cellular tissue factor.

The modification of tissue factor by peroxynitrite can bring about inhibition of its activity not only of the pure protein, but also when located in the cell membrane, as demonstrated by the experiments studying the influence of peroxynitrite on the procoagulant activity on the surface of monocytes. Under physiological conditions, an induction of tissue factor activity on the surface of vascular cells may arise during the inflammatory response to Gram-negative endotoxin (Ternisien et al., 1993; Colucci et al., 1983). A study by Polack et al., (1997) indicated that inhibition of NADPH oxidase or nitric oxide synthase, the major sources of active oxygen species, block the induction of tissue factor by LPS in blood monocytes, suggesting superoxide and nitric oxide participate in the cell surface induction of tissue factor. In addition, Gagnon et al., (1998) has shown leukocytes produce large amounts of peroxynitrite in response to LPS. Thus, the presence of both tissue factor and peroxynitrite within the vicinity of the cell is highly probable. The results presented in this study indicated that cell surface tissue factor was inhibited by peroxynitrite in a concentration-dependent manner. Gerlach et al., (1998) reported that the peroxynitrite generating agent SIN-1 also reduced tissue factor expression, and activity in human monocytes incubated with LPS, in a concentration - dependent manner, although nitric oxide alone had no effect. However, unlike the present study, the authors co-incubated SIN-1 and LPS with the monocytes, thus the effect seen could have been the result of peroxynitrite acting on existing tissue factor and/or on LPS itself to reduce its ability to induce tissue factor. As an extension of the results presented in section 4.2.7, which showed that LPS activity was destroyed by peroxynitrite, as measured by the Limulus amoebocyte lysate assay, the present
study (section 5.2.6) demonstrated that LPS exposed to peroxynitrite has a reduced capacity to induce monocytic tissue factor activity.

Increased nitration of cellular proteins may occur during normal physiological processes e.g. the activation of blood platelets by collagen or thrombin (Bruckdorfer et al., 1997). Indeed, low levels of nitration were detected in this study in the membranes and cytosol of monocytes, which were not exposed to exogenous peroxynitrite: an effect also observed in resting platelets (Bruckdorfer et al., 1997). However, a physiological role for nitration has yet to be established. The decrease in the amount of cytosolic nitrated protein, seen in this study with time parallels an observation by Bruckdorfer et al. (1997) on work completed on platelets, where the degree of nitration decreased to baseline 30 - 60 min following nitration with peroxynitrite. These results indicate that there may be a mechanism present that converts the nitro group to a product not recognised by the ELISA antibody. Gow et al., (1996) reported human plasma removed protein nitrotyrosine epitope in a concentration -, time -, and temperature - dependent manner, a result paralleled by Kamisaki et al. (1998) who demonstrated the loss of nitrotyrosine epitope in nitrated BSA was time and protein concentration-dependent. Greenacre et al., (1999) observed the loss of nitrated proteins in rat skin treated with peroxynitrite was biphasic, with nitrated proteins remaining in the skin for at least 24 h. However, the duration of the presence of nitrated proteins may be dependent upon the proximity to denitrating agents. Whiteman & Halliwell (1999) reported that hypochlorous acid, formed by myeloperoxidase at sites of inflammation caused a significant loss of 3 - nitrotyrosine. Furthermore, the denitration of nitrotyrosine has been demonstrated to be increased by treatment with LPS suggesting that the effect is inducible (Kamisaki et al., 1998). This may be an effect of nitrotyrosine denitrase that reverses protein nitration, and decreases the effect of peroxynitrite in vivo. There is evidence in the literature of mammalian de-nitrating enzymes including the putative protein nitratases (Kuo et al., 1999) and the nitroreductases, which act to reduce the nitro-group (e.g. of some environmental pollutants) to an amine, which can be further deaminated (Belisario et al., 1996). However, the subject of denitration of
peroxynitrite treated protein remains controversial, as it has not been investigated in detail for protein bound nitrotyrosine.

The results presented in this chapter indicate a positive role for peroxynitrite in inflammatory conditions such as atherosclerosis, by abolishing the ability of LPS to induce the inflammatory response, with regards to induction of tissue factor. Furthermore, the direct action of peroxynitrite on tissue factor, resulting in a reduction of procoagulant activity, appears to be primarily mediated by nitration of tyrosine residues. The question of whether the denitration of tissue factor tyrosine, would restore the procoagulant activity, is one that deserves further investigation.
CHAPTER 6

FINAL CONCLUSIONS
The initiation of the earliest atherosclerotic lesion, the fatty streak, is the migration of blood borne monocytes into the subendothelial space and their differentiation into macrophages (Ross, 1986). In the subendothelial space, LDL is modified through an, as yet, unidentified mechanism. In its oxidatively modified form, LDL is recognised by the scavenger receptors on the macrophage, which results in a rapid internalisation of the LDL, and the development of the foam cell. The removal of oxidised LDL from the intimal space by the macrophage may act to afford some protection to other arterial cells, including endothelial and smooth muscle cells, upon which oxidatively modified LDL has been shown to exert a cytotoxic effect (Cathcart et al., 1985). However, the internalisation of oxidised LDL by macrophages, also has a negative effect, in terms of haemostatic balance, through the induction of tissue factor from these cells (Lewis et al., 1995). Tissue factor, has been elucidated as the source of the thrombogenicity associated with atherosclerotic lesions (Wilcox et al., 1989; Toschi et al., 1997).

