THE EFFECTS OF ANTIOXIDANT VITAMINS ON THE EXPRESSION OF TISSUE FACTOR AND TISSUE FACTOR PATHWAY INHIBITOR GENES IN HUMAN MONOCYTIC AND ENDOTHELIAL CELL LINES

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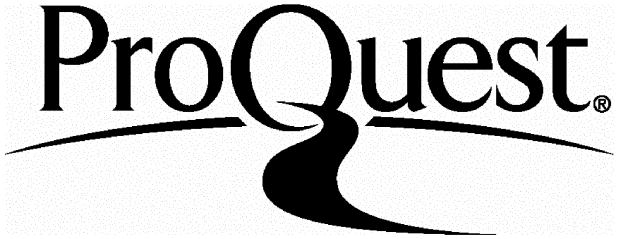

ABSTRACT

Oxidative stress has been implicated in the pathogenesis of various diseases including cancer, septic shock, stroke, hypertension and atherosclerosis. This manifests itself in oxidative modification of low-density lipoprotein (LDL) and the expression of specific proteins such as tissue factor (TF). It has been claimed that some of these effects can be reversed by dietary antioxidants and that at least some of these actions may be at gene level in vascular cells.

The aim of the project was to study the effects of minimally oxidised low-density lipoprotein (mmLDL) and the bacterial endotoxin – lipopolysaccharide (LPS) on the expression of genes for TF and TF pathway inhibitor (TFPI) in a human monocytic cell line (THP-1) and the cell line ECV 304 (once thought to be of endothelial origin). The effect of antioxidant vitamins on the expression of these genes was elucidated. The action of the antioxidant vitamins on TF protein activity was also investigated. The principal antioxidants studied were ascorbate, α-tocopherol and lutein. The mode of action of the antioxidants was also studied through the involvement of selected nuclear transcription factors (NFκB, CREB, ELK-1 and HIF-1).

In this study, Quantitative Competitive RT-PCR (QC RT-PCR) was successfully developed and applied for the determination of the changes in TF and TFPI expression. Some of the results were confirmed using Northern blot techniques. There was simultaneous expression of TF and TFPI mRNA in both THP-1 and ECV 304 cells after stimulation with a range of effectors. When an increase in TF expression was evident, a corresponding decrease in TFPI expression was noted in the case of LPS stimulation. The study confirmed that mmLDL and LPS are potent inducers of TF expression in THP-1 and ECV 304 cells. The treatment of cells with antioxidant vitamins significantly suppressed the induction of the TF gene and also induced the expression of TFPI mRNA. LPS and mmLDL also increased TF pro-coagulant activity on the surface of the cells and this activity was suppressed by antioxidants. Also, the antioxidants have effects on some of the nuclear transcription factors studied. The timing of the effects of antioxidants was given particular attention.

DEDICATION

TO GOD BE THE GLORY

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CHAPTER 1: MAIN INTRODUCTION

1.1 Atherosclerosis

Atherosclerosis is a progressive degenerative disease of the blood vessels characterised by a variable combination of changes such as the accumulation of lipids and fibrous elements in the intima of arteries followed by a progressive thickening of arterial intima and resulting in narrowing of the lumen (Fig.1.1). It is one of the most prevalent fatal diseases in Western societies and one major disease in which oxidative stress has been implicated.

Atherosclerosis was, at one time, thought to be an inevitable consequence of aging. For many years after, it was believed to be a response to injury to the endothelium caused by hypercholesterolemia or hypertension. Atherosclerosis now seems to be a chronic inflammatory condition that leads to an acute clinical event by the induction of plaque rupture, which in turn leads to thrombosis.

Diabetes, smoking, hypertension, hyperlipidemia and low antioxidant status are well established risk factors for the development of atherosclerosis but the relative abundance of the plasma lipoprotein appears to be of primary importance. Low density lipoprotein (LDL) diffuses passively through endothelial cell junctions and it is retained in the vessel wall. The retention in the vessel wall seems to involve interactions between the LDL constituent apolipoprotein B and matrix proteoglycans (Boren *et al*, 1998). In the vessel wall, LDLs undergo modification such as oxidation, lipolysis, aggregation and proteolysis (Lusis, 2000). Although the mechanism is not yet fully known, the most important modification that contributes to the atherosclerotic plaque formation is the oxidative modification of lipids in LDLs that are trapped in the extracellular matrix of the subendothelial space.

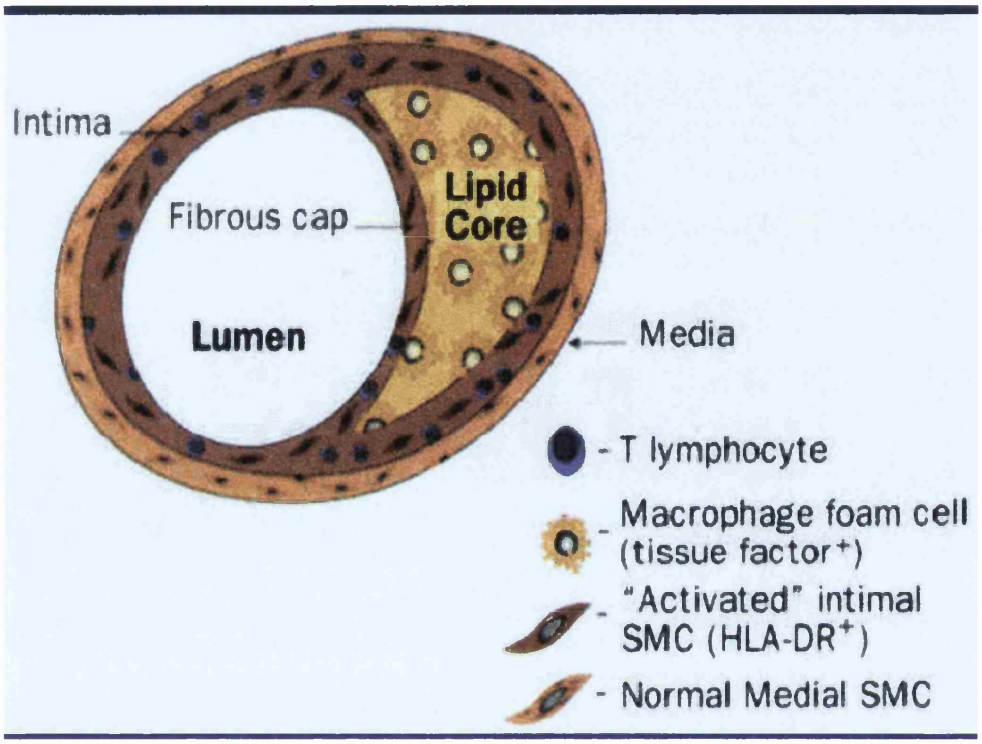


Figure 1.1 The development of atherosclerosis. An atherosclerosis plaque characterized by the thickening of arterial intima and narrowing of the lumen. Also showing the different cell populations in the atherosclerotic plaque (Libby, 1996).

Oxidative modification of LDL is believed to play an important role in the pathogenesis of atherosclerosis; it has been suggested that accumulation of oxidatively modified LDL in the intima contributes to monocyte recruitment and foam-cell formation (Lusis, 2000). Oxidatively modified LDL may alter gene expression in arterial wall cells. The oxidized LDL can activate transcription factors such as NFκB and induce the expression of genes containing the binding sites for the transcription factor. Minimally oxidized LDL can stimulates the transcription and secretion of monocyte chemotactic protein-1 (MCP-1) in cultured human aortic endothelial and smooth muscle cells (Cushing *et al*, 1990), and it can also stimulate the expression of tissue factor by monocytes and aortic endothelial cells (Drake *et al*, 1991).

Endothelial cells and monocytes/macrophages are of crucial importance to the development of atherosclerosis. The endothelium plays a central role in mediating inflammation due to its generation of effector molecules such as the adhesion molecules and growth factors that regulate thrombosis, vascular tone and inflammation. One of the earliest events in atherosclerotic plaques formation (Fig.1.2) is the attraction of blood monocytes to the endothelium. Circulating monocytes penetrate into the arterial wall where they differentiate to become macrophages. LDL also penetrate into the artery wall and undergoes oxidation to become oxidised LDL, which is then taken up by the macrophages via the scavenger receptor pathway to form foam cells.

The characteristic lesion of atherosclerosis is the fibrous plaque (Fig.1.3), which contains numerous lipid-laden macrophages and smooth muscle cells, as well as foam cells and extracellular cholesterol crystals. The atherosclerotic plaques can grow large enough to block blood flow but the most important clinical complication is an acute occlusion due to the formation of a thrombus; this results in myocardial infarction or stroke (Lusis 2000).

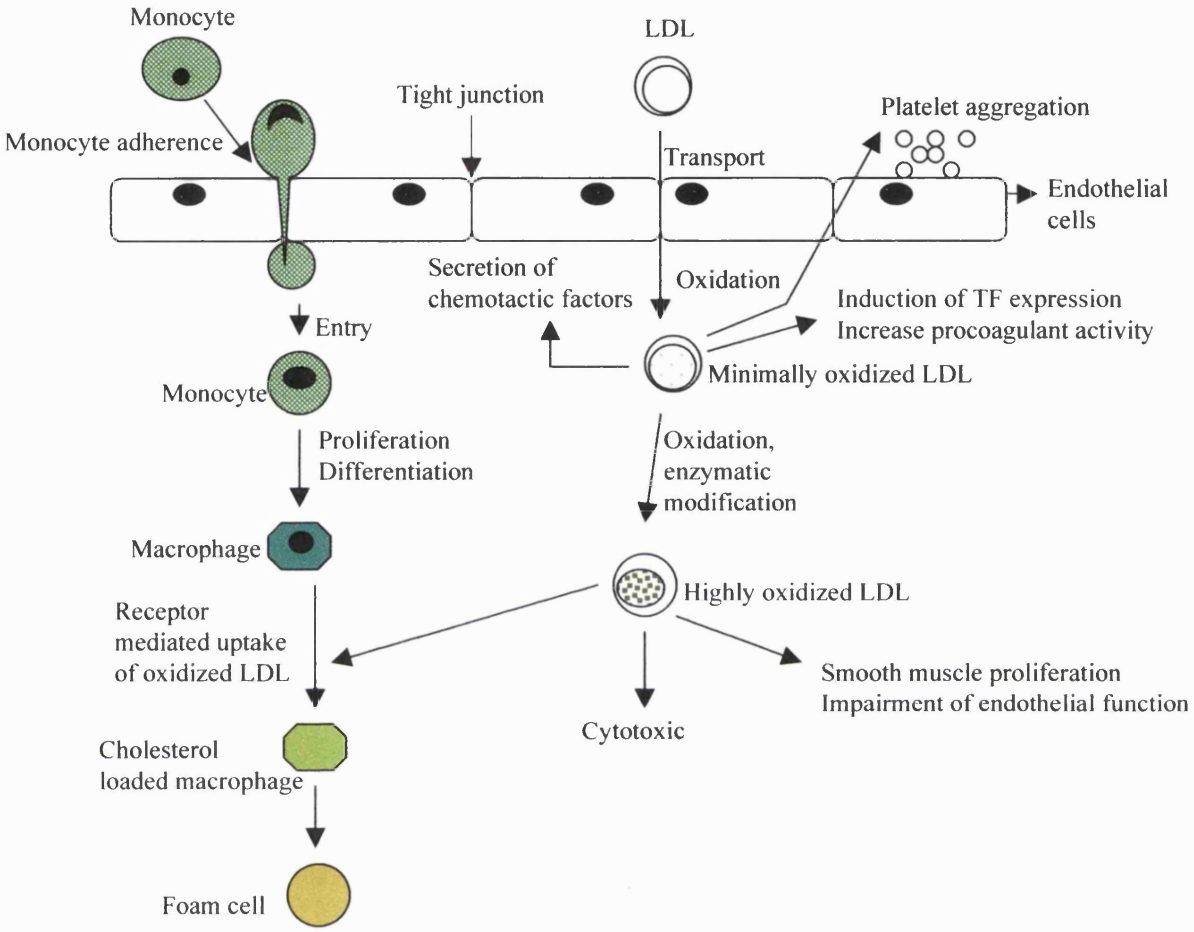


Figure 1.2 The early events in the development of atherosclerosis and the effects of oxidative modification of LDL on arterial cells.

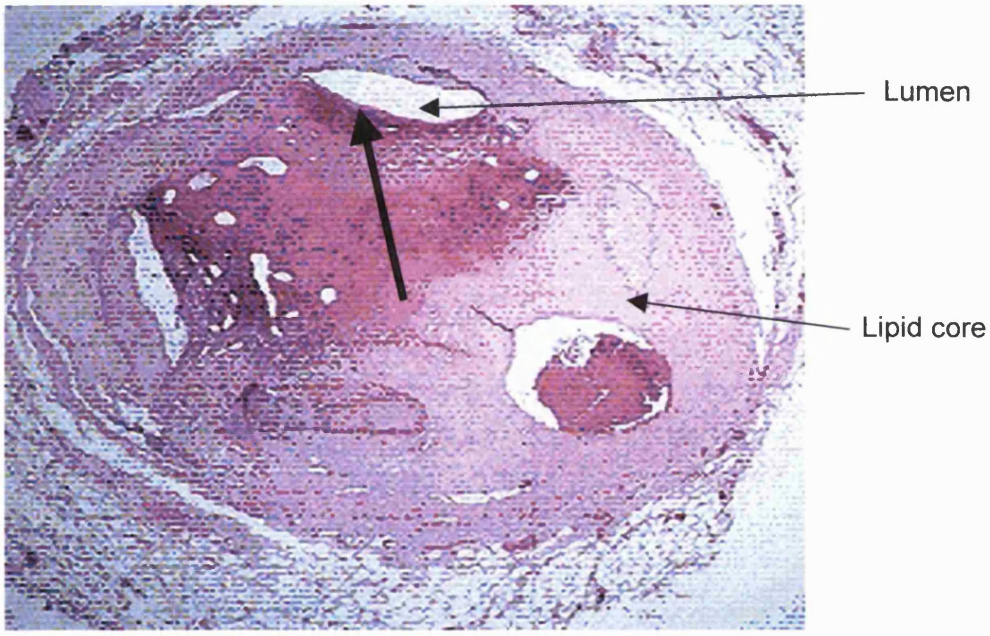


Figure 1.3 An atherosclerotic plaque
The plaque marked by the arrow has occluded most of the lumen of this blood vessel.

1.2 The plasma lipoproteins

The plasma lipoproteins are water -soluble particles secreted into the plasma by the liver and small intestine. The plasma lipids are insoluble and they are transported in plasma in association with specialized proteins called apolipoproteins in lipoprotein complexes. Lipoproteins contain triglyceride, cholesterol, phospholipids and proteins, and can be divided into five major density classes (Table 1.1): – chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL). The main cholesterol-carrying lipoproteins are LDL and HDL; LDL carries approximately 70% and HDL, 20% of the total plasma cholesterol in a normal individual. Chylomicrons and VLDL are the main triglyceride-carrying lipoproteins.

1.2.1 Chylomicrons

Chylomicrons are the largest of the lipoproteins ranging in size from approximately 100 to 1000nm. They are produced by the intestinal epithelium during absorption of dietary fat, and are responsible for the transport of dietary triacylglycerols and cholesterol to various cells of the body. They are degraded by the enzyme lipoprotein lipase into chylomicrons remnants which are taken up by the liver.

1.2.2 Very low density lipoproteins

Endogenously produced triglycerides are secreted into the circulation in very low density lipoproteins (VLDL). VLDL are large particles of about 30-90nm in diameter, and function to transport fatty acids in the form of triacylglycerol to various tissue of the body. VLDLs are degraded by lipoprotein lipase to intermediate density lipoproteins (IDL).

THE DENSITY CLASSES OF PLASMA LIPOPROTEIN

	Density (g/ml)	Mean diameter (nm)
Chylomicrons	< 0.95	500
Very Low Density Lipoprotein (VLDL)	< 1.006	43
Intermediate Density Lipoprotein (IDL)	1.006 – 1.019	27
Low Density Lipoprotein (LDL)	1.019 – 1.063	22
High Density Lipoprotein (HDL)	1.063 – 1.21	8

Table 1.1 Lipoprotein classes categorized on the basis of their relative density

1.2.3 Intermediate density lipoprotein

Intermediate density lipoproteins (IDL) have a mean diameter of approximately 27nm. They are the transient intermediate lipoproteins between the degradation of VLDL to LDL. IDL is in part converted in the liver to low density lipoprotein (LDL).

1.2.4 High density lipoprotein

The high-density lipoproteins (HDL) are 8 – 12nm in diameter. HDL has been suggested to have a protective effect against arteriosclerosis by removing excess cholesterol from peripheral tissues and inhibiting lipoprotein oxidation. The inhibition of LDL oxidation by HDL is attributable to the high content of antioxidants present in the HDL, to anti-oxidative properties of apoA-1 and also to the presence of paraoxonase (Nofer *et al*, 2002). Peroxides of both phospholipids and cholesteryl esters are reduced in the presence of apoA-1 (Garner *et al*, 1998). Paraoxonase is an esterase carried on HDL that is capable of degrading certain biologically active oxidised phospholipids (Shih *et al*, 2000). It prevents LDL from oxidation and also inhibits the oxidation of HDL (Mackness *et al*, 1991; Aviram *et al*, 1998). Paraoxonase catalyses the breakdown of oxidized phospholipids in LDL (Watson *et al*, 1995) and also decreases the lipid peroxide content in human coronary and carotid lesions (Aviram *et al,* 2000).

1.2.5 Low density lipoprotein

Low-density lipoproteins are spherical particles consisting of a core containing mostly cholesteryl esters (Fig.1.4), together with some triglyceride surrounded by a monolayer of phospholipid and unesterified cholesterol in which is embedded a single copy of apolipoprotein B-100 (Chatterton *et al*, 1995).

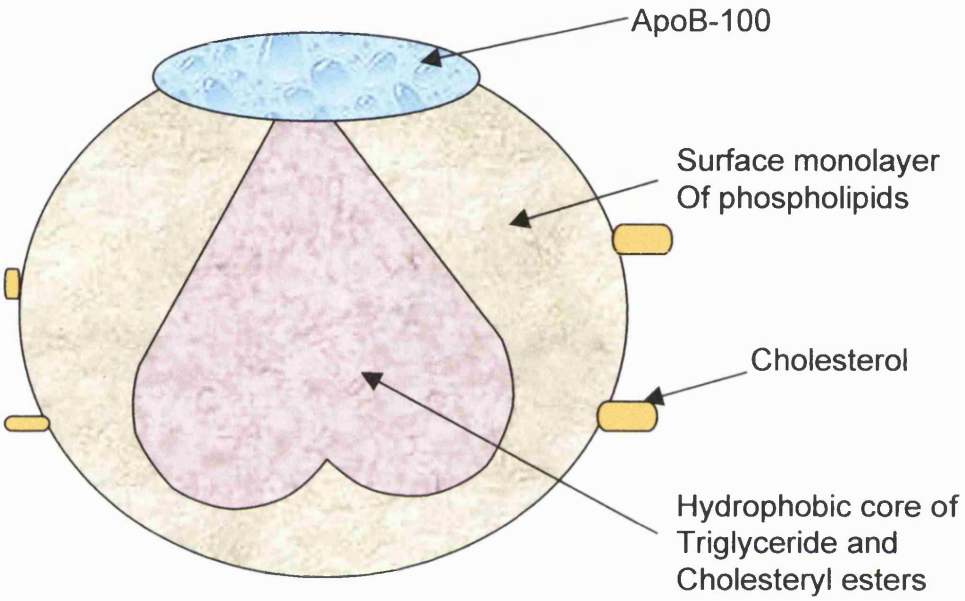


Figure 1.4 The structure of low density lipoprotein
Showing the surface monolayer of phospholipids and cholesterol, the hydrophobic core of triglyceride and cholesteryl, and a single molecule of apolipoprotein-B that encircles the lipoprotein.

The apolipoprotein B-100 (apoB-100) is a large, single-chain glycoprotein composed of 4536 residues (Knott *et al*, 1986). Cells take up LDL via receptor-mediated endocytosis. The apoB-100 is recognised by the LDL cell membrane receptor. Low-density lipoproteins (LDL) have a mean diameter of approximately 20nm and they are the major cholesterol-transporting lipoproteins. The serum LDL concentration is approximately 3 mg/ml and it carries about 60% of the total serum cholesterol (Myant, 1990).

Low-density lipoprotein (LDL) is known to be an important factor that contributes to the genesis and development of atherosclerosis. Because of the presence of polyunsaturated fatty acids in phospholipids and cholesterol esters (which form part of the lipoprotein structure) LDL is prone to oxidation. The oxidative modification of LDL enhances uptake by macrophage, the cellular accumulation of cholesteryl ester and foam cell formation.

1.3 Lipoproteins receptors

Specialised receptors on the surface of cells recognised specific apoprotein and are responsible for binding and internalising the lipoprotein. Lipoprotein receptors regulate cholesterol and triacylglycerol catabolism: they provide an effective mechanism for removing lipoprotein from the plasma and also provide an effective delivery system for supplying specific cells and tissues with cholesterol and triacylglycerols. Lipoprotein receptors have been implicated in the progression of atherosclerosis.

1.3.1 Apolipoprotein B/E receptor

The uptake of LDL by cells is through a receptor-mediated pathway. LDL receptors recognise apolipoprotein B-100 and apoE containing lipoproteins and are called Apolipoprotein B/E receptor. The LDL receptor supplies cells with

cholesterol and also removes cholesterol-rich lipoprotein particles from the bloodstream in order to prevent cholesterol accumulation in the circulation. The receptors are synthesized in response to a fall in cellular free cholesterol concentration.

1.3.2 The scavenger receptor

Oxidised LDL is not recognised by the apolipoprotein B/E receptor, it is rather internalised via the scavenger receptor that is present on the surface of macrophages. The scavenger receptors are a family of integral membrane glycoproteins that mediates binding and uptake of native and modified lipoproteins by the macrophage. Figure 1.5 illustrates the cellular uptake of modified LDL. 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. The uptake of modified LDL by macrophages is not downregulated by internalised LDL cholesterol and leads to lipid loading of these cells and the subsequent formation of foam cells in the intima. It has been suggested that smooth muscle cells and endothelial cells may express the scavenger receptor in the presence of oxidative stress and certain growth factors *in vitro* and *in vivo* (Mietus-Snyder *et al*, 1997; Li *et al*, 1995). The names of the scavenger receptors for oxidized LDL are listed in table 1.2.

1.3.3 Lectin-like ox-LDL receptor-1

An endothelial receptor for oxidised LDL called lectin-like ox-LDL receptor-1 (LOX-1) has been recognised. It has been suggested that ox-LDL uptake through this receptor may be involved in endothelial activation or dysfunction in atherogenesis (Sawamura *et al*, 1997), as well as increasing the generation of reactive oxygen species in endothelial cells (Cominacini *et al*, 2000).

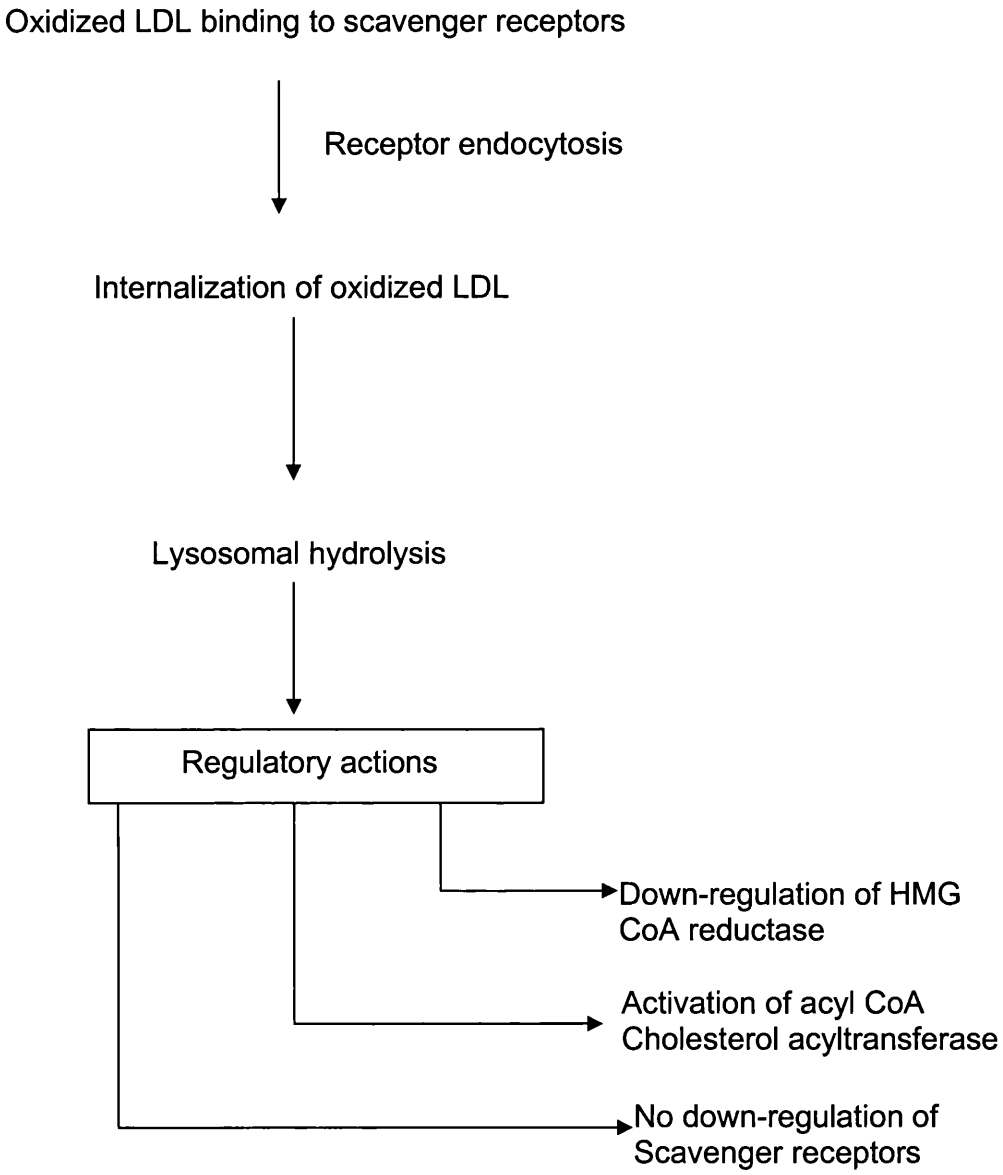


Figure 1.5. Mechanism of the cellular uptake of oxLDL.

Classification	Cell / tissue	Putative function	Ligand features
Class A SR			
SR-A1/11	macrophages	Innate immunity, oxidized LDL uptake, adhesion to charged surfaces and amyloid fibrils	Polyanions, LPS
MARCO	Spleen macrophages	Innate immunity?	Acetyl LDL, bacterial components
Class B SR			
SR-B1	Adrenal, liver, gonads,	Cholesterol transport	Native or modified lipoproteins
CD 36	Platelets, monocytes, endothelial cells,	Uptake of cell fragments, fatty acid delivery, binding thrombospondin	Modified lipoproteins, thrombospondin, plasmodium-infected red blood cells
Others			
SR-C1	Embryonic insect macrophages	Innate immunity? Phagocytosis of apoptotic cells?	polyanions
Macrosialin / CD 68	Macrophages / kupffer cells	Abundant in lysosomes, function unknown	Polyanions, apoptotic cells
Fcy receptor	macrophages	Immune complex binding	IgG1 and IgG2-containing immune complexes
SREC	endothelium	Signal transduction?	acetyl LDL, oxidized LDL
LOX-1	endothelium	Signal transduction?	Polyanions, oxidized LDL

Table 1.2 Oxidized Low Density Lipoprotein Scavenger Receptors
(Taken from Steinbrecher, 1999)

1.4 Oxidative stress

Oxidative stress is the term used to describe the condition of oxidative damage inflicted by reactive free radicals, when a balance between the free radical generation and antioxidant defences is not maintained. It arises when the normal redox homeostasis of the cell is disturbed and the rate of formation of reactive free radicals exceeds the capacity of the antioxidant defense system. This could either be due to deficiencies of antioxidants or increased formation of reactive free radicals. The balance between the reactive free radicals and the antioxidant defences determines the degree of oxidative stress.

Oxidative stress has been implicated in biological processes such as inflammation and ageing and also in the pathogenesis of various diseases including cancer, septic shock, stroke, hypertension and atherosclerosis. The cellular reactions to oxidative stress include activation or suppression of gene expression and apoptosis. Oxidative stress can result in tissue inflammation, DNA damage and protein modifications (Chao *et al*, 1999). It can increase leukocyte adhesion, activates platelets and stimulates platelet aggregation. It is also responsible for lipid peroxidation, the oxidation of low-density lipoproteins and vascular smooth muscle cell proliferation.

Oxidative stress can occur in tissues injured by trauma, infection, heat, radiation, hyperoxia, toxins, and excessive exercise. The injured tissues produce increased radical-generating enzymes such as cyclo-oxygenase and lipoxygenase. Activation of phagocytes, the release of free iron and copper ions or a disruption of the electron transport chains of oxidative phosphorylation can also produce excess reactive oxygen species (Rock *et al*, 1996). Many toxic agents also generate intracellular oxygen species.

1.4.1 Free radicals

A free radical is any species capable of independent existence that contains one or more unpaired electrons. When two free radicals meet, their unpaired electrons can join to form a pair, and both radicals are lost. Most molecules present in living organisms do not have unpaired electrons; any free radicals that are produced will likely react with non-radicals to generate new free radicals. Therefore, free radical reactions tend to proceed as chain reactions (Gutteridge & Halliwell, 1994; Sies, 1991).

1.4.2 Reactive oxygen species

Reactive oxygen species include oxygen containing free radicals such as superoxide anion radical, peroxyl, alkoxyl, hydroxyl radicals and nitric oxide, but also related species that do not themselves contain unpaired electrons but are often involved in the generation of free radicals or are reactive themselves. Such related species includes hydrogen peroxide, ozone, peroxynitrite and singlet oxygen.

Reactive oxygen species have both beneficial as well as deleterious roles. Many are useful when present in small amounts, but are toxic when present in excess. Moderate amounts of reactive oxygen species are known to play a role in signal transduction processes such as cell growth and post-translational modification of proteins (Kunsch and Medford, 1999). Reactive oxygen species are also used in host defence. Inflammatory cells such as macrophages and neutrophils produce a toxic amount of intracellular hydrogen peroxide and superoxide in response to invading organisms. When produced in excess, reactive oxygen species can cause oxidative damage to biological macromolecules such as DNA, lipids and proteins in the nucleus and cell membranes.

It is now well established that mitochondria are the main site of the generation of oxygen radicals (Morel and Barouki, 1999). Other sources of reactive oxygen species (Figure 1.6) include radiation, cytotoxic chemicals and drugs (Kannan and Jain, 2000). The membrane-associated NAD(P)H oxidases are physiological producers and major sources of reactive oxygen species in vascular tissue (Maytin *et al*, 1999; Griendling *et al*, 2000; Hancock *et al*, 2001). Enzymes such as xanthine oxidase (Harrison, 2000) and peroxidase (Bolwell *et al*, 1995) are also recognized as being able to produce ROS.

Reactive oxygen species were originally thought to only be released by phagocytic cells during their role in host defence, but is now clear that they have a cell signalling role (Hancock *et al*, 2001). ROS induce apoptosis or necrosis, induce or suppress the expression of many genes and activates cell signalling cascades (Hancock *et al*, 2001).

1.4.2.1 Superoxide radical

Superoxide is formed by adding one electron onto an oxygen molecule. The added electron pairs with one of the two unpaired electrons present in O_2, leaving one unpaired electron. Superoxide radical ($O_2^{\cdot}$) is formed in the human body in several ways with an estimated production of approximately 1.6kg / year.

In the human body, oxyhaemoglobin slowly releases superoxide radical and forms ferric haemoglobin, often called methaemoglobin. This release of $O_2^{\cdot}$ may happen only once in a thousand cycles of O_2 binding and release, but due to the large mass of haemoglobin in the body, the total body $O_2^{\cdot}$ production by this mechanism is significant. Superoxide can also be generated during oxidative phosphorylation in the respiratory chain in mitochondria. It is also generated by activated macrophages and some other immune system cells as a cytotoxic defense mechanism (Babior, 1987).

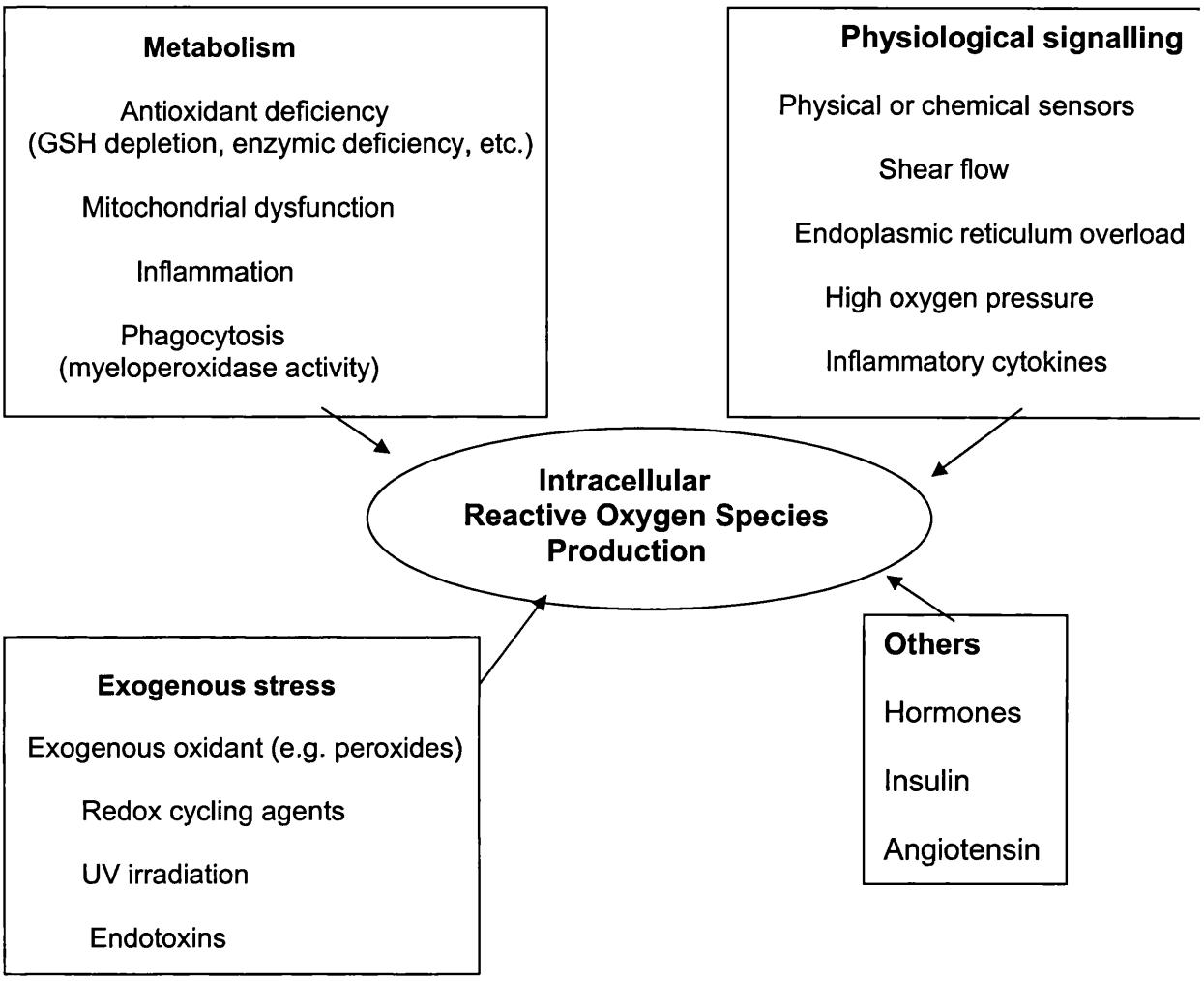


Figure 1.6 Factors that can trigger ROS generation directly or indirectly.
(Adapted from Morel and Barouki, 1999)

Mutations in mitochondrial DNA may block electron transport pathways and deflect electrons into superoxide formation (Elliott and Elliott, 1997). Superoxide is also generated via the cellular oxidase systems, xanthine oxidase, and NADH/NADPH oxidase (Maytin *et al*, 1999).

1.4.2.2 Hydroxyl radical

Hydroxyl radical is the most reactive oxygen radical. It attacks all biological molecules as soon as it comes into contact with them, usually setting off free

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$H_2O_2 \xrightarrow{Fe^{2+}/Fe^{3+}} OH^\cdot + OH^-$$

$$H_2O_2 + O_2^- \xrightarrow{Fe^{2+}/Fe^{3+}} OH^\cdot + OH^- + O_2$$

radical chain reaction. It can be generated after the conversion of superoxide to hydrogen peroxide. Superoxide dismutase catalyzes the conversion of superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2), but any hydrogen peroxide that escapes destruction can react with reducing transition metals especially Fe^{2+} or Cu^{1+} to form hydroxyl radicals (as shown in the equations above), which is the proximal agent of much oxidative damage to DNA (Halliwell and Aruoma, 1993; von Sonntag, 1987; Imlay and Linn, 1988).

1.4.2.3 Hydrogen peroxide

Dismutation of superoxide, which can occur spontaneously, especially at low pH generates H_2O_2 as shown in the equation below:

$$2O_2^\cdot + 2H^+ \rightarrow H_2O_2 + O_2$$

Although H_2O_2 is not a free radical, but further reactions may lead to the formation of hydroxyl radicals as shown in the equation below:

$$H_2O_2 + O_2\text{-} \rightarrow OH^{\cdot} + OH^{-} + O_2$$

1.4.2.4 Nitric oxide

Nitric oxide is synthesized from L-arginine by nitric oxide synthase (NOS). NOS isoforms have a similar overall catalytic scheme, involving the five-electron oxidation of the terminal guanido nitrogen of the amino acid L-arginine to form NO and L-citrulline, in a reaction involving molecular oxygen and NADPH as co-substrates, with other redox cofactors including enzyme-bound heme, reduced thiols, FAD, FMN and tetrahydrobiopterin (Michel and Feron, 1997).

Nitric oxide ($NO^{\cdot}$) is very useful in small amounts but toxic in excess. It is a cellular signaling molecule that plays a role in a number of biological processes. Nitric oxide is a free radical that contributes significantly to the killing of bacteria and tumor cells (Drapier and Hibbs, 1988). It regulates endothelial cell proliferation (Freedman *et al*, 1995). NO also regulates vascular tone, induces relaxation of smooth muscle cell, and inhibits platelet adhesion and aggregation. It is involved in the prevention of vascular thrombosis (Ziche *et al*, 1994). It is a neurotransmitter and neuromodulator in the central and peripheral nervous systems. It suppresses inflammatory cell-mediated injury (Pipili-Synetos *et al*, 1994)

The association of nitric oxide with cardiovascular disease has long been recognized and research showed both pro- and anti-atherosclerotic effects (Buttery *et al*, 1996; Leeuwenburgh *et al*, 1997; Patel *et al*, 2000).). Low levels of nitric oxide generated by endothelial nitric oxide synthase (eNOS) can inhibit lipid peroxidation (Volk and Kox, 2000). But when generated at elevated levels,

as in the case of inducible nitric oxide synthase (iNOS) expression in inflammation, nitric oxide may convert to pro-oxidant species such as peroxynitrite and nitrogen dioxide (O'Donnell and Freeman, 2001).

1.4.2.5 Peroxynitrite

Peroxynitrite is the product of the reaction between nitric oxide and superoxide anion (equation 1). The formation of peroxynitrite is dependent upon the relative concentration of nitric oxide and superoxide present at any one time. Peroxynitrite is capable of initiating lipid peroxidation, even in the presence of the biological antioxidants such as ascorbate and α-tocopherol (Patel *et al*, 1996). In its protonated form, peroxynitrous acid (ONOOH), it decomposes to form a species with the reactivity of the hydroxyl radical (equation 2).

$$NO^{\cdot} + O_2^{\cdot -} \longrightarrow ONOO^- \qquad \text{equation 1}$$

$$ONOO^- + H^+ \longleftrightarrow ONOOH \longrightarrow HO^{\cdot} + NO_2^{\cdot} \qquad \text{equation 2}$$

1.4.2.6 Singlet oxygen

The oxygen molecule (O_2) is essential for aerobic organisms, but it qualifies as a free radical because it contains two relatively stable unpaired electrons. Oxygen molecule is not a particularly reactive free radical because the unpaired electrons are arranged in such a way that O_2 oxidizes most things at a slow rate. But a simple rearrangement of its electrons can make it a reactive molecule, by converting it into singlet oxygen, which is a powerful oxidizing agent.

1.5 Lipid peroxidation

A free radical-mediated attack on lipid membranes can initiate a chain reaction that results first in lipid peroxidation and ultimately in functionally significant damage to membranes, enzymes, and nucleic acid (Bendich and Olson, 1989).

Lipid peroxidation *in vivo* provides a steady supply of free radicals since it is a chain reaction in which the chain is carried by free radicals. This process is generally represented as follows:

Initiation: production of $R^{\cdot}$ from a molecular precursor

$HO^{\cdot} + RH \Rightarrow H_2O + R^{\cdot}$ (!)

Propagation:

$R^{\cdot} + O_2 \Rightarrow ROO^{\cdot}$ (2)

$ROO^{\cdot} + RH \Rightarrow ROOH + R^{\cdot}$ (3)

Termination:

Antioxidant + $ROO^{\cdot}$ => non-radical products. (4)

$ROO^{\cdot} + ROO^{\cdot}$ => non-radical products. (5)

In this reaction scheme, RH represents the lipid and $R^{\cdot}$ the carbon-centered radical derived from it (Burton and Ingold, 1984; Liebler, 1993).

Lipid peroxidation occurs mainly in polyunsaturated fatty acids. The reaction of an initiating radical for an example a hydroxyl radical, when it captures a hydrogen atom from a methylene carbon in the alkyl chain of the polyunsaturated fatty, yields a carbon-centered radical (equation 1), which in turn adds oxygen to form a peroxyl radical (equation 2). The peroxyl radical gains a hydrogen atom from another polyunsaturated fatty alkyl chain to generate a hydroperoxide product, ROOH and another carbon-centered radical (equation 3) which subsequently induces the propagation of lipid peroxidation. Lipid peroxidation is therefore a branching chain reaction with potentially devastating effects on a living organism. Tocopherol and other lipid soluble antioxidants inhibit the chain reaction by inhibiting "reaction 3" as shown in equation 4. The antioxidant traps a peroxyl radical to form a non- radical

product. Also the chain reaction can be broken if two free radicals come together and form a non-radical product.

1.6 Lipid peroxidation in the pathogenesis of atherosclerosis

The initial oxidative modification of low-density lipoprotein lipids has been implicated in atherogenesis (Witztum and Steinberg, 1991; Lusis, 2000). Mild oxidation of LDL results in the formation of minimally oxidized LDL in the subendothelial space. The minimally oxidized LDL can induce the endothelium to express monocyte adhesion molecules, monocyte chemotactic protein-1 (Cushing *et al*, 1990) and macrophage colony stimulating factor (Rajavashisth *et al*, 1990), resulting in monocyte binding to the endothelium and subsequent migration into the sub-endothelial space where the monocytes differentiate into macrophages (Frostegard *et al*, 1990). The macrophages oxidize mmLDL into more oxidized form which is taken up by the macrophage scavenger receptor resulting in unregulated cholesterol accumulation and foam cell formation.

1.7 Biological properties of oxidatively modified LDL

Oxidatively modified LDL is more atherogenic than native LDL; it is chemotactic for circulating monocytes (Quinn *et al*, 1987) and cytotoxic for cells in culture (Cathcart *et al*, 1985). It has been demonstrated that oxidatively modified LDL can alter gene expression in arterial wall cells. It suppresses the expression of tumour necrosis factor-alpha mRNA in murine peritoneal macrophages (Hamilton *et al*, 1990) and stimulates the release of interleukin-1β from monocytes / macrophages (Thomas *et al*, 1994). Oxidatively modified LDL stimulates the expression of tissue factor by monocytes and aortic endothelial cells (Drake *et al*, 1991). It can promote procoagulant activity on the surface of human monocyte-macrophages by an increase in tissue thromboplastin activity (Schuff-Werner *et al*, 1989). It can also induce the gene expression of platelet-derived

growth factor and the platelet-derived growth factor receptor, c-fos and egr-1 (Sachinidis *et al*, 1993).

Oxidised LDL has been demonstrated to regulate many of the critical biological processes related to atherosclerosis. It stimulates vascular smooth cell proliferation, which is key event in atherogenesis (Ross, 1993). Oxidised LDL promotes the differentiation of monocytes into macrophages by enhancing the release of macrophage colony-stimulating factor from the endothelial cells (Rajavashisth *et al*, 1990). It inhibits endothelium-derived relaxing factor in the rabbit aorta (Jacobs *et al*, 1990; Plane *et al*, 1993) and also inhibits the endothelial cell-dependent arterial relaxation (Ohgushi *et al*, 1993). Oxidized LDL also stimulates platelet activation and aggregation (Aviram, 1989; Bruckdorfer, 1989; Naseem *et al*, 1993) resulting in the production of growth factors and cytokines that have been implicated in vessel inflammation and vascular smooth muscle cell proliferation and migration. It can also trigger humoral and cellular immune responses capable of modulating progression of atherosclerosis (Palinski and Witztum, 2000).

Kinscherf *et al* (1998) reported that oxLDL induced apoptosis in macrophages, and apoptosis may also play a role in the development of atherosclerosis (Kockx and Herman, 2000). The verification of a role of apoptosis in atherogenesis is the presence of apoptotic cells in atherosclerosis lesions (Geng and Libby, 1995; Han *et al*, 1995; Mallat *et al*, 1997). The characteristic features of apoptosis in atherosclerotic lesions include shrinkage of cell membranes, formation of apoptotic bodies and nuclear chromatin condensation (Napoli *et al*, 2000). The role of apoptosis in atherosclerosis is debatable. Apoptosis of arterial cells is believed to contribute to the progression of atherosclerosis. Apoptosis of intimal macrophages and smooth muscle cells leads to the progressive formation of necrotic core of atheromas (Ball et al, 1995; Ross.1999). Apoptotic lipid-laden macrophages may also sustain chronic inflammation by spilling toxic oxidized

products of LDL and lysosomal proteases into the plaque (Hegyi et al, 2001). Smooth muscle cells comprise the majority of the structural components of the fibrous cap; apoptosis of SMC will render atherosclerotic lesions mechanically unstable and prone to rupture. On the other hand, apoptosis within atherosclerotic lesions may act to reduce the number of inflammatory cells.

1.8 Antioxidant defence mechanisms

Animals have evolved intricate and interrelated processes for protecting against the effects of reactive radical species. Since many reactive free radical species are reactive forms of oxygen, the effective compounds that react with, and thereby scavenge, these radical species are often referred to as antioxidant.
Antioxidant defence mechanism is divided into two classes, the enzymatic and non-enzymatic protective mechanisms.

1.8.1 The enzymatic defence mechanism

1.8.1.1 Superoxide dismutases

Superoxide dismutases (SOD) are important components of human antioxidant defence. They catalyse the dismutation of superoxide anions into less toxic products (H_2O_2 and O_2). They contain metals essential for their catalytic function. There are two types of SOD in humans, the copper- and zinc- containing SOD (Cu,Zn-SOD) and the manganese containing SOD (Mn-SOD). The metals that are bound to SOD catalyze the dismutation of two $O_2^{\cdot}$ molecules with H^+ ions to form H_2O_2 and O_2. as shown in the equations:

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

The first stage of the reaction involves reduction of Cu^{2+} to Cu+ and in the second stage, Cu+ is re-oxidised in the presence of zinc, to Cu^{2+} by a second molecule of superoxide, with the resulting formation of peroxide (Cooper, 2001). This reaction occurs slowly at pH 7.4 but the SODs accelerate it 10,000 fold (Gutteridge and Halliwell, 1994).

1.8.1.2 Catalase

Catalase is an antioxidant enzyme that contains haem-bound iron at its active sites. Catalase converts H_2O_2 into H_2O and O_2 as follows:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

1.8.1.3 Glutathione and glutathione peroxidases

Peroxidases are enzymes that use H_2O_2 to oxidize a substrate (glutathione). Human tissues contain glutathione peroxidases as their major peroxide-removing enzymes. These enzymes remove H_2O_2 at high rate by using it to oxidize reduced glutathione (GSH) into oxidized glutathione (GSSG).

$$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$$

Glutathione reductase converts the GSSG back to GSH using NADPH as a source of reducing power.

$$GSSG + NADPH + H^+ \rightarrow NADP^+ + 2GSH$$

GSH is a thiol (sulphydryl) -containing molecule, found mostly within the cells. It helps protect cells against damage that can be caused by oxidants (H_2O_2 and

lipid hydroperoxides). It is also present, at low concentrations, in the blood and other body fluid.

1.8.2 Non-enzymatic defence mechanism

Non-enzymatic protective mechanism is essential because the enzymatic mechanism is not 100% effective in eliminating the formation of all free radicals. Even the very reactive hydroxyl free radical, is not eliminated by the enzymatic mechanisms (Rose and Bode, 1993).

The non-enzymatic protective mechanisms involve the use of an endogenous compound with the inherent trait of entering into redox reactions and contributing an electron to fill the outer shell of the reactive free radical species, thereby neutralize it to a non-reactive species. These effective compounds that react with and scavenge the radical species are often referred to as antioxidants.

Halliwell and Gutteridge (1990) defined an antioxidant as any substance which, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate. The term 'oxidizable substrate' includes almost everything found in living cells, including proteins, lipids, carbohydrates, and DNA.

For an antioxidant to be effective physiologically, it must react with a variety of reactive free radical species and be readily oxidized. It must have tolerable toxicity, be suitable for compartmentation and be readily available. It must be present in adequate amounts in the body, conserved by the kidneys and should be possible to regenerate (Rose and Bode, 1993).