Inflammatory conditions, including atherosclerosis, have been associated with an increase in the levels of nitric oxide and superoxide (Mugge et al, 1994), which can lead to the formation of the powerful oxidising species, peroxynitrite. Peroxynitrite has been demonstrated to oxidise LDL (Hogg et al, 1993b), which is capable of being bound and internalised by macrophage scavenger receptors (Graham et al., 1993), and may be the initiating factor for oxidised LDL in atherosclerotic lesions (Ylä-Herttuala et al., 1989). In addition to its lipid oxidising capability, peroxynitrite nitrates protein tyrosine residues, to generate 3 - nitrotyrosine (Beckman et al., 1994b), a reaction that can influence the biological activity of proteins (Yamakura et al., 1998; MacMillan-Crow & Thompson, 1999). Nitrotyrosine has been detected within atherosclerotic lesions (Beckman et al., 1994a: Buttery et al., 1996), although not in normal vessels. Furthermore, LDL oxidised by peroxynitrite (Leeuwenburgh et al., 1997b) has been shown to have elevated levels of nitrotyrosine when compared
to plasma LDL, thus implicating peroxynitrite and tyrosine nitration in the pathogenesis of atherosclerosis.

The source of peroxynitrite within atherosclerotic lesions may arise from constituent cells, as a number of cell types have been demonstrated to promote peroxynitrite formation, including rat macrophages (Ischiropoulos et al., 1992b; Zhu, et al., 1992) and bovine aortic endothelial cells (Kooy & Royall, 1994). Until recently, the question of whether peroxynitrite is released from human cells has been a matter of debate, as the detection of nitric oxide from human cells proved to be difficult. However, evidence does exist for the production of nitric oxide by human cells. One of the earliest indications came from a report that human mononuclear cells inhibited platelet aggregation, by releasing a NO-like factor (Salvemini et al., 1989). In 1994, Carreras et al., reported that the kinetics of nitric oxide and superoxide release from human neutrophils during the respiratory burst were favourable for peroxynitrite formation. More recently, Sharara et al., (1997) demonstrated interferon-α activated nitric oxide synthase (NOS) and promoted the release of nitric oxide from human monocytes, whilst a study by Gagnon et al., (1998) detected the production of peroxynitrite in human neutrophils, monocytes and lymphocytes challenged with LPS.

An alternative source for the production of peroxynitrite within the atherosclerotic lesion may arise from the presence of oxidised LDL. Lysophosphatidylcholine (lyso PC) is a prominent component of oxidised LDL (Steinberg et al., 1989). The oxidative modification of LDL is accompanied by approximately 40% of the LDL phosphatidylcholine (PC) content being converted to lyso PC, by phospholipase A₂ (PLA₂) (Steinbrecher et al., 1984). There is evidence for the presence of lyso PC (Portman & Alexander, 1969) and PLA₂ (Hurt-Camejo et al., 1997) in atherosclerotic lesions. Thus, the action of PLA₂ on extracellular deposits of LDL in the arterial wall may be the source of local release of pro-inflammatory factors such as lyso PC. Liu-Wu et al., (1998) reported that lyso PC induced the production IL 1–β in human monocytes. Indeed, IL 1–β has been detected within atherosclerotic lesions (Moyer
et al., 1991). This may be indicative of a potential source of plaque derived peroxynitrite, as Zou et al., (1998), detected peroxynitrite formation following the incubation of rat mesangial cells incubated with IL 1–β.

Thus, LDL within the structure of the plaque, upon oxidation generates Lyso PC, which in turn, can induce the production of IL 1–β. Through the activation of phospholipase A₂ (Gilman et al., 1998), IL 1–β may stimulate the synthesis of phospholipase A₂ activating protein (Bomalaski et al., 1992) leading to a positive feedback mechanism between lyso PC and IL 1–β, which could sustain the inflammatory process leading to peroxynitrite formation. The presence of peroxynitrite could also act to oxidise plaque LDL, promoting a vicious circle between lyso PC formation and the generation of peroxynitrite.

The present thesis has focused on the oxidative modification of LDL, using a variety of oxidising agents, including peroxynitrite, to determine the effect on the induction of tissue factor expressed in THP-1 monocyte-derived macrophages. Furthermore, the influence of native and oxidatively modified LDL on existing tissue factor activity expressed on the surface of monocyte-derived macrophages was studied, a scenario that may arise within the structure of the plaque if tissue factor activity is expressed on the surface of constituent cells following induction by cytokines or LPS.

6.1 The influence of LDL and modified LDL on tissue factor activity and expression

The effect of native and LDL oxidatively modified by a variety of agents, on tissue factor activity was studied using both recombinant tissue factor and cell surface tissue factor (Chapter 4). It was shown that in its native form, LDL acts as an anticoagulant (Figs. 4.3.1 & 4.3.2), inhibiting both isolated and surface tissue factor on THP-1 monocytes, respectively. This has been demonstrated to be the result of an interaction between positively charged residues within the receptor binding
domain of LDL and negatively charged residues within tissue factor, which mask amino acids that are essential for the binding and activation of factor VII. Upon oxidation of the LDL, basic amino acid residues are masked by lipid oxidation products, the inhibition is reversed (Ettelaie et al., 1995), and tissue factor activity is augmented. Thus, the oxidative state of LDL acts as an important determinant of the biological activity of the surface tissue factor activity in cells of the vasculature.

The effect of native and oxidatively modified LDL on the induction of cell surface tissue factor activity and antigen expression was studied using THP-1 macrophages, using the one-stage prothrombin time assay and flow cytometry respectively. Native LDL and LDL oxidised by air (minimally modified) both acted to reduce the procoagulant activity of tissue factor on the surface of THP-1 monocyte-derived macrophages. Conversely, the LDL that had been oxidised to a greater extent, all acted to increase the procoagulant activity on the surface of these cells (Fig. 4.4.2). However, this was not accompanied by an increase in cell surface tissue factor antigen levels, indicating that induction of tissue factor above that of basal levels had not occurred, and suggested that the effect detected with regards to procoagulant activity may have been caused by the direct influence of the lipoproteins on basal tissue factor.

Previous literature on the ability of oxidatively modified lipoproteins to induce tissue factor in monocytes monocyte-derived macrophages has been divided. A study by Brand et al., (1990) found that oxidatively modified LDL induced tissue factor activity in THP-1 monocytes, however, when the monocytes became adherent (Brand et al., 1994), oxidised LDL did not result in induction. However, in agreement with the results presented in this thesis, oxidised LDL acted to enhance tissue factor expression induced by LPS. Conversely, a study by van den Eijnden et al., (1999) reported that incubation of monocyte-derived macrophages with oxidised LDL did not lead to expression of tissue factor or change in procoagulant activity, an effect also noted by Penn et al. (1999), studying smooth muscle cells. The authors hypothesised that an increased tissue factor activity on the surface of such cells was
the result of a 2-step pathway, with lipoproteins regulating the synthesis of a latent form of tissue factor, which is then activated by oxidants.

The conflicting results presented in the literature may be explained, in part, by the presence of LPS as a contaminant within the preparations of LDL used. Induction of tissue factor expression following exposure to bacterial LPS has been demonstrated in a number of cell types in vitro (Colucci et al., 1983; Brand et al., 1991). The induction of tissue factor in vivo by LPS has been associated with aberrant blood clotting that accompanies inflammatory episodes, and was shown to be reduced by tissue factor pathway inhibitor (Creasey et al., 1993). In the present study, the contribution of LPS within samples was eliminated as all LDL preparations were routinely tested for LPS.

It may be that for oxidatively modified LDL to induce tissue factor activity in such cells that the duration of incubation needs to be lengthened as in vivo, within an atherosclerotic plaque, cells are likely to have continual exposure to oxidatively modified LDL as opposed to limited exposure, which the cells in the present study received. It would be of interest to continue the experiments undertaken in the present study to extend the duration of the incubation of THP-1 macrophages with the oxidatively modified LDL to determine whether it is capable of inducing tissue factor antigen over a longer period of time. Furthermore, the replacement of the cell line with peripheral blood mononuclear cells would provide a more physiological model in the study of lipoproteins in the induction of tissue factor expression and provide less basal tissue factor activity.

6.2 The influence of peroxynitrite on tissue factor activity

In view of data in earlier literature, which indicated the presence of peroxynitrite within atherosclerotic lesions, and the effect of exposing proteins to peroxynitrite, in terms of influencing the biological activity, the effect of exposing tissue factor to peroxynitrite was established.
The influence of peroxynitrite on the biological activity of tissue factor was studied in Chapter 5. The results presented in this study demonstrated that peroxynitrite effectively inhibited the procoagulant activity of recombinant (Fig. 5.4) and cell surface (Fig. 5.5) tissue factor activity. It is believed that nitration of tyrosine residues within the extracellular domain of tissue factor, is responsible for the effect as an increase of nitrotyrosine detected in samples incubated with peroxynitrite, accompanied the inhibition of tissue factor activity (Fig. 5.3). However, the influence of peroxynitrite on the nitration of phenylalanine (Fig. 5.1.4) and nitration or oxidation of tryptophan (Fig. 5.1.6) must also be taken into consideration.

A study by Schaeffer et al., (1997) reported that activated factor X induces release of nitric oxide and promoted relaxation of pre-contracted aortic rings. Papetropoulos et al., (1998), identified activated factor X stimulated endothelial cell nitric oxide release. The ability of factor Xa to induce the release of nitric oxide may counterbalance a local activation of coagulation by inhibiting platelet activation and promoting vasodilation (Moncada et al., 1991; Sessa, 1994). A study by Corseaux et al., (1998) reported that dietary L-arginine inhibited enhanced monocytic tissue factor following balloon angioplasty, in hypercholesterolaemic rabbits, which was previously shown to be accompanied by elevated levels of superoxide (Mugge et al., 1994). It is believed the formation of peroxynitrite is responsible for the inhibition of tissue factor activity in monocytes, incubated with LPS and nitric oxide (Gerlach et al., 1998). Thus, it is not unreasonable to hypothesise that in disease states such as atherosclerosis, with the associated high levels of superoxide, that factor Xa generated nitric oxide may promote the formation of peroxynitrite. This could then target tissue factor and inhibit procoagulant activity. This may indicate a potential feedback mechanism within the coagulation cascade that would limit the extent of coagulation, without the influence of physiological coagulation inhibitors.