1.8.2.1 Ascorbate

Ascorbate is the main water-soluble antioxidant in the plasma (Frei *et al*, 1989). Ascorbic acid (Fig.1.7a) also known as vitamin C, is synthesized by most animals except the guinea pig, primates (including man), the fruit bat, and some birds. The demand for ascorbic acid by man is met by dietary intake of this water-soluble vitamin.

Ascorbic acid has a strong reducing potential which makes it an excellent antioxidant, capable of scavenging a wide variety of different oxidants such as superoxide, hydroxyl radical, aqueous peroxyl radicals and singlet oxygen (Frei *et al*, 1989). It is considered to be the most important antioxidant in extracellular fluids (Uddin and Ahmed 1995). It protects against endogenous oxidative DNA damage in human serum. Several studies indicated that vitamin C more effectively protects lipids in human plasma and LDL against oxidative damage induced by oxidants (Frei *et al*, 1989; Esterbauer *et al*, 1989; Jialal *et al*, 1990). It is essential for the protection of humans against scurvy. Vitamin C is also useful in the regeneration of vitamin E after interacting with a free radical.

However, ascorbic acid has also been shown to have a pro-oxidant role *in vitro* (Borg and Schaich, 1989). In the presence of iron, ascorbate is used to initiate lipid peroxidation and, with copper, it is used to generate hydroxyl radicals, which are known to initiate lipid peroxidation. The natural sources of vitamin C are vegetables, oranges, lemons and other citrus fruits.

1.8.2.2 Carotenoids

Carotenoids are lipophilic antioxidants. They are a group of nearly 600 compounds, with about 50 having provitamin A activity (Palace *et al*, 1999). Human serum contains α-carotene, β-carotene, cryptoxanthin, lycopene, and

lutein as major components (Parker, 1989). Cryptoxanthin, α-carotene, and β-carotene are converted into vitamin A in humans, whereas lutein (Fig.1.7b) and lycopene are not.

Carotenoids, with or without provitamin A activity protect cells from oxidative stress by quenching free radical capable of causing cellular damage. The antioxidant activity is conferred by the hydrophobic chain of polyene units that can quench singlet oxygen, neutralize thiyl radicals and combine with and stabilize peroxyl radicals (Palace *et al*, 1999)

It has been proposed that dietary β-carotene in combination with other antioxidants protects against lipoprotein oxidation and thus may play a potentially important role in retarding the progression of atherosclerosis. Dietary β-carotene is reported (Leske *et al*, 1991) to afford protection against the progression of cortical, nuclear and mixed cataract of the lens. It is also been associated with the decreased risk of several neoplasms, cervical cancer, lung cancer, and squamous cell cancer in particular. Murakoshi *et al* (1989) indicated that β carotene protects against UV- and chemically-induced carcinogenesis and against tumor implants in a variety of regimens in mice, rat, and hamsters, it inhibited the proliferation of human neuroblastoma cell line and suppressed the level of N-myc messenger RNA.

Carotene interacts with peroxyl radical to produce carotene radical effectively terminating lipid peroxidation process. But as the oxygen tension increases, the carotene radical increasingly reacts with oxygen to generate end-products that are capable of propagating lipid peroxidation (Burton, 1989; Handelman *et al*, 1991; Rousseau *et al*, 1992; Lieblar, 1993).
The natural dietary source of carotenoids is plants particularly fruits and vegetables.

1.8.2.3 Tocopherols

Tocopherol, which is largely present as α-tocopherol (Fig.1.7c), is the only significant lipid-soluble, chain-breaking type of antioxidant present in human blood (Burton *et al*, 1989). Tocopherols, also known as vitamin E, seem to be essential for the protection of circulating lipoproteins against lipid peroxidation and the correct functioning of cell membranes. They inhibit lipid peroxidation by preventing the addition of a hydrogen atom to peroxyl radical thereby stopping the generation of lipid hydroperoxide products.

Tocopherols, mainly *α*-tocopherol, are present in the LDL at an approximate concentration of 6 moles/mole apoB (Bruckdorfer, 1995). It has been shown that raising the vitamin E concentration of LDL increases the resistance of the LDL to oxidation by cupric ions (Esterbauer *et al*, 1992; Reaven *et al*, 1993).

The tocopherol content of membranes often controls the susceptibility of microsomal membranes and hepatocytes to damage by peroxidizing agents such as hydroxyl radicals, peroxyl radicals, singlet oxygen and perhaps a number of oxygen metal complexes. Kinlay *et al* (1999) found a positive correlation between plasma concentration of α-tocopherol and endothelium-dependent vasodilator function.

Vitamin E also exhibits anti- and pro-oxidant activity for lipoprotein lipids depending on the degree of radical flux and the reactivity of the oxidant (Thomas and Stocker, 2000). The richest natural sources of vitamin E are vegetable oils, nuts, whole grain, and wheat germ oil.

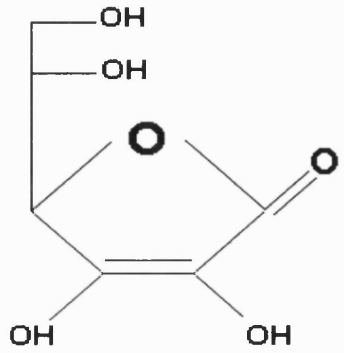


Fig. 1.7a L-Ascorbic Acid (Vitamin C)

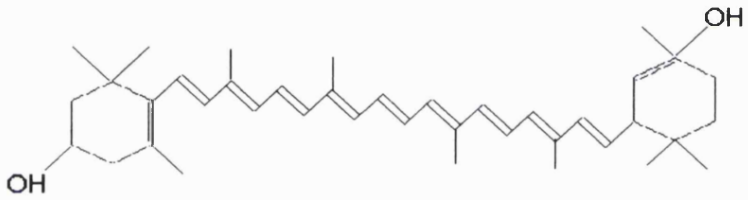


Fig. 1.7b Lutein

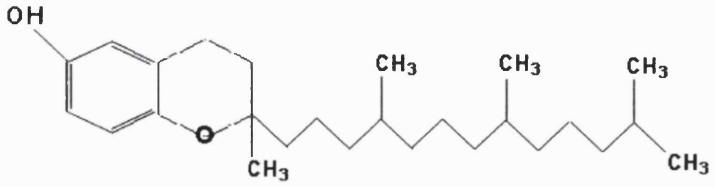


Fig. 1.7c a-Tocopherol (Vitamin E)

Figure 1.7: Antioxidant vitamins

1.8.2.4 Thiols

Antioxidant thiols act as intracellular antioxidant by scavenging free radicals through enzymatic reactions. Glutathione is the most important cellular thiol, which acts as a substrate for several transferases, peroxides and other enzymes that prevent and mitigate the deleterious effects of oxygen free radicals. Novi (1981) suggested that dietary glutathione may be an effective anticarcinogen against aflatoxin.

1.8.2.5 Flavonoids

Many flavonoids are found to be strong free radical scavengers and antioxidants. Flavonoids are a group of naturally occurring benzopyrene derivatives and are ubiquitous in photosynthesizing cells. The hydroxyl and superoxide anion scavenging activities and antioxidation of several flavonoids have been reported (Husain *et al*, 1987). Important dietary sources are green tea, red wine, chocolate and onions.

1.8.2.6 Minerals

The role of antioxidant minerals in the etiology of human cancer has been reviewed (Diplock, 1990). Evidence exists for the involvement of low levels of dietary intake of manganese, copper or zinc as risk factors in regards to cancer. Selenium, on the other hand, is emerging as a dietary factor that might prove to be of major significance as prophylactic agent against cancer (Diplock, 1991); it is present at the active sites of glutathione peroxidase enzymes.

1.9 Thrombosis and Haemostasis

Thrombosis is an integral component of atherosclerosis. Spontaneously ruptured

lipid-riched atheromatous plaques are sites of thrombus formation and a direct cause of the unexpected acute onset of stroke, myocardial infarction, unstable angina, and sudden death (Fuster *et al*, 1988 and Fuster *et al*, 1992). It also occurs as a complication of balloon angioplasty, atherectomy, and arterial bypass surgery. Thrombosis is initiated when the contents of a disrupted atherosclerotic plaque come into contact with circulating blood. Thrombus formation involves adherence of platelets to the subendothelium and activation of the coagulation cascade.

Haemostasis is a host defense mechanism that protects the integrity of the vascular system after tissue injury. Haemostatic systems are generally quiescent, but they are rapidly activated upon tissue injury or damage. A haemostatic balance is maintained by localised cross catalytic and inhibitory reactions connecting the intrinsic, extrinsic, and common coagulation pathways (Nemerson, 1992).

1.10 Blood coagulation

Initiation of blood coagulation has been divided into two pathways - the intrinsic and the extrinsic pathways (Fig.1.8.). The intrinsic cascade is initiated when blood comes into contact with an anionic surface, and it relies only on factors intrinsic to flowing blood while the extrinsic pathway is initiated by tissue factor (TF), a protein that is not normally in contact with the blood stream (Camerer *et al*, 1996).

Blood coagulation is initiated by the exposure of the circulating zymogen factor VII (FVII) to membrane bound tissue factor. The activation of FVII to its active form FVIIa and the formation of the TF-FVIIa complex in turn activate factors IX (FIX) and factor X (FX) generating trace amounts of thrombin (FIIa).

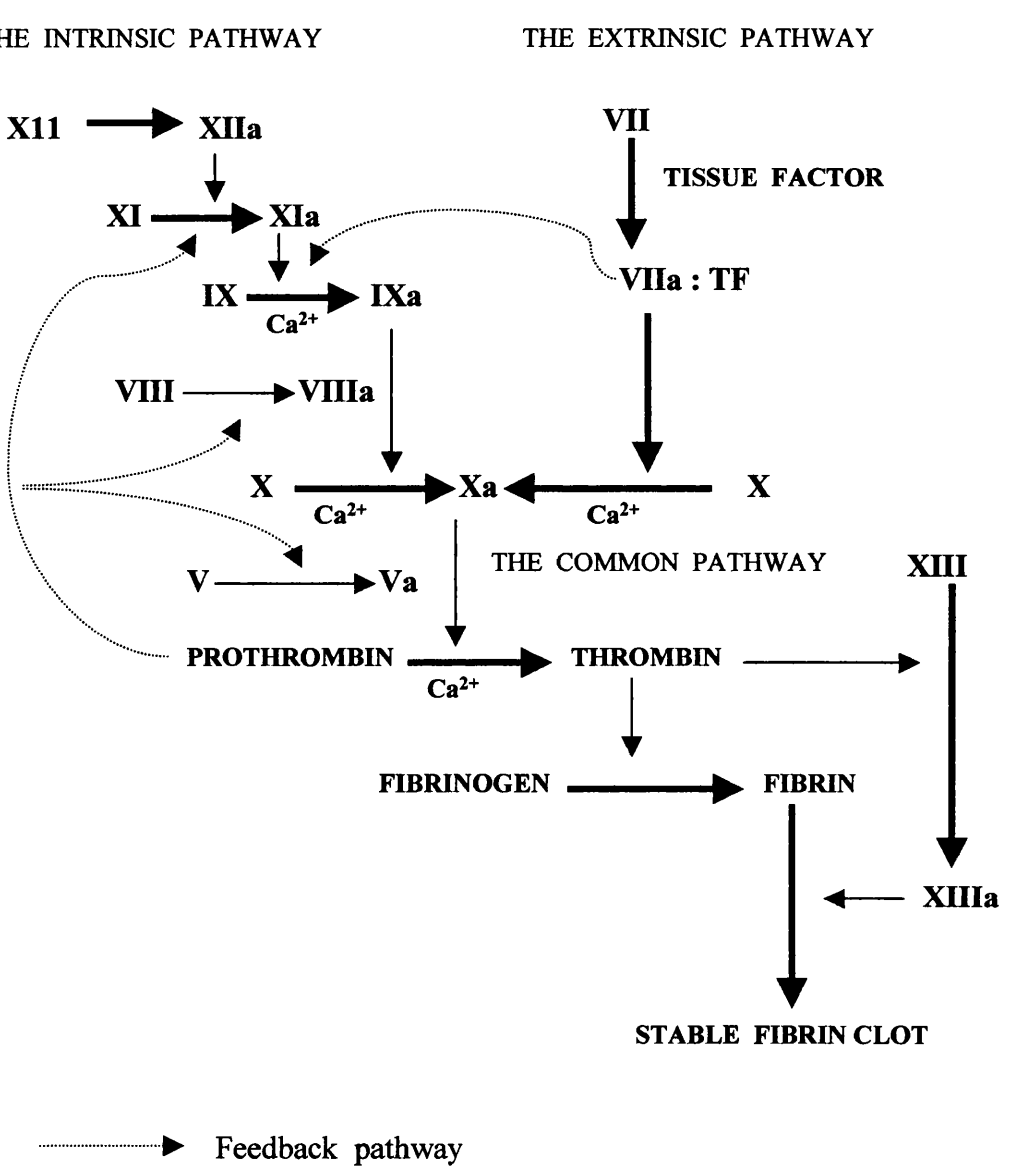


Figure 1.8 The blood coagulation cascade. Tissue factor initiates the extrinsic pathway of blood coagulation by participating as a cofactor in the activation of factor VII to its active form, factor VIIa. The factor VIIa-tissue factor complex activates factor X and factor IX in the presence of Ca^{2+} to promote clotting via the intrinsic pathway. Factor Xa activates prothrombin to thrombin, and thrombin converts fibrinogen to fibrin.

The thrombin formed back-activates the intrinsic pathway, thereby activating the formation of enough thrombin to generate a fibrin clot. Thrombin is the main effector protease of the coagulation cascade (Coughlin, 2000). Thrombin converts fibrinogen to fibrin monomer, which polymerizes to form a fibrin mesh at the site of injury. Tissue factor increases thrombin production via the initiation of blood coagulation.

1.11 Tissue factor

Tissue factor (TF) is a member of the cytokine receptor superfamily (Osterud, 1997). It is a 47-kDa transmembrane glycoprotein that functions in normal haemostasis as the high-affinity, cell-surface receptor and essential cofactor for factors vii and viia (Broze, 1982; Bach *et al.*, 1986). The exposure of TF on cell surface initiates the extrinsic pathway of coagulation and leads to the formation of a blood clot. It has been classified as an immediate-early gene, induced in the absence of de novo protein synthesis (Mackman, 1995). Expression of TF mRNA or antigen may not necessarily reflect functional availability of the active protein. The protein can be regulated by inhibitors such as annexin V (the high-affinity phospholipid-binding protein that can be used to identify plasma membrane damage because it specifically binds to phosphatidylserine that is exposed on the damaged membrane), antithrombin III or TFPI (Camerer *et al*, 1996).

Normally, TF is not detectable in the human blood-stream but in a number of pathological conditions, monocytes and endothelium cells express TF on their surfaces (Ernofsson *et al*, 1996). TF expression by monocyte-derived macrophages and vascular endothelium has been associated with pathological conditions such as cancer, infection, inflammation and atherosclerosis (Edwards *et al*, 1981; Osterud and Flaegstad, 1983; Semararo *et al*, 1985; Landers *et al*, 1994; Taudman *et al*, 1997). Cells of the vascular compartment do not express TF, but inflammatory mediators such as tumor necrosis factor -α, interleukin -1β,

and lipopolysaccharide are known to induce TF expression in monocytes, endothelial and epithelial cells. Tissue factor initiates the extrinsic pathway of blood coagulation by participating as a cofactor in the activation of factor VII to its active form, factor VIIa. The factor VIIa-tissue factor complex activates factor X and factor IX in the presence of Ca^{2+} to promote clotting via the intrinsic pathway. Binding of factor VII to TF induces a million-fold increase in the catalytic efficiency of factor X and IX activation (Ruf and Edgington 1994). Factor Xa activates prothrombin to thrombin, and thrombin converts fibrinogen to fibrin. The schema of the blood coagulation cascade is shown in figure 1.8 and the names of the coagulation factors are listed in table 1.3.

1.12.1 The structure of Tissue Factor

Tissue factor protein is an integral membrane glycoprotein of 295 amino acid polypeptide including a leader sequence. In its mature form, it consists of 263 amino acids organised into a 219 amino acid extracellular domain, a 23 amino acid transmembrane segment and a 21 amino acid cytoplasmic tail (Morrissey *et al*, 1987). The cytoplasmic tail contains three serine residues capable of being phosphorylated in response to cell activation and signal transduction (Rickles *et al*, 2000).

The human TF gene is 12.4 kb in size and it is located on chromosome 1. It is organised into six exons, the second through fifth exons encode the extracellular domain of the protein and the sixth exon encodes the transmembrane and cytoplasmic domains (Camerer *et al*, 1996, Carmeliet and Collen, 1998). TF serve as a cofactor for FVIIa by stabilising the active site of FVIIa and by co-ordinating the assembly of the macromolecular substrate. The interaction of factor VIIa with TF is predominantly mediated by protein-protein interactions, with phospholipids possibly influencing the interaction (Carmeliet and Collen, 1998).

Numeral	Common Name
Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Tissue Factor
Factor IV	Calcium
Factor V	Proaccelerin (Labile Factor)
Factor VII	Proconvertin (Stable Factor)
Factor VIII	Thromboplasinogen-Antihemophilic Globulin (AHG)
Factor IX	Plasma Thromboplastin Component (PTC)[Christmas Factor]
Factor X	Stuart-Prower Factor
Factor XI	Plasma Thromboplastin Antecedent (PTA)
Factor XII	Hageman Factor
Factor XIII	Fibrin Stabilizing Factor

Table 1.3 The common names of the coagulation factors.
(From Table of human blood plasma proteins. BEHRING Istitue Behring)

1.11.2 Regulation of Tissue Factor gene expression

The human TF promoter contains transcription factor binding sites that could contribute to the induction and repression of the gene. The binding sites for the transcription factors AP-1, NFκB, Egr-1 and Sp1 are all contained within TF promoter (Fig.1.9). Also within the 5' promoter region of TF gene lie 3 binding sites for the transcription factor HIF-1 (hypoxia-inducible factor).

The induction of TF mRNA in THP-1 cells involves both transcriptional and post-transcriptional regulation (Brand *et al.* 1991). Mackman *et al* (1991) indicated that binding of both AP-1 and NFκB/Rel protein to the transcription factor binding sites is required for LPS induction of the TF in monocytic THP-1 cells. Crossman *et al* (1990) showed that LPS induction of TF expression in HUVEC depends on the mRNA stability. Studies of Moll *et al* (1995), Bierhaus *et al* (1995) and Oeth *et al* (1997) demonstrated that LPS induces TF gene expression in monocytic cells and endothelial cells through activation of AP-1, Sp-1 and NFκB transcription factors. It has been shown by Cui and co-workers (1999) that both Egr-1 and Sp-1 transcription factors mediate the native and oxidised LDL induced TF gene expression in rat aortic smooth muscle cells.

The NFκB/Rel family of transcription factors includes NFκB1 (p50), NFκB2 (p52), RelA (p65), RelB and cRel (Cogswell *et al*, 2000; Schmitz *et al*, 2001); they can form homodimers or heterodimers. The cRel/p65 heterodimers are implicated in the inducible expression of TF in monocytes and endothelial cells (Mackman, 1995).

The AP-1 family of transcription factors is divided into two groups: the Fos related proteins that include c-Fos, FosB, Fra1, and Fra2 and the Jun proteins that include c-Jun, JunB, and JunD (Abate and Curran, 1990). The Jun and fos

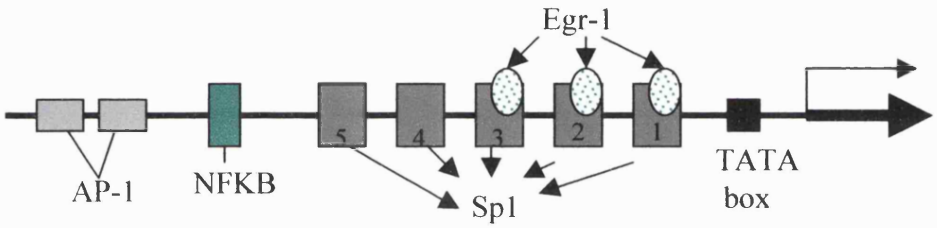


Figure 1.9 Transcription factor binding sites in the human TF promoter
The Transcription factor binding sites are shown, together with the TATA box, transcription initiation site and the direction of transcription of the gene

gene products form homodimeric (Jun/Jun) or heterodimeric (Jun/Fos) complexes (Schenk *et al*, 1994).

Hypoxia-induced factor-1 (HIF-1) is a heterodimeric transcription factor that consists of the constitutively expressed HIF-1β and the inducible protein HIF-1α (Wenger, 2000). Hypoxia-induced factor-1 is known to be involved in the regulation of gene expression in response to changes in cellular oxygen tension (hypoxia stress) but more recently HIF-1 have been shown to be activated by reactive oxygen species (Chandel *et al*, 2000). HIF-1 can transcriptionally regulate genes containing the recognition sequence in their regulatory region. It has been shown that plasminogen activator inhibitor-1 has the binding site for HIF-1 in its 5' promoter region and it is regulated by HIF-1 (Kietzmann *et al*, 1999); suggesting that HIF-1 is likely to be involved in the regulation of tissue factor mRNA.

Bochkov et al (2002) demonstrated that oxidized phospholipids stimulates TF expression in human endothelial cells via activation of PKC/ERK/EGR-1 and ca++/calcineurin/NFAT pathways rather than by NFκB-mediated transcription. It has also been shown that simvastatin prevented the up-regulation of TF expression and activity in human aortic smooth muscle cells through inhibition of Rho/Rho-kinase and activation of Akt (Eto et al, 2002).

Cyclopentenone prostaglandins are naturally occurring prostaglandin D_2 (PGD_2) derivatives that comprises prostaglandin J_2 (PGJ_2) and its metabolites Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ_2); these compounds exert anti-inflammatory effects in vivo. Eligini et al (2002) investigated the effect of 15d-PGJ_2 on TF expression in human macrophages and endothelial cells and showed a down-regulation effect on LPS- and TNFα-induced TF activity, protein and mRNA via inhibition of TF gene transcription. This effect of 15d-PGJ_2 was

targeted to the NFκB / IκB pathway and to the mitogen activated protein kinase ERKI/2.

1.11.3 Biological role of tissue factor

TF plays an essential role in haemostasis to limit haemorrhage in the event of vascular injury. It also plays non-haemostatic roles such as in tumor-associated angiogenesis (Contrino *et al*, 1996; Ruf and Mueller, 1996; Ott *et al*, 1998; Abdulkadir *et al*, 2000). TF contributes to the regulation of blood vessel development in early embryogenesis (Carmeliet *et al*, 1996). TF is also a member of the cytokine receptor superfamily (Osterud, 1997); the binding of factor VIIa to TF resulting in the transduction of cytosolic calcium signals in several cell types suggested that TF might also be involved in intracellular signalling (Rottingen *et al*, 1995). Tissue Factor is the principal initiator of coagulation and its un-controlled expression initiates the thrombotic episodes associated with atherosclerosis. Elevated TF levels in the circulation and in plaques are associated with increased thrombogenicity in the acute coronary syndromes.

1.12 Tissue factor pathway inhibitor

Tissue factor pathway inhibitor (TFPI) is a multivalent, Kunitz-type plasma protease inhibitor. It is the primary physiological inhibitor that regulates TF-induced blood coagulation (Pendurthi *et al*, 1999). TFPI directly inhibits activated factor X and produces feedback inhibition of the factor VIIa / tissue factor catalytic complex in a factor Xa-dependent fashion (Broze, 1995). It is an immediate early gene synthesised primarily in the liver, but also synthesised by endothelial cells and located on lipoproteins.

1.12.1 The structure of TFPI

TFPI contains three tandem Kunitz domains; the first two inhibits factor VIIa and Xa respectively. Although the role of the third Kaunitz domain is not fully understood, it has been suggested that it may be involved in the interaction with lipoproteins (Girard *et al*, 1989). Alteration of specific residues within the first two Kunitz domains decreases the inhibition of factor VIIa and also reduced the overall anticoagulant effect (Ettelaie *et al*, 1999).

The mechanism of TF inhibition by TFPI occurs in two steps. In the first step, factor Xa binds to the Kunitz second domain of the TFPI and in the second step, the first domain of the TFPI in the TFPI-Xa complex binds to the factor VIIa-TF complex (Fig.1.10), resulting in the formation of an inactive quaternary TF-VIIa-TFPI-Xa complex (Broze *et al*, 1988).

The mature TFPI protein contains 276 residues, with an acidic amino-terminal region followed by three tandem Kunitz-type protease inhibitory domains and a basic carboxyl-terminal region (Broze, 1995). Another Kunitz-type protein called TFPI-2 has recently been purified. Although TFPI-2 is structurally similar to TFPI, it differs significantly from TFPI in terms of target enzyme specificity. Unlike TFPI, TFPI-2 is a weak inhibitor of Xa (Rao *et al*, 2000).

1.12.2 Biological role of TFPI

It has been reported that TFPI can reduce smooth muscle cell migration as well as thrombosis (Sato *et al*, 1997). TFPI may help in regulating procoagulant activity and thrombotic events in atherosclerotic plaques (Drew *et al*, 1997; Caplice *et al*, 1998).

Hamuro and his colleagues (1998) suggested that TFPI may play a significant role in regulating the development of the vascular system during embryological development and angiogenesis by inducing apoptosis in vascular endothelial cells and by regulating human smooth muscle cell proliferation. TFPI has been shown to inhibit intimal hyperplastic lesion formation following various injuries to the arterial wall (Brown *et al*, 1996)

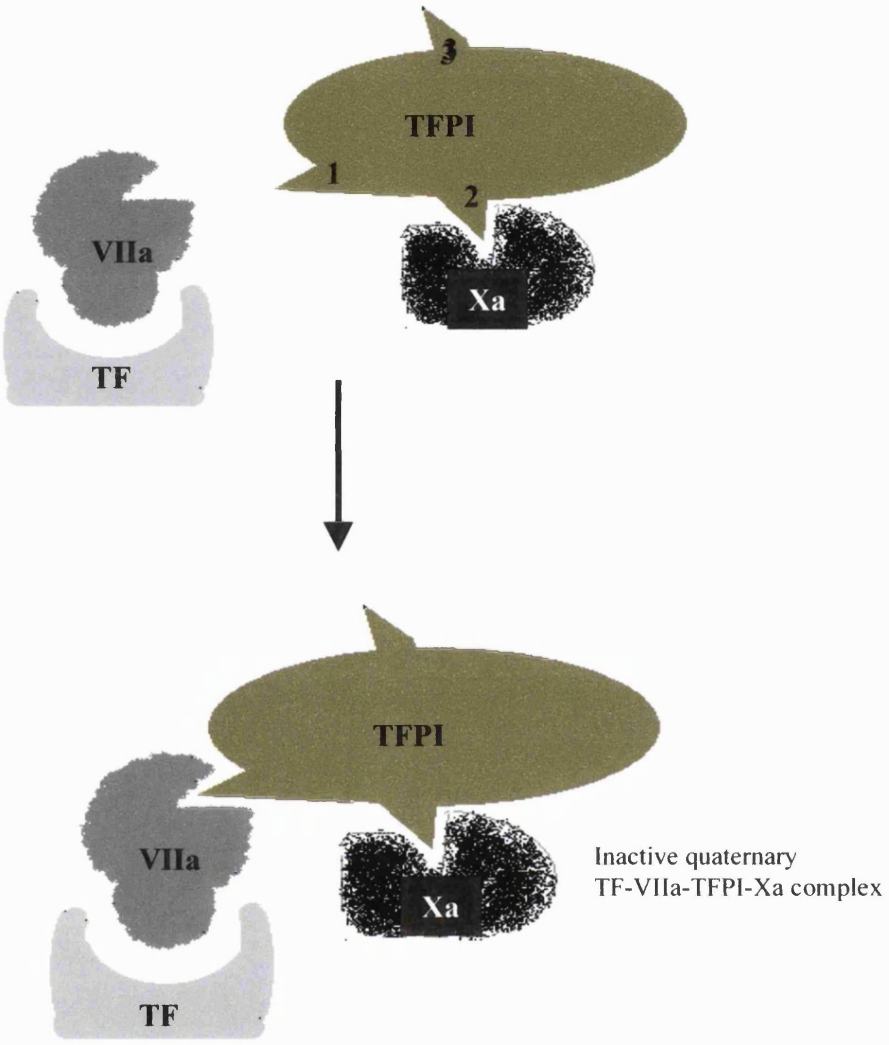


Figure 1.10 Mechanism of TF pathway inhibition by TFPI
TFPI's first and second tandem Kunitz domains inhibits factors VIIa and Xa respectively. The TFPI binds to factor Xa and factor VIIa-TF complex to form an inactive quaternary complex.

1.13 Regulation of gene expression

Activation of transcription factors stimulates gene transcription in response to specific stimuli. The relative balance of the activating and inhibiting actions of transcription factors could be the key factor in the regulation of gene transcription in a specific cell type and by a particular stimulus (Latchman, 2001). When a cell is treated with an agonist, the agonist activates or induces the specific transcription factor (Fig.1.11). The transcription factor stimulates specific gene expression by binding to the responsive binding sequences of the gene in the nucleus. Transcription factors can also inhibit gene expression through a negatively acting factor interfering with the action of a positively acting factor or by direct interaction with the basal transcriptional complex causing a reduction in the activity of the complex (Latchman, 2001).

The effect of reactive oxygen species on the expression and activity of transcription factors is complex and occurs at multiple levels. Although reactive oxygen species generally cause an increase in AP-1 and NFκB, oxidant stress can at the same time reduce the transcription activity of these molecules through the direct oxidation of critical cysteine residues contained within the DNA-binding domain (Allen and Tresini, 2000).

Cellular and molecular biology studies have demonstrated that reactive oxygen species and antioxidants can directly affect gene expression. The reactive oxygen species can play a role as second messangers regulating gene expression by the modulation of specific redox-sensitive signal transduction pathways and transcriptional regulatory events (Kunsch and Medford, 1999). The antioxidants could act in the cell by activating signal transduction pathways that can lead to the increase in the synthesis of endogenous antioxidants, or by blocking the cellular signaling, or by down regulating the genes which are induced in responses to oxidative stress.

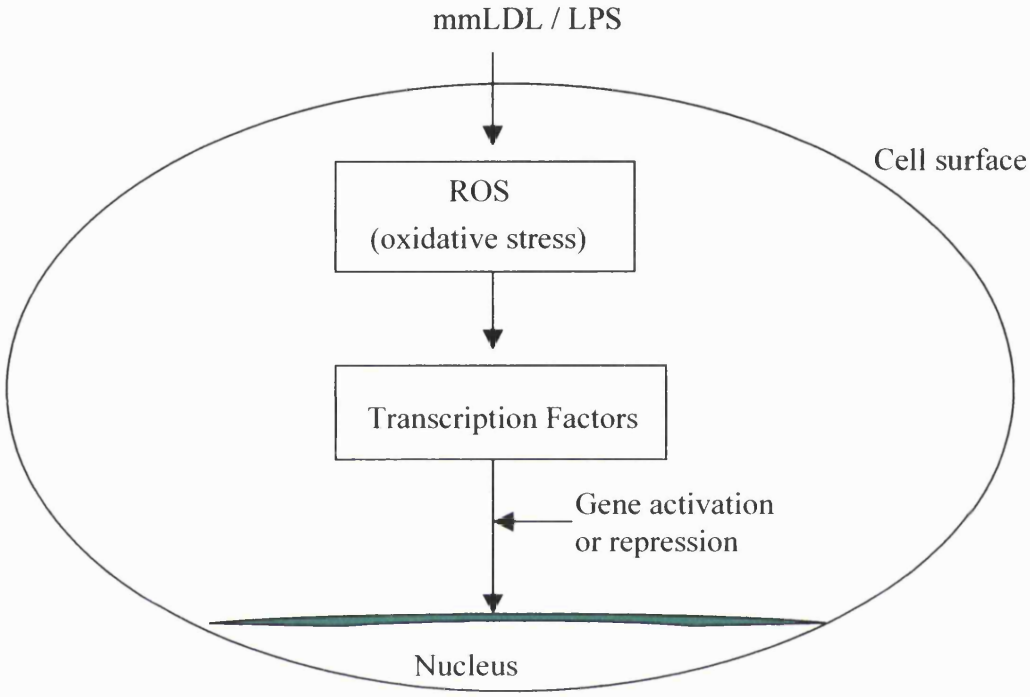


Figure 1.11 Effect of external stimulus on gene expression.
Upon stimulation by LPS or mmLDL, ROS are generated, which activate specific transcription factor. The transcription factor migrates to the nucleus to direct the transcription or repression of the cognate gene.

1.14 Aims of project

1. To establish a quantitative competitive RT-PCR system for the measurement of TF and TFPI messenger RNA levels in cells.
2. To study the effects mmLDL and the endotoxin- lipopolysaccharide (LPS) on the expression of genes for TF and TFPI in a human monocytic cell line (THP-1) and an endothelial cell line (ECV 304).
3. To investigate the effects of antioxidants on the expression of TF and TFPI in endothelial and monocytic cell lines.
4. To determine the effects of antioxidants on TF protein activity.
5. To elucidate the mode of action of the antioxidants through the involvement of selected nuclear transcription factors.

CHAPTER 2: GENERAL METHODS

2.1 The isolation of Low Density Lipoprotein (LDL)

LDL was separated from normal human plasma by preparative ultracentrifugation. Extra precautions were taken during the extraction to avoid LPS contamination; all reagents were prepared with ultra pure, pyrogen free water. All glassware used was thoroughly washed and soaked in a 1% E-Toxa clean, an endotoxin-cleaning agent [Sigma], overnight and baked at 200^{0}C prior to use. This measure of precaution has been proven in this research laboratory to be adequate for ensuring a LPS-free LDL preparation with less than 10pg endotoxim /ml.

Whole blood was added to ACD (113.8mM glucose, 29.9mM trisodium citrate, 72.6mM sodium chloride, 2.8mM citric acid, 48µM DTPA, pH adjusted to 6.4) in the ratio of 4:1 and then centrifuged at 2,500 rpm for 20 min, with maximum brake, in the IEC Centra GP8R centrifuge. The plasma fraction was removed using a clean plastic Pasteur pipette. The density of the plasma was increased by the addition of NaBr, which causes the lipoprotein to float on the surface during centrifugation. The density was adjusted to 1.3g/ml from 1.006g/ml by the addition of 0.4428g NaBr/ml of plasma. Ultracentrifuge tubes were half filled with normal saline (154 mM sodium chloride, 2µM DTPA) and this was underlayed with 10-15 ml plasma/NaBr. The tubes were centrifuged at 45,000rpm for 2.5h at 16^{0}C. The LDL that separated into a central band was removed to a clean tube. 6.5 ml of 1.151g/ml density solution (0.195M sodium chloride, 0.3mM EDTA, 5mM sodium hydroxide, 2µM DTPA, 195g NaBr/L), was put into a clean ultracentrifuge tube, overlaid with LDL and filled up with 1.063g/ml density solution (0.195M sodium chloride, 0.3mM EDTA, 5mM sodium hydroxide, 2µM DTPA, 74g NaBr/L). The tube was centrifuged at 45,000rpm for 16-18 h at 16^{0}C. The LDL, which had separated on the top layer of the tube, was removed and

desalted by passing it through PD-10 columns containing Sephadex G-25M (Amersham).

2.1.1 Determination of LDL concentration

LDL concentration was determined by measuring the concentration of its protein moiety - apo B100 using Bradford reagent (Sigma). The Bradford reagent and the protein form a complex, which alters the dye's absorption at 465 to 595 nm. The amount of absorption produced is proportional to the protein concentration.

For each assay, a standard curve was constructed by adding 3 ml of Bradford reagent to 100 μl of Bovine Serum Albumin (BSA) standard solutions (0–100 μg/ml). The absorbance at 595 nm was measured with the spectrophotometer and a plot of the average absorbance against the concentration of standard protein solutions was made (figure 2.1). Protein concentration of test samples were determined by comparing the A595 values against the standard curve using the straight- line regression constants.

2.1.2 Oxidative modification of LDL

Minimal oxidation was achieved by incubating the LDL in air for 18-96h at 37^0C under sterile conditions. This procedure initiates the earlier stages of oxidation with an increase in lipid peroxides concentration and just a slight shift in electrophoretic mobility.

2.1.3 Determination of extent of LDL oxidation

The extent of modification was assessed by measurement of lipid peroxide, conjugated dienes by absorbance at 234 nm and electrophoretic mobility on agarose gels in barbital buffer.

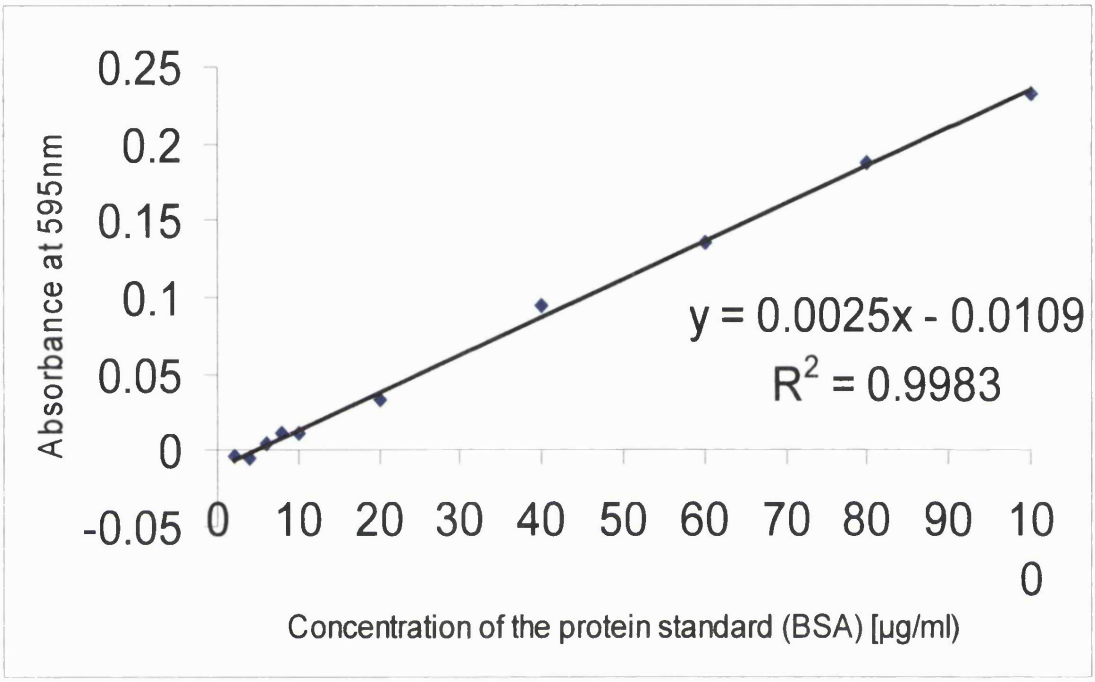


Figure 2.1: A representative Bradford standard curve for the measurement of protein concentration.

2.1.3.1 Spectophotometric absorbance at A234

The conjugated diene assay is a very simple and convenient assay for determining the extent of LDL oxidation using the ultra violet spectrophotometer. The LDL samples were diluted to 100μg protein/ml in sterile phosphate buffered saline (PBS) and the conjugated dienes were monitored at 234nm. Minimal oxidation was achieved when the A_{234} increased by 0.15-0.30 above the initial value.

2.1.3.2 Determination of lipid peroxides concentration

Lipid peroxide present in the LDL samples was measured by means of an iodometric assay using the colour reagent CHODE Iodide (El-Saadani *et al*, 1989). CHODE Iodide is one of the reagents contained in a cholesterol estimation Kit (Merck/BDH Chemicals, Poole, Dorset, U.K) which was developed for the colorimetric estimation of cholesterol. Its composition is listed in the table below:

Potassium phosphate, pH 6.2	0.2M
Potassium iodide	0.12M
Sodium azide	0.15mM
Polyethyleneglycol mono[p(1,1',3,3'-tetramethyl-butyl)-phenyl]ether	2g/l
Alkylbenzyldimethylammonium chloride	0.1g/l
Ammonium molybdate	10μM

Table 2.1 The reagent constituents of CHODE

This assay is based on the ability of lipid peroxide to oxidize iodide to iodine. The iodine produced is measured spectophotometrically at 365nm and there is a direct relationship between the amount of peroxides present and the concentration of the iodine produced.

Standard curves (figure 2.2) were constructed using serial dilutions of H_2O_2 (0-100 μM). 100 μl of 100 μg/ml LDL samples or the H_2O_2 were mixed with 1ml of the CHODE-Iodide reagent. The samples were incubated in the dark at room temperature for 1h before measuring the absorption at 365nm against a reagent blank. This method might not give an absolute estimation of lipid peroxide, but it gives a quick and clear indication of lipid peroxidation. It was used in conjunction with the other methods.

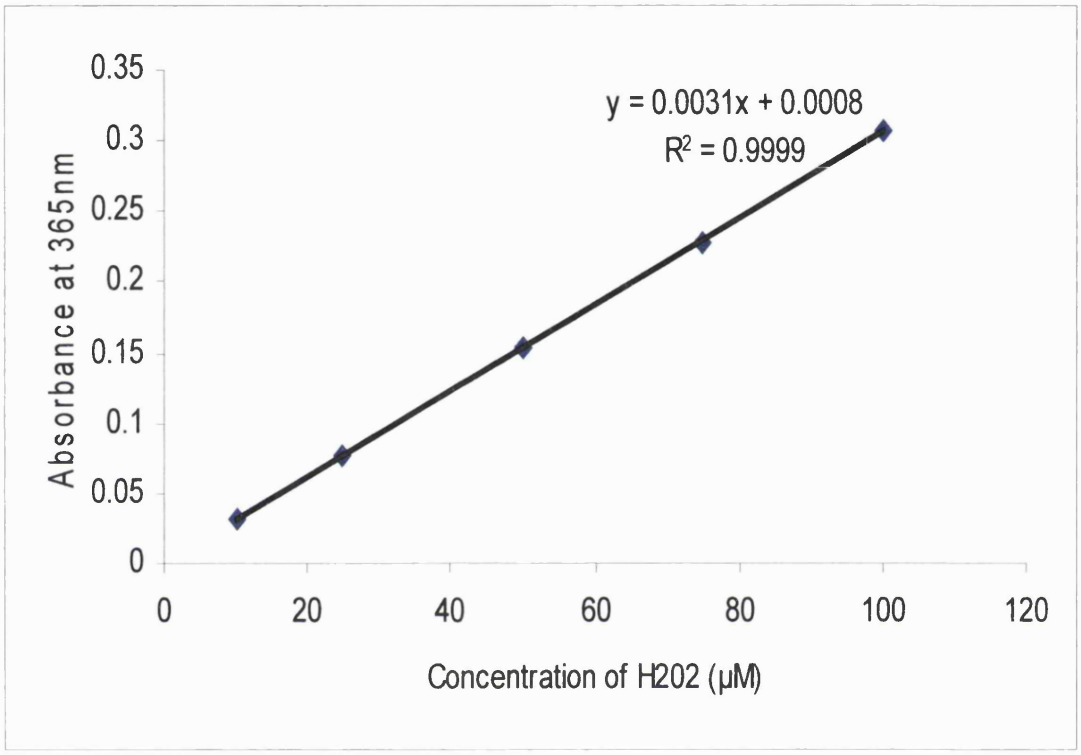


Figure 2.2: A representative standard curve for the measurement of lipid peroxidation by CHODE-iodide method.

2.1.3.3 Agarose gel electrophoresis of LDL

LDL has a net negative charge which increases upon oxidation due to the neutralization of the positive charges of the lysyl residues of apo B-100 as aldehydes bind to these (Rice-Evans *et al*, 1996). The oxidative status of LDL was determined by measuring the relative electrophoretic mobility of the LDL sample, which is the distance migrated by the oxidized LDL divided by the distance migrated by the native LDL.

Analysis was performed using the HYDRAGEL LIPO + Lp(a) kits (Sebia Inc. USA). The LDL samples were electrophoresed on agarose gel, in Tris-barbital buffer, stained with lipid specific Sudan black stain. The excess and background stain was removed from the gel by destaining in ethanol.

2.1.3.3.1 Reagents preparation for LDL electrophoresis

(a) Agarose Gel

Each agarose gel contains 8g of agarose in 1L of alkaline buffer pH 8.8 ± 0.1. The gels were stored in their protective packaging at room temperature.

(b) Tris-Barbital Buffer

Each vial (75 ml) of the stock buffer contains 2.45% barbital, 13.73% sodium barbital and 0.13% sodium azide. The electrophoresis buffer was prepared by making up each vial to 1L with distilled water and adjusting the pH to 9.2 ± 0.3.

(c) Sudan Black Stain

The working Sudan black stain solution was prepared 1h before use and it contained 160 ml of pure ethanol, 2 ml of Sudan black stock solution and 140 ml of distilled water all mixed together by gentle stirring.

(d) Destaining Solution

The destaining solution was prepared fresh, 30 min before each use and it contained 45% pure ethanol and 55% distilled water.

2.1.3.3.2 LDL electrophoresis procedure

LDL samples were loaded onto the gel using the sample application template. The samples were allowed 10 min to diffuse completely into the gel before putting into an appropriate electrophoresis tank with the samples on the cathodic side. The gel was electrophoresed for 90 min at a constant voltage of 50 V and initial current of 12 mA. After electrophoresis, the gel was placed in the oven to dry at 80^0C for at least 20 min. The dried and cooled gel was placed in the staining solution for exactly 15 min and destained for exactly 5 min. The gel was washed rapidly in distilled water and dried in the oven at 80^0C.

2.2 One stage prothrombin time assay

Procoagulant (TF) activity was measured by a one-stage prothrombin time assay in a coagulometer (Helena). Cells were harvested by centrifugation, washed and resuspended in PBS ($1x10^6$cells/100μl PBS). Control human plasma was resuspended in water and pre-warmed to 37^0C. 100 μl of cell suspension was incubated at 37^0C for 1 min in the coagulometer and 100 μl of normal human control plasma was added and reaction was incubated for another 1 min at 37^0C. 100 μl of 25 mM $CaCl_2$ was added to initiate clotting and the time it takes for a clot to form was recorded. The clotting time was converted to arbitrary TF units using the standard curve generated from the serial dilution of recombinant TF standard [DADE Innovin, Sysmex UK] (figure 2.3). 1 unit of TF activity was taken as the equivalent of a 1,000 fold dilution of the recombinant TF standard.

2.3 Flow cytometric determination of TF antigen expression

Cells were washed and resuspended in 1% BSA/PBS (w/v) at 2 x 10^6 cells/ml. The cells were incubated, in the dark, with 10 μl fluorescein isothiocyanate

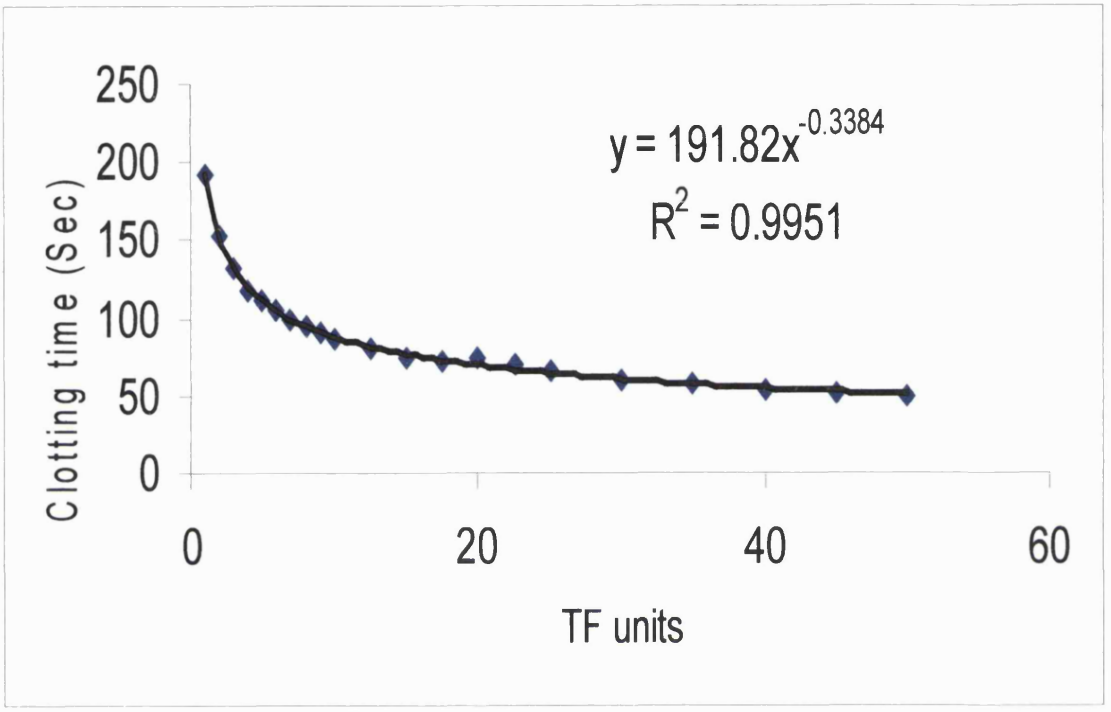


Figure 2.3: A representative recombinant TF standard curve.

(FITC) conjugated anti-tissue factor antibody (American Diagnostics Inc, USA) for 30 min on ice. The negative control cells were incubated likewise but with FITC conjugated mouse goat anti-mouse antibody (American Diagnostics Inc, USA). After incubation, the cells were washed and resuspended in 1% BSA/PBS and the fluorescence intensity was analyzed on a Coulter EPIC MCL flow cytometer.

2.4 Cell cultures

All the materials and chemicals used for the cell culture and RNA isolation were obtained from Sigma (UK) and Gibco (UK) unless otherwise specified. All reagents used were prepared with ultra pure (18.2Ω) water.

2.4.1 Isolation of Human Umbilical Vein Endothelial Cells (HUVECS)

Human umbilical vein endothelial cells, isolated from umbilical cords, are a popular source of endothelial cell. HUVECS were isolated from the vein of fresh human umbilical cords. The vein was washed with PBS before incubating with 0.1% collagenase solution for 20 min at 37^0C. The loosened HUVECS were collected with PBS, centrifuged and suspended in growth medium.

2.4.1.1 Culture of HUVECS

HUVECS were cultured in medium 199 with Glutamax 1, supplemented with 20% heat inactivated foetal calf serum, endothelial cell growth factor, heparin (20 units / ml) and antibiotic / antimycotic, in 5% CO_2 / 95% air at 37^0C. Cells were detached from the flasks using 0.125% trypsin and 0.2% EDTA in PBS, and split 1:2 every 4 days.

2.4.2 The cell line ECV 304

The isolation and culture of HUVECS have several disadvantages, including the risk of infection, exogenous growth factor requirement, low proliferative capacity and heterogeneity between cells isolated from different cords.

Early reports indicated that ECV 304 cells were spontaneously transformed human umbilical vein endothelial cell line. Since the project commenced a genetic similarity between ECV 304 and T24/83- a human bladder cancer cell line has been established. ECV 304 cells and T24/83 cells both express similar receptors positively coupled to adenylate cyclase (Brown et al, 2000). ECV304 cells do not express the endothelial cell marker von Willebrand factor (Hughes, 1996; Takahashi et al, 1990). Nevertheless, the cell line has been characterized as a good model for endothelial cells activation by cytokines (Bowie *et al*, 1995) they have also been described as a cellular model to study TF gene expression (Lopez-Pedrera *et al* 1997). They do not require special growth factors and they can be maintained in culture indefinitely. Some of the known characteristics of ECV 304 cells are listed in appendix B.

2.4.2.1 Culture of ECV 304 cells

ECV 304 cells were obtained from European collection of animal cell culture (ECACC) and maintained in DMEM medium supplemented with 10% heat inactivated foetal bovine serum and antibiotic/antimycotic, in 5% CO_2 / 95% air at 37^0C. Cells were detached from the flasks using 0.125% trypsin, 0.2% EDTA in PBS, and split 1:4 every 4 days.

2.4.3 The human monocytic cell line THP-1

The human monocytic cell line THP-1 cells possess cytological, histochemical, and functional properties of monocytes (Tsuchiya, *et al* 1980). The steady-state

levels of TF mRNA and TF protein in these cells, though high, have been shown to increase in response to endotoxin in a manner similar to that in human monocytes (Gregory, *et al*, 1989), indicating that they are a suitable model system to examine the expression of TF gene.

2.4.3.1 Culture of THP-1 cells

THP-1 cells were obtained from the European collection of animal cell culture (ECACC) and cultured in RPMI-1640 medium containing 10% heat inactivated foetal bovine serum, 2 mM glutamine, penicillin, and streptomycin, at 37^0C in 5% CO_2 / 95% air. The cell medium was changed every 7 days by spinning down the cells, discarding the old medium and resuspending the cells at 0.25 x 10^6 / ml in the supplemented medium. The cells were fed every 2-3 days by diluting the stock with fresh medium with the cell density not exceeding 1 x 10^6 / ml.

2.4.4 Cell treatment

The effects of antioxidant vitamins on gene expression were examined in cells that were pre-treated for 18h in culture medium containing 0 to 100μM of either L-ascorbic acid, lutein (in 100% ethanol; final ethanol concentration less than 0.1%), folic acid, or D-α-tocopherol (in 100% ethanol; final ethanol concentration less than 0.1%), before exposure to LDL / LPS in the continued presence of the antioxidants.

2.5 Determination of cell density and viability

2.5.1 Cell counting

Cell density was determined using a Bright Line counting chamber (Hausser Scientific Company, Horsham). Cell suspension was applied to the chamber and the number of cells / ml was calculated as 'the number of cells counted per

square millimeter' multiplied by the 'dilution factor of the cells' and multiplied by 10^4.

2.5.2 Trypan blue exclusion

Trypan blue was used to estimate the proportion of viable cells in a population of cells. The dye is negatively charged and does not react with the cell unless the cell membrane is damaged.

Equal volumes of trypan blue and cell suspension were mixed together and left to stand for 5min. The dye accumulates in dead cells while viable cells exclude it. The percentage of the viable cells was determined by counting the number of viable and non-viable cells.

% Cell Viability = (total viable ÷ total viable and non-viable) X 100.

2.6 Storage of cells

Cells to be stored were washed with PBS and the density adjusted to 1 x 10^6 cells / ml in FCS containing 10% dimethyl sulfoxide (DMSO). Aliquots of the cells were put in cryovials, wrapped in a roll of cotton wool, stored at -80^{0}C overnight and then transferred into the liquid nitrogen tank.

2.7 Recovery of stored cells

Cryovials containing the cells were removed from the liquid nitrogen tank and immediately transferred to a water bath at 37^{0}C for a few minutes to defrost. The defrosted cells were transferred to a centrifuge tube containing pre-warmed (37^{0}C) complete medium. The tube was centrifuge at 1200rpm for 5 min, the supernatant discarded and the cells resuspended in complete medium.

2.8 Preparation of total RNA from cells

RNA is a single stranded molecule with a little degree of stability and highly susceptible to degradation by ribonucleases (RNase). The use of high-quality RNA is critical for the success of Northern and RT-PCR analysis. Great care and precautions were taken to avoid introducing RNase contamination into RNA samples during and after isolation. Such precautions include the use of sterile disposable plastic ware, frequent changing of gloves and use of separate solutions and pipette dedicated to RNA work only.

Total RNA was isolated from cells using the RNeasy Mini Kits (Qiagen, UK). The cells were first lysed and homogenized in the presence of denaturing guanidinium isothiocyanate (GITC) containing lysis buffer, which inactivates RNase, and ensures isolation of intact RNA. Ethanol was added to provide appropriate binding conditions. The sample was then applied to a spin column where the total RNA binds to the silica-gel-based membrane; contaminants were efficiently washed away, and the RNA was then eluted in sterile water.

2.8.1 Cell Harvesting

(a) Cells grown in suspension

Cells grown in suspension were harvested by centrifuging for 5 min at 300 x g in a centrifuge tube and the supernatant was completely removed.

(b) Cells grown in a monolayer

The cell culture medium was discarded and the cells were washed with PBS to remove any traces of medium. The cells were detached from the culture flask with trypsin. PBS was added to the detached cells and the cells were transferred to a centrifuge tube, pellet by centrifugation at 300 x g for 5 min and the supernatant was completely removed.

2.8.2 Cell Disruption and Homogenization

The cell pellet was loosened by flicking the centrifuge tube and the cells lysed by the addition of lysis buffer. In order to create a homogeneous lysate and to reduce the viscosity of the lysate, the cell lysates were loaded onto the QIAshredder spin column sitting in a 2 ml collection tube and spun for 2 min at 13,000 x g in a microfuge.

2.8.3 Binding and Eiution of Totai RNA

Equal volume of 70% ethanol was added to the homogenized lysate and the sample was loaded unto a RNeasy mini spin column where the total RNA binds to the membrane. The column was washed with wash buffers to remove contaminants and the total RNA was eluted in RNase-free water and stored at – 80^0C.

2.8.4 Quantitation of RNA

RNA concentration was determined spectrophotometrically by measuring the absorbance at 260nm. The RNA samples were diluted 1:50 in water and the absorbance at 260nm was determined in a quartz cuvette. An absorbance reading of 1 at 260nm (A_{260}) is equivalent to 40μg/ml of RNA (Sambrook *et al*, 1989). The concentration of the RNA sample can be calculated as follows:

$$\text{Concentration } (\mu g/ml) = A_{260} \text{ X } 40 \text{ X dilution factor.}$$

The purity of the RNA samples were determined by the ratio between the readings taken at 260nm and 280nm (A_{260} / A_{280}).

As water is unbuffered and the same RNA sample may give different A_{260}/A_{280} ratios in different types of water, the RNA samples were diluted in 10mM

Tris.HCL, pH 7.5. An A_{260}/A_{280} ratio of 1.9-2.2 indicates a pure preparation. A value of less than 1.9 can indicate protein contamination and in such situation the RNA samples were further purified using phenol: chloroform: isoamylalcohol (section 2.16) followed by ethanol precipitation (section 2.17). The Integrity of the RNA was checked by denaturing – agarose gel electrophoresis (section 2.12.1).

2.9 Methods to prevent Ribonucleases contamination

Ribonucleases (RNase) are very stable and active enzymes that destroy RNA. Great care and precautions were taken to avoid introducing RNase contamination into RNA samples during and after isolation.

2.9.1 General Handling

All working surfaces, the centrifuges and pipettes were cleaned with RNaseZap (Qiagen) - a commercial reagent for inactivating RNase, before doing any procedure that involves RNA samples. Latex gloves were used and were frequently changed when handling reagents and RNA samples. Sterile RNase disposable eppendorfs and pipette tips were used throughout all procedures.

2.9.2 Glassware

All glassware used for RNA work were cleaned with detergent and thoroughly rinsed with diethyl pyrocarbonate (DEPC) treated water. The glassware were then filled with 0.1% DEPC, incubated at 37^0C overnight and then autoclaved to remove residual DEPC.

2.9.3 Electrophoresis Tanks

The designated RNA electrophoresis tank was cleaned, before each use, with 0.5% SDS, rinsed with water, dried with ethanol and then filled with 3% H_2O_2 for 10 min. The tank was then rinsed thoroughly with DEPC water.

2.10 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR is an *in vitro* technique. It involves the conversion of mRNA to complementary cDNA. This cDNA then serves as a template for the amplification of a specific DNA fragment that lies between two regions of specific DNA sequence referred to as primers (figure 2.4). The oligonucleotide primer is required to initiate cDNA synthesis. The primer anneals to the RNA and the cDNA is extended toward the 5' end of the mRNA. To minimize contamination and variations due to excessive pipetting, Ready-To-Go RT-PCR beads (Pharmacia, UK) were used for RT-PCR. These beads are designed as pre-mixed, pre-dispensed reactions, and provided as dried beads. Each bead when brought to a final volume of 50μl contains ~2.0 units of Taq DNA polymerase, 10mM Tris-HCL, 60mM KCL, 1.5mM MgCl2, 200μM of each dNTP, Moloney Murine Leukemia Virus Reverse Transcriptase, RNAguard Ribonuclease Inhibitor and stabilizers, including RNase/DNase-Free BSA.

2.11 Synthesis of RT-PCR primers using the oligosynthesiser

Some of the forward and reverse primers used for RT-PCR were synthesized on the oligosynthesiser. After completion of the synthesis, the synthesis column was removed from the instrument and a 5ml syringe was attached to one of the column. A second syringe containing 1ml of ammonia solution was attached to the other end of the column and the ammonia solution was passed through the column several times and left in the refrigerator overnight.

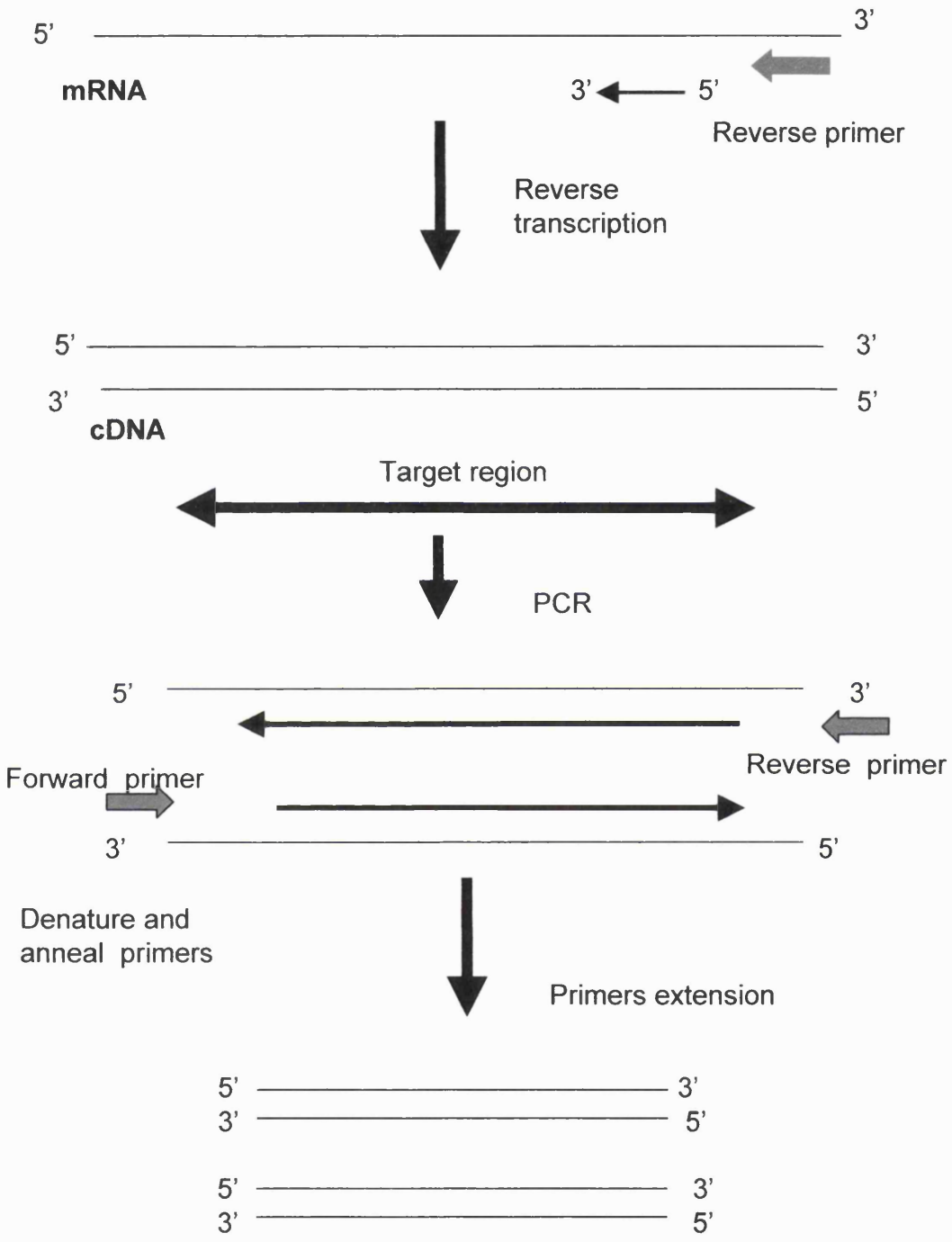


Figure 2.4 Principles of RT-PCR

The ammonia solution containing the oligonucleotides (primers) was transferred into a screw cap tube, the tube was sealed with parafilm and heated at 60^0C for 7h. The ammonia solution was cooled to room temperature, centrifuged at full speed for 30 min and the supernatant transferred into another tube. The solution was freeze dry and the primers were resuspended in nuclease free water.

2.12 Northern blot analysis

Northern blotting was first described by Alwine *et al* (1977) and it is an adaptation of the Southern blotting technique described by Southern in 1975. Northern blotting is the capillary transfer of electrophoretically analyzed RNA species from the agarose gel in a flow of liquid onto a membrane.

Northern blot analysis allows the size estimation and relative quantitations of specific RNA species. Figure 2.5 shows an outline of the Northern blot analysis. Total RNA are denatured and electrophoresed on formaldehyde – agarose gels. The RNA fragments are transferred to nylon or nitrocellulose membranes by the capillary method. Nucleic acid probe specific for the RNA in question is radiolabelled and hybridized to the membrane. Autoradiography of the membrane will reveal the fragment of RNA containing sequences homologous to the probe used.

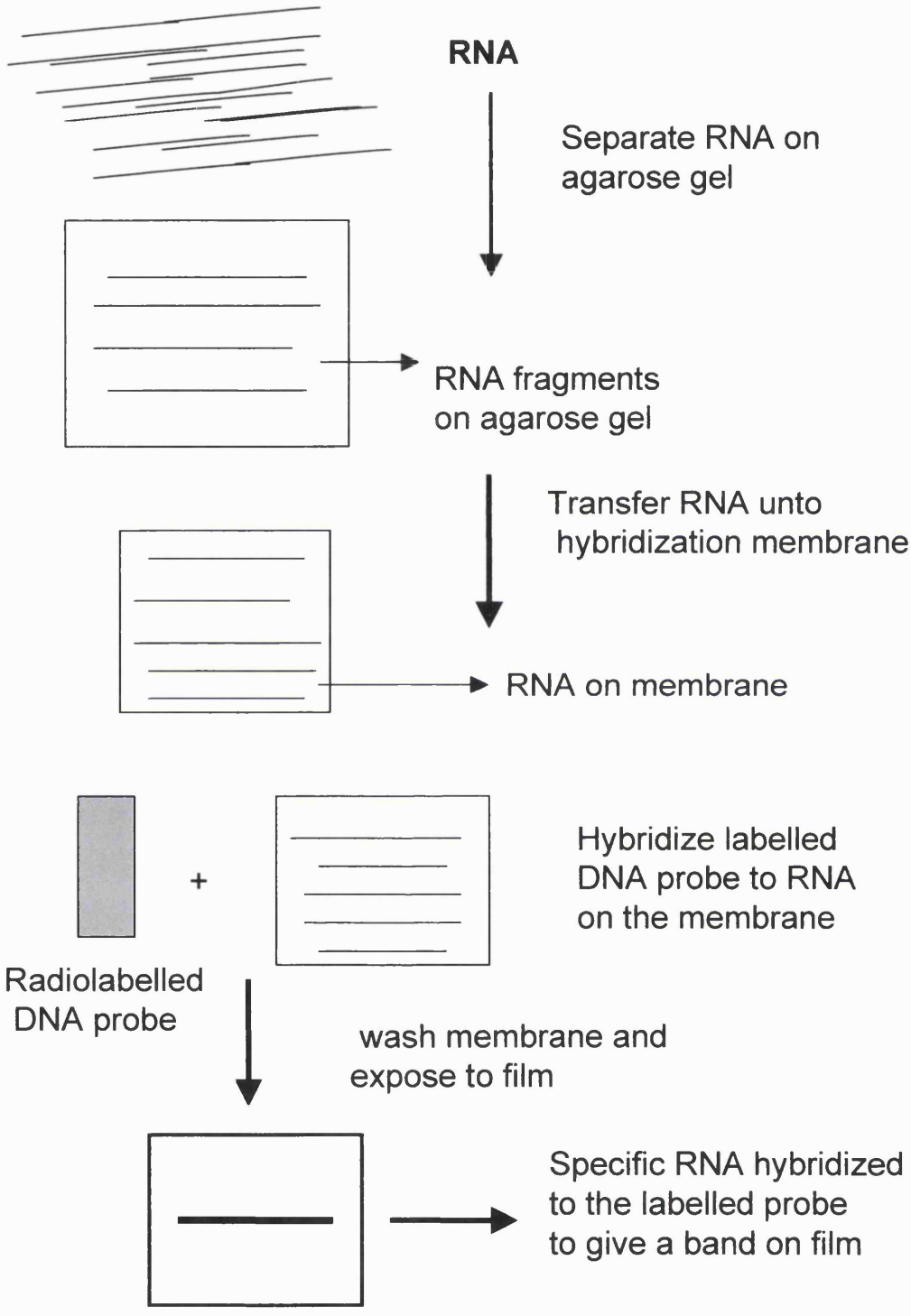


Figure 2.5: Principles of Northern Blotting

2.12.1 RNA agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation, size determination and quantitation of RNA transcripts. RNA is negatively charged and will migrate through the agarose gel towards the positive anode in an electric field. The RNA was visualized within the agarose gel by staining with ethidium bromide which intercalates into the nucleic acid and fluoresces under UV light allowing visualization.The RNA samples were analyzed on denaturing, 1% formaldehyde agarose gel because of RNA's high degree of secondary structure. Formaldehyde in the agarose gel disrupts secondary RNA structure so that RNA molecules can be separated by their charge migration. Great care was taken to avoid RNases contamination.

A 1% gel was prepared by combining 10% of MOPS 10X buffer [200mM 3(N-Morpholini) propanesulfonic acid, 50mM sodium acetate, 10mM EDTA, pH 7.0], 72.14% of DEPC-treated water and 1g of agarose powder in a flask. The components were mixed together and boiled to dissolve the agarose. The gel was cooled to about 55^0C before adding 17.86% of a 37% formaldehyde. The gel was mixed and poured onto the gel support with the gel comb carefully fitted. The gel was left to set for 1h at room temperature. The electrophoresis tank was filled with 1 X MOPS buffer and the gel was transferred into the tank before removing the comb.

RNA samples were prepared by mixing 1 part of RNA (10 or 20 μg) with 2 parts of RNA sample buffer (10ml deionized formamide, 3.5ml 37% Formaldehyde, 2 ml MOPS 5X buffer), heated at 65^0C for 5 min and chilled on ice. 2μl of RNA loading buffer (50% Glycerol, 1mM EDTA, 0.4% bromopheniol blue) was added and the samples were loaded onto the gel and electrophoresed at 5V/cm for approximately 3h or until the dye front had migrated three quarters of the length of the gel.

2.12.2 Transfer of RNA unto hybridization membrane.

After the electrophoresis of RNA in the formaldehyde gel, the gel was rinsed 5 times in DEPC treated water and soaked in 20X SSC for 45 min to remove excess formaldehyde. The blotting apparatus was set up as shown in figure 2.6. Two rubber bungs were placed on the bottom of a tray and a glass plate was placed on top. The tray was filled with 20X SSC and a sheet of 3 mm Whatman filter paper was placed on top of the glass plate so that it acts as wicks on the two sides of the plates. The Whatman paper was soaked with the SSC buffer and all air bubbles were removed. The gel was carefully placed on top of the Whatman paper and all air bubbles were removed by rolling a glass rod over the gel.

All the edges of the gel were covered with clingfilm to ensure that the SSC buffer passed evenly through the gel. Hybridization membrane (Hybond^{TM} $-N^{+}$nylon membranes) was cut to the size of the gel, wet in distilled water and soaked in 20X SSC before placing it on top of the gel. Again all air bubbles were removed. Three sheets of Whatman No.1 paper were cut the size of the gel, soaked in 20X SSC and placed on top of the hybridization membrane. A pile of blotting papers was placed on the Whatman No.1 papers, followed by a glass plate and then a 1kg weight.

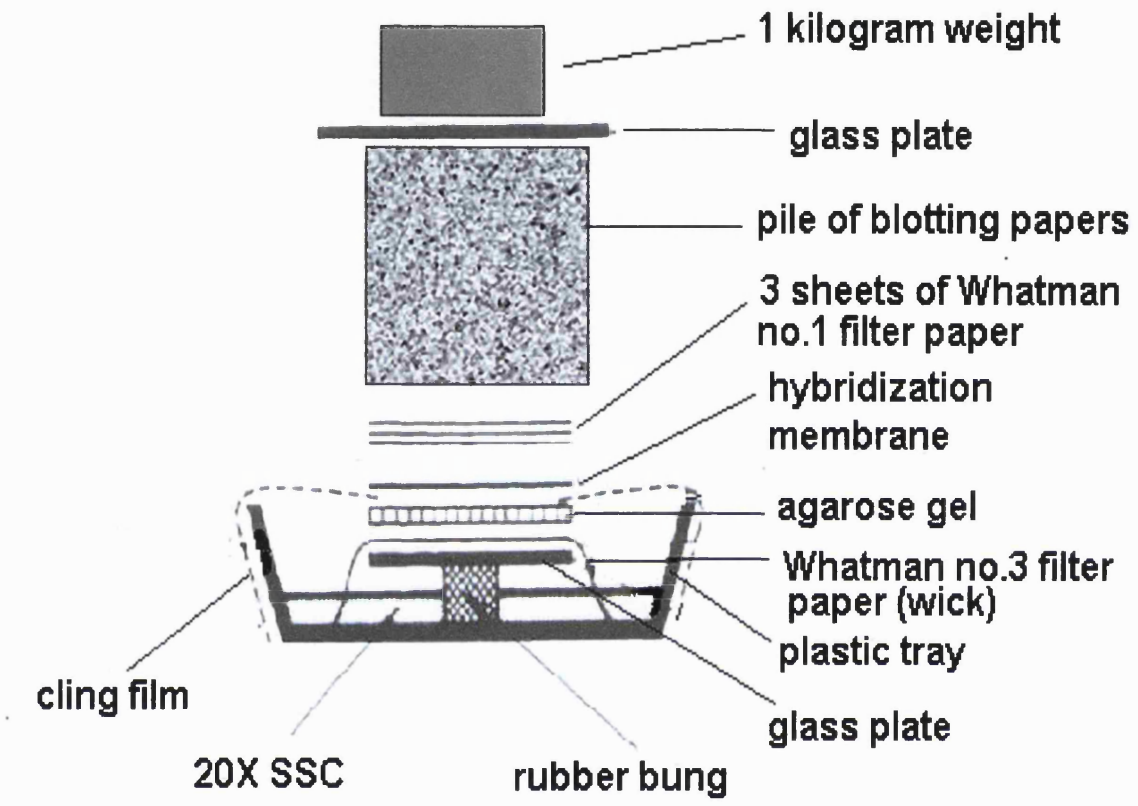


Figure 2.6: Apparatus for Northern blotting

2.12.3 Fixation of RNA

After blotting, the hybridization membrane was removed and washed once in 5 X SSC buffer. The membrane was dried by blotting it on filter paper. The RNA was fixed onto the hybridization membranes using UV cross- linker or by baking.

(a) UV cross-linking: The hybridization membrane was placed on a piece of filter paper with the RNA side up and irradiated for 120 mJoules in a Stratalinker UV Crosslinker.

(b) Baking: The blotting membrane was placed between two filter papers and baked at 80^0C for 2h.

The crosslinked membrane was placed between two filter papers and wrapped in aluminum foil pending hybridization.

2.12.4 DNA Probes

The DNA probes were prepared by cloning a specific DNA in plasmid vector and subsequent transformation of bacterial cells with the recombinant plasmid. The transformed bacterial cells were left to grow overnight in LB medium, harvested and lysed. Plasmid DNA was isolated and cut with restriction enzyme to release the specific DNA that was cloned into the plasmid. The restriction enzyme digest was electrophoresed on agarose gel and the cloned DNA was extracted from the agarose gel.

2.12.5 Probe labelling

DNA probes were radio-labelled using [α-^{32}P] dCTP (3000 Ci/mmol) (Amersham) in conjunction with the MegaPrime Labelling Kit (Amersham). Random nonamer

primers were added to 25 ng of the DNA to be labelled and heated to 100^{0}C on the dry heat block for 5 min. The reaction was placed on ice and the unlabeled dNTPs (ATP, GTP, and TTP), the reaction buffer and 2 units of DNA polymerase 1 Klenow fragment were added. Behind a glass screen, 5 µl of radiolabelled α-^{32}P dCTP was added to the reaction and incubated for 10 min at 37^{0}C. The reaction was stopped by the addition of 5 µl of 0.2 M EDTA. To reduce the background produced by the probe during hybridization, unincorporated radionucleotides were removed by passing the labelled probe through ProbeQuant™ G-50 Micro Columns (Pharmacia Biotech) containing Sephadex G-50 DNA Grade F.

2.12.6 Hybridization

The RNA immobilized on the hybridization membrane was hybridized to radiolabelled DNA probe in Rapid-hyb buffer (Amersham). The hybridization membrane was placed in a glass hybridization bottle and pre-hybridized for 1 h at 65^{0}C, with 10 ml of rapid hybridization buffer, in a hybridization oven with gentle rotation. The labelled DNA probe was denatured at 100^{0}C for 5 min, immediately chilled on ice and added at a concentration of 2 ng/ml, to the hybridization buffer. The membrane was hybridized overnight at 65^{0}C in the hybridization oven with gentle rotation.

Following hybridization, the membrane was washed (to remove non-incorporated probe and non specific hybridization) at room temperature with 2X SSC, 0.1% (w/v) SDS for 20 min (low stringency washes). Further washes were done for 2 x 15 min in 1.0 – 0.1 X SSC, 0.1% (w/v) SDS at 60^{0}C (high stringency washes). The washed membrane was wrapped in Saran Wrap™ and exposed to autoradiograph film at –70^{0}C using two intensifying screens.

2.12.7 Membrane stripping

The tight binding of RNA to the blotting membrane makes it possible to remove the DNA probe and re-use the blot in the subsequent hybridization reaction. The membrane was kept wet at all time, after the first hybridization, by keeping it wrapped up in saran film. To remove the DNA probe, the membrane was soaked in a boiling solution of 0.5% SDS for 5 min, and then allowed to cool to room temperature. The stripped membrane was autoradiographed to ensure the complete removal of the ^{32}P-labelled probe.

2.13 Cloning in piasmid DNA

Plasmids are small, circular, double-stranded DNA molecules that occur naturally in bacteria and yeast. Because of their small size and their antibiotic resistant genes, they are widely used as vectors in DNA cloning. The combined use of restriction enzyme and DNA ligase makes it possible to excise and graft any DNA fragment into the plasmid. The DNA fragment and the plasmid are joined together using DNA ligase. The procedure is illustrated in Figure 2.7. The hybrid plasmid is transformed into bacteria enabling propagation. The antibiotic resistance conferred on the bacterial cells by the plasmid selected for bacterial cells that have been transformed with the plasmid. As the bacteria multiply, the plasmid also multiplies to produce large amounts of the original DNA fragment. The plasmids are isolated from the bacteria and the original DNA fragments recovered from the plasmid using restriction enzyme.

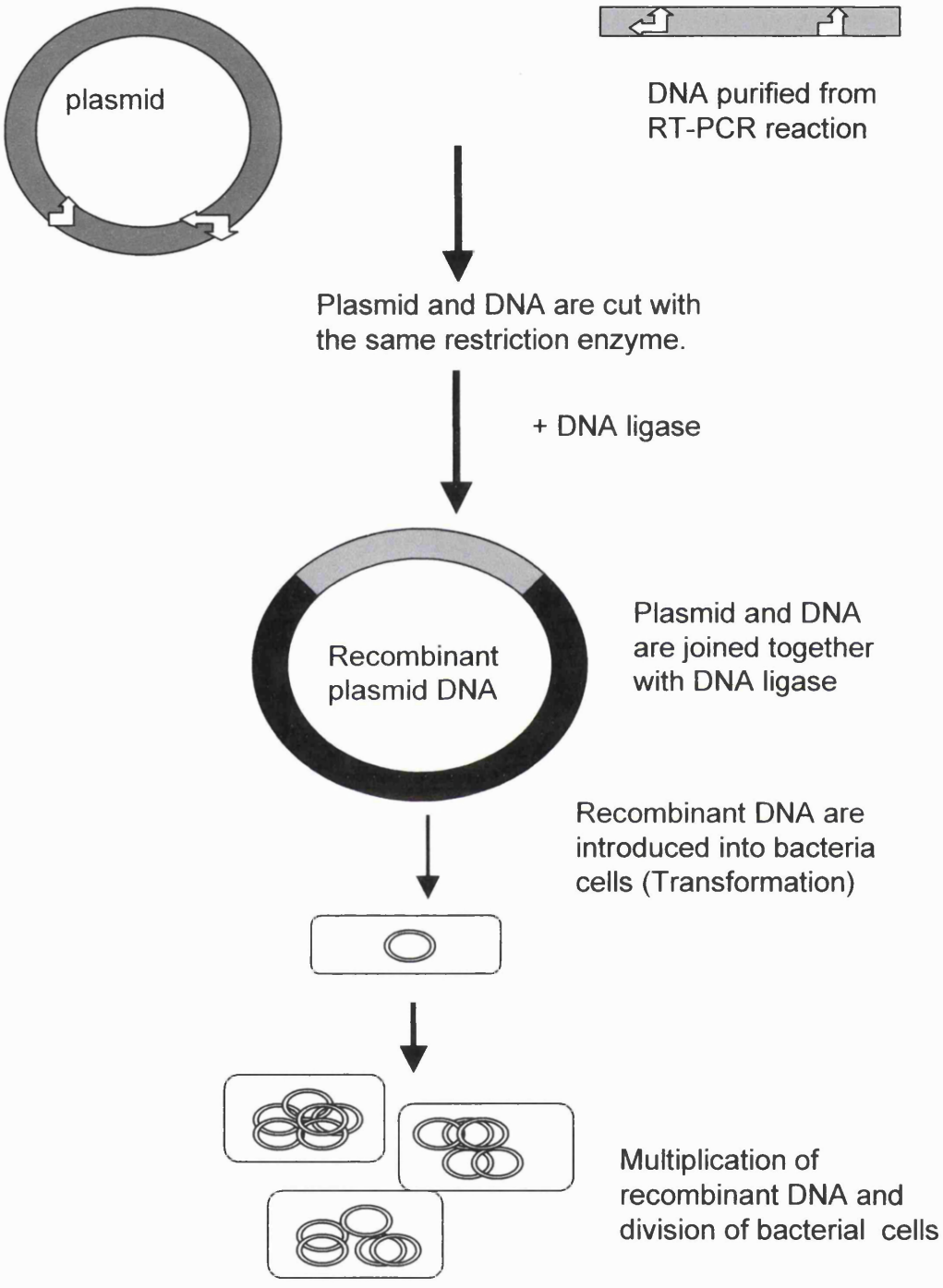


Figure 2.7: The principle of cloning DNA fragment into a plasmid.

2.14 DNA agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation, size determination and quantitation of DNA fragments. In most cases, DNA was separated on a 1% or 2% (w/v) agarose gel. The required amount of agarose was weighed out and added to the appropriate amount of 1X TBE buffer. The mixture was heated up in the microwave until all the agarose dissolved. The solution was allowed to cool to about 60^0C before adding ethidium bromide to a final concentration of 0.5μg/ml. The ethidium bromide intercalates into the DNA and fluoresces under UV light allowing visualisation. The gel was mixed gently and poured onto the gel support tray. An appropriate comb was inserted and the gel was left for about 45 min to set. The comb was removed from the gel, the gel was put in the electrophoresis chamber and sufficient volume of TBE buffer was put in the chamber to cover the surface of the gel. The DNA samples were loaded onto the gel along with DNA molecular weight maker, the gel apparatus was connected to an electrical power supply and appropriate voltage was applied. In an electric field, DNA migrates towards the anode due to the negatively charged phosphates along its backbone. The gel was removed from the chamber after electrophoresis, placed on the UV light illuminator and photographed.

2.15 DNA extraction from agarose gel

(a) Using siliconised glass wool

DNA was extracted from agarose gel using siliconised glass wool. Siliconised glass wool was prepared by soaking the glass wool in dimethyldichlorosilane that gives water-repellent properties to the glass. The soaked glass wool was placed on a paper towel, in the fume cupboard, and allowed to dry.

Under UV light, the DNA band of interest was excised from the agarose gel using a sterile scapel blade and placed into a small 0.5 ml Eppendorf tube which had been pierced at the bottom with a needle and contained a plug of siliconised glass wool. The small Eppendorf was placed into a larger 1.5 ml Eppendorf tube and centrifuged in a microfuge at maximum speed (13,000 x g) for 30 min. The eluate containing the DNA was collected in the larger Eppendorf tube, phenol extracted and ethanol precipitated.

(b) Using QIAEX DNA extraction kit

The extraction and purification of DNA, with the QIAEX extraction kit (Qiagen), is based on the solubilization of agarose and the adsorption of DNA to silica-gel particles in the presence of high salt.

The DNA band of interest was excised from the agarose gel using a sterile scapel blade. The gel slice was weighed and 300µl of QX1 buffer was added to every 100mg of gel together with 30µl of resuspended QIAEX 11 silica-particles. The sample was incubated at 50^0C for 10-30 min to solubilize the agarose. During the incubation period, the reaction was vortexed every 2 min to keep the gel in suspension. The sample was centrifuged at 13,000 x g for 30 s and the pellet was washed three times in the washing buffer and air dried for 15 min. The pellet was resuspended in sterile water and centrifuged at 13,000 x g for 30 s and the supernatant containing the DNA was transferred to a sterile tube and stored at -80^0C.

2.16 Nucleic acid phenol extraction

Since phenol and water are immiscible, in nucleic acid phenol extraction, the protein is extracted into the phenol layer and is removed from the nucleic acid, which is extracted in the aqueous layer. The procedure involves extraction firstly with phenol:chloroform;isoamylalcohol (ratio 25:24:1) and secondly with

chloroform. The phenol denatures and precipitates the protein thereby facilitating its removal from the RNA, while the second extraction with chloroform removes all residual traces of phenol.

To extract nucleic acid from a sample, an equal volume of acid phenol:chloroform:isoamyl alcohol (25:24:1) was added to the sample. The mixture was vortexed for 1 min to form an emulsion and centrifuged at maximum speed (13,000 x g) for 5 min in a bench-top microfuge resulting in an organic phase at the bottom of the tube, an aqueous phase at the top and an interphase containing proteins. The upper aqueous phase was transferred to a sterile Eppendorf tube and 1 volume of chloroform:isoamyl alcohol (24:1) was added to remove all traces of phenol, the mixture was vortexed again for 1 min and centrifuged for 5 min at maximum speed. The upper aqueous phase containing the nucleic acid was transferred to a sterile Eppendorf tube and ethanol precipitated.

2.17 Ethanol precipitation of nucleic acid

The recovery of nucleic acid from aqueous solution involves the precipitation of the nucleic acid with ethanol or isopropanol. DNA and RNA were routinely precipitated with ethanol in the presence of small concentrations of monovalent cations.

Nucleic acid was precipitated by adding 0.5 volume of 7.5 M ammonium acetate and 2.5 volume of 100% ethanol to 1 volume of samples. The mixture was vortexed and placed at – 80^0 C for a minimum of 60 min to precipitate. The mixture was centrifuged at the maximum speed for 20 min. The supernatant was discarded and the pellet was washed, to remove contaminating salts, by adding 1 ml of 70% ethanol, vortexed for 1 min and centrifuged for 30 min. The supernatant was carefully poured off, the pellet was air dried and resuspended in water.

2.17.1 Restriction enzyme digestion

Restriction enzymes are sequence-specific endonucleases that cut DNA at specific sites. The plasmid DNA (vector DNA) and the DNA fragment to be cloned (insert DNA) were digested with appropriate restriction enzyme in the presence of specific incubation buffer, at 37^0C overnight.

2.17.2 DNA ligation

The DNA fragment and the plasmid with compatible cohesive or blunt ends were joined together using T4 DNA ligase. After the vector and the insert DNA have been digested with the appropriate restriction enzyme, a ligation reaction was set up consisting of 50 – 200 ng of the vector, 50 ng of the insert, 1 unit of T4 DNA ligase, 1 μl of 10X ligase buffer and nuclease-free water to a final volume of 10 μl, in a sterile Eppendorf tube. The reaction was incubated at room temperature overnight.

2.18 Culture of E. coli bacterial cells

The Escherichia coli strain JM109 (Promega, UK) was used as host for the recombinant DNA. The bacterial cells were stored at -80^0C in 80% (v/v) Luria - Bertani broth (Promega) and 20% glycerol (Gibco). Bacterial cells were cultured in Luria - Bertani (LB) broth contained in sterile conical flasks at 37^0C with vigorous shaking (250 rpm).

2.19 Preparation of competent cells

The E. coli bacterial cells were treated with calcium chloride (Sigma) to make them more permeable or receptive to exogenous DNA or plasmids. Competent cells were prepared from a 250ml overnight culture of E.coli with an OD_{600}

reading of between 0.45 – 0.55. The culture was cooled to 0^0C on ice water for 30 min and the bacterial cells were pelleted by centrifugation at 2,500 x g for 15 min at 4^0C. The cells were resuspended in 100ml ice cold sterile 100mM $CaCl_2$ solution and incubated on ice for 10 min. The cells were pelleted again at 2,500 x g for 15 min at 4^0C and resuspended in 10ml of 100mM $CaCl_2$. Glycerol was added to the cells to a final concentration of 20% (v/v). The cells were aliquoted and stored at -80^0C.

2.20 Transformation of competent cells with plasmid

Transformation is the introduction of plasmid DNA into bacterial cells. For each transformation, 200µl aliquots of competent JM 109 bacterial cells were thawed on ice; 10-50 ng of plasmid DNA was added to the cells and gently mixed together. The cells were incubated on ice for 30 min, heat shocked at 42^0C for 50 s and placed on ice for 2 min. SOC medium [Promega] (up to 1 ml) was added and the cells were incubated at 37^0C for 1 h with shaking at 150 rpm.

2.21 Selection for transformed bacterial cells

All the plasmids used for the cloning carried an ampicillin resistance gene. After transformation, only bacteria that have taken up a plasmid will be resistant to ampicillin and are able to form colonies on an agar plate that contains ampicillin. To reduce the number of false positive transformants, a blue/white colour screening was performed to select bacterial cells with the recombinant plasmid. Bacterial cells containing recombinant plasmid appeared as white colonies, while those containing non-recombinant plasmid appeared as blue colonies.

2.21.1 Antibiotic seiection

LB agar plates for antibiotic selection were prepared by dissolving 18.5 g of LB agar in 500 ml sterile water. The mixture was autoclaved to dissolve and cooled down to about 50^0C before the addition of 100 μg/ml ampicillin. The solution was dispensed into culture plates and allowed to solidify before storing in the refrigerator (4^0C).

To select the transformants, one-fifth of the transformation mix was plated onto LB agar selection plate containing 100μg/ml ampicillin. Only the transformed cells formed colonies on the LB agar plate.

2.21.2 Blue/white colony selection

The plasmids, pUC19 and pT_7/T_3-18 [Promega], used for the cloning contain the regulatory sequences and coding information for β-galactosidase gene (lacZ) and within the coding region is the polycloning site. When plasmids with the regulatory sequences and coding information for lacZ are used to transform bacteria with sequences that code for β-galactosidase (Lac^+ bacteria), an α-complementation occurs, resulting in the synthesis of β-galactosidase. These bacteria are recognized because they form blue colonies in the presence of the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). When DNA fragments are inserted into the plasmid via the polycloning site, an amino-terminal fragment that is not capable of α-complementation is produced and the bacteria with the recombinant plasmid form white colonies.

LB agar selection plates were prepared by dissolving 18.5 g of LB agar in 500 ml sterile water. The mixture was autoclaved to dissolve and cooled down to about 50^0C before the addition of 100 μg/ml ampicillin, 0.1 mM IPTG and 40 μg/ml X-Gal. The solution was dispensed into culture plates and allowed to solidify before

storing in the refrigerator (4^0C). Alternatively, 50 μl of 50 mg X-Gal / ml and 100 μl of 0.1 M IPTG were spread onto previously prepared LB / antibiotic plates, and allowed to absorb for at least 1 h at 37^0C before usage.

2.22 Recovery of recombinant plasmid DNA

Single bacterial colonies were picked from the LB selection plate and added to 5 ml LB broth containing 100 μg ampicillin / ml, in sterile 30ml universal tubes and incubated at 37^0C with vigorous shaking (250 rpm) for 18 h. DNA was isolated from 1.5ml of these cultures, and digested with appropriate restriction enzymes Recombinations were identified by runing the digested DNA on 1% agarose gels and determining the presence and size of the cloned DNA. To obtain medium-scale DNA preparation, culture from one positive recombination was transfered to 100ml fresh LB broth in a conical flask and grown overnight at 37^0C with vigorous shaking (250 rpm).

Plasmid DNA were prepared from the bacterial cells using Wizard Plus Midipreps DNA Purification System (Promega). The overnight bacterial cultures were centrifuged to pellet the bacteria. The pellet was resuspended in cell resuspension solution (50 mM Tris-HCL, pH7.5; 10 mM EDTA and 100 μg/ml RNase A), and lysed in lysis buffer (0.2 M NaOH; 1 % SDS). Neutralization solution (1.32 M potassium acetate, pH 4.8) was added to the lysate and centrifuged to separate the DNA from the cell debris. The supernatant containing the plasmid DNA was transferred to a clean centrifuge tube by filtering it through Whatman No.1 filter paper. Resin was added to the plasmid DNA and the mixture was passed through a DNA Midicolumn. The plasmid DNA bound to the midicolumn was washed twice with column wash solution (80 mM potassium acetate; 8.3 mM Tris-HCL, pH 7.5; 4 0 μM EDTA; 55% ethanol) and the DNA was eluted in water.

2.23 DNA sequencing

The Sanger dideoxynucleotide DNA sequencing method was employed. After denaturing the DNA to be sequenced, and annealing M13 forward primer that is complementary to the plasmid sequence, the DNA is elongated from the primer using DNA polymerase. The reaction is run for a short time with the deoxynucleotides dTTP, dCTP, dGTP and the radiolabelled ^{35}S alpha dATP. The reaction is terminated by adding it to four separate tubes, each of which contains a different 2',3'-dideoxynucleotide (ddNTP). When a dideoxynucleotide is incorporated by the polymerase, the elongation stops because there is no 3'-hydroxyl group on the ddNTP. The products are electrophoresed on acrylamide gels that can separate the DNA molecules that differ in length by one molecule. Four lanes are run, one for each type of dideoxynucleotide used in the termination reaction.A ladder of bands will be seen when the gel is exposed to film and the DNA sequence is read from the bottom of the gel to the top.

The recombinant plasmids were sequenced to confirm that they had the inserted DNA fragment and the DNA fragment is of correct length and sequence and also to know the orientation of the cloned DNA within the plasmid.

2.23.1 DNA sequencing of recombinant plasmids

Recombinant plasmids were sequenced using the T7 sequenase version 2.0 DNA sequencing kit (United States Biochemicals, UK) to confirm the existence and the orientation of the cloned gene. The double-stranded plasmids (5μg) were denatured by incubating at 37^0C, with 0.1 volume of 2 M NaOH and 2 mM EDTA, for 30 min. The reaction was neutralized with the addition of 0.1 volumes of 3M sodium acetate (pH 4.5 - 5.5). The DNA was precipitated with ethanol and redissolved in 7μl of water. M13 forward primer and reaction buffer was added to

the denatured DNA for the annealing reaction and the mixture was heated at 65^0C for 10 min in a dry heat block. The heat block was turned off and the reaction, still inside the heat block, was allowed to cool slowly down to $<37^0$C (≈ 40 min) allowing the primer to anneal to the template.

Four Eppendorf tubes were labelled ddATP, ddTTP, ddGTP and ddCTP for the termination reaction and 2.5 μl of each ddNTP was put into the tubes. The labelling mix was prepared by combining together DTT, dG mix, water, ^{35}S alpha dATP, enzyme buffer and sequenase enzyme. The labelling mix was added to the annealing tube and incubated for 5 min at 37^0C. An aliquot of this labelling reaction was added to each of the termination tubes and incubated for 5 min at 37^0C. The reaction was stopped by the addition of blue formamide / stop dye.

2.23.2 Preparation of the sequencing gel

The two sequencing gel plates were cleaned with detergent and deionized water and dried with ethanol. The smaller plate was coated with dimethyldichlorosilane and fitted on top of the larger plate with the appropriate spacers and wedges in place. The plates were fitted tightly together. Gel solution (75 ml) was prepared by adding 8.3 M urea and 5% polyacrylamide in 1X TBE. 20 ml of the solution was transferred into a small beaker and 100 μl of 25% NH_4 persulphate and 100 μl of TEMED was added to the small beaker, stirred and poured immediately onto the Whatman strip in the casting tray. The gel assembly was fixed on top and left for 5 min to set. To the remaining 55 ml gel solution, 60 μl of NH4 persulphate and 60 μl of TEMED was added and stirred to mix before pouring in between the gel plates. The gel was left for 1 h to polymerize and was pre run for 90 min in 1X TBE for the temperature to get to 47^0C,

The samples were denatured at 100^0C for 3 min and placed on ice before loading onto the gel. The samples were loaded in the order G A T C.

Electrophoresis was carried out at ≈45^{0}C and 40 W constant power for 4 h in 1X TBE. The gel was transferred onto 3M chromatography paper and saran wrap was placed on top of it. The gel was vacuum-dried for 2 h at 80^{0}C using the BioRad gel dryer and exposed to X-ray film at –80^{0}C.

2.24 *In vitro* transcription of RNA

RNA transcripts were transcribed *in vitro* using the MAXIscript *In Vitro* Transcription Kit (Ambion Biotechnology, UK). During *in vitro* transcription reactions, RNA molecules were synthesized by run-off transcription from a DNA template. The DNA template was linearized by complete restriction enzyme digestion to ensure the synthesis of transcript of a uniform, defined length.

The recombinant plasmids were linearised with the appropriate restriction enzymes. The digested plasmids were electrophoresed on agarose gels to confirm complete digestion. One of the restriction enzymes used for the linearization of plasmids prior to transcription *in vitro*, Sac 1, produces 3' protruding ends which has been reported to produce extraneous transcripts, in addition to the expected transcript, when such templates are transcribed *in vitro*. The 3' protruding ends of the plasmids were converted to blunt ends. The linearised plasmids were treated with 150 μg/ml proteinase K and 0.5% SDS for 1 h at 50^{0}C, extracted with phenol and precipitated with ethanol. RNA was transcribed *in vitro* from the plasmids using 10 X Transcription buffer, 100 mM DTT, 2.5 mM each NTP (A,U,C&G) and either T_3 or T_7 RNA polymerase. The reactions were incubated at 37^{0}C for 90 min.

2.25 Removal of the DNA template

After *in vitro* transcription, RNase-free DNase was added at a concentration of 1U / μg template DNA and incubated at 37^{0}C for 30 min. The transcribed RNA

was phenol extracted and ethanol precipitated. The RNA was resuspended in water and stored in aliquots at -80^0C.

2.26 Converting the protruding ends of a double stranded DNA to blunt ends

Both the Klenow fragment of E. coli DNA polymerase 1 and bacteriophage T4 DNA polymerase (Promega) carry a 3'→ 5' exonuclease activity and a 5'→ 3' polymerizing activity that can be used to convert the protruding ends of a double stranded DNA to blunt ends.

2.26.1 Filling Recessed 3' Termini of DNA

Recessed 3' termini of double stranded DNA were filled by the polymerase activity of Klenow fragment of E. coli DNA polymerase 1. The Klenow fragment of E. coli DNA polymerase 1 catalyses the addition of mononucleotides onto the 3'OH end of double stranded DNA possessing a 5' overhang. In a 20 μl reaction, 1 μl of a solution containing each of the desired dNTPs at a concentration of 1mM was added to 0.2 – 5 μg of restriction enzyme digested DNA. 1 unit of the Klenow fragment of E. coli DNA polymerase per μg of DNA was added to the reaction and incubated at room temperature for 15 min. The reaction was stopped by heating it at 75^0C for 10 min.