It would be of interest to extend the experiments undertaken in the present study to look at the individual tyrosine residues within the extracellular domain of tissue factor and whether the nitration of key tyrosine residues at a particular site causes
the inhibition of tissue factor activity. Ruf et al. (1994) pinpointed the essential amino acids in tissue factor involved in the recognition and binding to factor VII. The results presented on the inhibition of cellular tissue factor activity (Fig. 5.5), following exposure to peroxynitrite and also the subsequent denitration of cellular nitrotyrosine (Fig. 5.6.1), over a 2 h period, invite further study to establish whether the inhibition of surface tissue factor activity could be reversed over a period of time.

6.3 Final comments

Overall the results presented in this thesis have indicated that the haemostatic balance of the vasculature, in the absence of injury or periods of inflammation, can be altered by the oxidative state of plasma LDL. In its native form, LDL limits coagulation through a direct interaction with tissue factor, inhibiting its procoagulant activity. This is reversed when the LDL becomes progressively more oxidised (Ettelaie et al., 1995). Although the exact mechanism for the oxidation of LDL in vivo has yet to be elucidated, the thesis has provided evidence that LDL oxidised by peroxynitrite, which is proven to exist in atherosclerotic lesions (Leeuwenburgh et al., 1997b), behaves in exactly the same manner, in terms of enhancing the procoagulant activity of tissue factor, as the previously tested air and copper oxidised LDL.

Thus, the presence of peroxynitrite in vivo, and the answer to the question whether peroxynitrite is a good guy or villain, in terms of blood coagulation, may depend upon the pathogenesis of atherosclerosis. In the early stages of this disease, formation of peroxynitrite, during periods of inflammation, may act in an antithrombotic manner, limiting the coagulation response through a direct action (nitration) on the extracellular domain of tissue factor, to diminish procoagulant activity. Furthermore, the influence of peroxynitrite on bacterial LPS would prevent further stimulation of tissue factor activity in vascular cells, thus reducing the inflammatory response further. However, with the progression of this disease and the accompanying deposition of LDL in the arterial wall, the beneficial effects of
peroxynitrite may be lost and a detrimental effect, in terms of coagulation, come into
effect, with the oxidation of LDL promoting the induction of tissue factor activity
from vascular cells and the development of a hypercoagulable state (Fig. 6.1).
Fig. 6.1 The positive and negative roles of peroxynitrite in atherosclerosis in terms of coagulation

-ve represents an antithrombotic role afforded by peroxynitrite through limitation of the coagulation response by its direct action on LPS and tissue factor protein; + ve represents a prothrombotic role of peroxynitrite, promoting coagulation through the oxidation of LDL, the induction of tissue factor activity and the enhancement of existing cell surface tissue factor activity.
APPENDICES
Appendix A: Abbreviations

ACAT - acyl co A cholesterol transferase
ACD - acid citrate dextrose
AFS - ammonium ferrous sulphate
Apo B - apolipoprotein B-100
BHT - butylated hydroxytoluene
BSA - bovine serum albumin
DMSO - dimethyl sulphoxide
DTPA - diethylenetriaminepenta acetic acid
EDTA - ethylenediaminetetra acetic acid
FBS - foetal bovine serum
FITC - fluorescein isothiocyanate
HDL - high density lipoprotein
HMG Co A - 3-hydroxy-3methyl-glutaryl Coenzyme A
4-HNE - 4 hydroxynonenal
HPBM - human peripheral blood monocytes
IDL - intermediate density lipoprotein
LAL - Limulus amebocyte lysate
LBP - LPS binding protein
LCAT - lecithin cholesterol acyl transferase
LDL - low density lipoprotein
LPL - lipoprotein lipase
LPS - lipopolysaccharide
MCP-1 - monocyte chemotractant protein
MDA - malonaldehyde
mm LDL - minimally modified LDL
n LDL - native LDL
ONO0 - peroxynitrite
ONOOLDL - peroxynitrite oxidised LDL
ox LDL - copper oxidised LDL
PBS - phosphate buffered saline
PMA - phorbol-12-myristate-13 acetate
PUFA - polyunsaturated fatty acids
REM - relative electrophoretic mobility
rTF - recombinant tissue factor
SIN-1 - 3-morpholinosydnonimine-hydrochloride
sin LDL - SIN-1 oxidised LDL
SR-A - scavenger receptor
TBARS - thiobarbituric acid reactive substances
TEP - 1,1,3,3, -tetraethoxypropane
TF - tissue factor
TFPI - tissue factor pathway inhibitor
Tris- Tris (hydroxymethyl) methylamine
VLDL - very low density lipoprotein
XO - xylene orange
Appendix B: Source of Materials

American Diagnostics Inc., Greenwich, USA.
FITC conjugated anti tissue factor antibody, Anti human TFPI antibody

Amersham International, Amersham, Bucks, U.K.
Anti rabbit IgG Polyclonal antibody

BDH Chemicals LTD., Poole, Dorset, U.K.
Chloroform, Ethanol, Ethylenetriamine tetraacetic acid (EDTA), Hydrochloric acid, Isopropanol, Methanol, Nitric acid, Orthophosphoric acid, Sodium hydroxide, Sulphuric acid