2.26.2 Removal of protruding 3' termini

Bacteriophage T4 DNA polymerase removes protruding 3' termini due to its very strong 3' exonuclease activity. The exonuclease activity was carried out by adding 1μl of a solution containing each of the desired dNTPs at a concentration of 2 mM. 2 units of the bacteriophage T4 DNA polymerase was added for each microgram of DNA in the reaction, the reaction was incubated at 12^0C for 15 min and stopped by heating to 75^0C for 10 min.

2.27 Assessment of transcription factor activation

The PathDetect *in vivo* signal transduction pathway *Cis*-reporting and *trans*-reporting systems (Stratagene) were used to study the effects of extracellular stimuli on the activation of signaling molecules that lead to the activation of transcription activators of the pathdetect system. The *Cis*-reporting plasmid, (figure 2.8a) consisting of an enhancer element (AP-1 or NFκB) and the binding sites that controls the expression of luciferase gene, was transfected into THP-1 cells and the activation of the luciferase gene would indicate the interaction of extracellular stimuli with the specific enhancer element.

For the *trans*-reporting system (figure 2.8b), a fusion trans-activator plasmid (pFA Jun, pFA Elk-1 or pFA CREB), consisting of the activation domain of the c-Jun, Elk 1, or CREB transcription activator fused with the DNA binding domain of yeast GAL4 and a reporter plasmid (pFR Luc, containing a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites that control the expression of firefly luciferase gene) were co-transfected into THP 1 cells. When the signal transduction pathways c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase (MAPK) or cyclic AMP-dependent kinase (PKA) is activated by upstream signals, JNK, MAPK or PKA translocate into the nucleus and activates the transcription factors c-Jun, CREB or Elk-1 respectively. The expression level of luciferase reflects the activation status of the signal transduction pathway.

2.27.1 Transfection of THP-1 cells by electroporation

Electroporation is the process by which genes / macromolecules are transferred into cells using an electric pulse or field. The electrical pulse permeabilizes the cell membranes for the uptake of the genes. THP-1 cells were electroporated, in

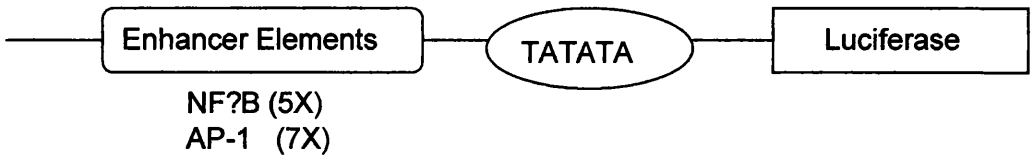


Figure 2.8a: General structure of the *Cis*- reporting plasmids.
The luciferase gene is controlled by one of the enhancer elements. The activation of the Luciferase gene indicates the interaction of extracellular stimuli with the specific enhancer element.

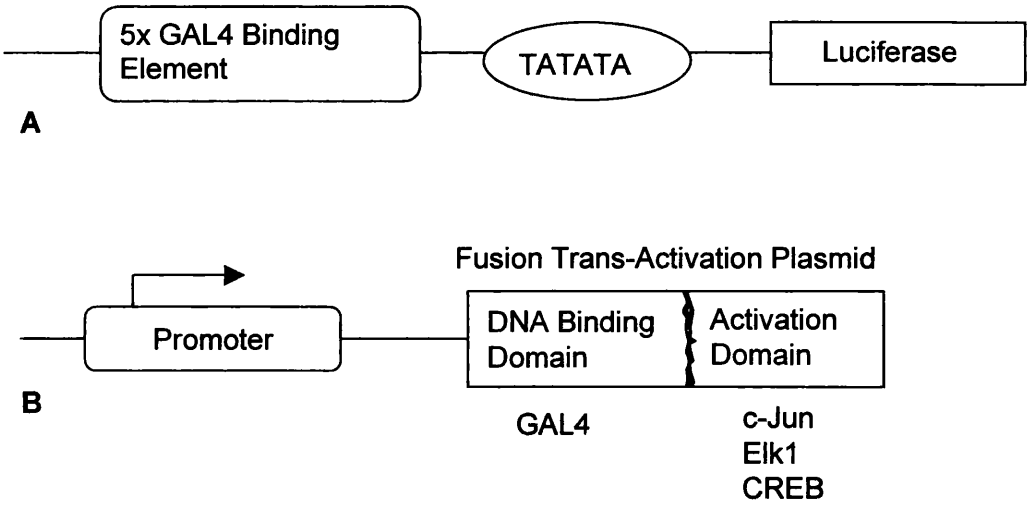


Figure 2.8b: General structure of the *trans*-reporting plasmids.
A is the reporter plasmid containing the entire coding sequence of the firefly luciferase gene downstream of a basic promoter element (TATA box) joined to 5 tandem repeats of the GAL4 binding element.
B is the pathway-specific fusion *trans*-activator plasmid that consists of the DNA binding domain of the yeast GAL4 protein and the activation domain of CREB,Elk1 or c-Jun. The fusion trans-activators bind to the promoter in the reporter plasmid.

sterile disposable 0.4 cm (inter-electrode distance) cuvettes, using the Gene Pulser 11 Electroporation system. The cell samples were centrifuged and then resuspended in 300 μl of PBS. The plasmids of interest were added to the cell suspensions and mixed thoroughly. The mixture was transferred into the electroporation cuvette and electroporated at 170 volts and at 950 uF capacitance. The samples were stood at room temperature for 2 min before resuspending in growth medium. The cells were incubated for 24 h before adding effector samples.

2.27.2 Transfection of THP-1 cells by chemical method

The commercially available transfection reagents contain cationic molecules that associates with negatively charged DNA. They form complexes with DNA through ionic interactions and facilitate the uptake of the DNA by cells. THP-1 cells were harvested by centrifugation and resuspended in 4 ml of growth medium. 5 μg of the desired plasmid was diluted to 150 μl in serum free growth medium and 20 μl of Superfect reagent (Qiagen) was added. The sample was mixed and incubated for 10 min at room temperature to allow complex formation. 1 ml of cell growth medium was added to the transfection complex before adding the complex to the cells. The cells were incubated for 24 h before adding effector samples.

2.28 Reporter gene assay

Promega's Luciferase Assay System was employed for the quantitation of the luciferase reporter gene. Light is produced by converting the chemical energy of luciferin oxidation to form oxyluciferin (figure 2.9). Firefly luciferase catalyzes luciferin oxidation using $ATP.Mg^{2+}$ as a cosubstrate. In the assay for luciferase, a flash of light was generated which was detected and measured by the plate reading luminometer. The cells to be assayed were centrifuged to remove the

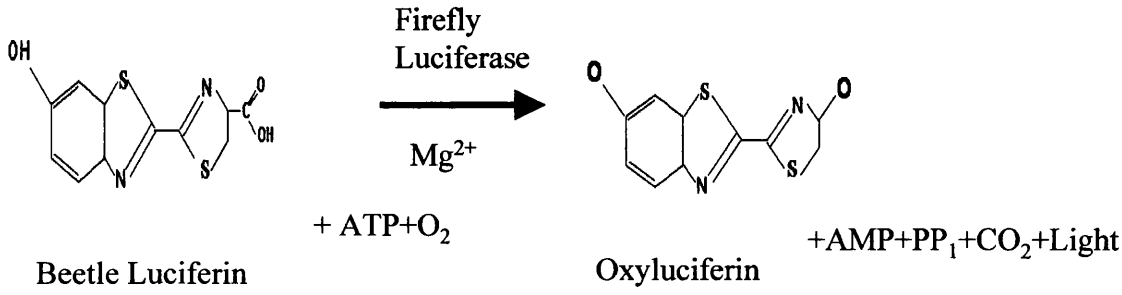


Figure 2.9: The bioluminescent reaction catalyzed by firefly luciferase

growth medium and 100 μl of lysis buffer was added to the cell pellet. The lysate was transferred to an Eppendorf, vortexed briefly and centrifuged at maximum speed for 15 s. A 96 well plate containing 20 μl of test sample per well was put into the TR717 microplate luminometer with injector. The luminometer's injector added 100 μl of luciferase assay reagent per well which was read immediately with a delay time of 2 s and 10 s reading time. Luciferase activity was expressed as relative light units (RLU) / 1 X 10^5 cells.

2.29 Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSA) determines the binding interaction between DNA and DNA-binding proteins. The assay is based on the observation that complexes of DNA and protein move more slowly than unbound DNA fragments or double-stranded oligonucleotides when electrophoresed through a non-denaturing polyacrylamide gel (Garner and Revzin, 1981; Fried and Crothers, 1981). The electrophoretic mobility shift assays is carried out by first radioactively labeling the specific DNA sequence, the protein binding properties of which are being investigated. The DNA-protein complexes are formed when the labeled DNA is incubated with the nuclear extract that contains the DNA binding proteins. The complexes are electrophoresed on a non-denaturing polyacrylamide gel and are visualized by autoradiography.

The competition reactions and/or supershift assay are set up along side of the EMSA to provide evidence of binding specificity and protein identity. In the supershift assay, antibodies which recognize the binding proteins are added to the reaction mixture and this produces even slower migrating complexes than the original DNA-protein complexes. In the competition reactions, unlabeled oligonucleotides are added to the EMSA reactions. Interaction between the binding protein and the unlabelled oligonucleotides will decrease the band intensity of the DNA-protein complexes.

2.29.1 Preparation of Nuclear Extracts

Nuclear extract was prepared based on the method of Dignam *et al* (1983). 10 x hypotonic pre-lysis buffer (100mM HEPES {pH 7.9}, 15mM $MgCl_2$, 100mM KCL) and 10 x pre-extraction buffer (20mM HEPES {pH 7.9}, 1.5mM $MgCl_2$, 0.42M NaCl, 0.2mM EDTA, 25% glycerol) were prepared. Fresh 1 x lysis buffer was prepared by diluting the 10 x pre-lysis buffer and adding complete protease inhibitor cocktail (Roche) and DTT. 20µl protease cocktail and 20µl 0.1M DTT was added to a 2ml lysis buffer. Fresh 1 x nuclear extraction buffer was also prepared by diluting the 10 x pre-extraction buffer and adding protease cocktail and DTT as above. All steps were carried out at 4°C and all solutions/reagents were kept on ice.

The cells were harvested and washed twice with ice-cold PBS by centrifugation at 10,000rpm for 5 min. 1ml of cell lysis buffer was added to the cell pellet and the cells were gently resuspended in the lysis buffer with a pipette. The cell suspension was incubated on ice for 15min and then centrifuged at 10,000rpm for 5min. The supernatant was discarded and the cell pellet was again resuspended in 2 volumes of lysis buffer. To disrupt the cells, the cells were transferred into a 1 ml syringe in the following sequence: a 25G needle was attached to the syringe, and the plunger of the latter was removed. With a 1ml pipette, the cells were transferred to the barrel of the syringe with the needle in a 1.5ml tube. The plunger was gently inserted and the cells were gently pushed through the needle into the 1.5ml tube. The broken cells were sucked up into the barrel of the syringe and rapidly ejected into the tube; this was repeated 20 times. The broken cell suspension was then centrifuged for 20min at 4°C in a microfuge at 10,000rpm. The supernatant was transferred to a fresh tube (cytosolic fraction), while the nuclear fraction was prepared from the pellet (nuclear pellet). The nuclear pellet (~75µl) was resuspended in 2/3 volume of nuclear extraction buffer (~50µl).The nuclei were broken by passing the nuclear

suspension through the needle 20 times as described above. The suspension was then incubated for 30min at 4°C on a shaker and was then centrifuged at 10,000rpm for 5min in a microcentrifuge. The supernatant (nuclear fraction) was collected and transferred to a fresh tube and stored at -80°C.

2.29.2 Preparation of consensus oligonucleotides

Consensus oligonucleotides representing the binding sites for NFkB (5'AGTTGAGGGGACTTTCCCAGGC3') was obtained from Promega and the consensus oligonucleotides representing the binding sites for hypoxia inducible factor (5'CGGTCACACCGTGGCTGA3') was synthesized by Oswel DNA Service (Southampton). All oligonuceotides were used at concentrations of 1.75pmol/µl.

2.29.3 Phosphorylation reaction

Consensus oligonucleotides were 5'-end labelled using T4 polynucleotide kinase (New England Biolabs). T4 polynucleotide kinase catalyzes the transfer of the γ phosphate of ATP to a 5' OH terminus of the DNA. The reaction was set up as follows;

Oligonucleotide (1.75pmol/µl)	2 µl
Polynucleotide kinase buffer (10X)	1 µl
[γ-^{32}P]ATP (3000Ci/mmol)	2 µl
Nuclease-free water	4 µl
T4 PNK (10units/µl)	1 µl

The mixture was incubated for 30min at 37°C. Phosphorylation was terminated by adding 1µl of 0.5 M EDTA, and the volume was made up to 100µl with Tris-EDTA buffer pH 8.0.

2.29.4 Preparation of non-denaturing polyacrylamide gel

Glass plates were thoroughly washed with detergent and rinse with distilled water, then wipe with 70% alcohol. A 6% gel (which was routinely used) was prepared by mixing the following in a clean 50ml Falcon tube

10X TBE	1 ml
30% acrylamide	4 ml
80% glycerol	625 µl
Ammonium persulphate (APS, 10%)	300 µl
Distilled water	14.375 ml
TEMED	20 µl

(TOTAL volume of gel {less APS and TEMED} was 20ml, and used for one 16x20cm slab gel of 0.5mm thickness). The mixture was thoroughly mixed with the aid of a 50ml syringe and transfer to gel casting stand (Protean II xi). The gel was allowed to set for 2 h at room temperature.

2.29.5 DNA Binding Reactions

A 5X binding buffer (50mM Tris-HCl {pH 7.5}, 250mM NaCl, 2.5mM DTT, 2.5mM EDTA, 5mM $MgCl_2$, 250µg/ml polydeoxyinosinic-deoxycytidylic acid, 20% glycerol) was prepared and approximately 10µg of nuclear extract was used for each binding reaction.

In 500µl tube, the following reactions were set up and reagents were added in the order:

1. **Negative control**

Nuclease-free water	7 µl
Gel shift binding buffer	2 µl
Nuclear extract	0 µl

2. Test sample without competitor oligonucleotide

Nuclease-free water	4.5 µl
DNA binding buffer	2.0 µl
Nuclear extract	2.5 µl

3. Test sample with specific competitor

Nuclease-free water	3.5 µl
DNA binding buffer	2.0 µl
Nuclear extract	2.5 µl
Unlabelled competitor	1.0 µl

4. Test sample with non-specific competitor (AP2 oligonucleotide)

Nuclease-free water	3.5 µl
DNA binding buffer	2.0 µl
Nuclear extract	2.5 µl
Unlabelled non-specific competitor	1.0 µl

In the supershift reactions, 1 µl of a polyclonal antibody to NFkB (p65 subunit), was added to reaction 2 and the amount of water added was adjusted accordingly. All reactions were then incubated at room temperature for 20min, after which 1µl of the respective labelled oligonucleotide was added. After a further incubation at the same temperature for 20min, 1µl of 10X loading buffer (250mM Tris-HCl {pH 7.5}, 0.2% bromophenol blue, 40% glycerol) was added to each reaction.

2.29.6 Polyacrylamide gel electrophoresis

The non-denaturing polyacrylamide gel was pre-run for 30min in 0.5X TBE at 250V. The wells were flushed using a plastic Pasteur pipette and the samples were loaded and run at 300V for 2-3 h at room temperature. When the dye-front

was ~3-5cm from the bottom, electrophoresis was stopped. The gel was transferred onto Whatman 3MM filter paper, covered with Saran wrap and dried on a vacuum dryer. The gel was then exposed to X-ray film for 2-12 h at -80°C with an intensifying screen. It was then developed according to standard techniques.

2.30 Statistical analyses

All experiments were performed at least three times. Data are presented as mean ± SEM. Statistical evaluation was performed using one way analysis of variance (ANOVA) followed by paired Student's *t*-test where appropriate. Values of $P<0.05$ were considered statistically significant.

CHAPTER 3:

The Establishment of a Quantitative Competitive Reverse Transcription Polymerase Chain Reaction (QC-RT-PCR) assay for the measurement of Tissue Factor and Tissue Factor Pathway Inhibitor gene expression.

3.1 Introduction

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is a reliable technique for the detection and quantitation of mRNA. The technique consists of two parts, first is the synthesis of cDNA from RNA by reverse transcription (RT), followed by the amplification of the cDNA by polymerase chain reaction.

Quantitation of gene expression requires that an accurate measurement of specific transcript be made. Gene expression can be studied by a variety of techniques including Northern blot analysis, RNA dot blots, in situ hybridization, RT-PCR and ribonuclease protection assay. But out of all RT-PCR is the most sensitive method for studying gene expression, especially genes expressed at low levels.

RT-PCR enables the detection and measurement of very low levels of mRNA which are below the detection limit of other conventional methods. Semi-quantitative or relative RT-PCR is a popular method, but the reliability of results obtained varies considerably. The method is prone to error, when comparing gene expression in different samples, because of the reaction variation in the reverse transcription and/or the amplification steps.

The main caveat of semi-quantitative RT-PCR is the inability to correct for the variation in samples due to tube to tube variability, making the data generated from such approach non-reproducible. The use of constitutively expressed housekeeping gene like GAPDH is only appropriate to correct for errors generated during the quantitation of the template RNA, since samples of equal concentrations should yield equal amounts of PCR products when amplified with GAPDH primers. However, there is no guarantee that the expression of the housekeeping gene is not affected by the experimental treatment. Also, it is very unreliable to compare levels of mRNA transcripts that have been amplified with different primer pairs.

Quantitative competitive RT-PCR is currently the most sensitive way to quantitate specific mRNA. The assay is based on competitive co-amplification of a specific target sequence together with known concentrations of a competitor in one reaction tube. The target is the RNA to be measured, and the competitor is the target modified by the removal of small fragment within it. The ratio of RT-PCR products obtained from the target and the competitor reflects the initial ratio of the target and the competitor in the reaction.

The competitor RNA and the target are amplified by the use of the same primers, but yield PCR products of different lengths. The presence of the competitor RNA in the PCR reaction allows for the quantification of the target and also serves as an internal control for the PCR reaction. When the competitor RNA is present in a RT-PCR reaction, a competition for amplification between the endogenous target and the competitor RNA is created. Both the target and the competitor amplicons compete equally well for primers, nucleotides, and enzyme in the reaction. Quantitation is performed by comparing the PCR signal of the target with the PCR signal obtained with the known concentration of the competitor using the competitor standard curve. The amount of the target RNA is calculated

as the ratio of target PCR product to competitor PCR product multiplied by the amount of competitor RNA in the reaction.

Potgens *et al* (1994) first described a quantitative RT-PCR assay for the measurement of TF mRNA. In their study, they employed a two-step quantification technique based on combination of PCR and Southern blotting. Due to the poor specificity of the primers they used for the PCR amplification, there was a need for the blotting of the RT-PCR products onto a membrane and subsequent hybridization with radiolabelled TF probe.

The first objective of the project was to establish a specific, sensitive, quantitative and reliable method for detecting and analyzing Tissue Factor (TF) and Tissue Factor Pathway Inhibitor (TFPI) genes. Hence, a quantitative competitive RT-PCR (QC RT-PCR) system for the measurement of TF and TFPI messenger RNA levels in cells was developed. TF and TFPI competitor RNAs for the QC RT-PCR were designed, transcribed *in vitro* and carefully quantified. For each competitor RNA a single standard curve was constructed from a serial dilution of the competitor RNA. This curve was used as a standard with which the data from test samples were analyzed.

A critical factor in RT-PCR is the selection of appropriate primers. Primers for TF and TFPI amplification were carefully designed for maximal efficiency and specificity. The primers also amplify PCR products across intron-exon junctions so that any contaminating genomic DNA in the QC-RT-PCR reaction would yield a PCR product differing in size from the target and the competitor RNA.

It has been proved (Zhang *et al*, 1997) that the PCR amplification efficiency of reverse transcribed target RNA and competitor RNA in the same reaction depends upon the ratio of the number of target RNA copies to competitor RNA copies, rather than the ratio of sizes of target RNA / competitor RNA or the ratio

of the amount of total RNA to competitor RNA. Nevertheless, tests were carried out to verify whether the designed competitor RNAs could actually act as an optimal competitor for the targets. A comparison between the amplification kinetics of the target RNA and the competitor RNA for each gene was made and there was comparable amplification kinetics for both target and competitor pairs.

3.2 Results

3.2.1 TF primer design

Selection of appropriate primers for maximal efficiency and specificity is a critical factor in RT-PCR. Primers for TF amplification (table 3.1) were 5'-TCGATCTCGCCGCCAACTGGTAGACATGGAGACC-3' (forward) and 5'-TTCAAAACTCTGAATTGTTGGCTGTCCGAG-3' (reverse). They were selected according to nucleotides spanning exon 1 and exon 4 (99 to 8525-bp region) of the TF gene sequenced by Mackman *et. al.* (1989). RT-PCR of the TF mRNA with these primers resulted in a 470bp-size fragment, corresponding to amino acids 32 through 117 in the mature TF protein. The competitor gave a 230bp-size fragment (figure 3.1a) while any contaminating genomic DNA would result in about 8 kb size fragment.

3.2.2 TFPI primer design

Specific primers for TFPI amplification 5'-GATTCTGAGGAAGATCAAGAA-3' (forward) and 5'-TCACATATTTTTAACAAAAAT-3' (reverse) were designed to amplify the 217- to 1047-bp region of the TFPI gene sequenced by Tze-Chein Wun *et.al.* (1988). RT-PCR of the TFPI mRNA with these primers resulted in a 830 bp size fragment (figure 3.1b), corresponding to amino acids 29 through 304 in the mature TFPI protein. The competitor resulted in a 705-bp size fragment and any contaminating genomic DNA would result in about 7 kb size fragment.

3.2.3 Construction of TF competitor RNA

Tissue factor competitor RNA for the QC-RT-PCR was designed from the plasmid pTF1 (kindly provided by Dr. Robert M. W. de Waal, Netherlands). The plasmid pTF1 was generated by cloning a 1.1 Kb TF cDNA, comprising the

Table 3.1a. Specific primers for tissue factor amplification.

Forward primer	5'-TCGATCTCGCCGCCAACTGGTAGACATGGAGACC-3'
Reverse primer	5'-TTCAAAACTCTGAATTGTTGGCTGTCCGAG-3'

Table 3.1b. Specific primers for tissue factor pathway inhibitor amplification.

Forward primer	5'-GATTCTGAGGAAGATGAAGAA-3'
Reverse primer	5'-TCACATATTTTTAACAAAAAT-3'

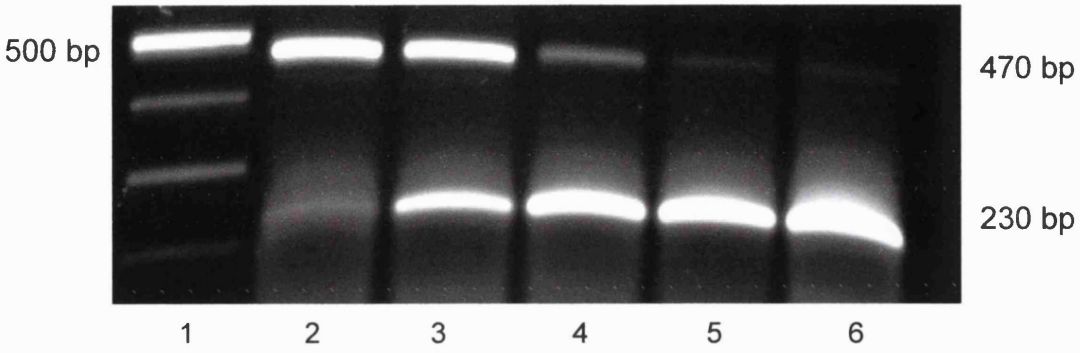


Figure 3.1a QC- RT-PCR of tissue factor.
Lane 1 is the molecular weight marker and lanes 2-6 are the QC-RT-PCR reactions. The top bands are the target amplicons (470bp) and the bottom bands (230bp) are the competitor amplicons.

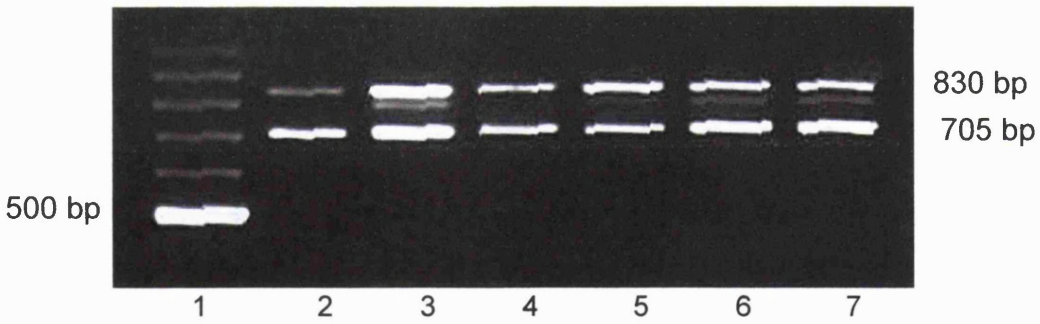


Figure 3.1b QC- RT-PCR of tissue factor pathway inhibitor.
Lane 1 is the molecular weight marker and lanes 2-7 are the QC-RT-PCR reactions. The top bands are the 830bp target amplicons and the bottom bands are the 705bp competitor amplicons.

complete human tissue factor protein coding region, into the Sma I site of the plasmid - pUC 19.

The manipulation involved in the construction of the TF competitor RNA is outlined in figure 3.2.

3.2.3.1 Cloning and modification of TF cDNA

The plasmid pTF1 containing the full length TF was digested (see section 2.17.1) with Bbs I, a restriction enzyme that cuts closely twice within the 1.1 Kb TF cDNA removing a 245bp fragment. The restriction enzyme digest was analyzed by running it on agarose gel (see section 2.14). Figure 3.3a shows the agarose gel analysis of the restriction enzyme digestion. The modified plasmid- pTFm1 was cut out of the agarose gel using a sterile scapel blade and extracted from the gel using siliconised glass wool (see section 2.15). Figure 3.3b shows the schematic diagram of the pTFm1 having non compatible protruding ends. The 5' protruding ends of the pTFm1 were filled with dNTPs by the polymerase activity of T4 DNA polymerase (see section 2.26.2). The plasmid was religated using T4 DNA ligase (see section 2.17.2), yielding a modified plasmid pTFm1 with a 855bp modified TF cDNA.

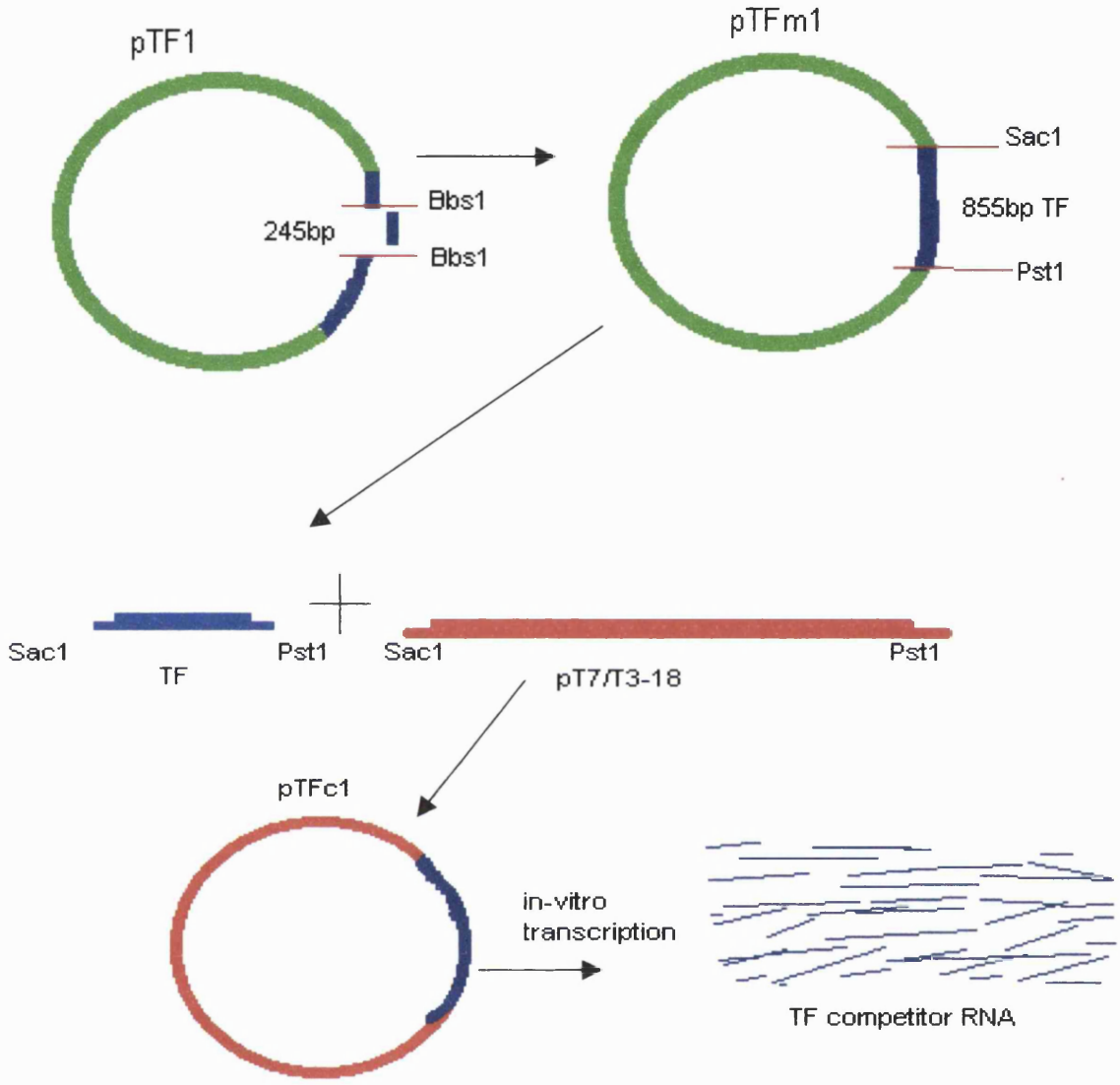


Figure 3.2. The construction of TF competitor RNA.
The plasmid pTF1 was digested with Bbs I, a restriction enzyme that cuts closely twice within the 1.1 Kb TF cDNA removing a 245bp fragment, yielding a modified plasmid pTFm1 with a 855bp TF cDNA insert. For the modified TF cDNA to be transcribed to a competitor RNA it has to be cloned into a transcription vector that can transcribe RNA. Hence, the 855bp insert of pTFm1 was recloned into vector pT7/T3-18 yielding the plasmid pTFc1.
The TF competitor RNA was transcribed *in vitro* from the plasmid pTFc1.

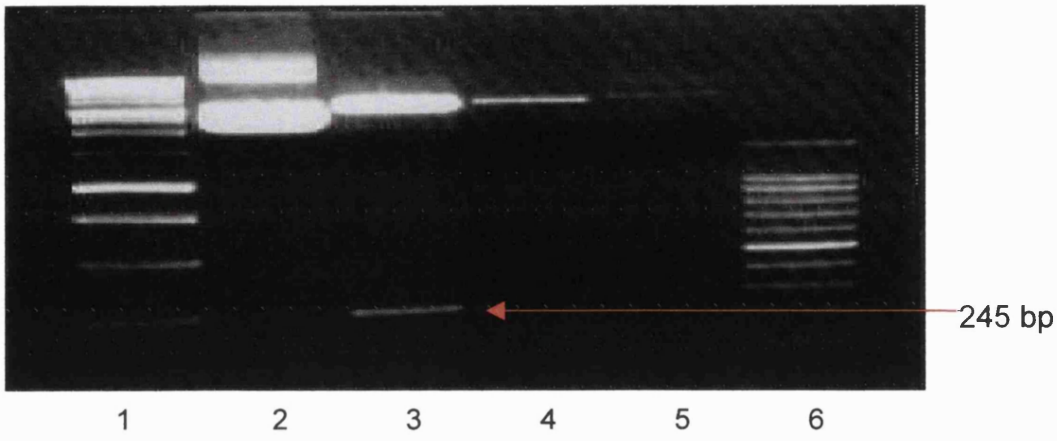


Figure 3.3a: Agarose gel electrophoresis of restriction enzyme digestion analysis of pTF1. Lane (1) 1Kb DNA ladder; Lane (2) uncut pTF1; lane (3) pTF1 digested with Bbs1, releasing a 245bp DNA fragment; lane (4) pTFm1 after gel extraction and purification; lane (6) 100bp DNA ladder.

5'-CCTGCTC GCACGGGTC-3'

3'-GGACGAGCCGA CCCAG-5'

Figure 3.3b: Schematic diagram of pTFm1 with non-compatible protruding ends

This result is a representative of at least 4 independent experiments .

For the modified TF cDNA to be transcribed to a competitor RNA it has to be clone into a transcription vector (pT7/T3-18) that can transcribe RNA. The modified plasmid pTFm1 was cut with the restriction enzymes Sac 1 and Pst 1 to release the 855bp modified TF cDNA insert. The restriction enzyme digest reaction was electrophoresed on agarose gel (Figure 3.4) and the modified TF cDNA (855bp) was excised from the gel and purified. The transcription plasmid pT7/T3-18 was cut with Sac 1 and Pst 1 and the 855bp modified TF cDNA was ligated (see section 2.17.2) into the plasmid pT7/T3-18 yielding the plasmid pTFc1. The plasmid was sequenced, using T7 sequenase version 2.0 DNA sequencing kit (see section 2.23.1), to confirm the presence and the orientation (5'-3') of the modified TF cDNA.

3.2.3.2 *In vitro* transcription of TF competitor RNA

The TF competitor RNA was transcribed *in vitro* from the plasmid pTFc1 (see section 2.24). The plasmid pTFc1 was linearized with the restriction enzyme Sac 1. The restriction enzyme digest was analyzed on agarose gel to confirm complete linearization. Since Sac 1 produces 3' protruding ends and extraneous transcripts have been reported to appear in addition to the expected transcript when such templates are transcribed, the linearized template ends were converted to blunt ends with Klenow DNA polymerase (see section 2.26.2).
Prior to transcription the linearized plasmid was treated with proteinase K, phenol -chloroform extracted (see section 2.16) and precipitated with ethanol (see section 2.17). And after the transcription, the DNA template was removed by treatment with RNase-free DNase (see section 2.25). The transcribed RNA was extracted with acid phenol: chloroform, precipitated with ammonium acetate/ethanol and washed with 70% ethanol. The RNA was resuspended in water, quantified by spectrophotometry (see section 2.8.4) and stored in a -70°C freezer. An aliquot of it was analyzed on agarose gels (see section 2.12.1) to confirm the integrity and the correct length of the competitor RNA.

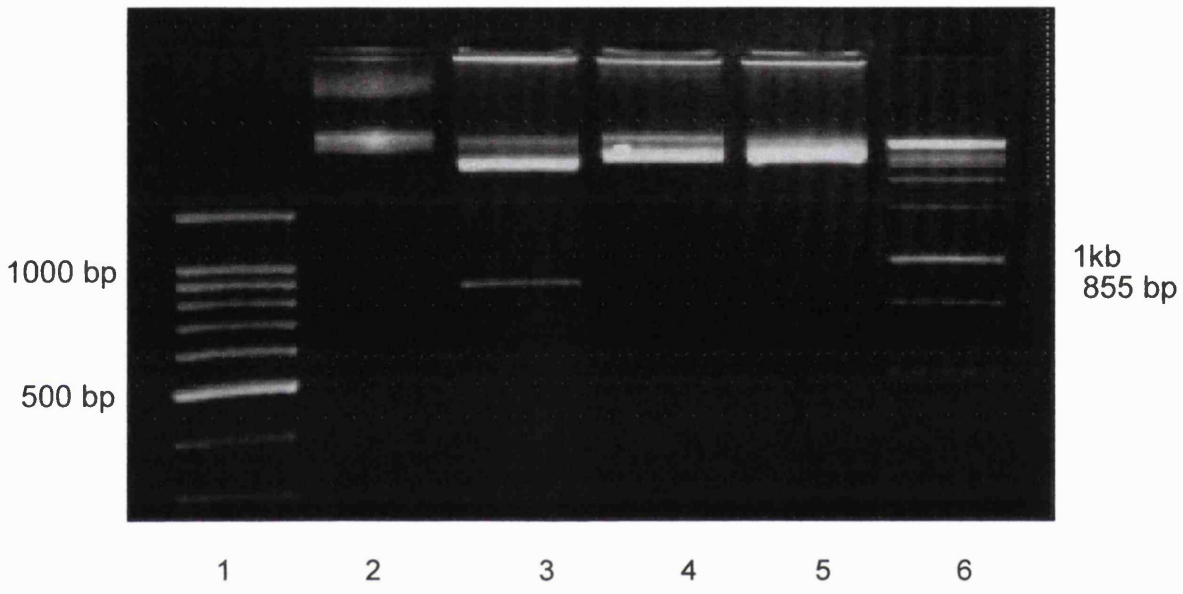


Figure 3.4: Agarose gel analysis of the restriction enzyme digestion of pTFm1 with Sac1 and Pst-1. Lane (1) 100bp DNA ladder; lane (2) uncut pTFm1; lane (3) pTFm1 cut with Sac1 and Pst1 generating a 855bp modified TF cDNA; lane (4) pTFm1 cut with Sac1 only; lane (5) pTFm1 cut with Pst1 only and lane 6 is the 1kb DNA ladder.

This result is a representative of at least 4 independent experiments.

Precautions were taken to avoid introducing RNase contamination into the competitor RNA (see section 2.9). In a typical *in vitro* transcription reaction, 1μg of the plasmid pTFc1 yielded 500ng of the competitor RNA. The ratio of the spectrophotometric absorbance taken at 260nm and 280nm (A260 / A280) was in the range of 1.8-2.0. When the competitor RNA was resolved on denaturing formaldehyde agarose gel (figure 3.5a) and on DNA agarose gel (figure 3.5b), a single band of the right molecular weight was observed (~900bp).

3.2.4 Construction of TFPI competitor RNA

TFPI competitor RNA was designed from the full-length TFPI cDNA. The manipulation involved in the construction of the TFPI competitor RNA is outlined in figure 3.6. The full-length TFPI cDNA (830bp) was prepared by reverse transcription and amplified by the polymerase chain reaction. The DNA was cloned into a T-vector plasmid (see section 2.13) yielding the plasmid pTFPI. The 830bp TFPI DNA inserts from pTFPI were cut out and recloned into the transcription vector pT7/T3-18 generating the plasmid mpTFPI. The plasmid mpTFPI was digested consecutively with single site-cutting restriction enzymes Cla 1 and Nsi 1, leaving out a 125bp fragment; the plasmid was religated yielding plasmid cpTFPI.
The TFPI competitor RNA was transcribed *in vitro* from the plasmid cpTFPI.

3.2.4.1 RNA isolation and RT-PCR of TFPI mRNA

Total RNA was isolated from ECV 304 cells. The RNA concentration was determined spectrophotometrically by measuring the absorbance at 260nm (section 2.8.4). The integrity of the RNA was checked by denaturing –agarose gel electrophoresis (section 2.12.1). Figure 3.7 shows the gel analyses of aliquots of total RNA samples.

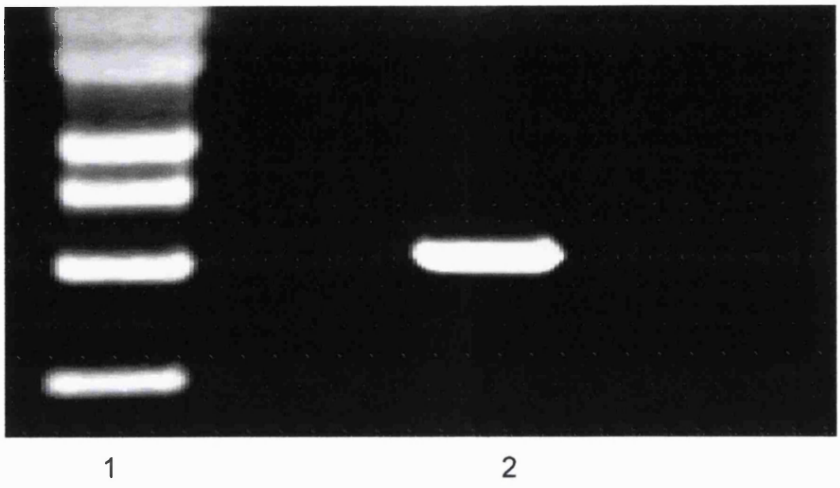


Figure 3.5a: TF competitor RNA on denaturing formaldehyde agarose gel. The first lane is the RNA molecular weight marker, lane 2 is the TF competitor RNA.

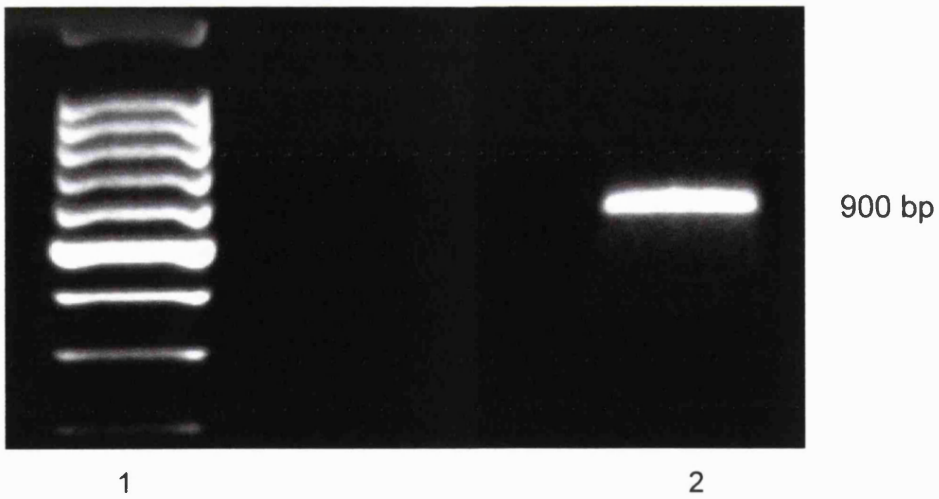


Figure 3.5b TF competitor RNA on agarose gel. Showing the absence of any genomic DNA. The first lane is the 100bp DNA molecular weight marker and lane 2 the TF competitor RNA.

Each result is a representative of at least 4 independent experiments.

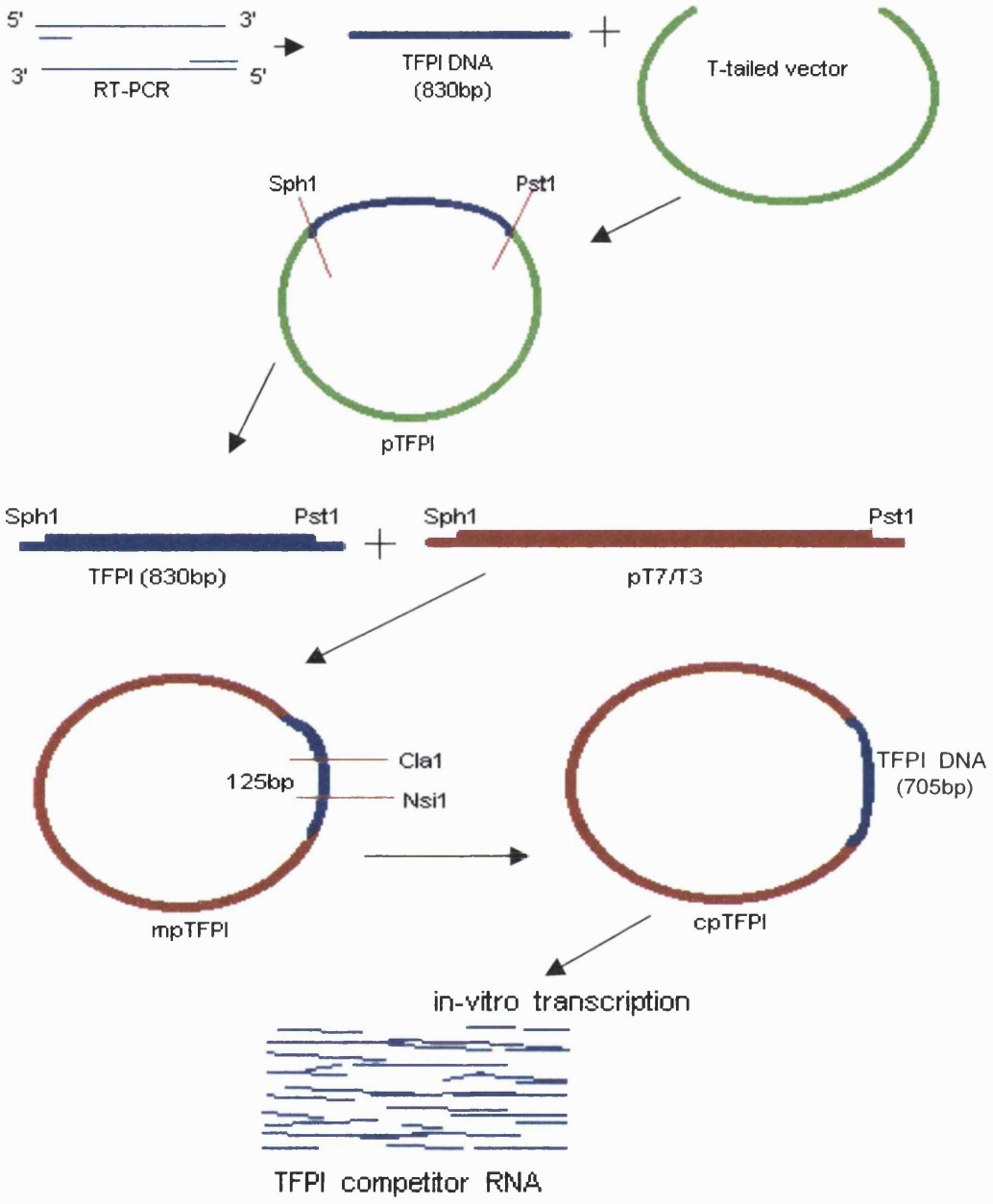


Figure 3.6: The construction of TFPI competitor RNA.
The full-length TFPI cDNA was prepared by RT-PCR. The 830bp TFPI DNA was cloned into T-vector plasmid yielding the plasmid pTFPI. The TFPI DNA inserts from pTFPI were cut out and recloned into the transcription vector pT7/T3-18 generating the plasmid mpTFPI. The plasmid mpTFPI was digested consecutively with single site-cutting restriction enzymes Cla 1 and Nsi 1, leaving out a 125bp fragment; the plasmid was religated yielding plasmid cpTFPI. The TFPI competitor RNA was transcribed *in-vitro* from the plasmid cpTFPI.

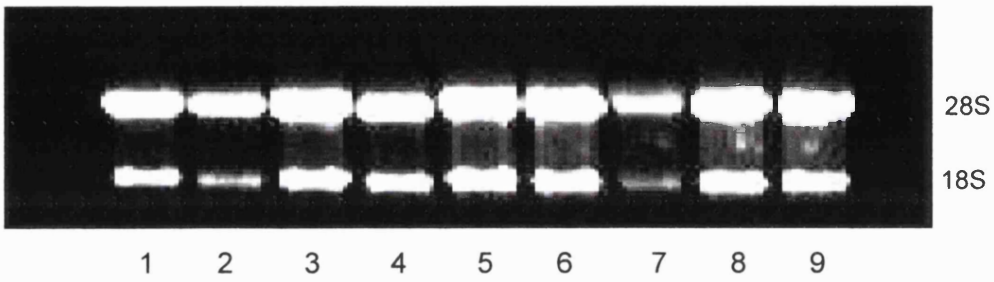


Figure 3.7: Picture of denaturing agarose gel electrophoresis of total RNA isolated from ECV 304 cells. Lanes 1-9 are aliquots from different total RNA preparations. 18S and 28S ribosomal RNA are labelled.

This result is a representative of at least 4 independent experiments .

The presence and the sizes of the two bands, corresponding to 28S and 18S ribosomal RNA, indicated the absence of RNA degradation usually caused by RNase contamination.

Full-length TFPI cDNA was prepared by reverse transcription- polymerase chain reaction (see section 2.10) using the specific primers for TFPI (table 3.1b). The RT-PCR reaction, containing the amplified TFPI DNA was analysed on agarose gel (figure 3.8a). The presence of the 830bp TFPI DNA fragment indicated the success of the RT-PCR.

3.2.4.2 Cloning of the full length TFPI cDNA.

The T-vector system provides an efficient system for the cloning of PCR products. The T-vector has a 3'-T overhang at its insertion site while the PCR product, amplified by Taq DNA Polymerase, has a template-independent single adenosine (A) at its 3'end. The full length TFPI cDNA was ligated into T-vector plasmid yielding the plasmid pTFPI. Subsequent to the ligation, the plasmid pTFPI was sequenced using T7 sequenase version 2.0 DNA sequencing kit (see section 2.23.1) to ensure that the TFPI cDNA was inserted into the vector in the correct orientation.

The TFPI cDNA was excised from the T-vector using the restriction enzymes Sph 1 and Pst 1 and electrophoresed on agarose gel (Figure 3.8b). The TFPI cDNA was extracted from the gel, purified and subsequently ligated into plasmid pT7/T3-18, which had been linearised by Sph 1 and Pst 1, generating the plasmid mpTFPI.

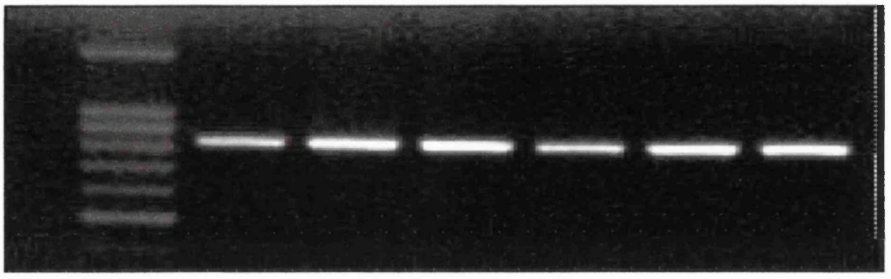


Figure 3.8a: Agarose gel analysis of TFPI DNA amplified by RT-PCR, showing the 830bp TFPI DNA.