Biowhittaker, Wokingham, Berkshire. U.K.
QCL 1000 100 chromogenic assay

Boehringer Mannheim, Lewes, East Sussex, U.K.
Nutridoma HU, Fatty acid free BSA

Corning Costar, High Wycombe, Bucks, U.K.
Cell culture plasticware

DADE Innovin, Sysmex, UK Ltd., Milton Keynes, U.K.
Recombinant tissue factor

Dako, UK
Biotinylated horseradish peroxidase

European Collection of Animal Cell Culture (ECACC), Salisbury, Wilts, U.K.
THP-1 monocytes
Gibco, Paisley, U.K.
L-glutamine, Mercaptoethanol, PBS, Penicillin/Streptomycin, RPMI 1640, Versene

Helena Biosciences, Sunderland, UK
Cascade -M- Coagulomter

Immune systems Ltd., Paignton, Devon, U.K.
Fast Read counting chambers

Immuno Ltd., Sevenoaks, U.K.
Anti human apolipoprotein B-100 antibody

Sartorius, Epsom, Surrey, U.K.
0.22 μm filters

Sebia, Issy-les-Moulineaux, France.
Agarose gels

Sigma Chemical Company, Poole, Dorset, U.K.
Ammonium Ferrous Sulphate, Butylated Hydroxytoluene (BHT), Calcium chloride, Citric acid, Copper sulphate, Coomassie blue, Diethylenetriamine pentaacetic acid (DTPA), E Toxa clean, Glucose, Hydrogen peroxide, lipopolysaccharide, Manganese oxide, Orthophenylenediamine, Ovalbumin, Perborate, Phorbol-12-myristate-13 acetate (PMA), Protein standard (BSA), Reconstituted human plasma, SIN-1, Sodium bromide, Sodium chloride, Sodium nitrite, Tetraethoxypropane, Thiobarbituric acid, Tris, Trisodium citrate, Trypan Blue, Tween, Xylenol Orange

TCS Biologicals Ltd., Botolph Claydon, Buckingham, U.K.
Polyclonal anti-nitrotyrosine IgG (rabbit)
Appendix C: Reagent preparation

All reagents prepared with ultra pure water

**LDL isolation**

**Acid citrate dextrose**
113.8 mM glucose (20.5 g/l)
29.9 mM trisodium citrate (8.79 g/l)
72.6 mM sodium chloride (4.24 g/l)
2.8 mM citric acid (0.59 g/l)
48 μM DTPA (0.019 g/l)
pH to 6.4

**Normal saline**
154 mM sodium chloride (9.0 g/l)
Add DTPA to 2 μM

**Density solutions**
Density solution 1.006 g/l
0.195 M sodium chloride (22.79 g/l)
0.3 mM EDTA (0.11 g/l)
5 mM sodium hydroxide (0.2 g/l)
Add DTPA to 2 μM

To the 1.00 g/l density solution add:
- 74 g sodium bromide to 1 litre - 1.063 g/l
- 195 g sodium bromide to 1 litre - 1.151 g/l
pH to 7.4

**Tris/NaCl buffer**
12.5 mM Tris (hydroxymethyl) methylamine (1.15 g/l) (TRIS)
140 mM sodium chloride (8.18 g/l)
Add DTPA to 2 μM
pH to 7.4
 Vacuum degas.
Protein determination

Bradford reagent
100 mg coomassie blue (G250) dissolved in 93.4 ml absolute ethanol and 200 ml isopropanol (Stir for 15 min).
Add 100 ml orthophosphoric acid (Stir for 60 min).
Make up to 2 litres with water.

Peroxide determination

FOX reagent
1 ml ammonium ferrous sulphate (10 mM stock: 3.92 mg/ml)
2.5 ml 1M sulphuric acid
5.5 ml H$_2$O
1 ml xylene orange (10 mM stock: 7.604 mg/ml)
90 ml methanol (90%), containing 0.88 mg/ml BHT

N.B. ADD IN THIS ORDER

TBARS determination

Thiobarbituric acid

0.67% 2-thiobarbituric acid dissolved in 50 mM H$_2$SO$_4$

Cell culture

Medium A
RPMI 1640, 10% FBS, 2 mM L-glutamine, 20 $\mu$M mercaptoethanol, 100 units/ 100 $\mu$g/ml penicillin/streptomycin

Medium B
RPMI 1640, 1% Nutridoma HU, 2 mM L-glutamine, 20 $\mu$M mercaptoethanol, 100 units/ 100 $\mu$g/ml penicillin/streptomycin
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Modification of tissue factor by peroxynitrite influences its procoagulant activity

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Abstract Peroxynitrite, a reactive oxidising species resulting from a reaction between nitric oxide and the superoxide anion, modifies proteins by nitration of certain amino acids such as tyrosine. Tissue factor (TF), a transmembrane protein, is expressed on cells under inflammatory conditions and initiates the coagulation cascade. The extracellular domain of TF is rich in tyrosine. Exposure of recombinant TF and cellular TF to peroxynitrite was associated with a reduction in procoagulant activity. This was accompanied by an elevated level of nitrotyrosine residues. Peroxynitrite may have a protective role by attenuation of the thrombogenic properties of TF.

Key words: Atherosclerosis; Peroxynitrite; Tissue factor; Procoagulant activity; Nitration

1. Introduction

Nitric oxide reacts with the superoxide anion to form the potent oxidising species peroxynitrite [1]. Peroxynitrite modifies proteins by nitration of specific amino acid residues, in particular the ortho positions of tyrosine residues to form nitrotyrosine [2]. Phenylalanine may also be nitrated and tryptophan to a limited extent whereas sulphhydryl groups are readily oxidised to sulphinoximes. These events may, in turn, influence the biological activity of proteins [3]. Nitrotyrosine has been detected in a number of human pathologies including Alzheimer’s disease [4] and rheumatoid arthritis [5] and thus could be a useful marker for the presence of peroxynitrite in vivo. Nitrotyrosine residues have also been detected in atherosclerotic plaques, but not in normal blood vessels, suggesting peroxynitrite plays a role in atherosclerosis [6]. Peroxynitrite oxidises low density lipoprotein, making it recognisable to the macrophage scavenger receptor leading to foam cell formation [7]. There is evidence that NO and peroxynitrite are formed by bovine aortic endothelial cells [8], by rat macrophages [9] and more recently by human macrophages [10]. Stimulated human neutrophils are reported to release NO and O$_2^-$ at rates that favour the formation of peroxynitrite [11] and may also be a source of this oxidant in vivo. Protein bound nitrotyrosine has been detected using an ELISA technique in normal human plasma, in the range of 120 nM, at least half of which is bound to albumin [12].

Tissue factor (TF), a transmembrane glycoprotein (47 kDa) which initiates the blood coagulation by acting as a receptor for coagulation Factor VII, may have a separate role in angiogenesis and neointima formation [13,14]. TF is expressed on monocytes following exposure to bacterial lipopolysaccharide [14,15], and from endothelial cells and macrophages exposed to oxidised LDL [16,17]. It has an extracellular (active) domain of 219 amino acids, which initiates the coagulation cascade, a short membrane spanning domain and an intracellular domain of 21 amino acids [18,19]. The extracellular domain of TF has 12 tyrosine residues, eight of which are exposed and thus susceptible to attack by peroxynitrite. The importance of specific tyrosine residues in this domain of TF and the coagulation response has been reported as well as phenylalanine and possibly tryptophan [19].

In this study, peroxynitrite was shown to attenuate the procoagulant activity of TF in parallel with the increased nitration of its tyrosine residues measured by ELISA.

2. Materials and methods

Materials and cells were obtained from the following sources: recombinant TF (rTF) (Baxter Diagnostics Inc., Deerfield, Illinois, USA), polyclonal anti-nitrotyrosine IgG (TCS Biologicals Ltd, Botolph Claydon, Bucks, UK), THP-1 monocytes (European Collection of Animal Cell Culture, Salisbury, Wilts, UK), cell culture reagents, phosphate buffered saline (PBS) and Versene (Gibco, Paisley, Scotland), phorbol-12-myristate-13 acetate (PMA) (Sigma Chemical Co., Poole, Dorset, UK), Nutridoma HU (Boehringer Mannheim UK, Lewes, Sussex, UK). All solutions were prepared with de-ionised HzO.

2.1. Preparation of peroxynitrite

Peroxynitrite was prepared by mixing NaNO$_2$, H$_2$O$_2$ and HNO$_3$ and passing the solution into NaOH: excess HzO$_2$ removed by filtering through a manganese oxide column, as described by others although traces may remain [20]. Authentic peroxynitrite was maintained at pH 10, but on dilution at pH 7.4 and addition to recombinant TF (rTF), large losses would have occurred in the amount of peroxynitrite delivered. Decomposed peroxynitrite was prepared by neutralisation at pH 7.4: the pH was then adjusted back to pH 10 by addition of NaOH and this was used as a control. The concentration of peroxynitrite in the stock solutions was determined by measuring its absorption at 302 nm using the ε of 1670 mol/1-cm$^{-1}$.

2.2. Exposure of rTF to peroxynitrite

rTF was diluted in H$_2$O to give a stock solution of 100 μg protein/ml and exposed to peroxynitrite or decomposed peroxynitrite at concentrations between 0-3 mM, diluted from a stock solution into phosphate buffered saline (PBS) and added within the space of 5 s by rapid vortexing. The controls were rTF treated with PBS pH 7.4.

2.3. Spectrophotometric analysis of tissue factor exposed to peroxynitrite

The UV and visible absorption spectra of rTF exposed to peroxynitrite and decomposed peroxynitrite, were scanned between 320-620 nm on a Beckman DU70 spectrophotometer, against a blank of HzO$_2$, following neutralisation to pH 7. This was compared to the changes in absorbance when peroxynitrite was added to free L-tryptophan and L-tyrosine.

The fluorescence spectra of L-tryptophan and rTF were also measured with emission wavelength of 340 nm [21] and scanning from 320-
410 on a Perkin Elmer MPF44B fluorimeter and repeated on rTF before and after exposure to peroxynitrite or its decomposed control. In all cases, the effects of hydrogen peroxide were determined on these spectra, in the event that traces remained in the peroxynitrite. Similar traces of the peroxide would also be found in the decomposed peroxynitrite.

2.4. Measurement of nitrotyrosine residues

Immuno-reactive nitrotyrosine residues in rTF following exposure to various concentrations of peroxynitrite were quantified by competitive ELISA assay as recently described [22]. A standard curve was constructed using nitrated bovine serum albumin (NO2-BSA) at a series of dilutions. Competition assays were performed by addition of appropriate samples of peroxynitrite treated rTF instead of NO2-BSA and inhibition of the antibody binding determined from the standard curve.