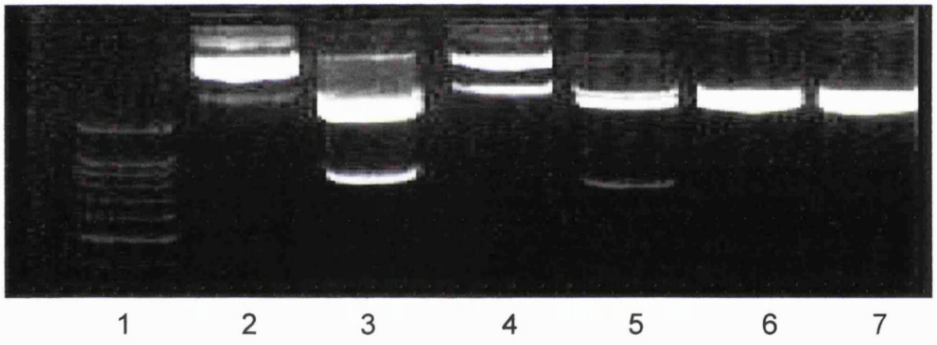


Figure 3.8b: Gel analysis of the restriction enzyme digest of pTFPI and pmTFPI. Lane (1) 100bp DNA ladder; lane (2) uncut plasmid pTFPI; lane (3) pTFPI digested with Sph1 and Pst1 showing the 830bp TFPI cDNA insert; lane (4) uncut pmTFPI; lane (5) pmTFPI digested with Sph1 and Pst1 showing the 705bp modified TFPI cDNA insert; lane (6) pmTFPI digested with Sph1 alone; lane (7) pmTFPI digested with Pst1 alone

Each result is a representative of at least 4 independent experiments.

The plasmid mpTFPI was digested consecutively with single site-cutting restriction enzymes Cla 1 and Nsi 1, leaving out a 125bp DNA fragment (figure 3.9a). Cutting the plasmid with the two enzymes resulted in the plasmid having non-compatible ends. Figure 3.9b shows the schematic diagram of the mpTFPI with non-compatible protruding ends. The 5' protruding end generated by Cla 1was filled with dNTPs and the 3' overhang produced by Nsi 1 was converted to blunt end using Klenow DNA polymerase 1 and T4 DNA polymerase (see section 2.26). The plasmid was religated yielding plasmid cpTFPI with a 705bp TFPI cDNA insert.

3.2.4.3 *In vitro* transcription of TFPI competitor RNA

The TFPI competitor RNA was transcribed *in vitro* from the plasmid cpTFPI (see section 2.24). The plasmid cpTFPI was linearized with the restriction enzyme Hind 111. The restriction enzyme digest was analyzed on agarose gel (see section 2.14) to confirm complete linearization. Prior to transcription the linearized plasmid was treated with proteinase K, extracted with phenol : chloroform (see section 2.16) and precipitated with ethanol (see section 2.17). After the transcription, the DNA template was removed by treatment with RNase-free DNase (see section 2.25). The transcribed RNA was extracted with acid phenol: chloroform, precipitated with ammonium acetate/ethanol and washed with 70% ethanol. The RNA was resuspended in water, quantified by spectrophotometry (see section 2.8.4) and stored at -70°C. An aliquot of this RNA was analyzed on an agarose gel to confirm the integrity and the correct length of the competitor RNA.

Great care was taken to avoid introducing RNase contamination into the competitor RNA (see section 2.9). The ratio of the spectrophotometric absorbance taken at 260nm and 280nm (A260 / A280) was in the range of 1.8-2.0.

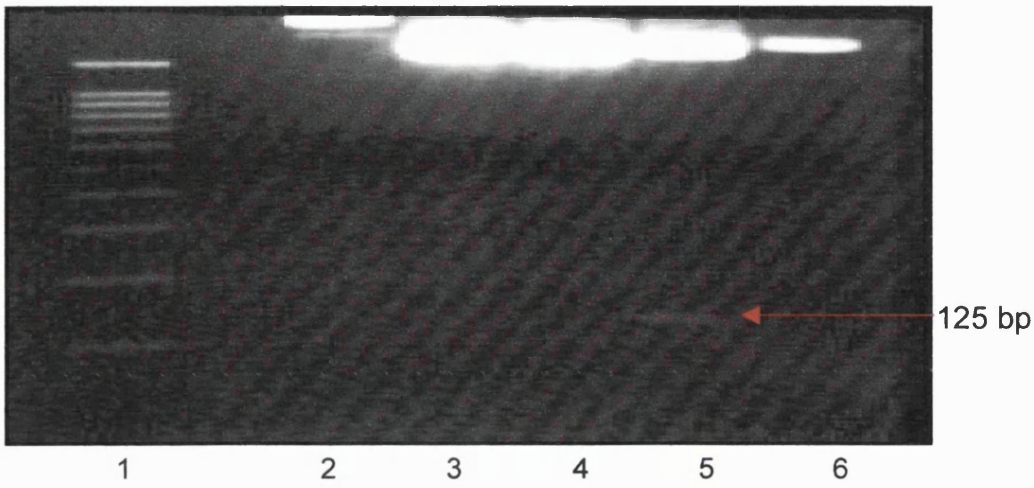


Figure 3.9a: Restriction enzyme digest of plasmid pTFPI with Cla1 and Nsi. Lane (1) 1Kb DNA ladder; lane (2) uncut plasmid pTFPI; lane (3) plasmid pTFPI cut with Cla1 alone; lane (4) plasmid pTFPI cut with Nsi alone; lane (5) plasmid pTFPI cut with both Cla1 and Nsi, leaving out a 125bp DNA fragment; lane (6) mpTFPI after gel extraction and purification.

5'-TTATGCA CGAT-3'
3'-AAT TA-5'

Figure 3.9b: Schematic diagram of mpTFPI with non-compatible protruding ends

This result is a representative of at least 4 independent experiments.

When the competitor RNA was resolved on denaturing formaldehyde agarose gel (figure 3.10) a single band of the correct molecular weight (~750bp) was observed.

3.2.5 Standard curves

Quantitation of gene expression in QC RT-PCR was performed by comparing the PCR signal of the target with the PCR signal obtained with the known concentration of the competitor RNA using a standard curve generated from the serial dilutions of the competitor RNA.

3.2.5.1 Generating a standard curve for TF

The standard curve for TF competitor RNA was constructed from a dilution series of the competitor. Varying amounts (1, 10, 100, 1000 and 10000pg) of the TF competitor RNA were amplified using 28 PCR cycles. PCR products were analyzed by electrophoresis on a 2% agarose gel. The DNA bands were photographed using a Polaroid camera and the intensity of each band (Figure 3.11a) was determined using the Alpha imager. A graph of the integrated density values (IDV) against the amount of the competitor (log10) was generated (Figure 3.11b). There was a linear correlation; the amount of PCR product is proportional to the initial amount of competitor RNA in the sample.

3.2.5.2 Generating a standard curve for TFPI

A 10-fold serial dilution (0.1, 1, 10, 100, 1000 and 10000pg) of the TFPI competitor RNA was reversed transcribed and amplified. The PCR products were resolved on agarose gel (figure 3.12a) and the intensities of the bands were determined.

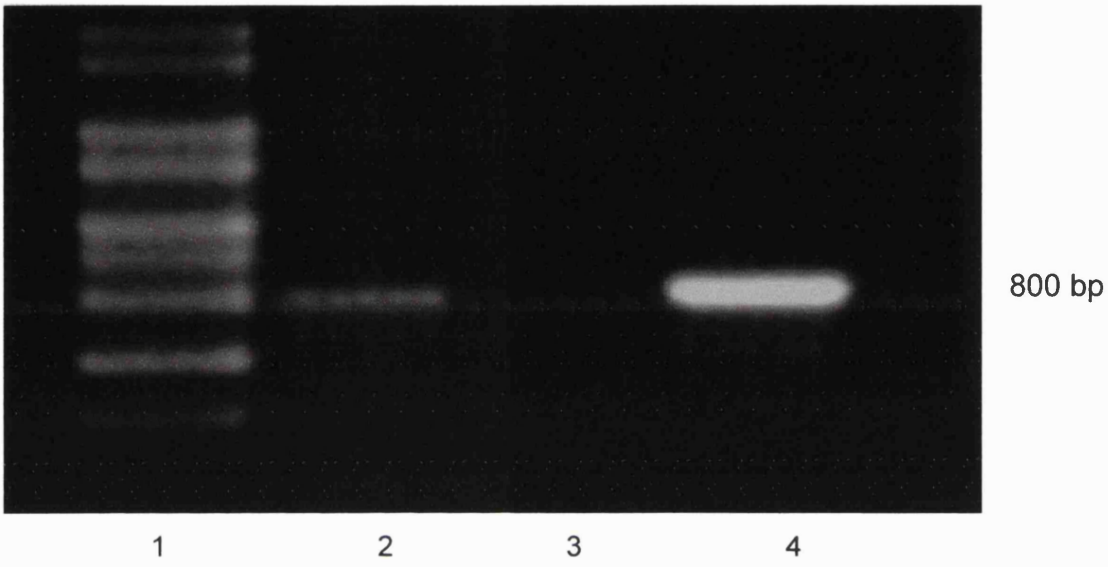


Figure 3.10: TFPI competitor RNA on denaturing formaldehyde agarose gel.
Lane 1 is the RNA molecular weight marker while lanes 2 and 4 are aliquots of the TFPI competitor RNA (different concentrations). Lane 3 is empty.

This result is a representative of at least 4 independent experiments.

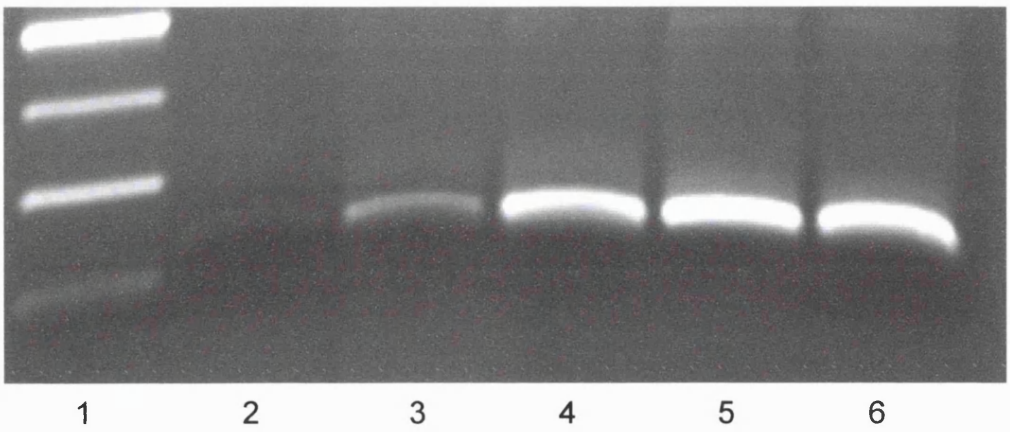


Figure 3.11a: RT-PCR of the serial dilution of TF competitor RNA.
Lane 1 is the molecular weight marker and the brightest band correspond to 500bp fragment. Lanes 2-6 are 1pg, 10pg, 100pg, 1000pg and 10000pg competitor RNA consecutively.

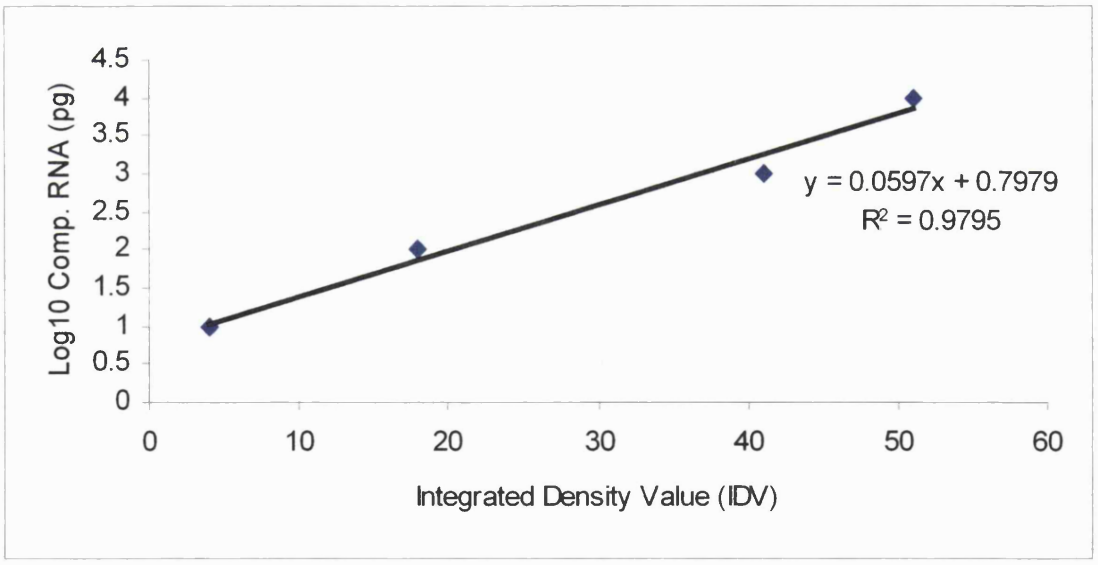


Figure 3.11b: Standard curve for TF competitor RNA.
A typical standard curve was constructed from a dilution series of the competitor RNA. 1pg - 10000pg of the TF competitor RNA were amplified using 28 PCR cycles and the logarithm of the RNA was plotted against the integrated density value.

Each result is a representative of at least 4 independent experiments.

The base 10 logarithms of the amount of competitor RNA were plotted against the integrated density values (IDV) and a linear plot was obtained as expected (figure 3.12b).

3.2.6 Determination of the optimal conditions for the QC-RT-PCR

3.2.6.1 Titration experiment for the determination of the optimal amount of template RNA for QC-RT-PCR of TF gene

Titration experiments were carried out to determine the optimal amount of total RNA to be used for the QC-RT-PCR of TF gene. In this study, 100μg of competitor RNA and various amount (1, 2.5, 5, 10, and 15μg) of total RNA was amplified by RT-PCR in a single tube using RT-PCR beads from Pharmacia (see section 2.10).

Higher input of the total RNA gave more of the target's PCR product relative to the competitor's PCR product. The ratio of the target to the competitor increased with a higher input of total RNA. The plot of the ratio of the target product / the competitor product against the amount of the total RNA used gave a linear curve as expected (figure 3.13a).

The amount of TF mRNA in the total RNA used in this experiment was determined from 3 points on the plot, using the constructed TF standard curve. When 2.5 μg total RNA was used, the calculated amount of TF mRNA was 5.28 pg/μg total RNA. The value was 4.49 pg/μg when 5 μg total RNA was used and 5.23 pg/μg with 10 μg total RNA. The mean value was 5 pg/μg total RNA, with a standard deviation of 0.44. This experiment also confirmed that QC-RT-PCR could be used to measure TF mRNA levels reproducibly.

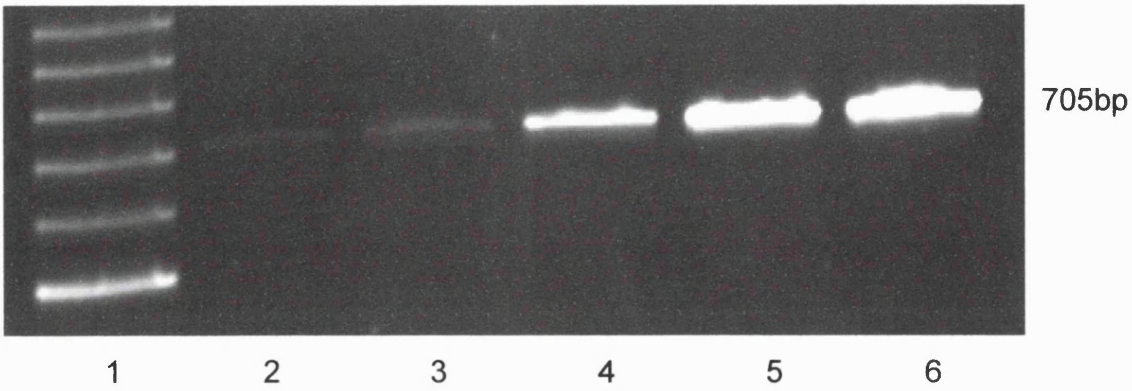


Figure 3.12a: RT-PCR of the serial dilution of TFPI competitor RNA.
Lane 1 is the molecular weight marker and the brightest band correspond to 500bp fragment. Lanes 2-6 are 1pg, 10pg, 100pg, 1000pg and 10000pg competitor RNA consecutively.

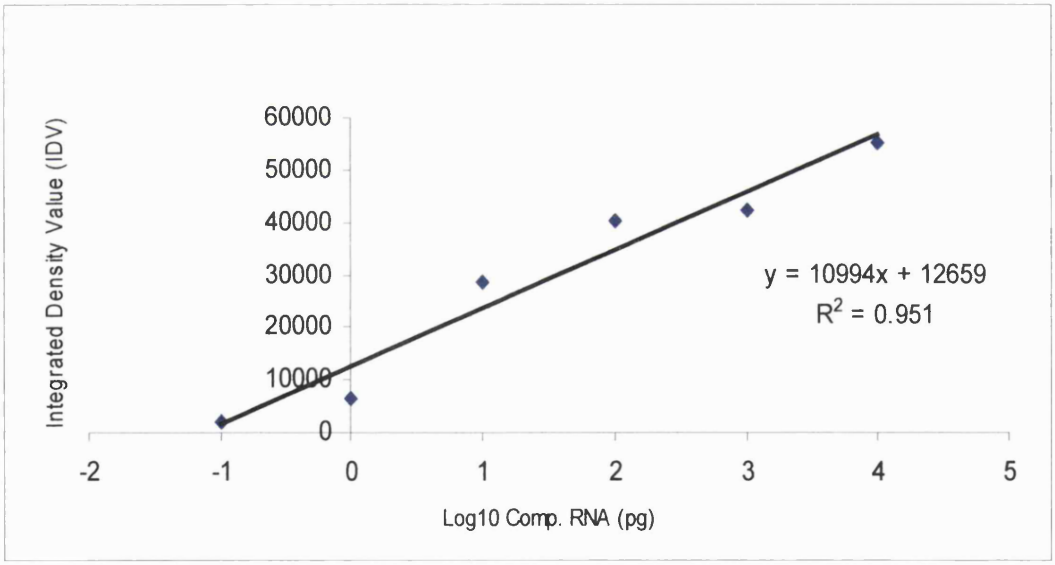


Figure 3.12b: Standard curve for TFPI competitor RNA.
A typical standard curve was constructed from a 1pg - 10000pg dilution series of the competitor RNA. The logarithm of the amount of RNA was plotted against the IDV.

Each result is a representative of at least 4 independent experiments.

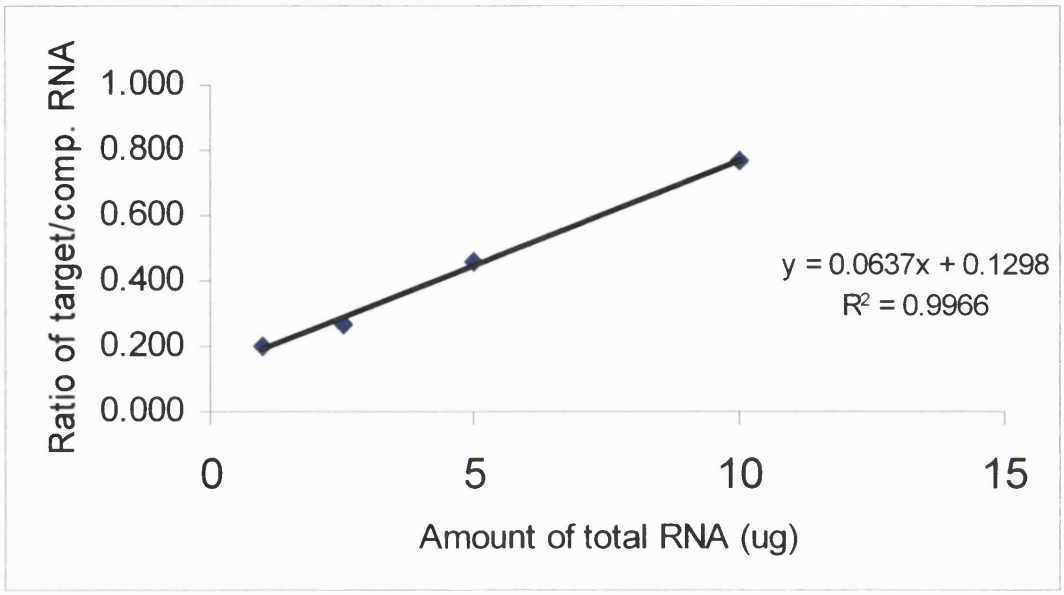


Figure 3.13a: The ratio of target:competitor RNA against amount of total RNA used over a linear range.

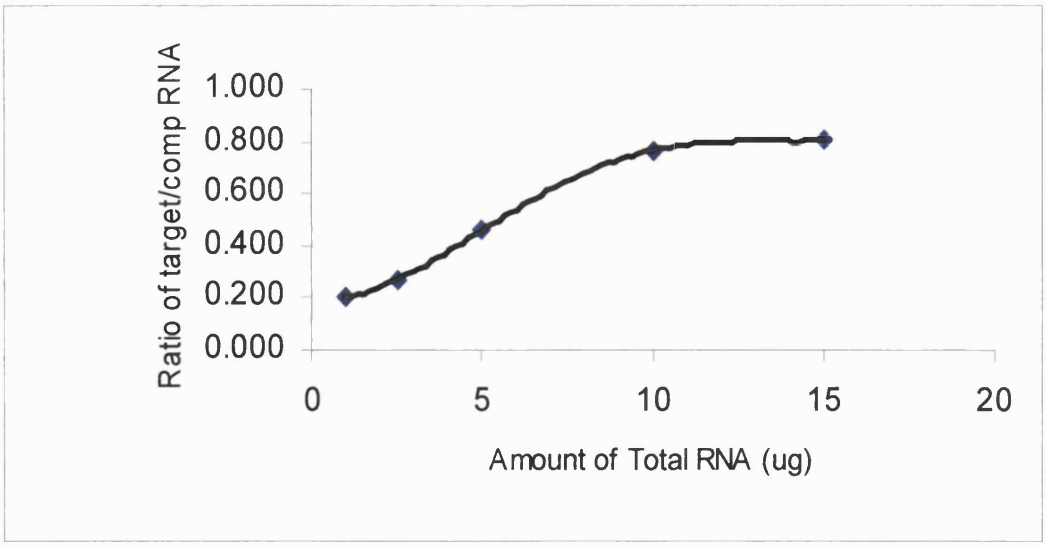


Figure 3.13b: The plot of the ratio of target:competitor RNA against amount of total RNA used over an extended range.

Each result is a representative of at least 4 independent experiments.

In this experiment the appropriate amount of total RNA for the QC-RT-PCR was determined. When less than 2.5μg total was used for the QC-RT-PCR, the target RNA band was below the detection limit on the agarose gel. Also when more than 10μg was used, the amplification was not proportional and saturation occurred (figure 3.13b). The amount of total RNA for QC-RT-PCR for TF was fixed at 3 or 5μg.

3.2.6.2 Determination of the optimal amount of template RNA for QC-RT-PCR of TFPI gene

In this study, varying amounts of total RNA (0.5, 1, 2, 4 and 8μg) were mixed with fixed (10pg) amounts of competitor RNA and amplified by RT-PCR. The RT-PCR products were analyzed on agarose gel (figure 3.14a). Band intensities were determined and the data were plotted as a ratio of the target product / the competitor product against the amount of the total RNA (figure 3.14b). When amount of total RNA was increased from 0.5μg to 8μg, the increase in PCR products was linear.

The appropriate amount of total RNA for the QC-RT-PCR was determined. The amount of total RNA for QC-RT-PCR for TFPI was fixed at 1μg. However, the cell line THP-1 (see section 2.4.3) expresses very low copies of TFPI mRNA. Hence the amount of total RNA from this cell line was fixed at 5μg.

3.2.6.3. Determination of the optimal amount of the TF competitor RNA for QC-RT-PCR reaction

The competitor RNA added to the QC-RT-PCR reaction acted as an internal control and it also allowed the quantitation of the target RNA. To determine the optimal amount of the competitor for the assay, a 10 fold serial dilution of the

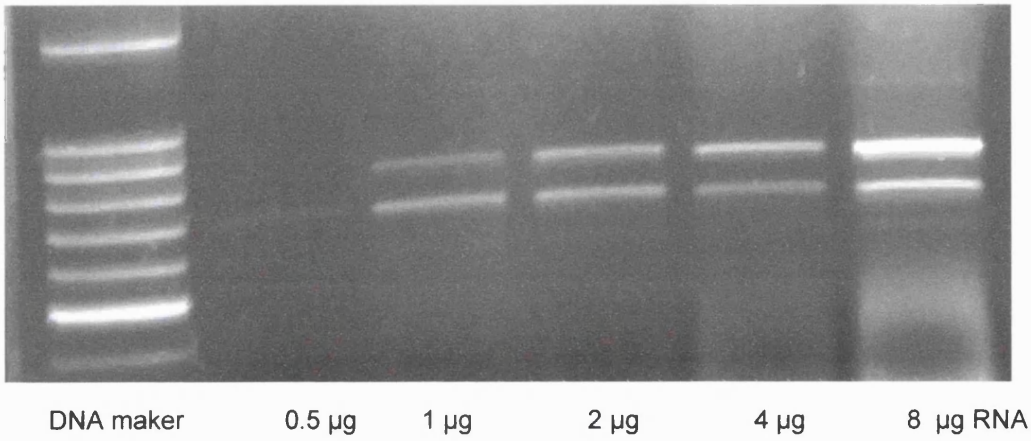


Figure 3.14a: Gel analysis of QC-RT-PCR with various amount of total RNA. Total RNA (0.5, 1, 2, 4 and 8µg) and 10pg TFPI competitor RNA were reverse transcribed and amplified by PCR.

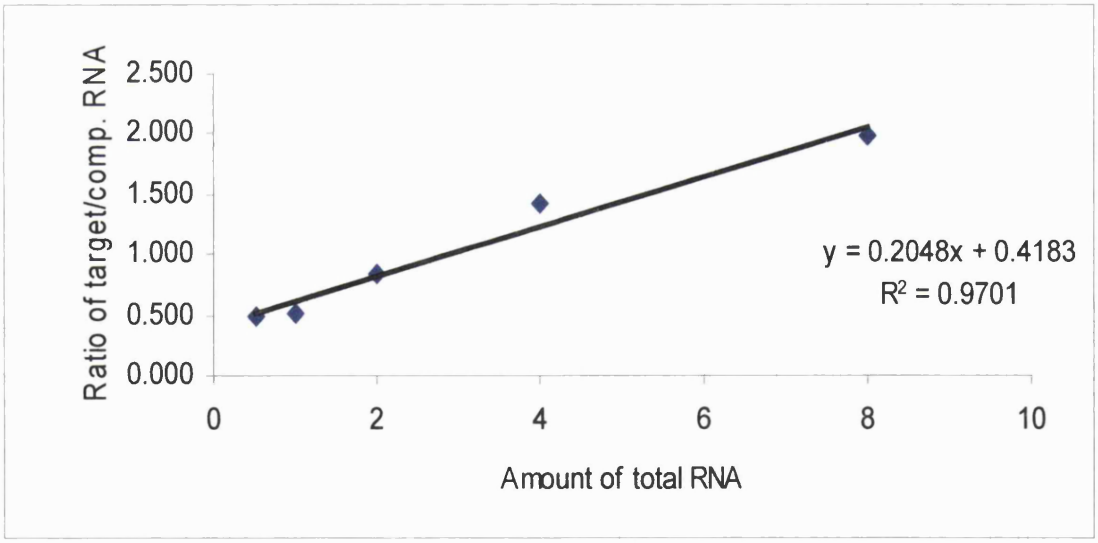


Figure 3.14b: QC-RT-PCR of TFPI with various amounts of total RNA. Band intensities of the picture above (fig.10a) were determined and the data was plotted as a ratio of the target product / the competitor product against the amount of the total RNA in the reactions. There was an exponential increase in PCR product with the increase in the amount of total RNA.

Each result is a representative of at least 4 independent experiments .

competitor RNA (10, 100, 1000, 5000 and 10000pg) containing a fixed amount of the total RNA (5μg) was reversed transcribed and amplified.

Following RT-PCR, aliquots of the RT-PCR samples were electrophoresed on the agarose gel. Figure 3.15a shows the agarose gel profile of the amplified products. Higher input of the comp RNA yielded more of the 230 bp competitor PCR product relative to the 470bp target PCR product. The base 10 logarithms of the ratios of the competitor to the target were plotted against the logarithms of the amount of competitor RNA added, as shown in Figure 3.15b. As expected, the plot was linear.

The optimal amount of competitor RNA used for the QC-RT-PCR was 100pg. This was close to the amount of the competitor RNA around the equivalence point, that is the point were the logarithm of the ratio of target to competitor is equal zero.

3.2.6.4 Determination of the optimal amount of the TFPI competitor RNA for QC-RT-PCR

To determine the optimal amount of TFPI competitor RNA for the QC-RT-PCR assay, 10 fold serial dilutions of the competitor RNA (1, 10, 100, 1000 and 10000pg) were mixed with 1μg total RNA and subjected to RT-PCR. The PCR products were resolved on agarose gel and the intensities of the bands were determined. The plot of the logarithms of the ratio of the target to competitor RNA against the logarithms of the amounts of the competitor RNA in the reaction is shown in figure 3.16a. When 10000pg competitor RNA was included in the reaction, the amplification was not exponential and saturation occurred.

The initial amount of target TFP1 mRNA is the point on the graph where the amount of product from the target is equal to that of the competitor.

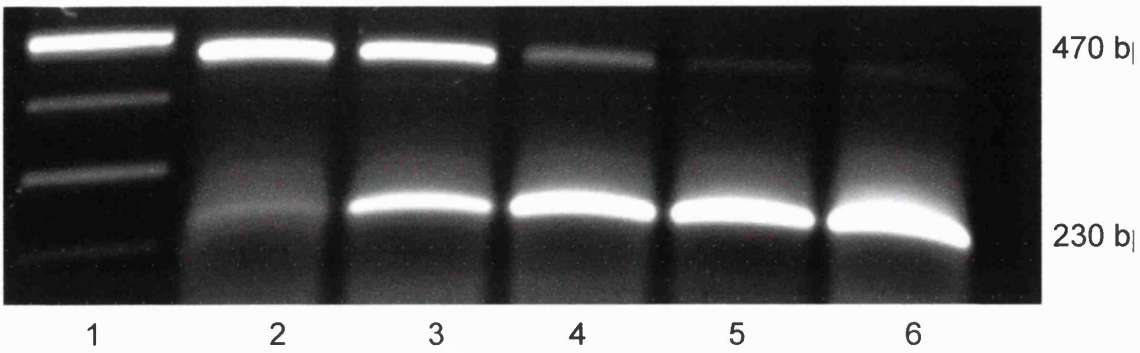


Figure 3.15a: QC-RT-PCR of TF using a fixed amount of total RNA
Fixed amounts of the total RNA (5μg) were amplified with increasing concentrations of competitor RNA. Lane 1 is the molecular weight marker, the brightest band correspond to 500bp fragment. Lanes 2 to 6 have 5μg total RNA and 10pg, 100pg, 1000pg, 5000pg and 10000pg competitor RNA consecutively. Higher input of the comp RNA yielded more of the 230 bp competitor PCR product relative to the 470bp target PCR product.

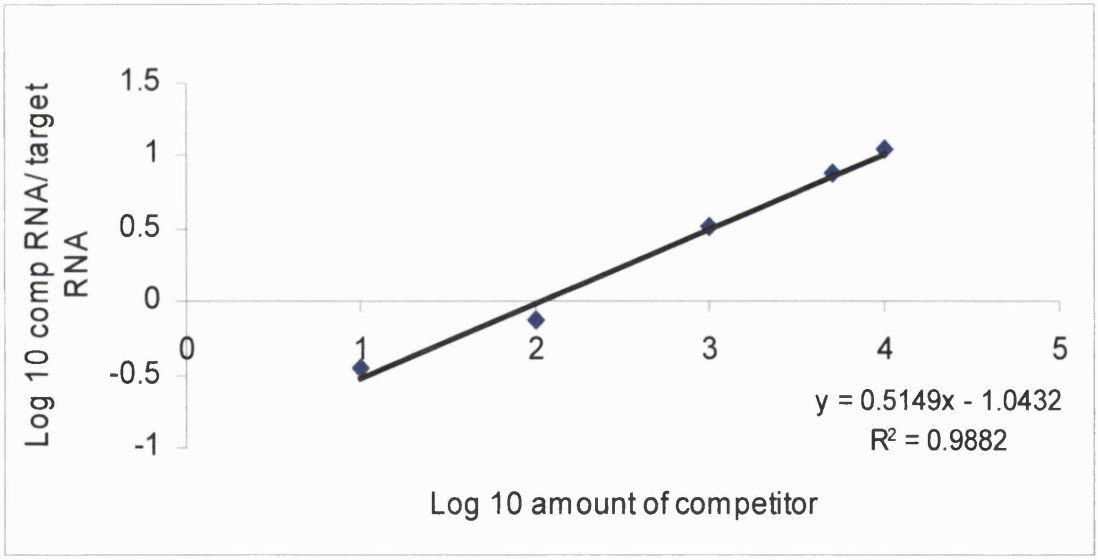


Figure 3.15b: QC-RT-PCR of TF using different amounts of competitor RNA. The linear plot of the base 10 logarithms of the ratios of the competitor to the target against the logarithms of the amount of competitor RNA.

Each result is a representative of at least 4 independent experiments.

The initial amount of TFPI mRNA, in this case, was calculated from the graph when the amplification was in the exponential phase (figures 3.16b & 3.16c), and the value was 5.5pg / μg total RNA.
The amount of competitor RNA used in the QC-RT-PCR was fixed at 10pg, but for total RNA prepared from THP-1 cells, 100fg competitor RNA was used in the assay because the cells express very low copies of the TFPI transcript.

3.2.7 Determination of the optimal number of PCR cycles for TF and TFPI amplification

PCR reactions go through two phases, the exponential phase and the plateau phase. In the exponential phase every cDNA is denatured, bound by a primer and extended by the polymerase. In the plateau phase, the components of the reaction become limited and the polymerase might lose its activity and there is no exponential accumulation of products.

Since quantitation of the PCR product must be limited to the exponential phase of amplification, this study was carried out to determine the optimal number of PCR cycles for the amplification of TF and TFPI. RT-PCR experiments with a fixed amount of total RNA and the competitor RNA with increasing numbers of PCR cycles were carried out.

3.2.7.1 TF RT-PCR cycle profile

For the TF amplification, 5μg of total RNA was added to 100pg of the competitor RNA. The PCR was undertaken for 24, 26, 28, 30, 32 and 34 cycles and the PCR products were analyzed on agarose gel (figure 3.17a). A plot of the ratio of integrated density value of target : competitor RNA against the cycle number is shown in figure 3.17b. Linear amplification was observed up to 32 cycles.
The RT-PCR cycle profile for TF is shown in figure 3.18.

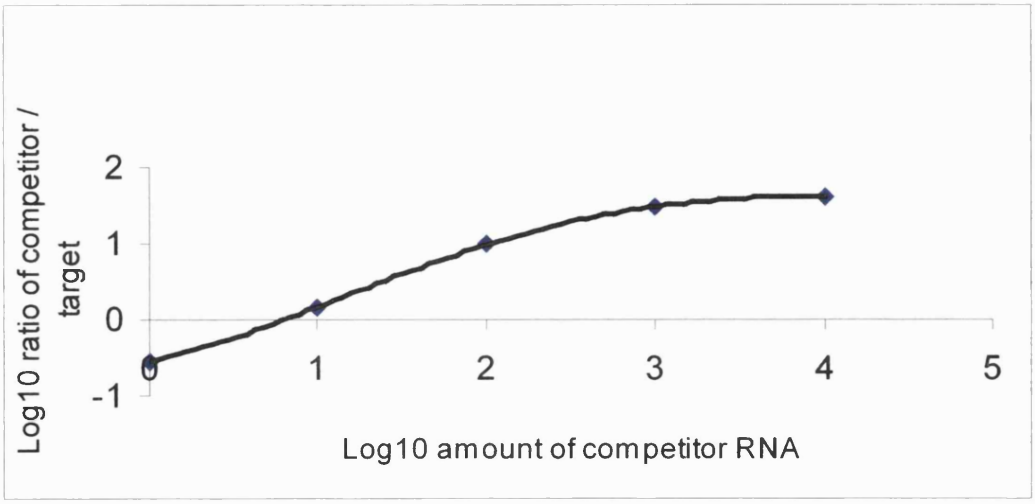


Figure 3.16a: QC-RT-PCR of TFPI with different amounts of competitor RNA. Logarithms of the ratio of the target to competitor RNA was plotted against the logarithms of the amounts of competitor RNA. When 10000pg competitor RNA was included in the reaction the amplification was not exponential and saturation occurred.

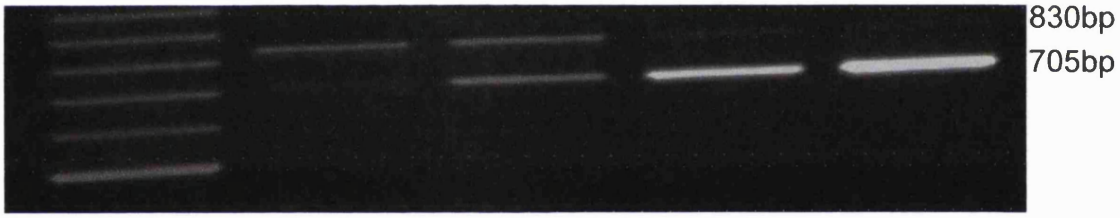


Figure 3.16b: Gel analysis of the QC-RT-PCR of TFPI products in the exponential phase

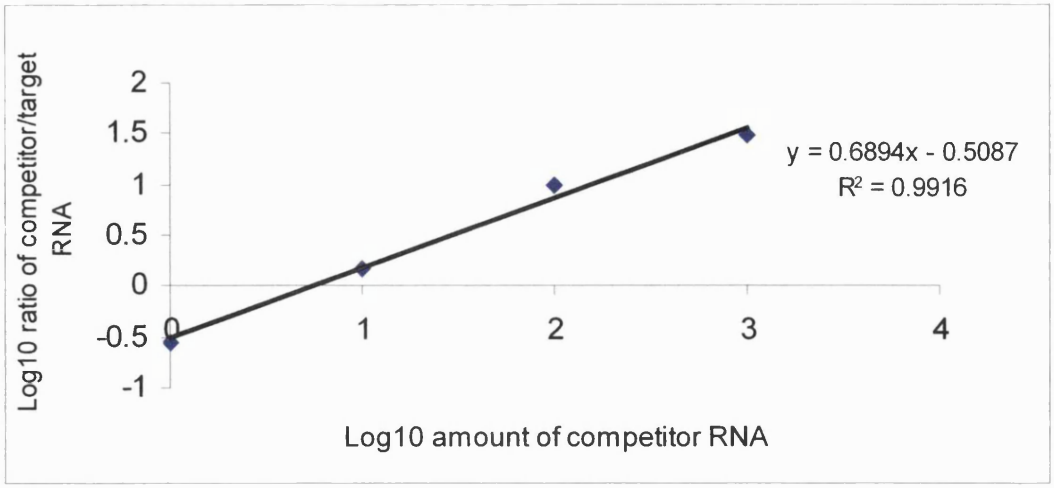


Figure 3.16c: The linear plot of the logarithms of the ratio of the target to competitor RNA against the logarithms of the amounts of TFPI competitor RNA.

Each result is a representative of at least 4 independent experiments .

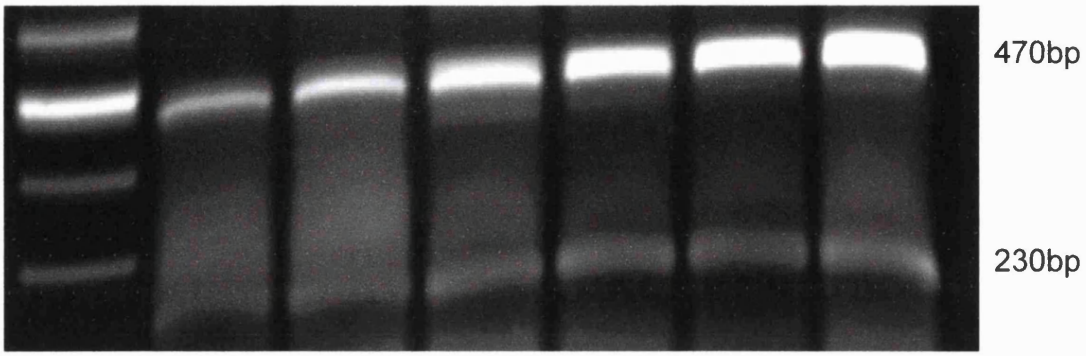


Figure 3.17a: QC-RT-PCR with increasing PCR cycle numbers
Gel analysis of QC-RT-PCR with constant amounts of total RNA and competitor RNA but with increasing PCR cycle numbers

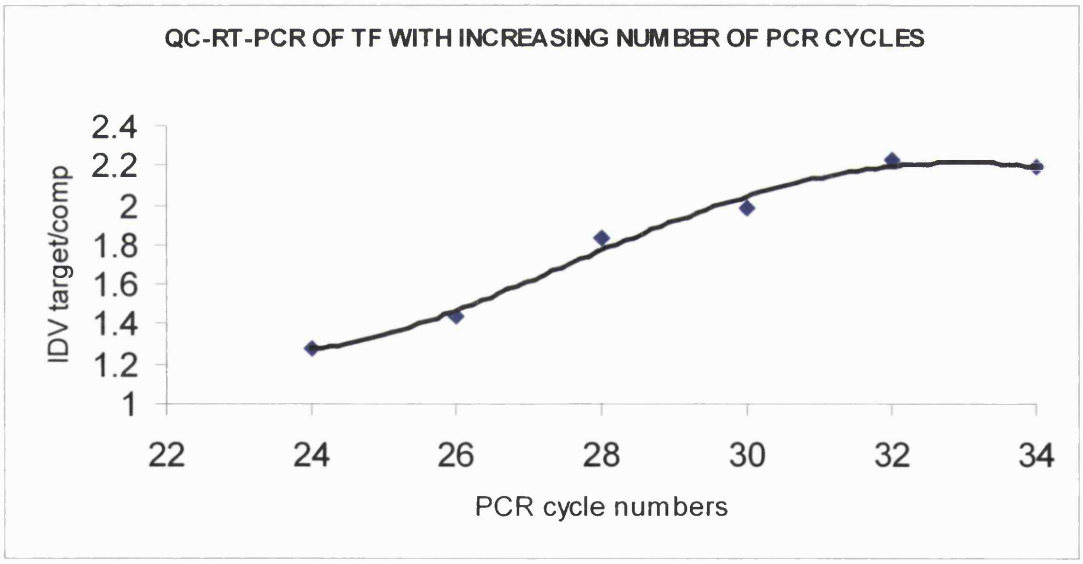


Figure 3.17b: QC-RT-PCR of TF with increasing PCR cycle numbers
A plot of the ratio of integrated density value of target : competitor RNA against the PCR cycle numbers. There is linear amplification up to 32 PCR cycles.

Each result is a representative of at least 4 independent experiments.

3.2.7.2 TFPI RT-PCR cycle profile

For TFPI amplification, 1μg of total RNA was added to 10pg of the competitor RNA. The PCR was undertaken for 24, 26, 28, 30, 32 and 34 cycles and the PCR products were analyzed on agarose gel (figure 3.19). Linear amplification was observed up to 32 cycles.
The RT-PCR cycle profile for TFPI is shown in figure 3 20.

3.2.8 Verification that the designed competitor RNA acts as an optimal competitor for the target in the QC-RT-PCR system

QC-RT-PCR assay demands a comparable amplification efficiency of the target and the competitor RNA. The amplification efficiency was determined by analysis of amplification kinetics on each primer-template set. It has been proven that the PCR amplification efficiency of reverse transcribed target RNA and competitor RNA in the same reaction depends upon the ratio of copies of mRNA rather than the ratio of sizes of target RNA /standard RNA (Zhang *et al*, 1997).

3.2.8.1 A comparison of the amplification kinetics of the target TF mRNA and the TF competitor RNA.

The experiment described in section 3.2.7.1 was used to compare the amplification efficiency of the target TF mRNA and the competitor RNA. After the RT-PCR with fixed amount of total RNA and the competitor RNA with increasing numbers of PCR cycles were carried out, the PCR products were resolved on agarose gel. A plot of the base 10 logarithm IDV of the amplified products (target and comp.) against the number of PCR cycles was made (Figure 3.21).

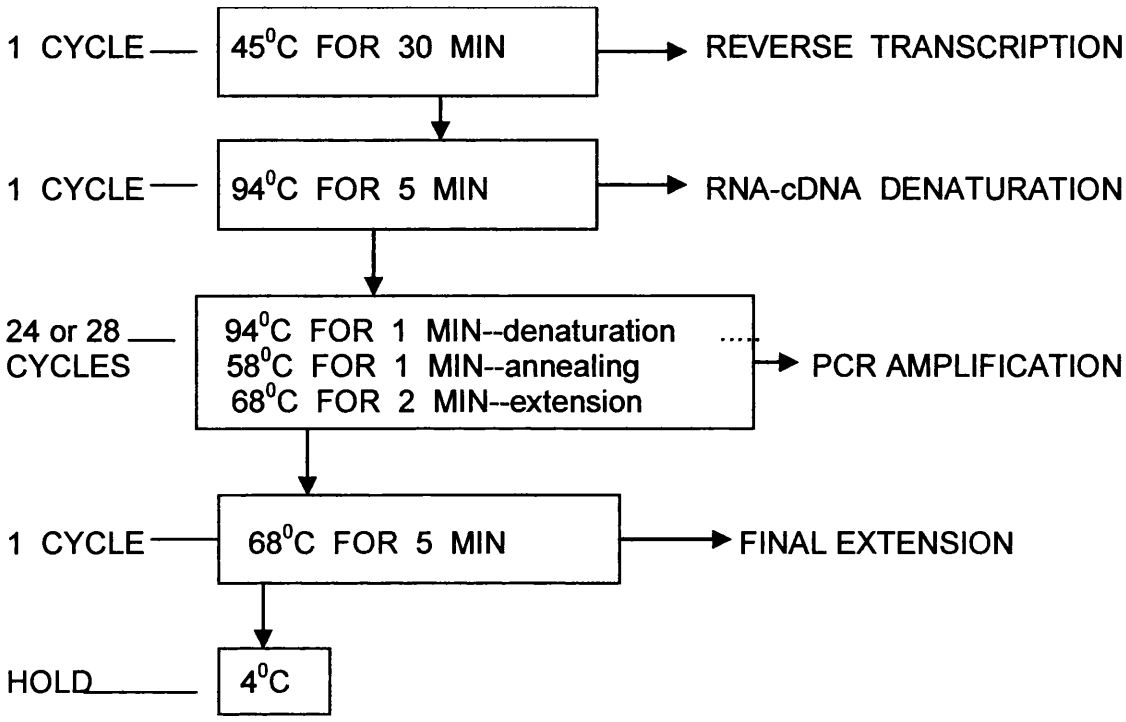


Figure 3.18: RT-PCR cycle profile for TF amplification.

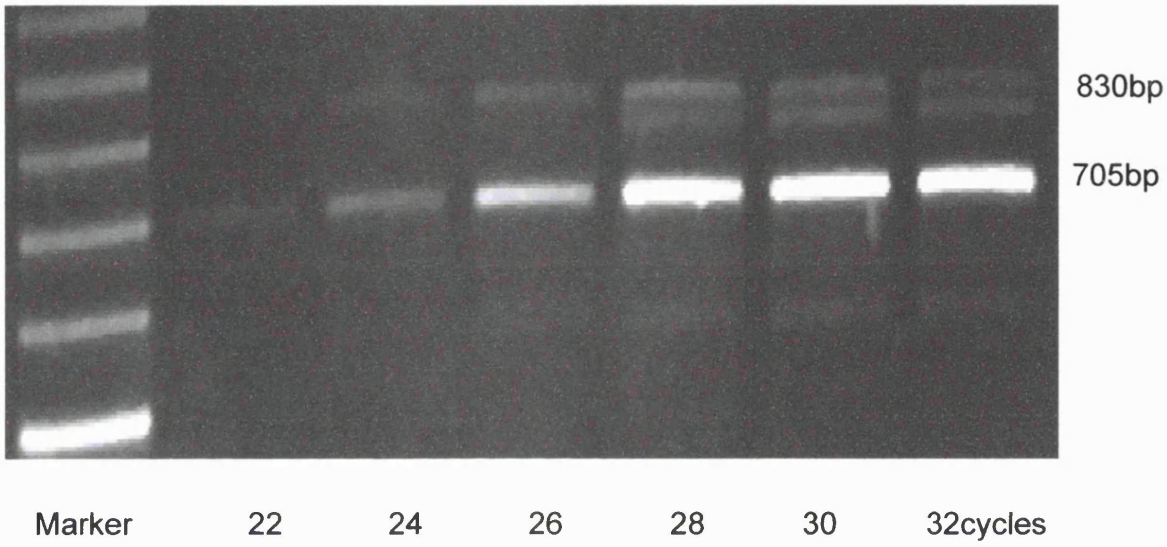


Figure 3.19: QC-RT-PCR of TFPI with increasing PCR cycle numbers
Gel analysis of QC-RT-PCR of TFPI with constant amounts of total RNA and competitor RNA but with increasing PCR cycle numbers. Linear amplification was observed up to 32 cycles.

This result is a representative of at least 4 independent experiments .