Measurement of nitrotyrosine was also made on the cytosolic and membrane proteins of human THP-1 monocytes following exposure to peroxynitrite (0-100 µM). The cells were sonicated by exposure to two intervals of ultrasound in a one-minute period, followed by centrifugation at 2000×g for 10 min to separate the cytosol and the total membrane fraction. The membrane fraction was washed 3× with PBS prior to solubilisation in 1% CHAPS buffer [23].

2.5. Measurement of procoagulant activity

The procoagulant activity of rTF which had been exposed to peroxynitrite and decomposed peroxynitrite was measured using the one-stage prothrombin time assay [24]. rTF samples were diluted (final concentration of 2 µg protein/ml) and the procoagulant activity quantified by reference to a rTF standard curve constructed by plotting log TF (units/ml) versus log t (clotting time in s). These data were compared to the control values (rTF exposed to PBS) and the degree of inhibition of procoagulant activity calculated from the equation:

\[
\% \text{inhibition} = \frac{\text{initial activity} - \text{residual activity}}{\text{initial activity}}
\]

All the assays were completed within 10 min of addition of peroxynitrite to the rTF.

2.6. Induction of tissue factor activity in THP-1 monocytes

THP-1 monocytes were cultured as described previously [25]. Cells were seeded 24 h prior to experiment in serum free media supplemented with Nutridoma HU (1% v/v), at 0.5×10⁶ cells/ml in 25 cm² flasks. Cells were stimulated with 10 nM PMA for 4 h and adherent cells removed by the addition of Versene. The cells were washed once in PBS and resuspended to 10⁷ cells/ml and were exposed to 0-100 µM peroxynitrite and decomposed peroxynitrite, which had been diluted from stock solution in PBS. The procoagulant activity was determined by the one-stage prothrombin time assay and compared to control cells (PBS addition only). The percentage inhibition was determined as described previously.

2.7. Measurement of lipid peroxides

rTF is dispersed in a small amount of lipid which is necessary for the biological activity of this protein. Lipid peroxidation was estimated by the FOX assay [26] within 10 min of addition of the peroxynitrite.

3. Results

3.1. Spectrophotometric measurements after nitration of rTF with peroxynitrite

The UV absorption spectrum of rTF shows two major peaks at 230 and 278 nm which are reminiscent of those of tryptophan, but may include peaks of tyrosine at neutral pH; these did not change significantly on addition of peroxynitrite up to 3 mM (not shown). In the visible region, rTF did not show strong absorbance (Fig. 1a), but on addition of 3 mM peroxynitrite a peak appeared at 350 nm with a smaller peak at 410–440 nm region (Fig. 1b), the latter corresponding to the visible absorption spectra of nitrotyrosine (Fig. 1c). Addition of 3 mM decomposed peroxynitrite to rTF (Fig. 1d) led to the appearance of the peak at 350 nm which may be attributable in part to a contaminant in peroxynitrite: there was no peak at 410–440 nm. Lower concentrations of peroxynitrite (30–300 µM) showed little change in absorption at any of these wavelengths.

The fluorescence spectra of rTF suggest that tryptophan was modified to a very limited extent by the addition of 300 µM peroxynitrite (Fig. 2) but was largely eliminated by the addition of 3 mM peroxynitrite. Decomposed peroxynitrite decreased fluorescence to some extent, but less than for active peroxynitrite. No change in fluorescence was noted in the presence of H2O2 (not shown) at concentrations which were the maximum assuming no steps were taken to remove it during peroxynitrite synthesis, 0–12 mM.

3.2. Elevated levels of nitrotyrosine residues in TF exposed to peroxynitrite

The spectrophotometric determinations were insufficiently sensitive for quantification of the extent of nitration, but gave an indication that modifications of tyrosine and tryptophan may occur with addition of peroxynitrite. The sensitive competitive ELISA procedure for nitrotyrosine was used,
was measured against the control where only buffer was added. The experiments.

Extent of inhibition or activation of the rTF following modification was measured using the one-stage prothrombin time assay. The results are expressed as the means ± S.E.M. of at least four independent experiments.

Therefore, as a convenient procedure for detection of protein modification by peroxynitrite at low concentrations. Only low levels of nitration of tyrosine in rTF were observed at 300 μM peroxynitrite, suggesting a high proportion of this nitrating species was lost during mixing (Fig. 3). Exposure of rTF to 3 mM peroxynitrite showed a large increase in nitrotyrosine. Decomposed peroxynitrite did not cause nitration tyrosine residues in rTF compared to controls when only buffer was used. There were no increases in the oxidation of the lipid associated with the rTF as detected by the assay for lipid peroxides 10 min after addition of the peroxynitrite (not shown).

Nitration of proteins was determined in THP-1 cells to which 100 μM peroxynitrite was added, but not with decomposed peroxynitrite. Low levels of nitration were detected in the membranes of these cells (100 nmol nitrotyrosine/mg protein) which was increase 200-fold by the addition of peroxynitrite. Levels of nitrotyrosine were below the limits of detection in the cytosolic proteins and were not increased to a significant extent. At lower concentrations of peroxynitrite (30 μM), small rises in nitration of the membrane fraction were detectable, but did not reach statistical significance.