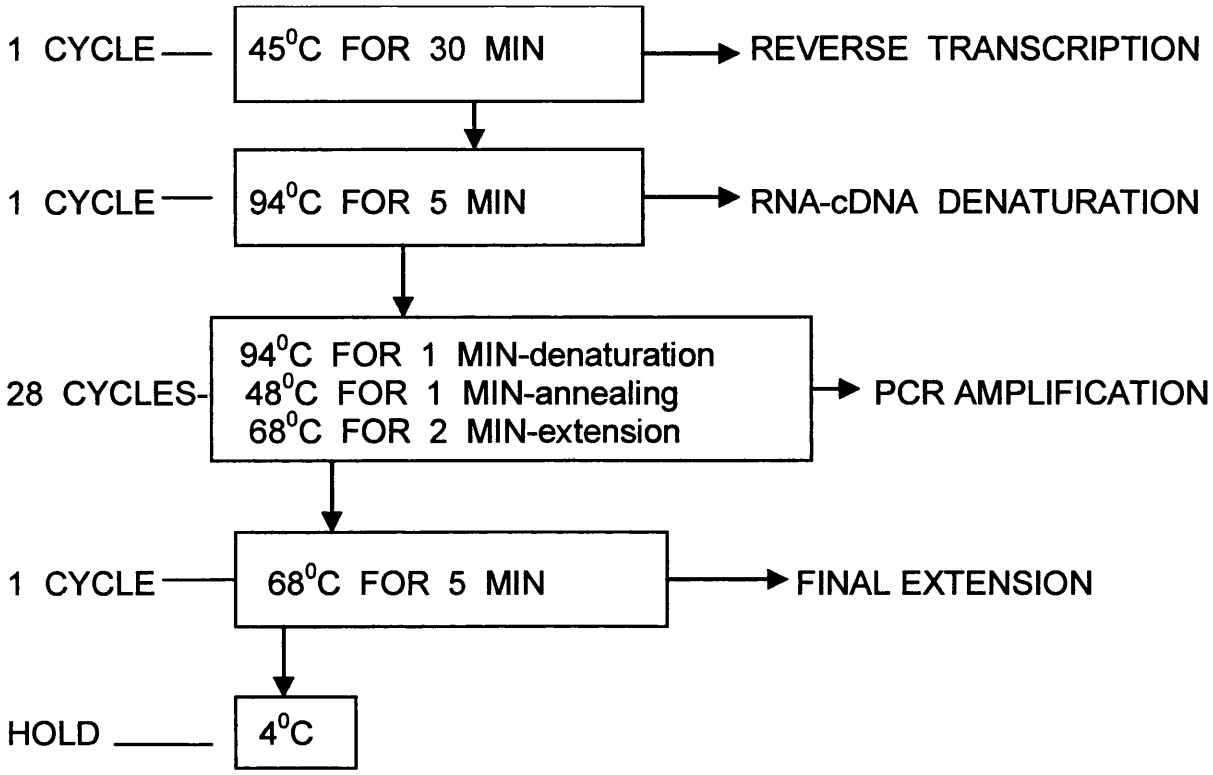


Figure 3.20: RT-PCR cycle profile for TFPI amplification.

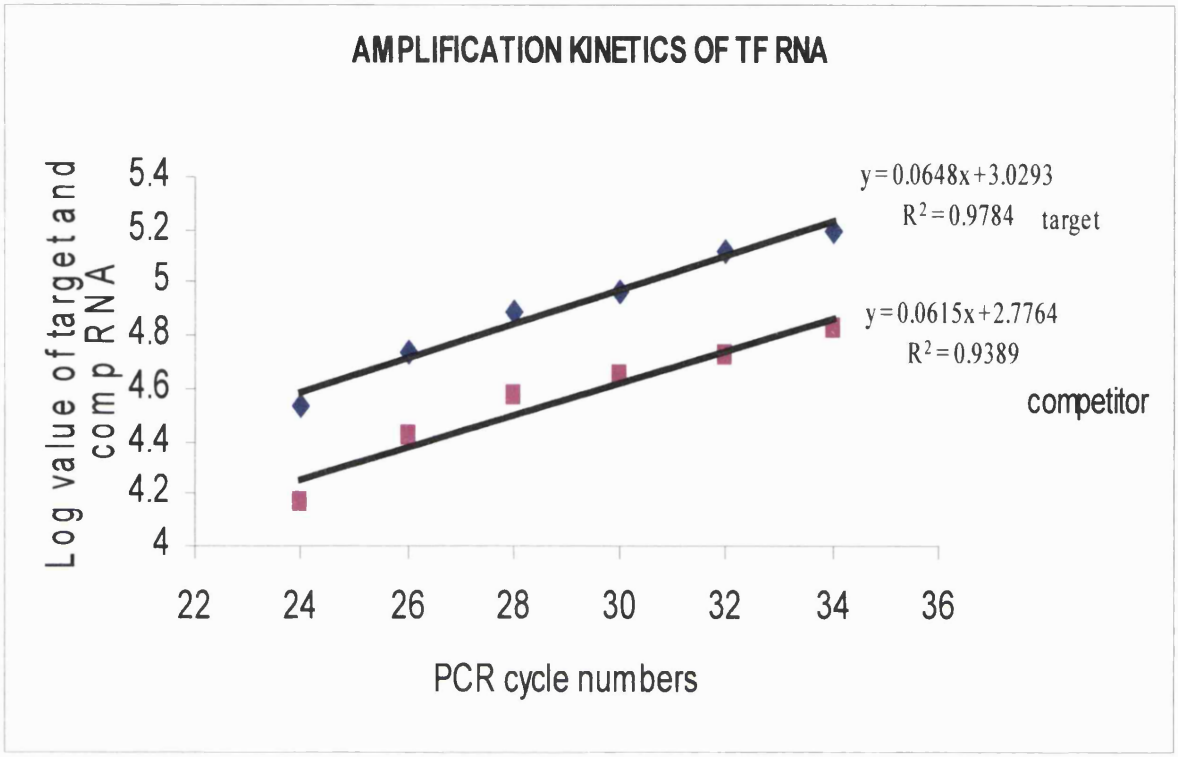


Figure 3.21: Comparison of the amplification efficiency of the target TF mRNA and the competitor RNA.
After the RT-PCR with fixed amount of total RNA and the competitor RNA with increasing numbers of PCR cycles were carried out, the PCR products were resolved on agarose gel. The base 10 logarithm IDV of the amplified products (target and comp.) were plotted against the number of PCR cycles.

The equations of regression lines were:
$Y = 0.0648X + 3.0293$ ($R^2 = 0.98$, Target RNA)
$Y = 0.0615X + 2.7764$ ($R^2 = 0.94$, Competitor RNA)

This result is a representative of at least 4 independent experiments.

The regression lines for the target RNA and the competitor RNA had comparable slopes, indicating similar amplification efficiency for the target and the competitor.

3.2.8.2 A comparison of the amplification kinetics of the target TFPI mRNA and the TFPI competitor RNA

To compare the amplification kinetics of TFPI target RNA with the competitor RNA, the procedure described in section 3.2.7.2 was utilized. After the RT-PCR the PCR products were resolved on agarose gel and a plot of the base 10 logarithm IDV of the amplified products (target and comp.) against the number of PCR cycles was made (figure 3.22). Again, the regression lines for the target RNA and the competitor RNA had comparable slopes, indicating similar amplification efficiency for the target and the competitor.

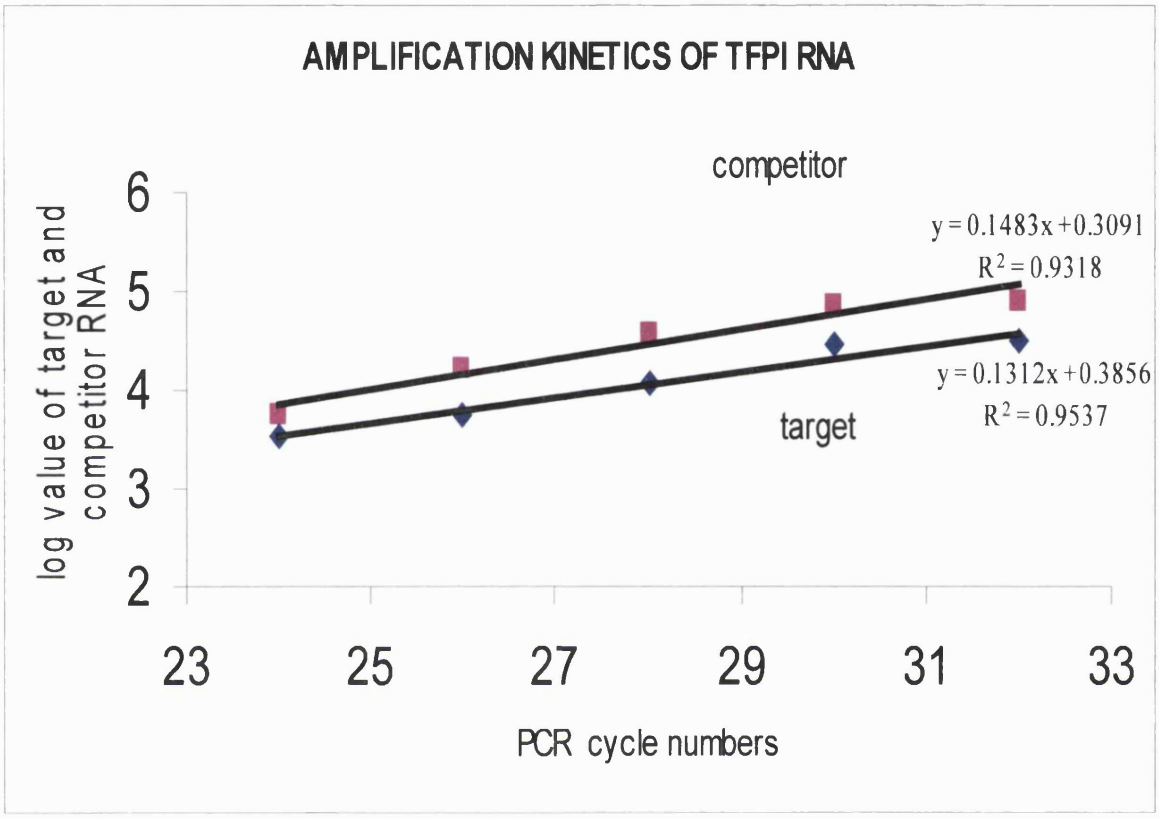


Figure 3.22: Comparison of the amplification efficiency of the target TFPI mRNA and the competitor RNA. After the RT-PCR with fixed amount of total RNA and the competitor RNA with increasing numbers of PCR cycles were carried out, the PCR products were resolved on agarose gel. The base 10 logarithm IDV of the amplified products (target and comp.) were plotted against the number of PCR cycles.

The equations of regression lines were:
$Y = 0.1312X + 0.3856$ ($R^2 = 0.95$, Target RNA)
$Y = 0.1483X + 0.3091$ ($R^2 = 0.93$, Competitor RNA)

This result is a representative of at least 4 independent experiments.

3.3 Discussion

Quantitative competitive RT-PCR is currently the most sensitive way to measure the abundance of specific mRNA. The assay is based on competitive co-amplification of a specific target sequence together with known concentration of a competitor in one reaction tube. Quantitative competitive RT-PCR had been used by different authors in the past to measure the abundances of different specific mRNAs (Potgens et al, 1994; Zhang and Byrne, 1997; Zhang et al, 1997; Gattel et al, 1997).

In this study, quantitative competitive RT-PCR had been successfully developed for the absolute quantification of TF and TFPI gene expression. The QC-RT-PCR system has proved to be a reliable quantitative system for the analysis of TF and TFPI mRNA transcripts. It was accomplished by the design and synthesis of competitor RNA that could be amplified in the same reaction tube, with the same pair of primers, same conditions and also, with a similar efficiency as the target RNA.

Primers for TF and TFPI amplification were carefully designed to amplify PCR products across intron-exon junctions so that any contaminating genomic DNA in the QC-RT-PCR reaction would yield a PCR product differing in size from the target and the competitor RNA.

For each competitor RNA a single standard curve was constructed from a dilution series of the competitor RNA. This curve was used as a standard with which the data from test samples were analyzed. The competitor RNA and the target mRNA were amplified by the same primers, but gave PCR fragments of different sizes. The differences in the sizes of the target and the competitor amplicons allowed the two products to be identifiably resolved by gel electrophoresis. By comparing the target with the competitor, the band intensity

of the PCR products on the agarose gel was used to estimate the initial amount of target mRNA available for the reaction, that is, the amount of gene expression for the particular gene.

Although Zhang and his colleagues (1997) declared that the RT-PCR amplification efficiencies of target RNA and competitor RNA in the same reaction depends upon the ratio of the copies of target RNA to competitor RNA copies, rather than the ratio of sizes of target RNA to competitor RNA, tests were carried out to verify whether the designed competitor RNAs could actually act as optimal competitor for the targets. A comparison between the amplification efficiencies of the target RNA and the competitor RNA for each gene was made and there were comparable amplification efficiencies for both target and competitor pairs.

Polymerase Chain Reaction goes through two distinct phases, the exponential phase and the plateau phase. In the exponential phase, every cDNA is denatured, bound by a primer and copied by the polymerase giving a linear accumulation of PCR products. In the plateau phase, components of the reaction mixture become limiting; unlike in the exponential phase, equal amounts of initial template give widely varying amounts of PCR products Titration experiments were carried out to determine the optimum conditions of the QC-RT-PCR for TF and TFPI. In the experiments to determine the exponential amplification phase, experiments were done with a fixed amount of total RNA and the competitor RNA with increasing numbers of PCR cycles. The optimal PCR cycle number was taken as the number of PCR cycles, within the exponential phase, that was required to enable visualization of the target and competitor PCR products on the agarose gel.

In the study to determine the optimal amount of total RNA for the QC-RT-PCR, titration experiments were carried out using fixed amount of competitor RNA and various amounts of total RNA. The results show that, with higher input of the

total RNA, more of the target PCR product relative to that of the competitor was achieved. The ratio of the target to the competitor increased with a higher input of total RNA and the plot of the ratio of the target product / the competitor product against the amount of the total RNA gave a linear correlation as expected.

In the study with dilution series of total RNA; when 2.5µg, 5µg and 10µg total RNA was amplified with 100µg competitor RNA, there was an approximately equal levels of TF mRNA , showing that this assay is reproducibly, reliable and applicable to a broad range of RNA concentration.

Experiments were also carried out to determine the optimal amount of competitor RNA for the QC-RT-PCR. The competitor RNA acted as an internal control for the reaction and also allowed the quantification of the target RNA. The higher input of the competitor RNA yielded more of the competitor PCR product relative to the target PCR product as expected. The amount of competitor RNA that was similar to the amount of target RNA found in the particular cell line was taken as the optimal amount of competitor for the assay.

These evaluations permitted the use of the QC-RT-PCR assay, in the following chapters, with confidence.

CHAPTER 4

The Modulation of Tissue Factor and Tissue Factor Pathway Inhibitor Gene Expression in Endothelial and Monocytic Cell Lines.

4.1 Introduction

Atherosclerosis is the primary cause of heart disease and stroke, and it is the underlying cause of about 50% of all deaths in the western societies (Lusis, 2000). While diabetes, smoking, hypertension, hyperlipidemia and low antioxidant status are well-established risk factors for the development of atherosclerosis, the relative abundance of the plasma lipoprotein is of primary importance. The increase in plasma cholesterol content and the cholesterol deposits in the fatty streaks and in atherosclerotic plaques has been attributed mainly to increase in levels of low-density lipoprotein (Esterbauer *et al*, 1989).

Oxidative modification of low-density lipoprotein (LDL) is believed to play an important role in the pathogenesis of atherosclerosis, but the mechanism is not yet fully known. Formation of lipid hydroperoxides is generally accepted as an early event in LDL oxidation (Girotti, 1998); the peroxides can decompose to further radicals and to aldehydes such as malondialdehyde and 4-hydroxynonenal which can in turn react with lysine amino groups on proteins to give adducts (Kawamura *et al*, 2000: Refsgaard *et al*, 2000). In atherogenesis, the oxidative modification of LDL is thought to occur in two stages. In the first stage, only the lipid in the LDL is oxidised with little change in apo B100, and the LDL is referred to as minimally oxidised LDL. In the second stage the lipids are oxidised more extensively and the apo B100 form adducts with the oxidation products. Monocytes are recruited to the atherosclerotic lesion and are further transformed into macrophages, which by their oxidative capacity oxidise the LDL further (Berliner *et al*, 1995). During atherogenesis, the intimal macrophages and

smooth muscle cells take up large amount of the oxidised LDL (Napoli *et al*, 2000), and the death of these cells then leads to the progressive formation of a necrotic core of atheromas (Ross, 1999).

LDL oxidation involves the conversion of polyunsaturated fatty acids in the LDL lipids into lipid hydroperoxides, which are the major initial products of lipid peroxiation. In the early stages of LDL oxidation that involve the lipids only, there is the generation of products such 4-hydroxynonenal (Jurgens *et al*, 1986) and the hydroxy- and hydroperoxy-derivatives of linoleic and arachidonic acids (Lenz *et al*, 1990; Thomas and Jackson, 1991). Approximately 40% of phosphatidylcholine present in native LDL are converted to lysophosphatidylcholine during oxidative modification of LDL (Hirata *et al*, 1995).

Lysophosphatidylcholine is a lipid component of oxidized LDL that is released from the oxidation of lipids in the LDL. It is the product of phosphatidylcholine hydrolysis by phospholipase A_2 (Golfman *et al*, 1999). The concentration of lysophosphatidylcholine in the plasma is normally low, but high amounts of lysophosphatidylcholine are found in oxidatively modified LDL (Steinberg *et al*, 1989). Lysophosphatidylcholine is found in atherosclerotic and inflammatory lesions of vascular vessels and it could be an important signal molecule that impairs endothelium-dependent relaxation of blood vessels (Witztum and Steinberg, 1991). The estimated contents of lysophosphatidylcholine in 1mg lipoprotein is 20μg in native LDL and 220μg in oxidised LDL (Meyer *et al*, 1996)

The uptake of LDL by cells is through apolipoprotein B/E receptor, but oxidised LDL is not recognised by this receptor; it is rather internalised via the scavenger receptor that is present on the surface of monocytes and macrophages. This could be as a result of the derivatization of apoB lysine residues by oxidised lipids (Steinbrecher *et al*, 1989) or breakdown products such as malondialdehyde (Haberland *et al*, 1982). Also the apoB lysine modification increases the negative charge of the protein, which

may result in conformational changes required for receptor recognition (Haberland *et al*, 1984). The uptake of modified LDL by macrophages is not downregulated by internalised LDL cholesterol and leads to lipid loading of these cells and the subsequent formation of foam cells in the intima.

Oxidation of LDL creates significant imbalances in the normal mechanism of the vasculature (Bruckdorfer, 1998) and it regulates many of the critical biological processes related to atherosclerosis. Oxidised LDL has been implicated in smooth muscle proliferation, leukocyte infiltration into arterial wall, impairment of endothelial function ((Ross, 1993; Andrews *et al*, 1987; Plane *et al*, 1990; Liao *et al*, 1995; Rangaswamy *et al*, 1997), formation of macrophage-derived foam cells (Lusis, 2000), stimulation of platelet aggregation (Naseem *et al*, 1993) and vascular cell death (Siow *et al*, 1999). Oxidised LDL can induce the gene expression of platelet-derived growth factor- c-fos and the platelet-derived growth factor receptor- egr-1 (Sachinidis, 1993). It stimulates the release of interleukin-1β from monocytes/macrophages (Thomas *et al*, 1994). Oxidised LDL can inhibit the release of NO from the endothelium (Bruckdorfer et al, 1997), and can also increase the production of superoxide anion in endothelial cells (Tsao *et al*, 1996) and fibroblasts (Maziere *et al*, 2000). The increase in lipid peroxidation end products (TBARS) may be attributed to the hydroperoxides arising from the polyunsaturated fatty acids of LDL (Maziere *et al*, 2000). The ability of the hydroperoxides to generate alkoxyl radical inside cells has been documented (Pryor, 1978) and this alkoxyl radical can induce the peroxidation of the cellular lipids.

White *et al* (1994) suggested that peroxynitrite might play a role in the formation of atherosclerosis. Peroxynitrite, the product of the reaction between nitric oxide and superoxide anion, could be responsible for the oxidative modification of LDL *in vivo*. Although peroxynitrite is not a radical, it is much more reactive than its radical precursors (Beckman *et al*, 1990). It is an oxidant that can decompose to

form hydroxyl radical and nitrogen dioxide. Ma *et al* (2000) described peroxynitrite as a 'double-edged sword' with both the ability to promote myocardial damage and also capable of protecting the myocardial cells from injury. Peroxynitrite is capable of initiating lipid peroxidation even in the presence of the biological antioxidants such as ascorbate and α-tocopherol (Patel *et al*, 1996). It can also contribute to the modification of LDL by releasing copper from caeruloplasmin thereby promoting lipid peroxidation (Swain *et al*, 1994).

Homocysteine is recognised as an independent risk factor for atherosclerotic vascular disease and for venous thrombosis (Refsum *et al*, 1998). As much as 10% of all cardiovascular risk may be attributable to high levels of homocysteine (Boushey *et al*, 1995). Low folate intake is an important determinant of elevated blood levels of homocysteine (Verhoef *et al*, 1998). Khajuria and Houston (2000) postulated that homocysteine induces thrombosis via the induction of tissue factor expression in circulating monocytes.

Tissue factor is the primary initiator of the coagulation protease cascades (Bach, 1988). Tissue factor is expressed by many cell types, such as the epidermis, brain, bronchial epithelium and hepatocytes that are not directly exposed to flowing blood (Luther *et al*, 1996). Tissue factor is not expressed in the intima or media of normal adult blood vessels (Drake *et al*, 1989; Wilcox *et al* 1989), but inflammatory mediators such as lipopolysaccharide (Mackman *et al*, 1991) and growth factors (Taubman *et al*, 1997) are known to induce tissue factor expression in monocytes and endothelial cells. Studies have revealed that tissue factor is synthesised by most cellular elements contained in atherosclerotic plaques, including endothelial cells, smooth muscle cells and macrophages (Cui *et al*, 1999; Drake *et al*, 1989). Tissue factor is the critical determinant of thrombin generation in normal haemostasis (Yang and Loscalzo, 2000). An increase in thrombin generation at sites of vascular injury plays a critical role in the pathogenesis of atherosclerosis (Deguchi *et al*, 1997). Thrombus formation

in the circulatory system is prevented by factors such as antithrombin III, protein C receptor, tissue factor pathway inhibitor and thrombomodulin (Van-Hinsbergh, 2001)

Tissue factor pathway inhibitor is the primary physiological inhibitor that regulates tissue factor-induced blood coagulation (Pendurthi *et al*, 1999). Tissue factor pathway inhibitor is present in vascular endothelium, smooth muscle cells, platelets, monocytes and macrophages (Bajaj *et al*, 1990; van der Logt *et al*, 1994; Caplice *et al*, 1998). In atherosclerotic carotid arteries, where tissue factor expression is abundant, tissue factor pathway inhibitor expression is found to be limited thereby resulting in predominant tissue factor activity (Caplice *et al*, 1998), whereas in plaque where tissue factor pathway inhibitor expression is the greatest, tissue factor activity is attenuated. The tissue factor pathway inhibitor present within the human atherosclerotic plaque may modulate the plaque thrombogenicity by attenuating the tissue factor activity (Caplice *et al*, 1998). It has been shown that tissue factor pathway inhibitor treatment in a rabbit atherosclerotic model reduced restenosis (Jang *et al*, 1995), while a deficiency of tissue factor pathway inhibitor in mice has been shown to promote atherosclerosis and thrombosis (Westrick *et al*, 2001). Sato *et al* (1997) demonstrated that tissue factor pathway inhibitor inhibits smooth muscle cell migration induced by TF/FV11a complex and also prevents smooth muscle cells proliferation (Kamikubo *et al*, 1997). The imbalance between tissue factor and tissue factor pathway inhibitor expression in plaque may be responsible for the prothrombotic phenotype associated with atherosclerosis and the inhibition of tissue factor activity by tissue factor pathway inhibitor may inhibit vascular remodelling (Singh *et al*, 2001).

The endothelium plays key role in thrombosis and haemostasis by protecting against vascular injury and maintaining blood fluidity, but studies with vascular cells provided evidence for the central role of the endothelium in mediating

inflammation. The endothelium mediates inflammation due to its generation of effector molecules such as adhesion molecules and growth factors that regulate thrombosis, vascular tone and inflammation. Studies have also revealed that blood derived inflammatory cells, particularly monocyte/macrophages, have a key role in atherogenesis (Lusis, 2000). The monocyte is one of the crucial and readily accessible cell types in the arterial intima during the genesis of the atherosclerosis lesion and it is present during all stages of atherogenesis (Devaraj and Jialal, 1998).

Infectious agents have been reported as risk factors for atherosclerotic vascular diseases and cardiovascular events (Valtonen, 1991). Endotoxin lipopolysaccharide (LPS) is a bacterial cell wall component released by gram-negative bacteria. It is a potent activator of monocyte or macrophage function leading to responses that are both protective and injurious to the host (Ulevitch and Tobias, 1999). It is a proinflammatory xenobiotic and is known to alter vascular function (Gunnett *et al*, 1999). Endothelial cells when stimulated with LPS express tissue factor (Mackman *et al*, 1991) as well as interleukin 1 (Libby *et al*, 1986), and plasminogen activator inhibitor 1 genes (Crutchley and Conanan, 1986). It can also induce human monocytes to express pro-inflammatory mediators such as cytokine tumor necrosis factor alpha (Guha *et al*, 2001).

The aim of this study was to investigate the influences of minimally modified LDL and LPS on the relative expression of tissue factor and tissue factor pathway inhibitor genes in human monocytic (THP-1) and endothelial (ECV 304) cell lines.

4.2 Results

4.2.1 Study to confirm the simultaneous induction of tissue factor and tissue factor pathway inhibitor mRNA in THP-1 and ECV 304 cells using Northern hybridisation

Northern hybridisation analysis were carried out to determine if the chosen cell lines (THP-1 and ECV 304) do express tissue factor and tissue factor pathway inhibitor when stimulated with LPS and mmLDL. mmLDL was initially prepared from the pooled plasma of 4-9 healthy subjects but afterwards, it was prepared from pooled plasma of 4 regular subjects.

4.2.1.1 Northern hybridisation analysis of tissue factor mRNA in ECV 304 and THP-1 cell lines.

ECV 304 cells and THP-1 cells were cultured in DMEM and RPMI 1640 medium respectively. Each cell line was exposed to either 50µg of protein/ml mmLDL or 10µg/ml LPS for 1 h. These concentrations of mmLDL and LPS were able to induce the expression of TF without killing the cells. Total RNA was extracted from the cells (section 2.8) and RNA concentration was determined by measuring the absorbance at 260nm (section 2.8.4). Northern blot analysis of the total RNA prepared from THP-1 and ECV 304 cells revealed a basal expression of tissue factor in these cell lines. Analysis of the total RNA extracted from the cells exposed to either mmLDL or LPS showed that the tissue factor gene is upregulated in the cells by mmLDL and LPS. Figure 4.1 represents the blot analysis of total RNA from ECV 304 cells, stimulated with LPS and mmLDL, showing the tissue factor mRNA band of ~ 2.2kb. The northern blot analysis of the total RNA from THP-1 cells (Figure 4.2) also shows a major tissue factor mRNA of ~2.2kb (lane 1) when stimulated by mmLDL but when the cells were stimulated with LPS (lane 3) another band of approximately 3.2kb was found.

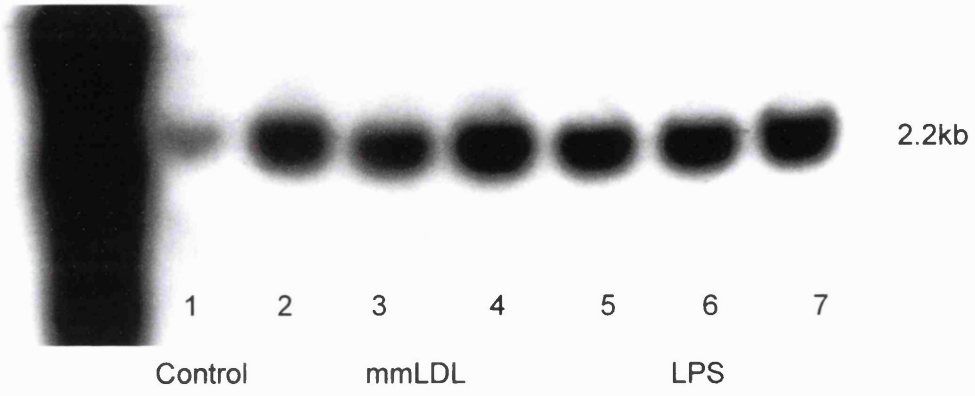


Figure 4.1: Northern Blot Analysis of mmLDL and LPS induced TF mRNA in ECV 304 cells. ECV 304 cells were exposed to LPS (10µg/ml) and mmLDL (50µg/ml) for 1hr. Total RNA was isolated and TF induction levels was determined by Northern blot analysis. Lane 1 is the control cells, lanes 2-4 are aliquots from mmLDL treated cells and lanes 5-7 are aliquots from the LPS treated cells. Full length TF cDNA fragment was used as probe. This result is a representative of at least 4 independent experiments.

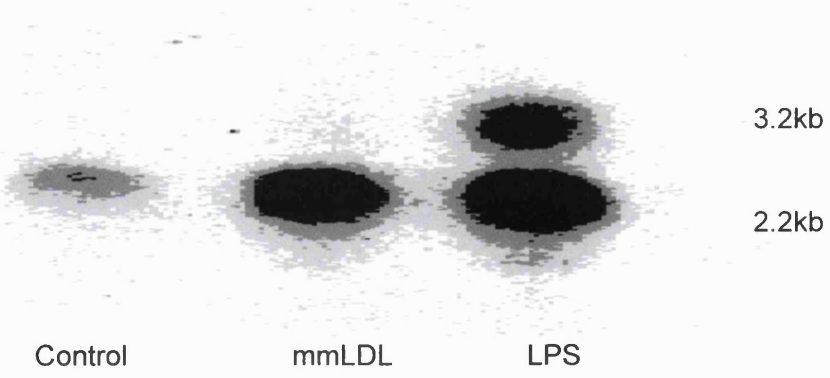


Figure 4.2: Northern Blot Analysis of TF mRNA induced by mmLDL and LPS in THP-1 cells. THP-1 cells were exposed to mmLDL (50µg/ml) and LPS (10µg/ml) for 1hr. Total RNA was isolated and TF induction levels was determined by Northern blot analysis. Full length TF cDNA fragment was used as probe. This result is a representative of at least 4 independent experiments.

The 3.2kb mRNA has been shown to be produced by the retention of the first intron of the human tissue factor gene, due to an alternative RNA splicing and does not encode a functional tissue factor protein (Edgington *et al*, 1991). The 2.2 kb message codes for the tissue factor protein while no protein is produced from the 3.1 kb transcript (van der Logt *et. al.* 1992).

4.2.1.2 Northern hybridisation analysis of tissue factor pathway inhibitor mRNA in ECV 304

ECV 304 cells and THP-1 cells were cultured and treated as described above. Total RNA was prepared from control cells and cells stimulated with either 50µg protein/ml mmLDL or 10µg/ml LPS for 1 h. Northern blot analysis, using radiolabeled full length tissue factor pathway inhibitor cDNA, of the total RNA prepared from ECV 304 cells revealed a basal expression of tissue factor pathway inhibitor in the cell line. ECV 304 cells express two tissue factor pathway inhibitor transcripts, a 4kb and a 1.4kb (Figure 4.3). Tissue factor pathway inhibitor mRNA was not detectable in THP-1 cells by Northern blot analysis even after mmLDL / LPS treatment.

4.2.2 Investigation into the effect of LPS on tissue factor activity, antigen and gene expression.

Having confirmed the presence of tissue factor mRNA in THP-1 and ECV304 cells and its up-regulation by LPS, studies were carried out to quantify tissue factor mRNA in control and stimulated cells using Quantitative Competitive RT-PCR (QC RT-PCR). Experiments were also carried out to determine if the induced gene expression of tissue factor is accompanied by an increase in the tissue factor surface antigen and activity.

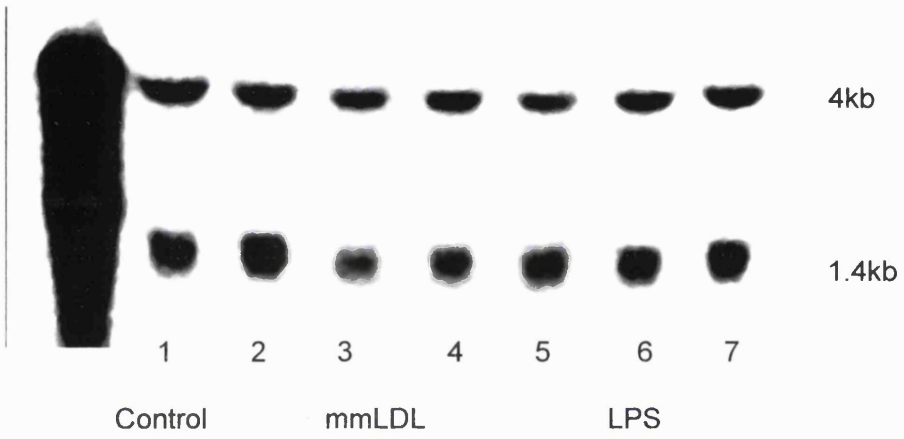


Figure.4.3: Northern Blot Analyses of TFPI mRNA. Total RNA was prepared from ECV 304 cells treated with mmLDL (50µg/ml) and LPS (10µg/ml) for 1 hour. Full length TFPI cDNA was used as probe for the Northern blot analysis. Lane 1 is the control cells, lanes 2-4 are aliquots from mmLDL treated cells and lanes 5-7 are aliquots from the LPS treated cells This result is a representative of at least 4 independent experiments.

4.2.2.1 The induction of tissue factor antigen by LPS in THP-1 cells

THP-1 cells were exposed to 10µg/ml LPS for 2 h. The cells were harvested, washed in PBS and subsequently stained for tissue factor antigen. Tissue factor antigen was measured as described in section 2.3 using the flow cytometer. The control THP-1 cells did express tissue factor antigen (figure 4.4) which increased upon stimulation with LPS (figure 4.5) from the basal level of 8.413 ± 0.925 to 10.49 ± 2.64 (mean of n = 3; ± SD).

4.2.2.2 Effect of LPS on tissue factor activity in THP-1 cells

The tissue factor protein activity of THP-1 cells was determined using the one-stage prothrombin time assay. The human monocytic cell line THPI was cultured as described in section 2.4.3.1. After the addition of 10µg/ml LPS, cells were incubated for a period up to 54 h. The cells were harvested, washed in PBS buffer and resuspended in PBS buffer at a concentration of 1 X 10^4 cells/µl. To 100 µl of the cells was added 100 µl of 20mM $CaCl_2$ and 100 µl control plasma at 37^0C and the time needed for clot formation was recorded on the coagulometer. The clotting time was converted to tissue factor unit using the standard curve generated from serial dilutions of the recombinant tissue factor (see section 2.2). One unit of tissue factor activity was assigned arbitrarily as the 1,000 fold dilution of the recombinant tissue factor. Stimulation of THP-1 cells with 10µg/ml LPS caused an increase in cell surface tissue factor pro-coagulation activity, which was maximal at 6h causing an enhancement of the tissue activity by an average of 50.07% ± 3.63 (figure 4.6). Total tissue factor activity of the cells was also looked into by measuring the activity in the cell lysate. Less than 20% of the total tissue factor activity is attributable to cell surface activity. The total tissue activity (figure 4.7) was maximal at 4h and remained fairly constant for up to 8h with a percentage enhancement of 78 – 100%.

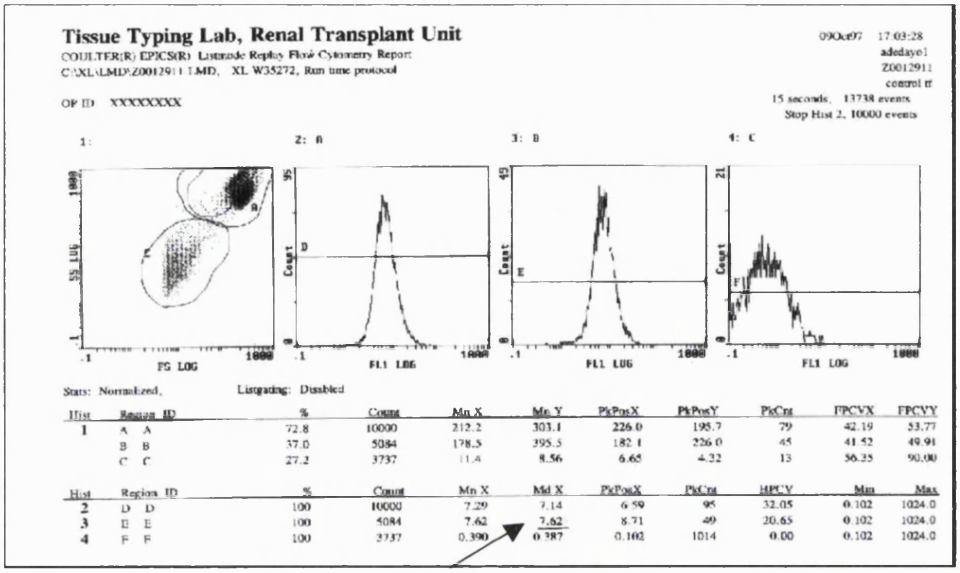


Figure 4.4: Tissue factor antigen analysis of control THP-1 cells.

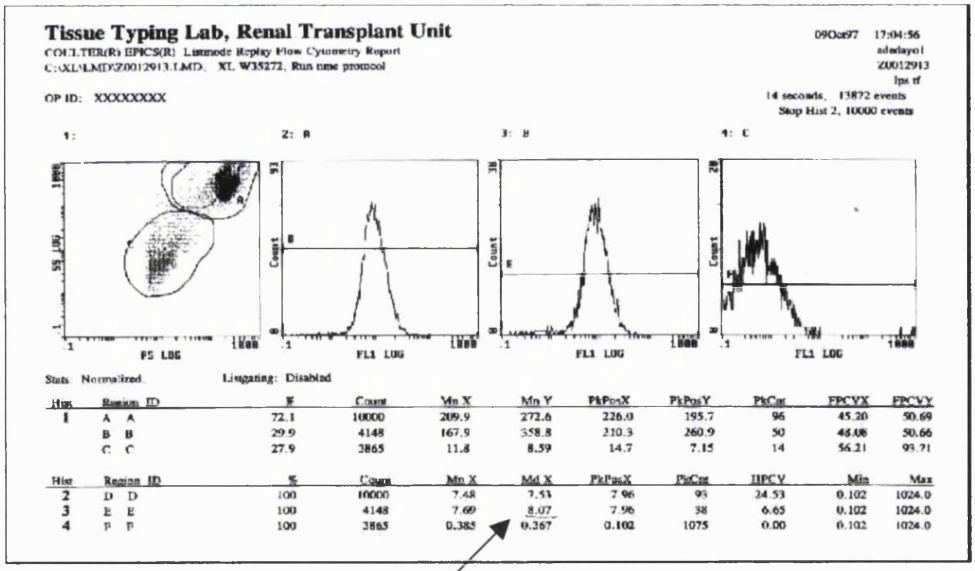


Figure 4.5: Tissue factor antigen analysis of THP-1 cells following 2h LPS (10µg/ml) incubation.

These histograms are representative display of flow cytometic analysis of the measurement of tissue factor antigen on the surface of THP-1 cells in control cells (figure 4.4) and in cells incubated with LPS for 2 h (Figure 4.5). Forward and side scater parameters were collected logarithmically and the cells were gated to exclude debris and microparticles (gate c). Fluorescence histograms of the gated cells (a & b) were used to asses tissue factor antigen. These results are representatives of 3 independent experiments.

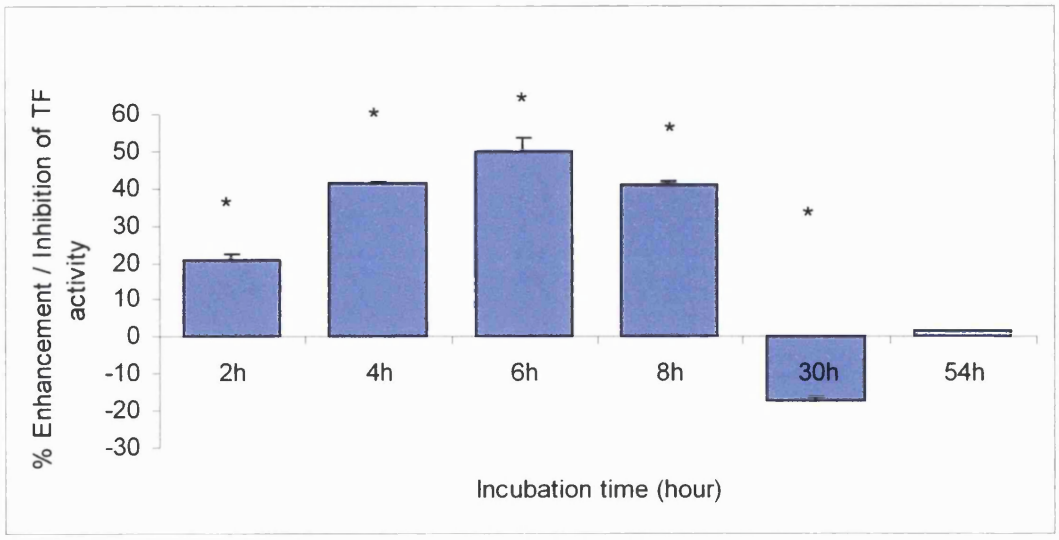


Figure 4.6: Time course of TF surface activity. THP-1 cells were incubated with 10μg/ml LPS for the specified length of time. The cell surface tissue factor activity was determined by the one-stage prothrombin time assay. The clotting time was converted to TF units using the recombinant tissue factor standard curve. Result represent the % enhancement (+ve values) or % inhibition (-ve values) of procoagulant activity compared with control (mean ± SEM of 3 independent experiments, $p < 0.050$).

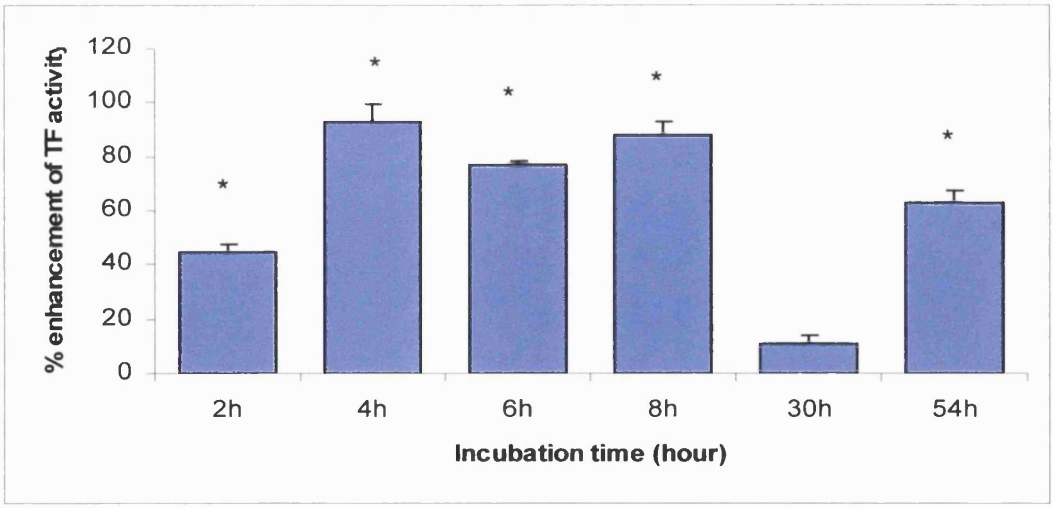


Figure 4.7: Time course of TF total activity. THP-1 cells were incubated with 10μg/ml LPS for up to 54 hours. The cell's total tissue factor activity was determined by the one-stage prothrombin time assay. Result represent the % enhancement of tissue factor activity compared with control (± SEM of 3 independent experiments). $p < 0.050$

The exposure of THP-1 cells to different concentration of LPS revealed that the effect is concentration dependent (figure 4.8). An increase, above the basal levels, in the cell surface tissue activity was observed when the cells were treated with 0.1µg/ml LPS. Cell surface tissue factor activites increased gradually with the increase in LPS concentration (0.1 - 10 µg/ml). A peak was reached at a concentration of 10µg/ml, after which a further increase in the concentration of LPS resulted in a decline of cell surface tissue factor activity.

4.2.2.3 Time course of LPS-induced expression of tissue factor in THP-1 cells

Tissue factor is an immediate early gene. To determine the time point of optimal tissue factor gene expression in response to LPS, the cells were incubated with (10µg/ml) LPS for various times up to 6 h. Figures 4.9 a & b represent data obtained from QC-RT-PCR of the total RNA extracted from THP-1 cells exposed to 10µg/ml LPS. There was a transient increase in the accumulation of tissue factor mRNA. Tissue factor expression increased after 30min, maximum levels of tissue factor mRNA were observed about 60min after stimulation and it declined after 90min.

4.2.2.4 Effect of LPS on tissue factor gene expression in THP-1 cells and ECV 304

To study the effects of LPS on tissue factor gene expression in THP-1 cells and ECV 304 cells, the cells were incubated with 10µg/ml LPS for 60 min. Total RNA was prepared from the cells and amplified for tissue factor using QC-RT-PCR. Figure 4.10 shows the effect of LPS on the expression of tissue factor in THP-1 and ECV 304 cells. The exposure of ECV 304 and THP-1 cells to LPS resulted in an up-regulation of tissue factor mRNA.