Fig. 3. The nitration of the tyrosine residues of rTF by peroxynitrite. rTF (100 μg protein/ml) was incubated with peroxynitrite (0, 30, 300 and 3000 μM) or decomposed peroxynitrite (300 μM) for a period of 10 min after which the extent of tyrosine nitration was determined by an ELISA procedure using nitrated bovine serum albumin as the standards. The values are expressed as the means ± S.E.M. of at least four independent experiments.

3.3. Reduction in the procoagulant activity of rTF exposed to peroxynitrite

The exposure of rTF (100 μg protein/ml) to 0–3 mM peroxynitrite resulted in a concentration dependent inhibition of procoagulant activity compared to that of the control sample, almost complete inhibition attained at 3 mM peroxynitrite and significant inhibition at 30 μM and above (Fig. 4). Exposure to decomposed peroxynitrite resulted in a small enhancement in procoagulant activity (Fig. 4). This enhancement is not caused by nitrate or nitrite, or indeed a mixture of the two, when these were tested separately (not shown), nor was it an effect of H₂O₂ 0–12 mM (not shown).

3.4. Reduction in cell surface tissue factor activity following exposure to peroxynitrite

Monocytic THP-1 cells were pre-treated with 10 nM PMA for 4 h to induce an increased expression of the tissue factor activity on the cell surface. These cells exhibit some procoagulant activity even when unstimulated. This activity was absent if Factor VII deficient plasma was used in the coagulation assay, showing that the procoagulant activity was due to tissue factor (Fig. 5). Inhibition of PCA of cell surface TF was attained at concentrations as low as 10 μM peroxynitrite and an increase in inhibition accompanied higher concentrations of peroxynitrite. The addition of peroxynitrite at concentrations above 100 μM resulted in some cell lysis and therefore measurements at higher concentrations were not made.

4. Discussion

The nitration of proteins has been associated extensively with pathological conditions related to inflammation such as atherosclerosis and arthritis [5,6]. However, this may only be appropriate when there is an excess of nitration. Excessive formation of peroxynitrite may give rise to extensive modifications of proteins and lipids, and ultimately become toxic to the cells. Nitration of tyrosine residues may arise as the result of the release of peroxynitrite from normal cells at low concentrations, but other sources of nitration are possible, e.g. from prostaglandin H synthase [27] and myeloperoxidase [28]. Indeed, low levels of nitration were detected in this study in...
the membranes and cytosol of monocytes which were not exposed to exogenous peroxynitrite: this was also observed in resting platelets [29]. The fact that the nitrated proteins are present in normal plasma and other biological fluids [12], strongly suggests that low levels of peroxynitrite may be released from cells in vivo or that nitration arises from the enzymic processes mentioned above. Increased nitration of cellular proteins may occur during normal physiological process, e.g. the activation of blood platelets by collagen or thrombin [29]. A physiological role of nitration has not been established.

In the current study, the nitration of tyrosine residues was associated with the reduction in the procoagulant activity of TF. However, at lower concentrations of peroxynitrite there was some inhibition of procoagulant activity with only very modest increases to the nitration of tyrosine. This may be because only certain key tyrosine residues are important to the procoagulant activity, e.g. tyrosine 71 [19] and that the additional nitration are superfluous. Peroxynitrite has other actions on proteins, such as nitration or oxidation of tryptophan, phenylalanine and cysteine. The modification of tryptophan was suggested by the spectrophotometric data, at least at higher concentrations of peroxynitrite. The observed effects on tissue factor may not, therefore, be exclusively related to nitration of tyrosine. There are no free thiol in the extracellular domain of tissue factor [19], but tryptophan residues may contribute to the interactions of tissue factor with Factor VII as part of the WKS motif [19]: nitration of these residues may lead to the partial loss of the procoagulant activity of tissue factor. Phenylalanine is also important in the interactions of Factor VII with tissue factor but at present their nitration cannot be detected by the spectrophotometric assay or the ELISA. Free nitrophenylalanine competes with nitrated bovine serum albumin to a lesser extent than free 3-nitrotyrosine in the ELISA which in turn is weak related to protein bound nitrotyrosine [22]. A contribution from nitrated phenylalanine residues in tissue factor cannot be excluded and deserves further investigation.

Peroxynitrite also oxidises lipids and sugars [1], but the lipids associated with recombinant tissue factor were not oxidised when recombinant tissue factor was exposed briefly to peroxynitrite. As there are no carbohydrate residues in recombinant tissue factor, this was not relevant to the impairment of procoagulant activity, but it may influence the glycosylated cellular TF. None of the observed effects of peroxynitrite could be attributed to the presence of residual H₂O₂, probably because of the brief duration of the experiments.

It can be concluded that modification of TF by peroxynitrite can bring about inhibition of its activity not only of the pure protein, but also when located in the cell membrane. This modification may occur through actions on different amino acids, but the nitration of tyrosine appears to be representative of the changes.

Modification of tissue factor protein may affect a self-regulatory process in which unwanted surface procoagulant activity may be limited by the release of NO and formation of peroxynitrite: human monocytes are also capable of NO synthesis in substantial quantities [10]. This modification may not be a permanent in vivo as the nitration of tyrosine residues in platelet cytosolic proteins has been shown to decrease after the initial action of peroxynitrite [29]. Ultimately the effects of endogenous peroxynitrite from macrophages or other cell types on TF function will need to be demonstrated.

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References