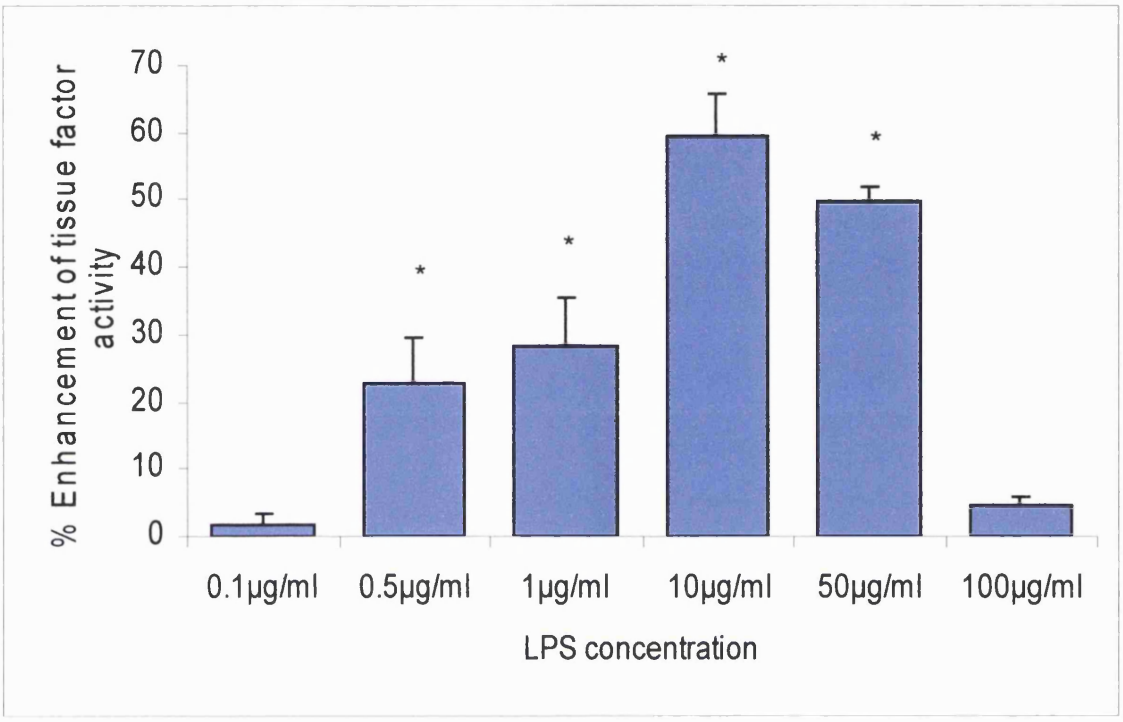
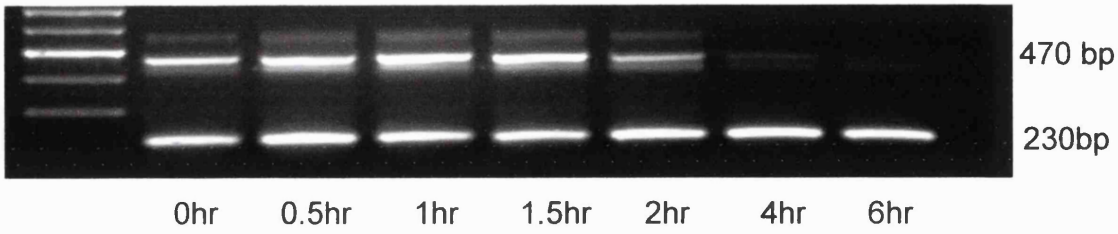
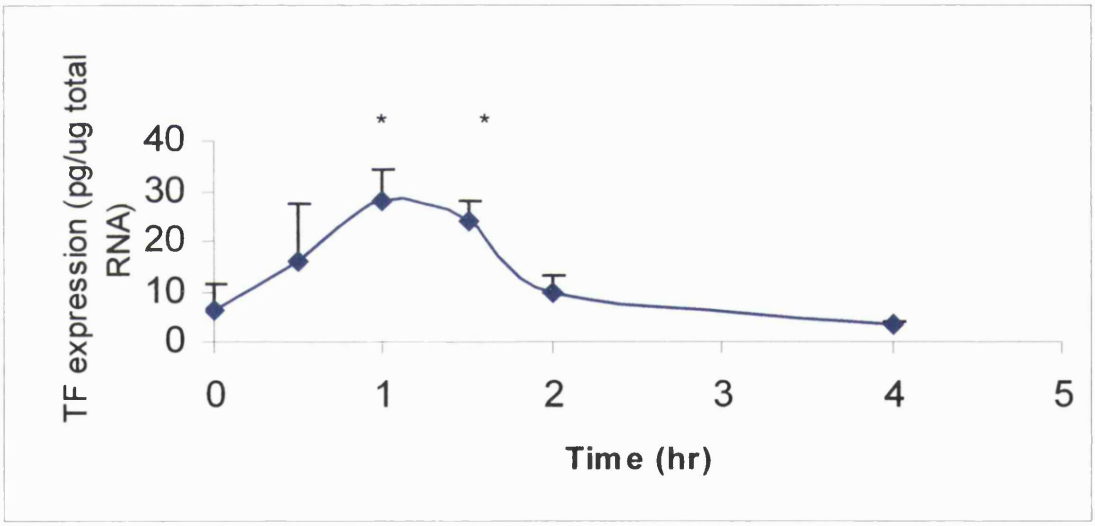


Figure 4.8: Concentration dependent induction of tissue factor activity by LPS. THP-1 cells were incubated with the specified concentration of LPS for 4 hours. The cell surface tissue factor activity was determined by the one-stage prothrombin time assay. The clotting time was converted to TF units using the recombinant tissue factor standard curve..Result represent the % enhancement (+ve values) of procoagulant activity compared with control (mean $\pm$ SEM of 3 independent experiments, $p < 0.050$).



a



b

Figure 9a & b: Time course of TF gene expression induced in THP-1 cells exposed to 10µg/ml LPS. The LPS was added to THP-1 cells for the time indicated. Total RNA was isolated and TF mRNA levels were determined by QC-RT-PCR. 'a' is a representative agarose gel analysis for the QC-RT-PCR. These results are representative of at least 4 independent experiments. (± SEM of 3 independent experiments * $p < 0.050$)

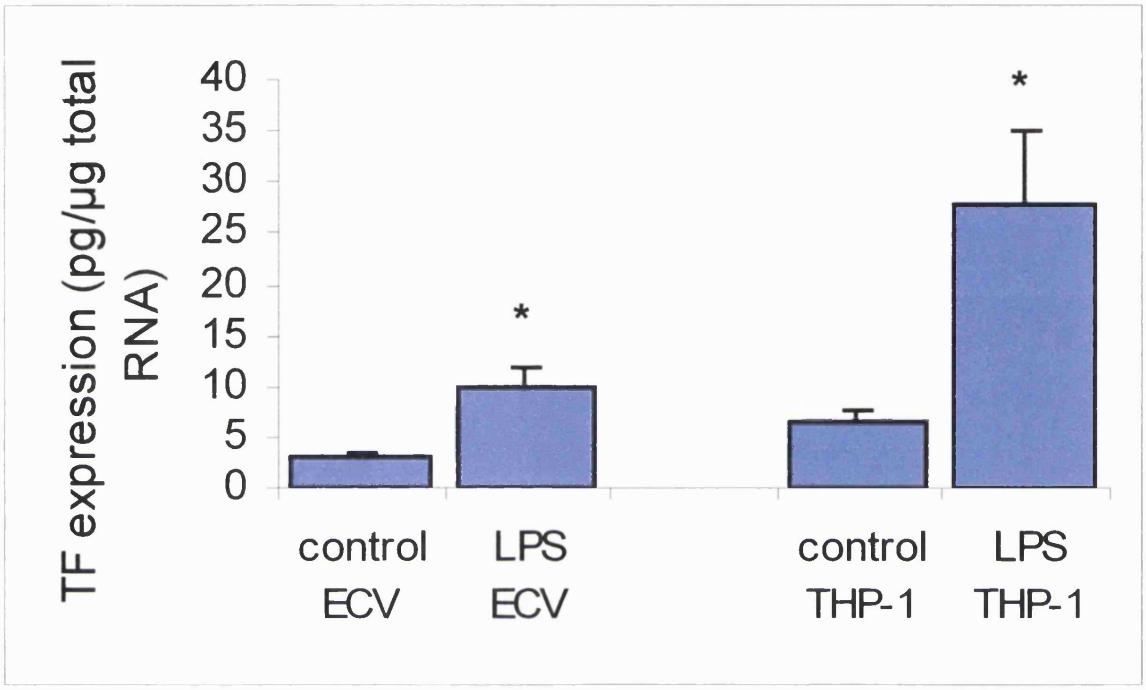


Figure 4.10: Effects of LPS on the expression of TF gene in THP-1 and ECV 304 cells. THP-1 and ECV 304 cells were incubated for 1 hour with 10 µg/ml LPS. Total RNA was isolated and TF mRNA levels were determined by QC-RT-PCR. Values denote mean ± SEM n=12 (THP-1) / n=8 (ECV 304) independent experiments. * $P < 0.050$

LPS caused an up-regulation of tissue factor mRNA from the mean basal level of 3.21 ± 0.25 pg/μg total RNA to 10.082 ± 1.55 pg/μg total RNA in ECV 304 cells. The effect of LPS on the expression of tissue factor is more pronounced in THP-1 cells compared to ECV 304 cells. LPS caused an up-regulation of tissue factor mRNA from the mean basal level of 6.51 ± 1.1 pg/μg total RNA to 27.99 ± 6.94 pg/μg total RNA in THP-1 cells.

4.2.2.5 Effect of LPS on the expression of tissue factor pathway inhibitor gene in THP-1 and ECV 304 cells

The effect of LPS on tissue factor pathway inhibitor gene expression in THP-1 cells and ECV 304 cells was investigated as above. The cells were incubated with 10μg/ml LPS for 60min, total RNA was prepared from the cells and amplified for tissue factor pathway inhibitor using QC-RT-PCR. Figures 4.11a and 4.11b show the effect of LPS on the expression of tissue factor in ECV 304 and THP-1 cells respectively. The exposure to LPS resulted in a 3 fold up-regulation in tissue factor pathway inhibitor gene in ECV 304 cells, increasing from mean basal level of 24.5 ±19.7 pg/μg total RNA to 79.3 ± 48.1 pg/μg total RNA. In THP-1 cells, the basal expression of tissue factor pathway inhibitor fell slightly from 12.29 ± 1.49 fg/μg total RNA to 10.64 ± 2.21 fg/μg total RNA when treated with LPS.

4.2.3 An investigation into the effect of minimally modified LDL on tissue factor activity and the gene expression of both tissue factor and tissue factor pathway inhibitor.

LDL was prepared from blood of healthy volunteers as described in section 2.1. In order to inhibit oxidative modification of the LDL by transition metal ions during preparation, 2μM DTPA was present throughout the isolation procedure.

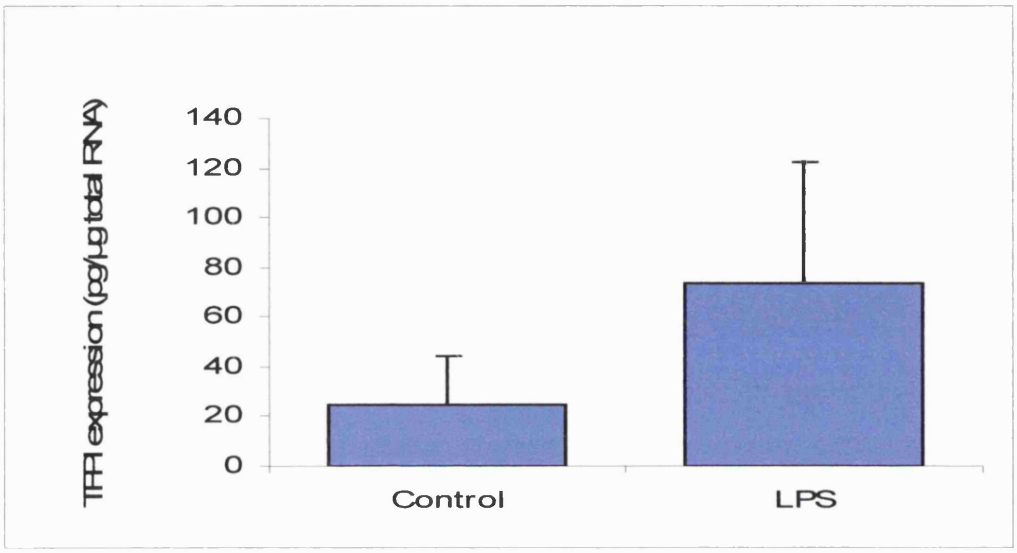


Figure 4.11a: Effect of LPS on TFPI gene expression in ECV 304 cells. ECV 304 cells were incubated for 1 hour with 10 µg/ml LPS. Total RNA was isolated and TF mRNA levels were determined by QC-RT-PCR. Values denote mean ± SEM n=8 independent experiments.

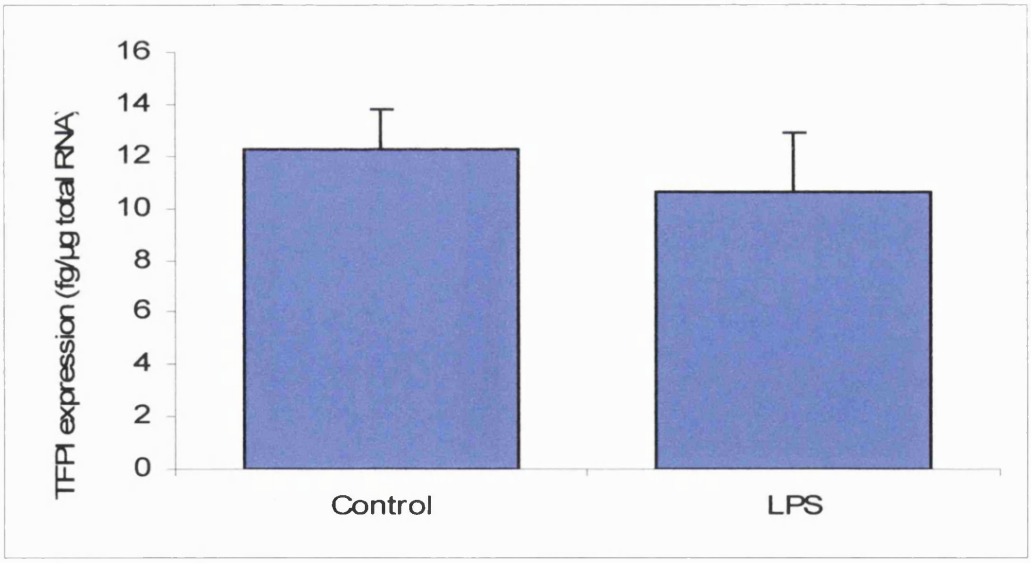


Figure 4.11b: Effect of LPS on TFPI gene expression in THP-1. Cells were incubated for 1 hour with 10 µg/ml LPS. Total RNA was isolated and TF mRNA levels were determined by QC-RT-PCR. Values denote mean ± SEM n=12 independent experiments.

4.2.3.1 Study of the kinetics of LDL oxidation

Native LDL was minimally oxidised in air at 37°C under sterile conditions. The kinetics of oxidation of LDL was measured by monitoring the change in the 234nm diene absorption (section 2.1.3.1), lipid peroxide (section 2.1.3.2) and electrophoretic mobility on agarose gels (section 2.1.3.3) over a 7day period.

Oxidative modification of LDL renders it more negatively charged and thereby increases the anodic electrophoretic mobility of the LDL on agarose gel. The 7-day time-course of LDL oxidation, monitored by the electrophoretic mobility on agarose gel (Figure 4.12) shows a slight increase in the distance migrated by the LDL samples. The relative electrophoretic mobility increased to approximately 1.25 versus native LDL on day 7. The lipid peroxide content of the sample (Figure 4.13) shows two phases as the oxidation proceeds over 7 days. There is a lag phase during which the lipid peroxide does not increase or only slightly increased, indicating that lipid peroxidation is low, and a propagation phase during which the lipid peroxide increases rapidly indicating lipid peroxidation. These data only show the early phase of LDL oxidation, excluding the later phase in which there is little or no further increase in the lipid peroxidation over time.

The duration of the lag phase varies within LDL isolated from different individuals and it could depend largely on donor-specific factors or the endogenous antioxidants contained in the LDL samples. Minimally oxidised LDL in this study is defined as the LDL in transit from lag phase to the propagation phase, which has lost all its antioxidants. It is the LDL in the earlier stage of oxidation with an increase in lipid peroxides concentration and just a slight shift in electrophoretic mobility. This level of LDL oxidation was chosen to cause minimum damage to cells.

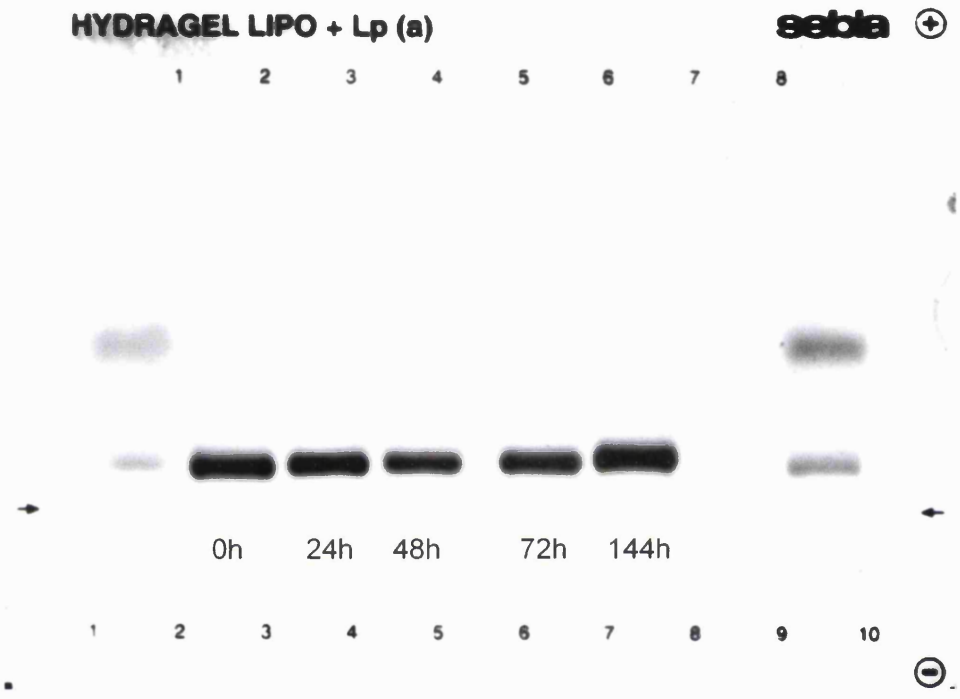


Figure 4.12 Comparison of changes in electrophoretic mobility of mmLDL oxidised over a 7-day period. Native LDL was oxidized in air for a period up to 7days. Samples were taken out at the indicated time point and 1μM DTPA was added to prevent further oxidation.
This result is a representatives of at least 4 independent experiments.

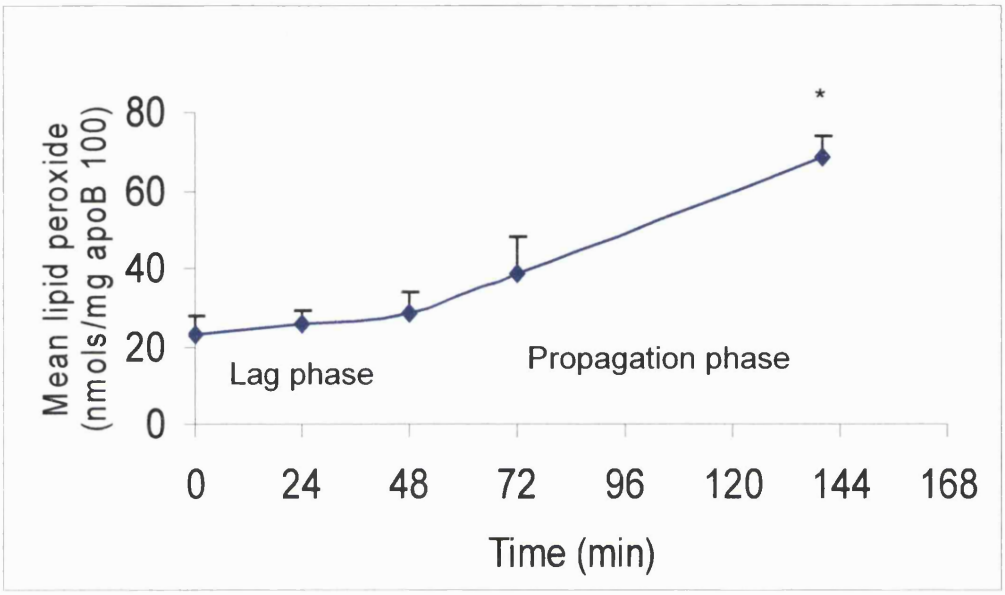


Figure 4.13: Time course of LDL oxidation: This data shows the relationship between lipid peroxide content of mm LDL and extent of oxidation. The data shows the beginning of propagation phase (full oxidation of LDL takes several hours). This result is a representatives of at least 4 independent experiments using different LDL preparations.

4.2.3.2 Study to confirm the minimal oxidation of LDL

In order to have comparable results, it is important to use LDL forms with similar levels of oxidative modification. Minimal oxidation of LDL was achieved by incubating the LDL in air for 18-96 h at 37°C under sterile conditions. LDL samples were withdrawn at intervals for analysis of lipid peroxide, conjugated dienes and relative electrophoretic mobility. Table 4.1 shows the range of the values in different preparations of native and minimally oxidised LDL.

	Relative electrophoretic mobility (versus native)	Lipid peroxide (nmol / mg apo B100)	A 234
Native LDL.	1	10.0 – 25.0	0.005 – 0.029
mm LDL.	1.05 – 1.2	30.0 – 100	0.030 – 0.060

Table 4.1 Methods used to confirm the minimal oxidation of LDL. The extent of LDL oxidative modification was assessed my measurement of lipid peroxide using the CHODE-iodide method, conjugated dienes by absorbance at 234nm and relative electrophoretic mobility on agarose gels.

At the point the A234 readings of the LDL increased by at least 0.02 above the initial value and the concentration of lipid peroxide increased above the initial values, but within the range defined in table 4.1, the oxidation was stopped and LDL defined as minimally oxidised LDL. The minimal oxidation was also confirmed by the relative electrophoretic mobility of the LDL samples. A comparison of the lipid peroxide content of the different preparations of native and minimally oxidised LDL is shown in Figure 4.14. There was a significant increase in the lipid peroxide content of LDL after minimal oxidation ($p<0.050$; $n=8$) and an increase in the diene absorbance at 234nm (Figure 4.15) was also evident.

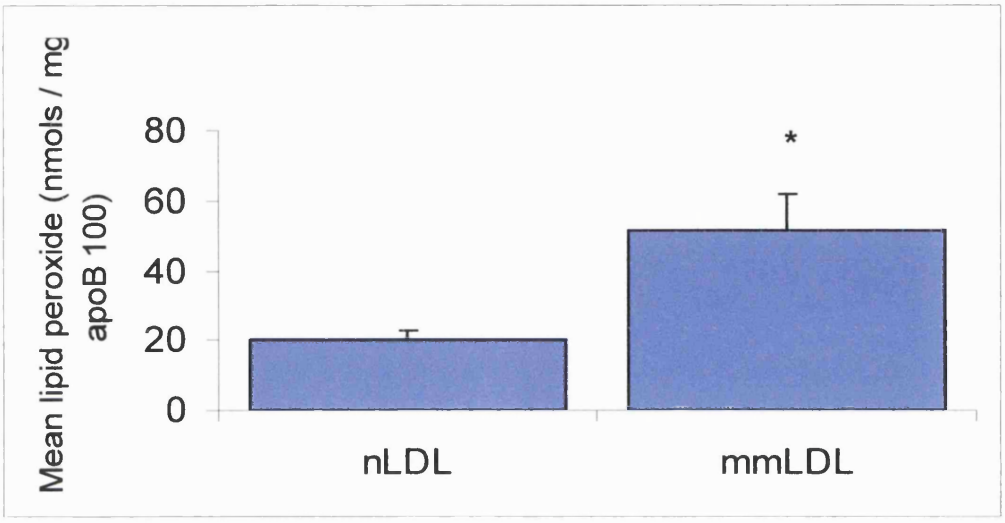


Figure 4.14: Comparison of the lipid peroxide content of native and minimally oxidised LDL. (N=8, * p<0.050)

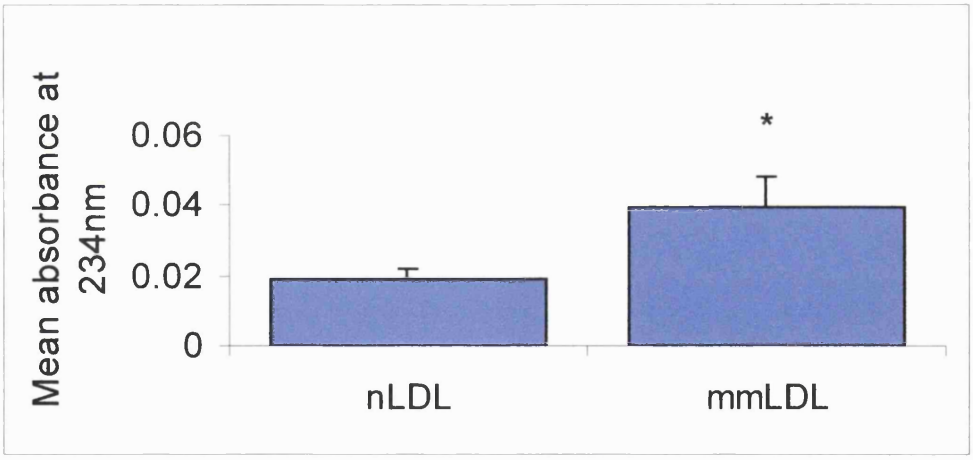


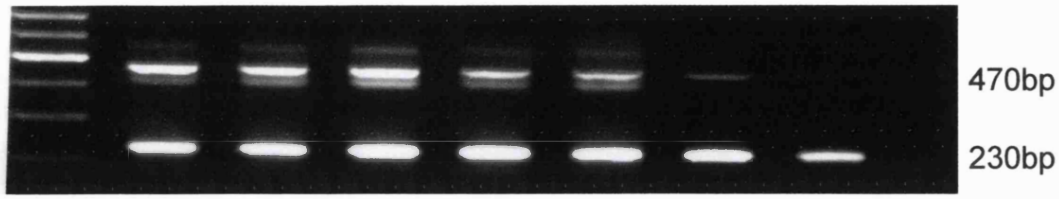
Figure 4.15: Comparison of the absorbance at 234nm of the native and mmLDL (N=8, * p<0.050).

4.2.3.3 Time course of mmLDL effect on the expression of tissue factor in THP-1 cells

Following LDL minimal oxidation, mmLDL (50µg protein/ml) was added to THP1 cells and incubated for up to 6 h. After incubation, the cells were washed in PBS and total RNA was prepared from the samples. Figure 4.16 represents the qualitative (a) and quantitative (b) data obtained from QC-RT-PCR of the total RNA. The tissue factor expression induced by 50µg mmLDL protein/ml increased after 30min, reached a plateau at 45 min, falling at 90 min and then returned to basal levels at 2 h.

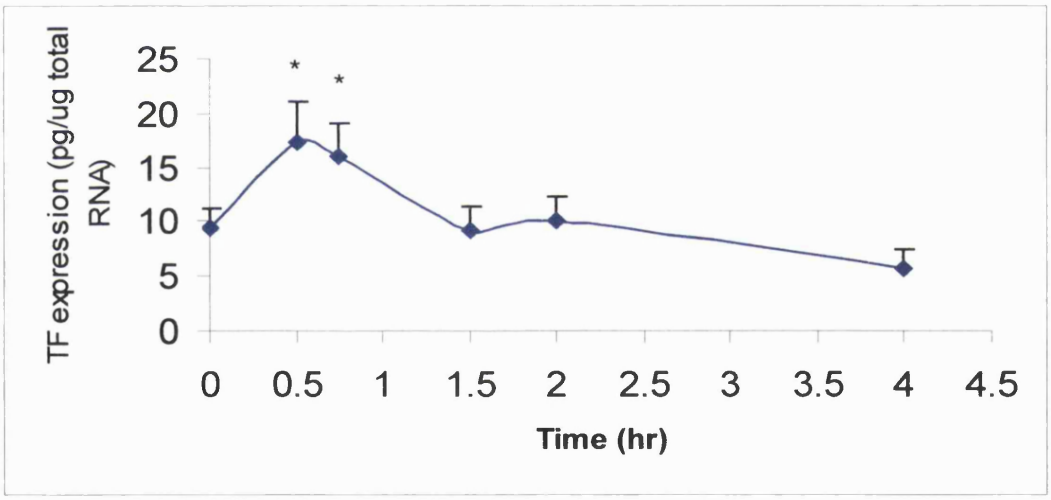
4.2.3.4 The effects of mmLDL on tissue factor and tissue factor pathway inhibitor gene expression in ECV 304 cells

ECV 304 cells were incubated with 50µg protein/ml mmLDL for 60 min. Total RNA was prepared from the cells and amplified for tissue factor and tissue factor pathway inhibitor using QC-RT-PCR. In ECV 304 cells, the basal levels of tissue factor mRNA varied from 2.27 – 3.99 pg/µg total RNA with a mean value of 2.89 pg/µg total RNA. When stimulated with mmLDL, the expression of tissue factor rose above the basal level to 8.34 ± 0.936 pg/µg total RNA (Figures 4.17). Basal tissue factor mRNA was considerably higher in the THP-1 cells, with mean basal level of 5.20 pg/µg total RNA and it increased to 13.60 ± 2.78 pg/µg total RNA when stimulated by mmLDL. Figure 4.18 shows the effect of mmLDL on the expression of tissue factor pathway inhibitor. In contrast to LPS, minimally oxidised LDL caused an increase in the expression of tissue factor pathway inhibitor from the basal level of 23.33 ±11.69 pg/µg total RNA to 49.20 ±27.97 pg/µg total RNA in ECV 304 cells and from 15.98 ± 4 fg/µg total RNA to 39.82 ± 19.68 fg/µg total RNA in THP-1 cells.



0hr 0.5hr 0.75hr 1.5hr 2hr 4hr 6hr

a



b

Figure 4.16: Time course of TF gene expression induced in THP-1 cells exposed to 50µg/ml mmLDL. mmLDL at a concentration of 50µg/ml was added to ECV 304 cells and incubated for the time indicated below each lane. Total RNA was isolate from the cells and TF was measured using QC-RT-PCR.
'a' is a representative agarose gel analysis for the QC-RT-PCR.
(mean ± SEM of 3 independent experiments * $p < 0.050$)

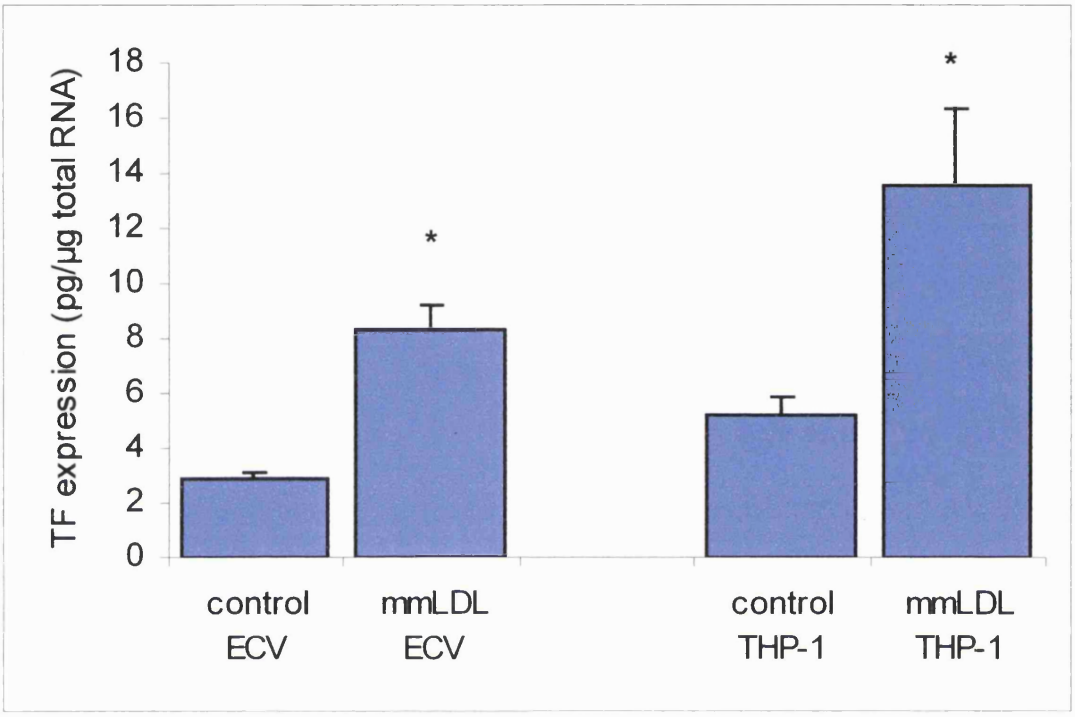


Figure 4.17: Effects of mmLDL on the expression of TF gene in THP-1 and ECV 304 cells. THP-1 and ECV 304 cells were incubated for 45 mins with 50 µg protein/ml mmLDL. Total RNA was isolated and TF mRNA levels were determined by QC-RT-PCR. Values denote mean ± SEM n=12 (THP-1) / n=8 (ECV 304) independent experiments. * $P < 0.050$

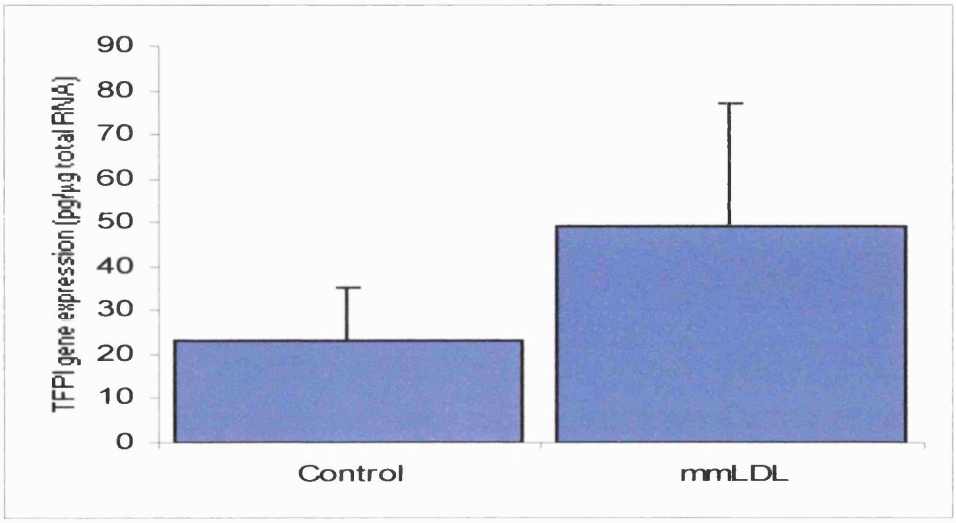


Figure 4.18a: Effect of mmLDL on TFPI gene expression in ECV 304 cells. ECV 304 cells were incubated for 1 hour with 50 µg protein/ml mmLDL. Total RNA was isolated and TF mRNA levels were determined by QC-RT-PCR. Values denote mean ± SEM n=8 independent experiments.

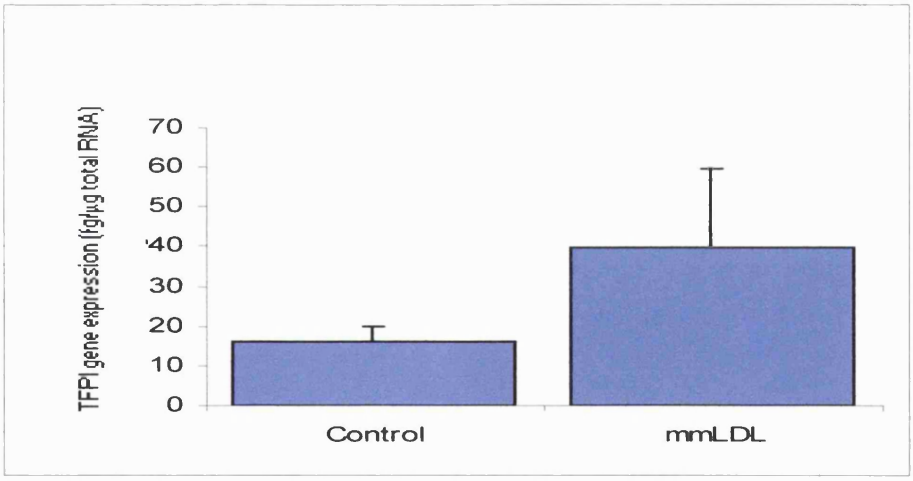


Figure 4.18b: Effect of mmLDL on TFPI gene expression in THP-1. Cells were incubated for 1 hour with 50 µg protein/ml mmLDL. Total RNA was isolated and TF mRNA levels were determined by QC-RT-PCR. Values denote mean ± SEM n=12 independent experiments.

4.2.3.5 An investigation into the influence of extent of oxidative modification of LDL on the expression of tissue factor mRNA

The effect of the extent of oxidative modification of LDL on the expression of tissue factor was studied in THP-1 and ECV 304 cells. The cells were treated with native LDL (nLDL), minimally oxidised LDL (mmLDL) and oxidised LDL (oxLDL). Oxidised LDL in this context refers to minimally oxidised LDL kept for a period of more than 12 weeks and thereby becoming more oxidised and not conforming to the values indicated in table 4.1. Figure 4.19 is a representative picture of LDL agarose gel comparing the distances migrated by the native (lanes2&3), minimally oxidised (lanes 4&5) and oxidised LDL (lanes 6&7). The mobility of the LDL increased with the degree of oxidation. While the native LDL hardly moved from the application point, there was a slight shift in the distance migrated by the mmLDL. The relative electrophoretic mobility of the mmLDL showed an increase from 1 to ≈1.1 when compared to the native LDL while the relative electrophoretic mobility of the oxidised LDL compared to the native was ≈1.4. The lipid peroxide content was found to be higher in minimally oxidised LDL compared with the oxidised LDL.

Studies were carried out to observe the effect of native LDL, minimally oxidised LDL and oxidised LDL on the expression of tissue factor in THP-1 and ECV 304 cells. Generally, the same effect was noted in the two cell lines. Native LDL did not significantly increase tissue factor gene expression. Native LDL from some donors actually suppressed the expression of tissue factor, whereas those prepared from other donors had little or no effect at all on this expression: samples from some donors did induce tissue factor expression. There was an increase in the expression of tissue factor in cells treated with minimally oxidised LDL (figure 4.20a). In THP-1 cells, minimally oxidised LDL caused an increase in the expression of tissue factor from the basal level of 4.34 ± 0.53 pg/μg total RNA to 12.07 ± 2.39 pg/μg total RNA.

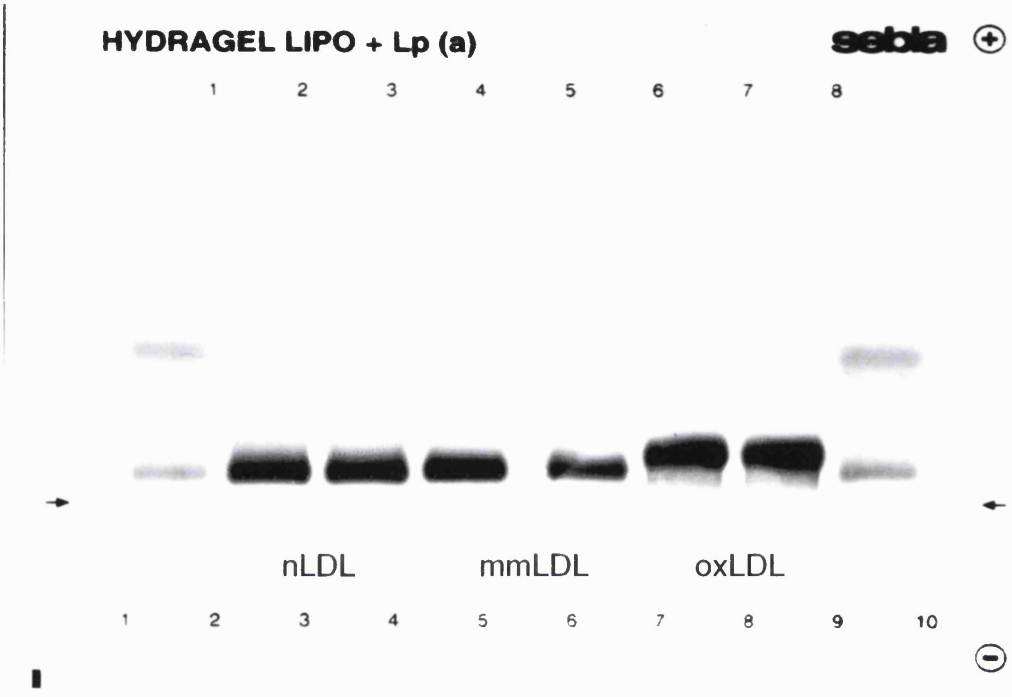


Figure 4.19: A representative agarose gel showing the electrophoretic mobility of native LDL, minimally oxidised LDL and more extensively oxidized (ox) LDL. mmLDL was oxidized by exposure to air at 37^{0}C for 72 h while the oxLDL was a once mmLDL that was kept in the refrigerator for 12 weeks. The lipid peroxide content of the native was well within the range of 10-25 nmol/mg apoB 100; in the mmLDL the lipid peroxide content was between 30 and 100 nmol/mg apoB100 (table 4.1), while the lipid peroxide content of the oxLDL was greater than 110nmol/mg apoB 100.

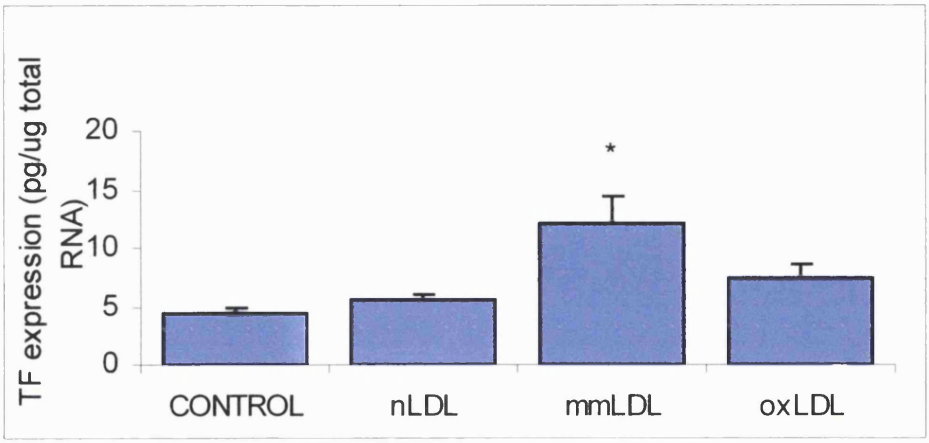


Figure 4.20a: Effects of native (n), minimally modified (mm) and extensively oxidised (ox) LDL on TF gene expression. THP-1 cells were incubated for 45min with 50 µg protein/ml native, minimally modified or extensively oxidised LDL. Total RNA was isolated and TF mRNA levels were determined by QC-RT-PCR. Values denote mean ± SEM n=3 of different LDL preparations. $P < 0.050$

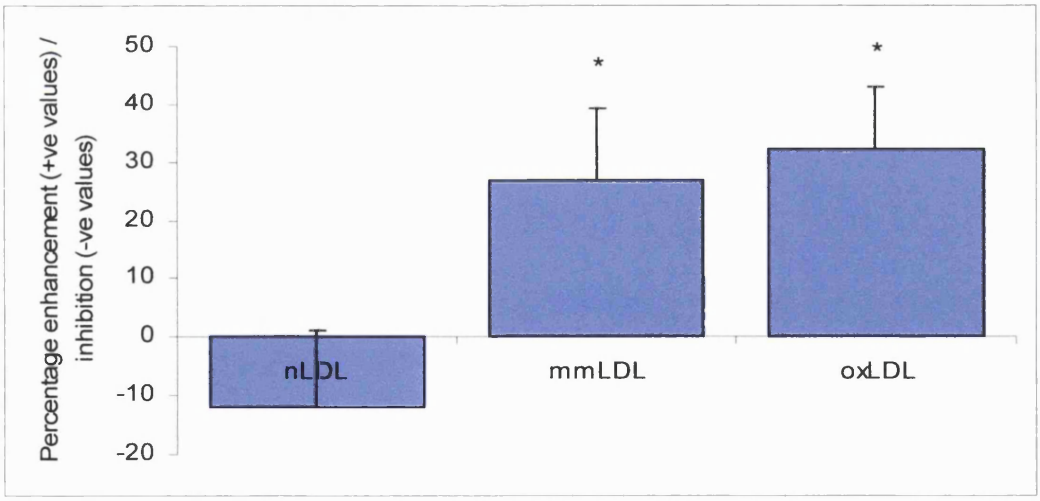


Figure 4.20b: Effects of native (n), minimally modified (mm) and extensively oxidised (ox) LDL on TF surface activity in THP-1 cells. THP-1 cells were incubated for 4 hours with 50 µg protein/ml nLDL, mmLDL or oxLDL. Cell surface tissue factor activity was determined by one-stage prothrombin time assay. The clotting time was converted to TF units using the recombinant tissue factor standard curve. The results represent the % enhancement (+ve values) or inhibition (-ve values) of procoagulant activity compared with controls. Values are presented as mean ± STD n=9; * $p < 0.050$

Oxidised LDL also increased the expression of tissue factor but to a lesser extent (7.47 ± 1.24 pg/µg total RNA) when compared with mmLDL. Similar observations were made with the tissue factor surface activity (figure 4.20b). Tissue factor activity was inhibited to a small extent by native LDL, by an average of 12% ± 13.03. As the oxidative modification of LDL progressed, the ability of the LDL to inhibit tissue factor activity was lost. While the native LDL suppressed the cells tissue factor activity, mmLDL and oxLDL enhanced tissue factor activity in THP-1 cells by up to 32% after 4 h.

4.2.3.6 The effect of different populations of mmLDL on the expression of tissue factor and tissue factor pathway inhibitor mRNA

There was a wide variation in the amount of mmLDL-induced tissue factor (Figure 4.20a). To determine whether this variation was due to donor-specific factors, LDL was prepared from the plasma of healthy donors and minimally oxidised in air. The LDL samples were not pooled together but were tested individually for the induction of tissue factor mRNA. Figure 4.21 shows the effect of 3 different preparations of mmLDL from different individuals on the expression of tissue factor in THP-1 cells. mmLDL preparations 1 and 2 had little or no effect at all on the expression of tissue factor while preparation 3 increased the accumulation of tissue factor mRNA.

Since the effect of mmLDL was tested on different populations of cells, the variations in the response of the cells to mmLDL could be due to cell-specific factors. Hence, experiments were carried out to test the effect of mmLDL preparation 1 on the expression of tissue factor in THP-1 cells with the inclusion of endotoxin lipopolysaccaride (LPS) as a positive control. The data illustrated in figure 4. 22 shows while the mmLDL had little effect on tissue factor expression, LPS (10µg/ml) increased the accumulation of tissue factor mRNA.

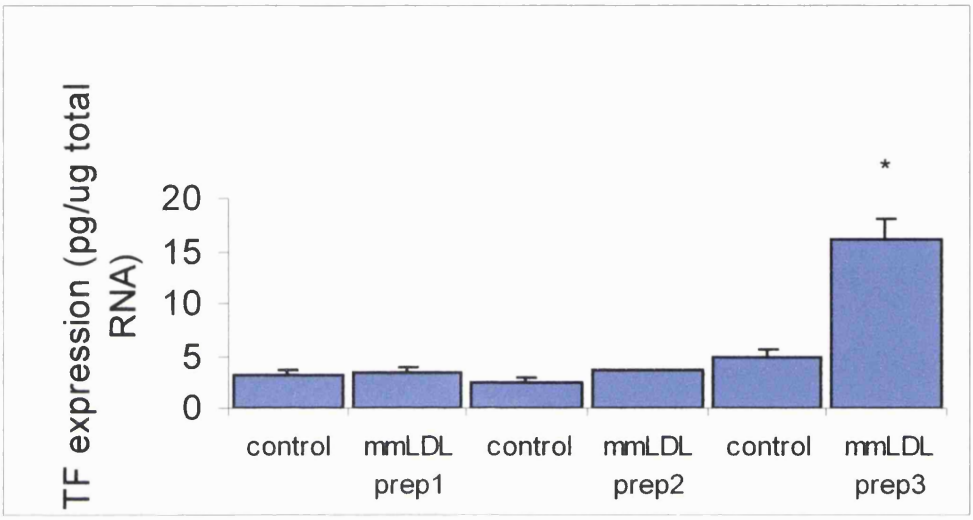


Figure 4.21 Effect of different preparations of mmLDL on TF expression in THP-1 cells. LDL preparations from 3 individual donors were tested for the induction of TF gene expression. The LDL samples were minimally oxidised in air and all the mmLDL preparation had comparable lipid peroxide content and relative electrophoretic mobility on agarose gel. mmLDL was added to THP-1 cells at a concentration of 50µg protein/ml for 45 min. Total RNA was prepared from the cells and subjected to QC RT-PCR. Values denote mean ± SEM n=3 different LDL preparations. * $P < 0.050$

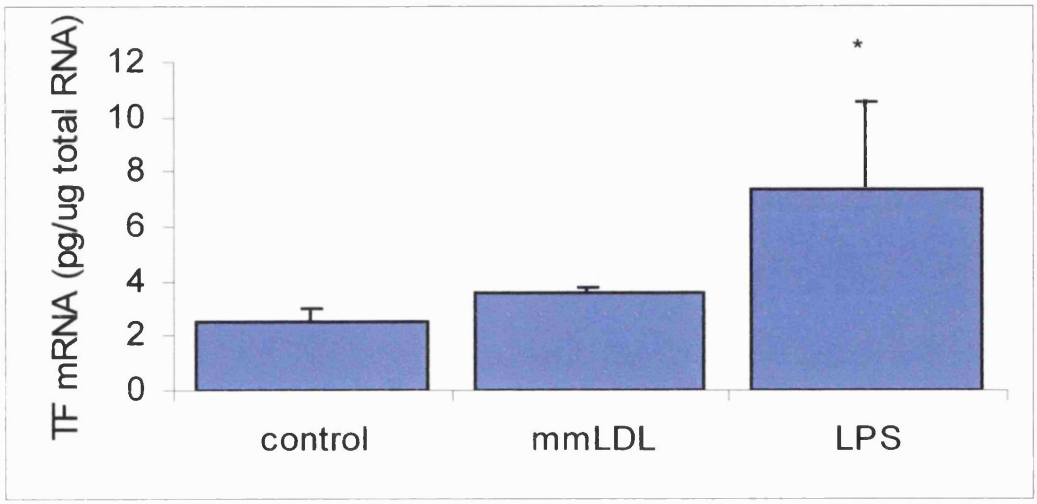


Figure 4.22: Effect of mmLDL prep 1 and LPS on the expression of TF gene in THP-1 cells. THP-1 cells were incubated for 45min with 50 µg protein/ml mmLDL prep 1 or 10µg/ml LPS. Total RNA was isolated and TF mRNA levels were determined by QC-RT-PCR. Values denote mean ± SEM n=3 different LDL preparations. * $P < 0.050$

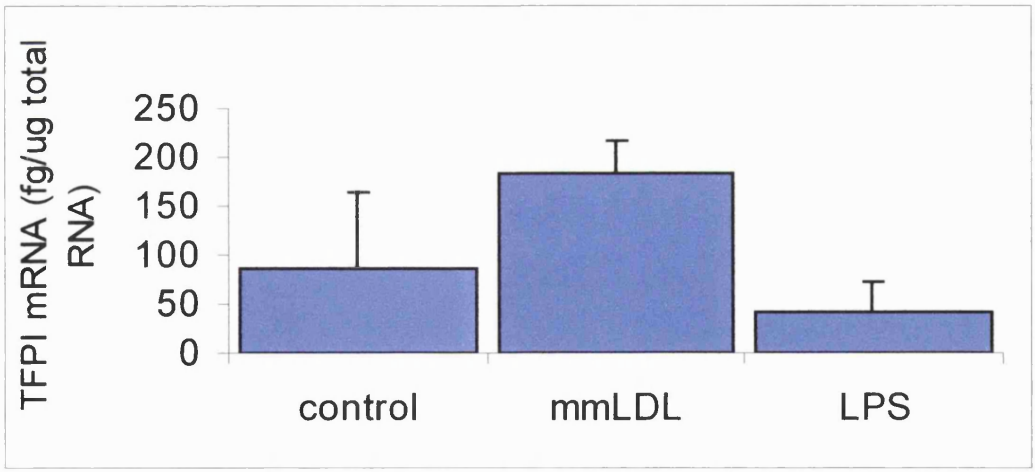


Figure 4.23: Effect of mmLDL prep 1 and LPS on the expression of TFPI gene in THP-1 cells. THP-1 cells were incubated for 45min with 50 µg protein/ml mmLDL prep 1 or 10µg/ml LPS. Total RNA was isolated and TFPI mRNA levels were determined by QC-RT-PCR. Values denote mean ± SEM n=3 different LDL preparations.

It is interesting to note that, while the mmLDL preparation 1 had no effect on tissue factor expression, it caused a rapid increase in tissue factor pathway inhibitor expression (Figure 4.23). While there was a sharp increase of tissue factor mRNA in the LPS stimulated cells, no increase in tissue factor pathway inhibitor mRNA was observed.

4.2.3.7 The effects of lysophosphatidylcholine and phosphatidylcholine on tissue factor and tissue factor pathway inhibitor gene expression

It is not clear whether the induction of gene expression by mmLDL is due to compounds generated during the lipid peroxidation. To test this, the involvement of lysophosphatidylcholine and phosphatidylcholine in the induction of tissue factor and tissue factor pathway inhibitor mRNA by mmLDL was studied. Experiments were carried out to study the effects of lysophosphatidylcholine (a lipid component of oxidised LDL) and phosphatidylcholine (found in native LDL) on the expression of tissue factor and tissue factor pathway inhibitor in THP-1 and ECV 304 cells. Neither lysophosphatidylcholine nor phosphatidylcholine, at a concentration of 0μM-200μM, had any significant effect on the expression of tissue factor and tissue factor pathway inhibitor (not shown).

4.2.3.8 Effect of peroxynitrite on the expression of tissue factor and tissue factor pathway inhibitor genes.

It has been suggested that peroxynitrite might play a role in the development of atherosclerosis (White *et al*, 1994) and the oxidative modification of LDL *in vivo* could be due in part to peroxynitrite. Studies were therefore carried out to investigate whether peroxynitrite may also directly influence the expression of tissue factor. Cells were exposed for 1h to SIN-1 (3-morpholinosydnonimine), which slowly releases the precursors of peroxynitrite (Ma *et al*, 1997).

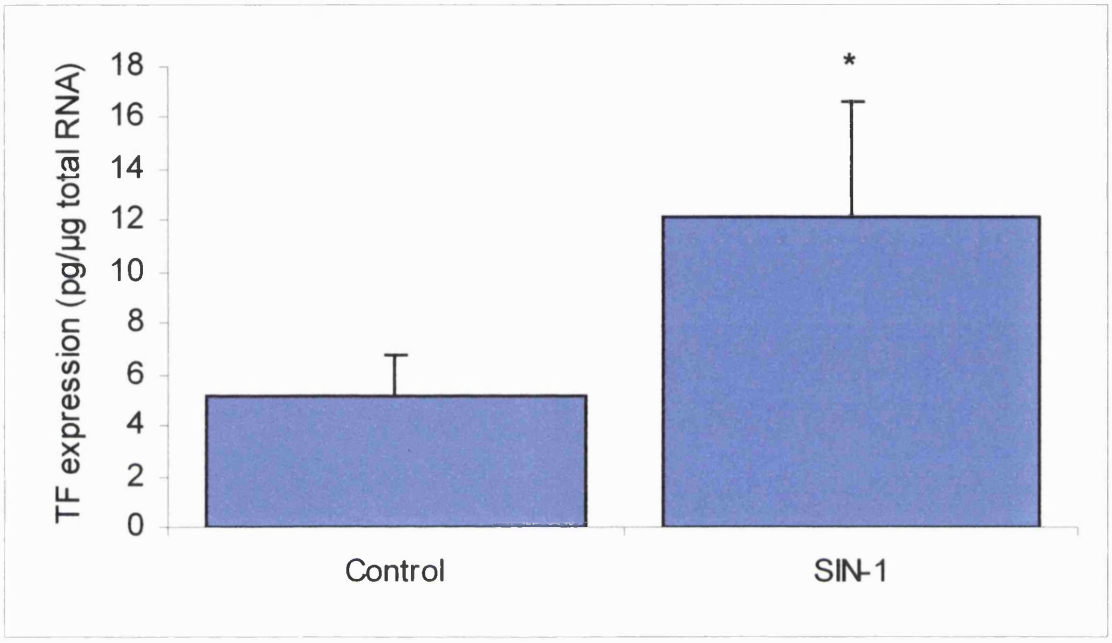


Figure 4.24: Effects of SIN-1 on the expression of TF gene in THP-1. THP-1 cells were incubated for 1 hour with 50µM SIN-1. Total RNA was isolated and TF mRNA levels were determined by QC-RT-PCR. Values denote mean ± SD of 5 independent experiments, * $p<0.050$

Total RNA prepared from the cells was assayed for tissue factor and tissue factor pathway inhibitor using QC RT-PCR. Figure 4.24 shows the effect of SIN-1 on the expression of tissue factor in THP-1 cells. SIN-1-induced the expression of tissue factor, from a mean basal level of 5.13 ± 1.65 to 12.11 ± 4.54, in the experimental model of endothelial cell but it had no effect on the expression of TFPI.

4.2.4 Tissue factor and tissue factor pathway inhibitor gene expression in other cell types.

The effects of LPS and mmLDL on the expression of tissue factor and tissue factor pathway inhibitor were observed in endothelial and monocytic cell. Studies were also carried out to determine if the observed effects can be reproduced in other cell lines. The actions of LPS and mmLDL on the expression of the genes were studied in human umbilical vein endothelial cells (HUVEC), fibroblasts (AG-1) and mouse skeletal muscle cells (C_2C_{12}). LPS and mmLDL had comparable effects on HUVEC and AG-1 cells with those obtained from ECV 304 cells. The mouse skeletal muscle cell had a high basal level of tissue factor pathway inhibitor mRNA, but did not express tissue factor gene, even after stimulation with LPS and mmLDL.

4.3 Discussion

Oxidatively modified LDL as well as tissue factor expression by monocyte-derived macrophages and vascular endothelium, has been associated with the development of atherosclerosis. Cell surface tissue factor activity has also been implicated in apoptosis (Greeno *et al*, 1996). The initial experiments using Northern hybridisation revealed that the chosen endothelial and monocytic cell lines used in this study expressed tissue factor at basal levels, which was further up-regulated in the presence of mmLDL and LPS. Northern blot analyses of ECV 304 and THP-1 cells incubated for 1h with either 10 µg/ml LPS or 50 µg mmLDL protein/ml revealed an exaggerated up-regulation (up to 30-fold increase) of TF. The unreliability of the Northern hybridisation techniques called for the development of the QC-RT-PCR.

The expression of tissue factor mRNA may not necessarily reflect functional availability of the active protein, because activity is regulated by inhibitors such as annexin V, antithrombin III or tissue factor pathway inhibitor (Camerer *et al*, 1996). Studies were carried out to investigate whether the up-regulation of tissue factor mRNA is accompanied by an increase in tissue factor antigen and cellular tissue factor activity. Tissue factor antigen increased upon treatment with LPS in parallel with tissue factor cell surface and total activity. The increase in tissue factor activity induced by LPS and mmLDL was time-dependent with maximum induction at 6 h. This is in agreement with the study by Crossman and co-workers (1990), in which the maximal induction of tissue factor activity in endothelial cells was found to be 6 h post treatment with LPS.

The increase in cell surface tissue factor activity could result from changes in the availability of tissue factor as a result of increased gene expression or as a result of the exposure of functional tissue factor that presumably existed in an encrypted form. The tissue factor activity on the surface of the cell was less than

20% of the total tissue factor activity from the cell lysates, suggesting that some of the tissue factor was encrypted and hence not active on the surface of the cell. It was evident from the cell culture experiments that cultured monocytes exhibited spreading in response to external stimuli (data not shown). Incubation of THP-1 cells with LPS and mmLDL did moderately promote the adherence of the cells to the culture flasks, although they cells were easily dislodged in PBS by shaking. Monocyte tissue factor activity may be stimulated in part by the adherence of monocytes to the culture vessel leading to cell signal transduction via activation of phospholipases (Lefkowith *et al*, 1992).

Tissue factor mRNA is very unstable with a half-life of 60-90min. The time course of induction of tissue factor gene expression in THP-1 cells revealed the presence of tissue factor mRNA in non-stimulated THP-1 cells. Treatment with 50μg/ml mmLDL caused an increased in the levels of tissue factor mRNA that peaked between 30 min to 1 h and dropped to basal levels after 90min. Treatment of THP-1 cells with 10μg/ml LPS also increased the expression of tissue factor after 30min, reached a plateau at 45min and fell at 90 min reaching basal levels at 2h. Using the microarray technology, Mikita *et al* (2001) demonstrated the up-regulation of 41 genes in LPS stimulated THP-1 cells, which exhibit peak up-regulation at 1 h. These genes include the pro-inflammatory TNF alpha, IL-1 α, IL-1 β and COX-2.

Brand *et al* (1991) showed that transcriptional and post-transcriptional regulations played roles in LPS induction of tissue factor mRNA. Similarly, Cui *et al* (1999) postulated that the accumulation of tissue factor mRNA could be due to an increase in the rate of transcription. Crossman *et al* (1990) showed that the accumulation of tissue factor mRNA in endothelial cells was dependent on increased mRNA stability. The upregulation of tissue factor in cells exposed to LPS results, at least in part, from mRNA stabilization that affects both the rates at which mRNA disappears following transcriptional repression, and its

accumulation following transcriptional induction (Crossman *et al*, 1990; Ross, 1995).

LDL oxidation

There was a variation in the susceptibility of LDL isolated from different donors to oxidation. LDL from some donors required a long exposure to air before becoming minimally oxidised, while LDL preparations from other donors oxidised rapidly in air. This could be due to the different amounts of antioxidant present in the LDL samples. LDL contains endogenous antioxidants, particularly α-tocopherol, which affords some protection from oxidation. Jessup *et al* (1990) showed that addition of α-tocopherol to LDL, or ingestion of large doses of it, increased the lag time for initiation of oxidation. Also there is a great tendency that donor-specific factors in LDL may increase or decrease the LDL's resistance to oxidation. Human plasma LDL is a heterogeneous collection of particles, which vary in size, buoyant density, lipid and apolipoprotein composition, ultracentrifugal flotation rate and particle diameter. Tribble *et al* (1992) found that LDL preparations characterised by smaller and denser LDL particles oxidised more readily compared to LDL with larger and more buoyant particles. Different concentration of endogenous lipid peroxides and different amounts of unsaturated fatty acids in the LDL may also influence the susceptibility of LDL to oxidation. Reaven *et al* (1991) demonstrated that LDL rich in polyunsaturated fatty acid are more readily oxidised than LDL rich in saturated fatty acids or monounsaturated fatty acid.

The methods used to oxidise LDL vary between different laboratories and the classification of LDL based on its degree of oxidation is not well defined. In this study, LDL was mildly oxidised using the method established in this research laboratory (Naseem et al, 1997). Minimal oxidation of LDL was achieved by incubating the LDL in air at 37^0C and monitoring the conjugated diene and the peroxide contents every 6 h. Previously, mmLDL was prepared by leaving it at

4^0C for several weeks. LDL is minimally oxidised so that only the lipid is slightly oxidised without any significant modification of the protein. Great effort was made to oxidise all the LDL used for the experiments, to the same extent by monitoring the LPO content, the conjugated dienes, and the relative electrophoretic mobility of each preparation. The exposure of native LDL to air at 37^0C for periods varying from 18-72 h generally resulted in a comparable increase in the conjugated dienes absorbance at 234nm and also a significant increase of lipid peroxide compared with native LDL.

Effect of LDL oxidative modification on gene expression

The mechanisms inducing the oxidation of LDL *in vivo* are uncertain. The degree of LDL modification in the arterial wall may vary. It has been suggested that the cells in early atherosclerotic lesions are mainly exposed to less extensively oxidized LDL (Napoli *et al*, 2000); because LDL extracted from advanced, necrotic lesions showed extensive apolipoprotein fragmentation (Palinski *et al*, 1989) and the onset of apoptosis due to moderately oxidised LDL appears to occur in earlier stages of lesions (Kockx and Herman, 2000).

The results presented in section 4.2.3.1 demonstrated the effect of the extent of LDL oxidation on the expression of tissue factor. Native LDL had little effect on the expression of tissue factor gene. Some preparations of the native LDL used in the experiment actually suppressed the expression of tissue factor, while others had no effect at all on this expression; only in a minority of preparations was the expression of tissue factor slightly increased. This finding contrasts with that of Cui *et al* (1999) who reported that native LDL significantly induced tissue factor gene expression in cultured rat aortic smooth muscle cells. This discrepancy may reflect the differences in blood donors and the criteria used to define native LDL.

The effect of native LDL on the expression of tissue factor mRNA could depend on the antioxidant status of the donor since vitamin E and carotenoid are carried within the LDL particles and this could prevent oxidation of the LDL. It is possible that the native LDL that did induce the expression of tissue factor had low levels of endogenous antioxidant as well as small amounts of peroxides and became oxidatively modified during isolation procedures. The samples that suppressed the expression of tissue factor might have had high levels of endogenous vitamins.

In most cases, minimally oxidised LDL significantly increased the expression of tissue factor, in agreement with studies by Drake *et al* (1991) and Fei *et al* (1993). The studies by Drake *et al* (1991) showed that the expression of tissue factor in endothelial cells was induced by mmLDL and not by highly oxidized LDL. It is known that the biological properties of the lipids in mmLDL are different from the biological properties of the lipids in oxLDL and while the lipids in highly oxidized LDL are cytotoxic, the lipids in mmLDL are not cytotoxic or less so (Cathcart *et al*, 1991). Highly oxidised LDL also promotes human monocyte differentiation and adhesion, giving rise to a macrophage-like monocyte (Frostegard *et al*, 1990).

The wide variation in the amount of mmLDL-induced tissue factor and tissue factor pathway inhibitor mRNA could be due to donor-specific factors and/or the extent of LDL oxidation. Because the chemical, physico-chemical, functional and biological properties of LDL change continuously during the process of oxidation, it is impossible to have LDL samples with defined constant composition and with reproducible biological properties (Esterbauer *et al* 1992). Most experiments reported by others use LDL oxidised by exposure to copper ions, but the levels of copper found in plasma and arteries are a million-fold smaller than those used *in vitro* for LDL oxidation (Napoli, 1996). Copper oxidation may produce a more uniform oxidation, but because of the high level of copper, some of which may

remain bound to the LDL even after dialysis, copper oxidation was not used. Also, there is a report that copper on its own induces tissue factor mRNA in cells (Crutchley and Que, 1995).

Factors influencing the effect of LDL on gene expression

The evidence for the influence of LDL donor-specific factors on the effects of LDL on gene expression comes from the observation that LDL from some donors did not induce tissue factor mRNA after minimal oxidative modification. The second line of evidence came from the studies of Edwards and Perla (1984), Østerud (1995), Amirkhosravi *et al* (1996) and Nijziel *et al* (2001). Edwards and Perla (1984) showed that serum and plasma enhanced monocyte tissue factor generation in cultures of normal peripheral mononuclear cells. Østerud (1995) observed that monocytes of some individuals express more tissue factor than monocytes of others and called it the 'high and low responder phenomenon'. This phenomenon was defined as a 50-fold difference in monocyte tissue factor activity between the highest responder and the lowest responder in LPS-stimulated whole blood (Østerud, 1995). Amirkhosravi and co-workers (1996) suggested that platelets could be responsible in part for this phenomenon because they were able to show that platelet-rich plasma induced more tissue factor in LPS stimulated monocytes than platelet poor plasma. But Nijziel *et al* (2001) showed that the differences in tissue factor activity between the high responders and low responders were due to factors in the plasma, independent of platelets, granulocytes and LPS.

The variations in the effect of mmLDL on gene expression could also be due, in part, to the cells used in the experiments. In the 'high and low responder phenomenon' of Østerud (1995), it was observed that monocytes of some individuals express more tissue factor than monocytes of others. A cell-specific factor may exist that influences the response of cells to external stimuli, but the data that showed the effects of mmLDL and LPS on the expression of tissue

factor and tissue factor pathway inhibitor in a side by side experiment (figures 4.22 & 4.23) using identical cells do not seem to support this idea. While there was little or no induction of tissue factor gene by these particular preparations of mmLDL, LPS did significantly increase the expression of tissue factor. Also with these preparations of mmLDL, when there was little induction of tissue factor, an increase in the accumulation of tissue factor pathway inhibitor mRNA ocurred. However, LPS suppressed the expression of tissue factor pathway inhibitor in the cells, showing that the responses of the cells were complex.

Discrepancy in TF and TFPI gene expression

It was found that there was a balance in the expression of tissue factor and tissue factor pathway inhibitor in the mmLDL-treated THP-1 cells. When the cells were treated with mmLDL, there was an up-regulation of tissue factor pathway inhibitor mRNA with an increase in tissue factor expression. In the case of LPS treated THP-1cells, there was an imbalance between tissue factor and tissue factor pathway inhibitor expressions. LPS increased tissue factor mRNA and suppressed tissue factor pathway inhibitor mRNA in THP-1 cells.

This imbalance between tissue factor and tissue factor pathway inhibitor expression in plaque may result in a prothrombotic phenotype associated with atherosclerosis while the inhibition of tissue factor by tissue factor pathway inhibitor may inhibit vascular remodelling (Singh *et al*, 2001). Attenuation of increased thrombogenicity and neointima formation by overexpression of tissue factor pathway inhibitor provides evidence for therapeutic approaches for mitigating both the enhanced thrombogenicity and vascular remodelling associated with vascular diseases (Singh *et al*, 2001).

Unlike the response in ECV 304 cells, LPS suppressed the expression of tissue factor pathway inhibitor gene in THP-1 cells. There was an increase in the expression of tissue factor gene when THP-1 cells were treated with mmLDL or

LPS. A decrease in the expression of tissue factor pathway inhibitor mRNA by LPS has also been observed in cultured mouse endothelial cells and in cardiomyocyte cell lines (Shimokawa *et al*, 2000). The decrease in the expression of tissue factor pathway inhibitor may contribute to an increase in the procoagulant activities in inflammation and septic shock. About 70-85% of the plasma tissue factor pathway inhibitor content is bound to apoB containing lipoproteins (Rapaport and Rao, 1995). Studies by Hansen *et al* (1997) indicated that the anticoagulant function of tissue factor pathway inhibitor in human plasma is limited to its free form. They demonstrated that LDL-bound tissue factor pathway inhibitor isolated from human plasma did not possess inhibitory function in a tissue factor -induced coagulation assay *in vitro.*

Factors responsible for the effects of mmLDL and LPS on gene expression

It is not clear whether the induction of tissue factor by mmLDL is due to a single compound or a combined action of all the compounds generated during the lipid peroxidation process. The effect of lysophosphatidylcholine and phosphatidylcholine on the expression of tissue factor and tissue factor pathway inhibitor genes was studied both in the THP1 and ECV 304 cells. Lysophosphatidylcholine and phosphatidylcholine at a concentration of 0μM-200μM did not have any significant effect on tissue factor and tissue factor pathway inhibitor gene expression. Hence the ability of mmLDL to induce tissue factor mRNA does not rely on lysophosphatidylcholine. Studies in human monocytes reported that lysophosphatidylcholine suppresses tissue factor activity and also inhibits LPS-mediated tissue factor expression in human monocytes (Engelmann *et al*, 1999).

It has been demonstrated that peroxynitrite may oxidise LDL *in vitro* to a form that is not recognisable to the macrophage scavenger receptor (Graham *et al*, 1993). This effect could be a result of the decomposition of peroxynitrite to nitrogen dioxide and hydroxyl radicals that has been shown to initiate lipid

peroxidation (Hogg *et al*, 1992). SIN-1 does not release measurable nitric oxide in physiological solutions *in vitro* (unless in the presence of superoxide dismutase), but it is known to release peroxynitrite (Ma *et al*, 1997). Gerlach *et al* (1998) observed that NOC18, a pure NO donor had no effect on TF expression and activity. They noted that lower doses (125 and 250μM) of SIN-1 increased TF antigen expression in human monocytes, whereas higher doses (1,000 and 2,000 μM) had a reducing effect. They also observed an up-regulation of TF mRNA when the cells were treated with 500 μM SIN-1 while 1,000 and 2,000 μM SIN-1 had no effect on TF mRNA. This indicates that the reduction of TF antigen observed with the high doses of SIN-1 did not take place at the transcriptional but at the post-transcriptional levels.

The biological importance of LDL oxidation is not limited to atherogenesis. Minimally oxidized LDL formed in atherosclerotic lesions may activates a broad cascade of oxygen radical-sensitive signaling pathways affecting apoptosis and other processes influencing the evolution of plaques (Napoli *et al*, 2000). Kinscherf *et al* (1998) reported that oxidised LDL induces apoptosis in macrophages. The presence of apoptotic cells in atherosclerosis lesions (Geng and Libby, 1995; Bjorkerud and Bjorkerud, 1995; Mallat *et al*, 1997) supports a role of apoptosis in atherogenesis. The characteristic features of apoptosis in atherosclerotic lesions include shrinkage of cell membranes, formation of apoptotic bodies and nuclear chromatin condensation (Napoli *et al*, 2000).

Oxidative stress is important to the development of atherosclerosis. Mice lacking 12/15- lipoxygenase have considerably diminished atherosclerosis, suggesting that this enzyme may be an important source of reactive oxygen species in LDL oxidation (Cyrus *et al*, 1999). Reactive oxygen species may also be an important intracellular mechanism by which LPS induces tissue factor expression. Studies have demonstrated that LPS enhances monocytes formation of oxidant species (Landmann *et al*, 1995; Rosen *et al*, 1995) and also increases the production of

hydrogen peroxide in B cell lines (Schreck *et al*, 1992). This study confirmed both mmLDL and LPS as potent inducers of tissue factor expression in THP-1 and ECV 304 cells. It is possible that LPS, due to bacterial infection, and mmLDL increases thrombogenesis in atherosclerosis through a tissue factor dependent, oxidant-mediated mechanism.

CHAPTER 5:

The effect of antioxidant vitamins on the induction of tissue factor and tissue factor pathway inhibitor expression in monocytes and endothelial cells

5.1 Introduction

Tissue factor expression by monocyte-derived macrophages and vascular endothelium is associated with atherosclerosis (Semararo *et al*, 1985; Landers *et al*, 1994; Taudman *et al*, 1997). Endotoxin lipopolysacharide (LPS) is known to be capable of enhancing the production of free radicals in vascular cells leading to oxidative stress (Landmann *et al*, 1995; Rosen *et al*, 1995; Schreck *et al*, 1992) which is also an important factor in the development of atherosclerosis. Low-density lipoprotein is oxidatively modified during the development of atherosclerosis and this is considered to be an important event in the initiation and progression of disease (Steinbrecher *et al*, 1990; Witztum and Steinberg, 1991). The studies described in the previous chapter confirmed both minimally oxidised LDL and LPS as potent inducers of TF expression in monocytes and endothelial cells, raising the possibility that LPS and mmLDL increase the thrombogenesis associated with atherosclerosis via a tissue factor dependent, oxidant-mediated mechanism.

Antioxidants are compounds that delay or inhibit oxidation of an oxidizable substrate (Halliwell and Gutteridge, 1990). They are known to inhibit oxidant formation, intercept oxidants once they are formed and repair oxidant-induced injury (Tribble, 1999; Catapano and Tragni, 1999). Several lines of investigation have indicated that antioxidants have protective effects against oxidative damage and may be useful in the treatment of atherosclerosis. Antioxidants have been shown to retard the progression of atherosclerosis (Hodis *et al*, 1995;

Steinberg, 1997) and reduce the risk of coronary heart disease (Losonczy *et al*, 1996). Bruckdorfer *et al* (1995) observed that the antioxidant probucol not only reversed atherosclerosis seen in adult Watanabe hereditary hypercholesterolaemic rabbits, but also partly restored the severe loss of vasodilatory properties seen in the vessels of these animals.

The main water-soluble antioxidant in the plasma is ascorbate (Frei *et al*, 1989), while urate, bilirubin, and protein sulfhydryl species provide less-efficient antioxidant protection (Frei *et al*, 1988). Important lipid-soluble antioxidants include α-tocopherol, α-carotene, β-carotene, cryptoxanthin, lycopene, and lutein (Burton *et al*, 1983; Bendich and Olson, 1989), which are transported in lipoproteins in the circulation.

The tocopherols, also known as vitamin E are the most significant lipid-soluble, chain-breaking type of antioxidants present in human blood (Burton *et al*, 1983). The plasma total vitamin E content in healthy population varies from 20.07 – 28 .3 μM (Gey 1986) and about 30-40% of it is contained in LDL at an approximate concentration of 6 moles/mole apoB (Esterbauer *et al*, 1989; Bruckdorfer, 1995). There are several mechanisms by which α-tocopherol is thought to reduce atherosclerosis. Esterbauer *et al* (1992) showed that raising the vitamin E concentration of LDL increases the resistance of the LDL to oxidation by cupric ions; but the basal α-tocopherol did not correlate with endogenous resistance to Cu^{2+} oxidation. There is a positive correlation between plasma concentration of α-tocopherol and endothelium dependent vasodilator function (Kinlay *et al*, 1999). Chan (1998) reported that α-tocopherol prevents inflammation and monocyte / macrophage adhesion to the endothelium; it reduces the formation and uptake of cholesteryl ester in macrophages (Suzukawa *et al*, 1994). It is known to protect LDL from oxidation (Reaven *et al*, 1993; Rajendran *et al*,1996; Mabile et al, 1999) and reduces the uptake of oxidized LDL by inhibiting CD36 scavenger receptor expression in cultured aortic SMCs (Ricciarelli *et al*, 2000). It

also reduces cholesterol esterification and uptake of acetylated LDL in macrophages (Shige *et al*, 1998). Mabile et al (1999) found moderate supplementation with *α*-tocopherol effective in inhibiting LDL oxidation. In their studies, they found that supplementation with 75IU protected LDL against oxidation while Rajendran *et al* (1996) gave the minimum dose of *α*-tocopherol required to significantly reduce LDL oxidation as 400IU/day.

Carotenoids are another type of lipophilic antioxidants present in human blood. Human serum contains α-carotene, β-carotene, cryptoxanthin, lycopene, and lutein as major components of carotenoids (Parker, 1989). Lycopene and β-carotene are found predominantly in the serum, while lutein which is more polar than the other carotenoids predominates in both red blood cells and peripheral blood mononuclear cells (Fotouhi *et al*, 1996). Carotenoids are transported in the blood in association with lipoproteins, primarily with low-density lipoprotein (Auletta and Gulbrandsen, 1974). They protect cells from oxidative stress by quenching free radicals capable of causing cellular damage. It has been proposed that dietary β-carotene, in combination with other antioxidants, protects against oxidative stress and thus may play a potentially important role in retarding the progression of atherosclerosis (Gey *et al*, 1993; Street *et al*, 1994; Martin *et al*, 1996).

Ascorbate, also known as vitamin C, is the main water-soluble antioxidant in the human plasma (Frei *et al*, 1989). It is considered to be the most important antioxidant in the extracellular fluids (Uddin and Ahmed, 1995). Low plasma vitamin C concentration has been implicated as an independent predictor of unstable coronary artery disease (Vita *et al*, 1998). Epidemiological studies revealed a correlation between low dietary levels of vitamin C and ischaemic hearth disease and cancer (Gey *et al*, 1987). Vitamin C is believed to more effective in protecting LDL and lipids in human plasma against oxidative damage induced by oxidants (Frei *et al*, 1989; Esterbauer *et al*, 1989; Jialal *et al*, 1990).

It protects human vascular smooth muscle cells against apoptosis induced by mmLDL (Siow *et al*, 1998; Siow *et al*, 1999) and improves endothelium-dependent vasodilation (Taddei *et al*, 1998). The physiological concentration range of vitamin C in human plasma is 30-150μM (Frei *et al*, 1989), while a plasma concentration of 40-50μM has been associated with minimal risk of cardiovascular disease and cancer (Gey *et al*, 1994). A plasma vitamin C levels less than 11.4 μmol / l represent deficiency (Jacob, 1990).

Vitamin C is transported into most cells in the oxidized form, dehydroascorbic acid (DHA), via glucose transporters; and as ascorbic acid in some cells, by sodium-dependent ascorbic acid transporters (Tsukaguchi et al, 1999). When transported as DHA, vitamin C is rapidly reduced inside the cell and accumulates as ascorbic acid.Vitamin C can function as a modulator of cytokine signal transduction pathways. In human monocytic U937 cell line, vitamin C inhibited the granulocyte macrophage-colony stimulating factor (GM-CSF) -induced p-MAPK p42/44 and also suppressed the phosphorylation of the transcription factor Stat5 (Carcamo et al, 2002). The GM-CSF- dependent p-MAPK is activated via the ras/raf signalling pathway.

Some polyphenolic compounds extracted from green tea leaves have been found to be good antioxidants against lipid peroxidation (Salah *et al*, 1995; Guo *et al*, 1996). Epigallocatechin gallate is a polyphenolic compound extracted from green tea leaves. It is an active compound with known anti-inflammatory, anticarcinogenic and antioxidant properties (Wang *et al*, 1994; Feng *et al*, 2001; Jung *et al*, 2001). Epigallocatechin gallate suppresses inducible nitric oxide synthase, peroxynitrite and mitochondrial ROS production (Feng *et al*, 2001), Some recent reports have suggested that the antioxidant mechanism of epigallocatechin gallate involves trapping the initiating radicals and / or the propagating peroxyl radical, and reducing α-tocopheroxyl radical to regenerate the endogenous α-tocopherol (Liu *et al*, 2000).

Antioxidant and pro-oxidant role for nitric oxide (NO) in the development of atherosclerotic plaques have been reported (Buttery *et al* 1996; Leeuwenburgh *et al*, 1997). NO derived from vascular endothelium has been shown to be a critical modulator of local vascular tone and thrombus formation and its deficiency has been demonstrated in cardiovascular diseases such as in hypertension, atherosclerosis and unstable angina (Britten *et al*, 1999; Drexler and Hornig, 1999; Panza, 1997). NO is synthesized by a calcium/calmodulin-dependent NO synthase (eNOS) present in endothelium but during inflammatory episodes, a cytokine-inducible NO synthase (iNOS) is expressed throughout the vessel wall which results in the production of larger quantities of NO (Bogle and Vallance, 1996). Balla et al (1991) described hemin, a ubiquitous iron-containing compound, as a possible physiological mediator of LDL oxidation *in vivo*. NO can act as an antioxidant by binding to ferrous ions and heme proteins and diminishing their pro-oxidant effects (Kanner et al, 1991; Dee et al, 1991) and also by removing superoxide radicals (Wink et al, 1993).

Folate is a B-vitamin that is present in a wide variety of foods such as in liver, yeast, banana, milk products and green vegetables. Folic acid is the synthetic form of the vitamin, which is present in supplements and fortified foods. In 1996, the Food and Drug administration issued a regulation requiring all enriched grain products to be fortified with folic acid (140µg/100g) to reduce the risk of neural-tube defects in newborn babies (Jacques *et al*, 1999). Recent studies suggest that folic acid has some antioxidant effects (Verhaar *et al*, 1998; Verhaar *et al*, 1999). Although there has been no hard evidence that increased dietary folate or supplements could reduce cardiovascular risk (Verhaar and Radelink, 1999), folic acid has been shown to have beneficial effect on endothelial function in patients with hyperhomocysteinemia (van den Berg *et al*, 1995; Usui *et al*, 1999; Verhaar *et al*, 1999) and could lower cardiovascular risk in homocysteinuric patients (Boushey *et al*, 1995).

Epidemiological data have shown that antioxidant vitamins are associated with reduced risk of cardiovascular disease and cancer (Albanes, 1999; Kushi, 1999; Rimm and Stampfer, 2000); however recent interventional trials (Omenn *et al*, 1996; Stephens *et al*, 1996; Yusuf *et al*, 2000; Boaz *et al*, 2000) did not show such a protective effect. The results of some of the interventional trials shall be discussed later in the thesis. The aim of the present study was to investigate the effect of antioxidant vitamins on the expression of tissue factor and tissue factor pathway inhibitor in monocytes and endothelial cells when stimulated with 50µg protein/ml mmLDL and 10µg/ml LPS.

5.2 Results

5.2.1: An investigation into the effects of antioxidant vitamins on the expression of TF and TFPI mRNA using Northern hybridisation.

The effect of antioxidant vitamins on the gene expression of tissue factor and tissue factor pathway inhibitor in THP-1 monocytic and ECV 304 endothelial cell lines was first investigated using Northern blot hybridisation. The cells were pre-treated for 18h with 50μM vitamin C or 10μM vitamin E (in 100% ethanol; final ethanol concentration less than 0.1%) and afterwards exposed to LPS or mmLDL for 1 h. Total RNA was extracted from the cells and tissue factor as well as tissue factor pathway inhibitor mRNA was determined by Northern blot analysis using radiolabeled, full length tissue factor and tissue factor pathway inhibitor cDNAs. Figures 5.2.1.1 and 5.2.1.2 represent the membranes obtained from the Northern blot assays of the total RNA extracted from THP-1 cells exposed, for 1 h, to 10 μg/ml LPS and 50 μg protein/ml mmLDL respectively. Both LPS and mmLDL induced the gene expression of TF in the THP-1 cells; the pre-treatment of the cells with ascorbic acid and vitamin E inhibited the expression of TF in the cell line. Same observation was noted in ECV 304 cell line (data not shown). Blots were stripped and reprobed to determine GAPDH mRNA levels to confirm comparable loading of RNA samples. TFPI mRNA was not detectable in THP-1 cells. In the ECV 304 cells, LPS and mmLDL both increased the expression of TFPI mRNA but the antioxidant vitamins had little or no effect on the induced transcripts (data not shown).

5.2.2: An investigation into the effects of vitamin C on the expression of TF and TFPI mRNA using Quantitative Competitive RT-PCR.

Vitamin C is the main water-soluble antioxidant in the human plasma and it is considered to be the most important antioxidant in the extracellular fluids; protecting lipids in human plasma against oxidative damage.

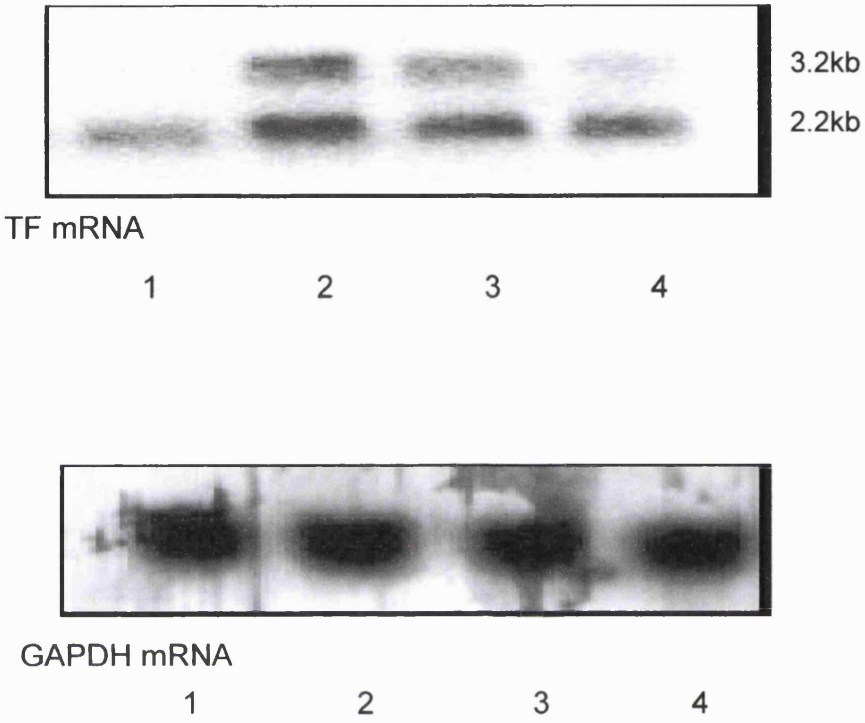


Figure 5.2.1.1: Northern Blot Analysis of effect of antioxidant vitamins on LPS induced TF in THP-1 cells. THP-1 cells were pre-treated for 18h with 50μM vitamin C or 10μM vitamin E. The cells were afterwards exposed to LPS (10μg/ml) for 1hr. Total RNA was isolated. TF and GAPDH gene induction levels were determined by Northern blot analysis. Lane 1 is the control cells, lane 2 is LPS treated cells and lane 3 is the LPS treated cells in the presence of 50μM vitamin C and lane 4 is the LPS treated cells in the presence of 10μM vitamin E. Full length TF and GAPDH cDNA fragments were used as probes. This result is representative of 3 experiments.

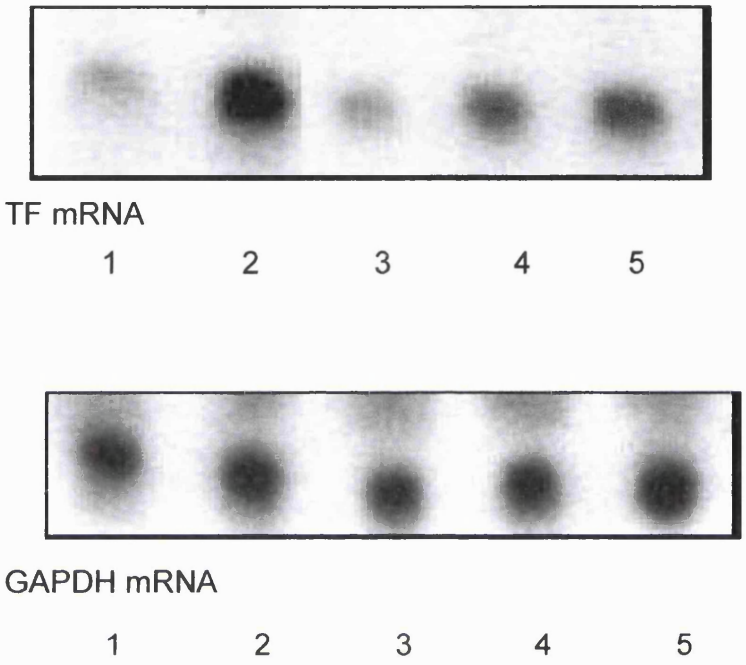


Figure 5.2.1.2: Northern Blot Analysis of effect of antioxidant vitamins on mmLDL induced TF gene expression in THP-1 cells. THP-1 cells were pre-treated with antioxidants vitamins for 18h before the incubation with mmLDL (50µg/ml) for 1hr. Total RNA was isolated. TF and GAPDH gene induction levels were determined by Northern blot analysis. Lane 1 is the control cells, lane 2 is mmLDL treated cells, lane 3 is the LPS treated cells in the presence of 50µM vitamin C; lane 4 is the mmLDL treated cells in the presence of 10µM vitamin E and lane 5 is the mmLDL treated cells in the presence of 10µM lutein. Full length TF and GAPDH cDNA fragments were used as probe. This result is representative of 3 experiments.

5.2.2.1: Effect of Vitamin C on TF and TFPI gene expression simultaneously induced in response to mmLDL in THP-1 cells.

Vitamin C plasma concentration of 40-50μM has been associated with minimal risk of cardiovascular disease and cancer (Gey *et al*, 1994). THP-1 cells were incubated with mmLDL for 1 h in the presence or absence of 50μM /100 μM vitamin C. The antioxidant was either added to the cells at the same time as the mmLDL or added 18 h prior to exposure to mmLDL; to determine the effect of the vitamin on mmLDL-induced TF when the vitamin and mmLDL are added simultaneously to the cells and when the cells are pre-treated with the antioxidant.

Vitamin C significantly inhibited TF expression induced by mmLDL in both ECV 304 and THP1 cells. Exposure of cells to mmLDL caused a significant increase in TF expression. Concurrent or pre-treatment of the cells with antioxidant vitamin C (50 and 100μM) inhibited the expression of TF (figure 5.2.2.1a), producing approximately 90-100% inhibition of mmLDL-induced TF expression. Figure 5.2.2.1b shows the effect of vitamin C on mmLDL-induced TFPI mRNA in THP-1 cells; pre-treatment of the cells with the vitamin at a concentration of 100μM caused an apparent non-significance increase in the expression of TFPI.

5.2.2.2 Effect of Vitamin C on TF and TFPI gene expression induced in response to LPS in THP-1 cells

THP-1 cells were incubated with LPS in the presence or absence of 50μM and 100 μM vitamin C for 1 h. The antioxidant was added to the cells at the same time as the mmLDL or added 18 h before the treatment of the cells with LPS. The incubation of cells with LPS for 1 h induced the up-regulation of TF mRNA compared with control cells. The addition of vitamin (50 and 100μM) 18 h before or at the same time as the LPS, resulted in a significant decrease of the TF mRNA (figure 5.2.2.2a).

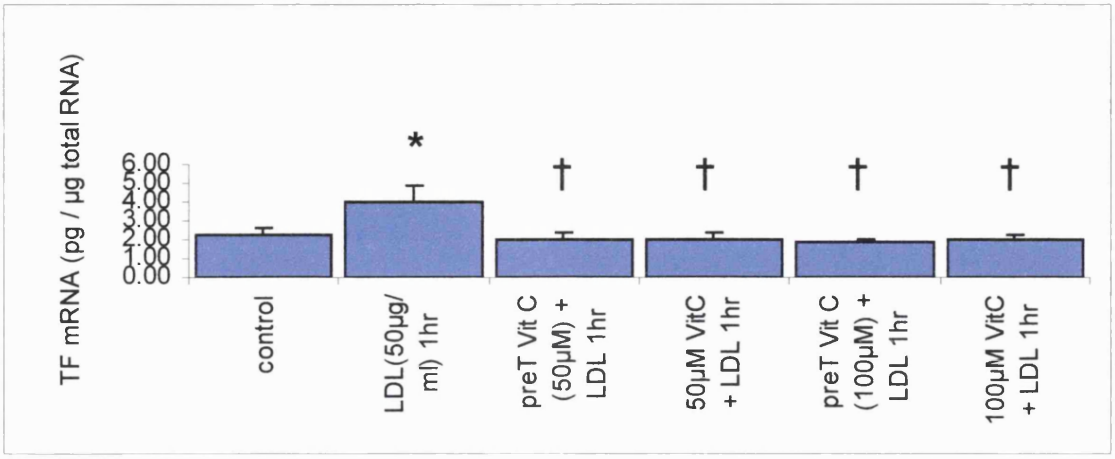


Figure 5.2.2.1a: Effect of vitamin C on mmLDL induced tissue factor mRNA in THP-1 cells. THP1 cells were incubated with either mmLDL(50µg protein/ml) or 50µg protein/ml mmLDL plus vitamin C (50µM or 100µM). The vitamin C was added either simultaneously or 18 hour prior (preT) to the addition of mmLDL. The data shows that mmLDL caused ~ 2-folds increase in the expression of TF mRNA.The treatment of cells with vitamin C inhibited the mmLDL induced expression of TF m RNA. (mean ± SEM, n=4; * $p<0.05$ compared to control : † $p<0.05$ compared to mmLDL alone)

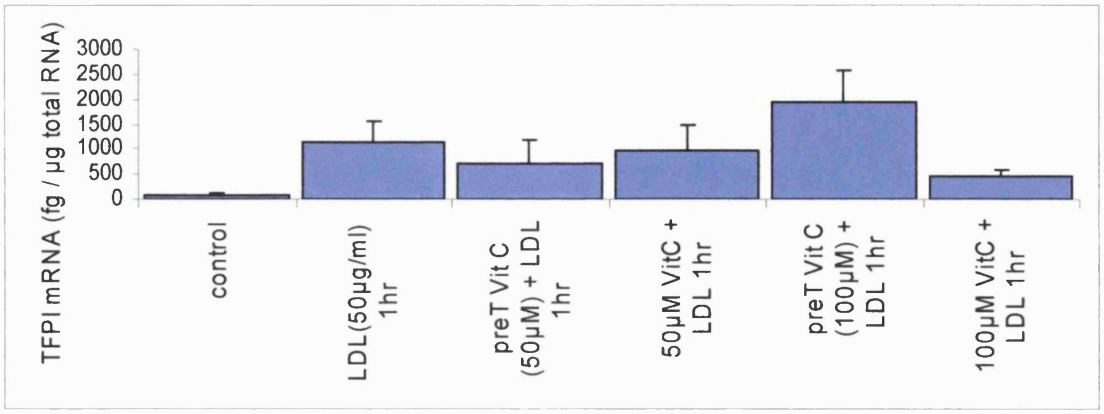


Figure 5.2.2.1b: Effect of vitamin C on mmLDL induced TFPI mRNA in THP-1 cells. THP1 cells were incubated with either mmLDL(50µg/ml) alone or together with vitamin C (50µM or 100µM). The cells were either pre-treated (preT) with the vitamin C for 18 hours before the treatment with mmLDL or the vitamin C added to the cells simultaneously with the mmLDL. The effect of vitamin C on mmLDL induced TFPI mRNA is less evident. But pre-treating the cells with 100µM vitamin C did caused a marked increase in the accumulation of TFPI m RNA.

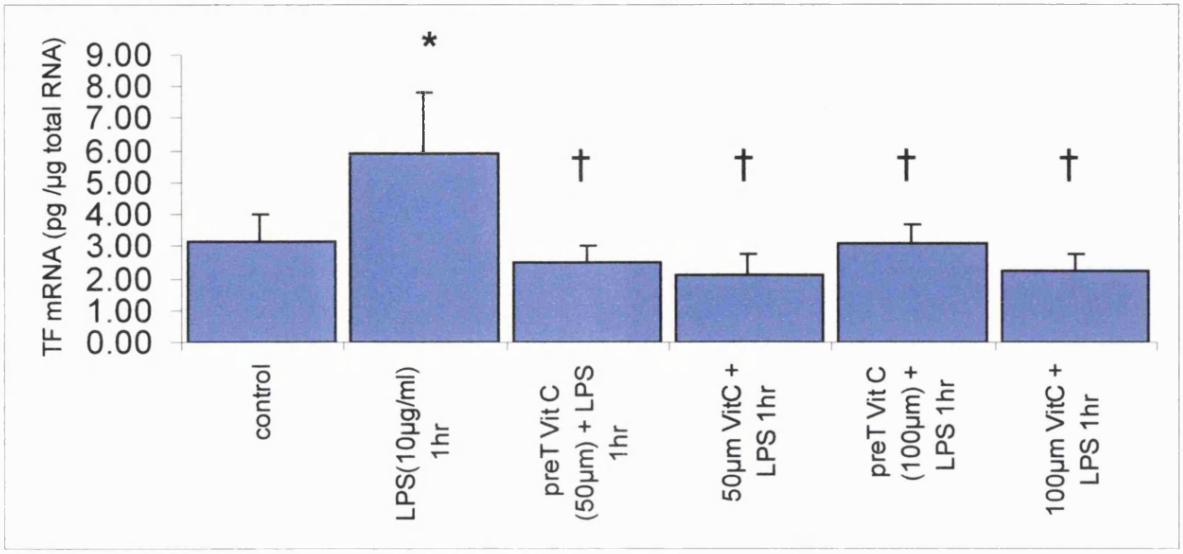


Figure 5.2.2.2a: Vitamin C and LPS induced tissue factor mRNA in THP-1 cells. THP1 cells were treated with either LPS(10µg/ml) alone or together with vitamin C for 1 hour. The vitamin C was added simultaneously or 18 hour prior (preT) to the addition of LPS. The result shows that LPS caused an up-regulation of TF expression which can be completely inhibited by treating the cells with vitamin C. * $p<0.05$ compared to control; † $p<0.05$ compared to LPS alone (mean ± SEM, n=4)

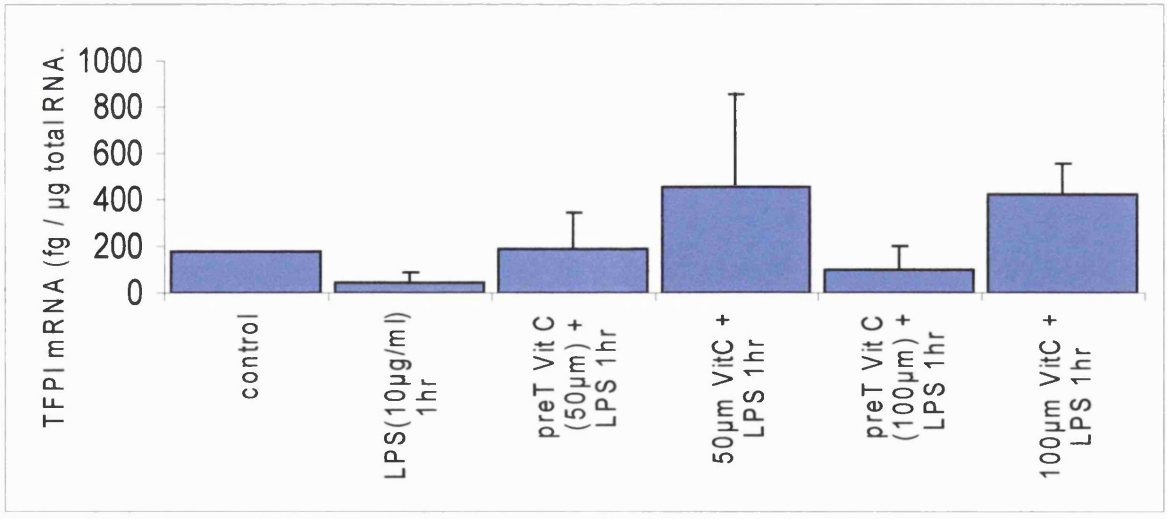


Figure 5.2.2.2b: Vitamin C and LPS induced TFPI gene expression in THP-1 cells. THP1 cells were treated with either LPS(10µg/ml) alone or together with vitamin C for 1 hour. The vitamin C was added to the cells either simultaneously or 18 hours prior (preT) to the addition of LPS to the cells.

Vitamin C significantly inhibited TF expression induced by LPS in THP1 cells. Similar observations were made in ECV 304 cells when treated with LPS. Figure 5.2.2.2b shows the effect of vitamin C on the expression of TFPI mRNA in THP-1 cells treated with 10 µg/ml LPS. Vitamin C consistently, although not significantly, caused an up-regulation of TFPI mRNA in the LPS stimulated cells.

5.2.2.3: Concentration dependent effects of vitamin C on the expression of tissue factor gene.

The physiological concentration range of vitamin C in human plasma is 30-150µM (Frei *et al*, 1989). Studies were carried out to look at the effect of vitamin C at a concentration range of 15µM – 200µM, on the expression of tissue factor. Tissue factor gene expression was measured using the quantitative competitive RT-PCR. The effect of vitamin C on mmLDL-induced TF expression is dose dependent (figure 5.2.2.3a). At low concentrations up to 25µM, vitamin C had little or no effect on mm-LDL-induced TF gene expression. The inhibitory effect of the vitamin increased gradually from 15µM with ~90-100% at 50 and 100µM concentration (figure 5.2.2.1a). When the concentration was increased further to 200µM, the vitamin had no effect on TF expression. This observation suggests a circumstantial non-beneficial effect or even pro-oxidant role of the vitamin at a high concentration. In the LPS-treated cells (figure 5.2.2.3b), LPS caused an increase in TF expression and pre-treating the cells with vitamin C (25-200µM) significantly suppressed this induction. The inhibitory effect of vitamins C on TF induction increased gradually from a concentration of 15µM and was maintained up to a concentration of 200µM.

5.2.2.4 Effect of vitamin C on SIN-1-induced TF expression in THP-1 cells.

SIN-1 is a compound that decomposes in solution and releases nitric oxide and superoxide thereby generating peroxynitrite.

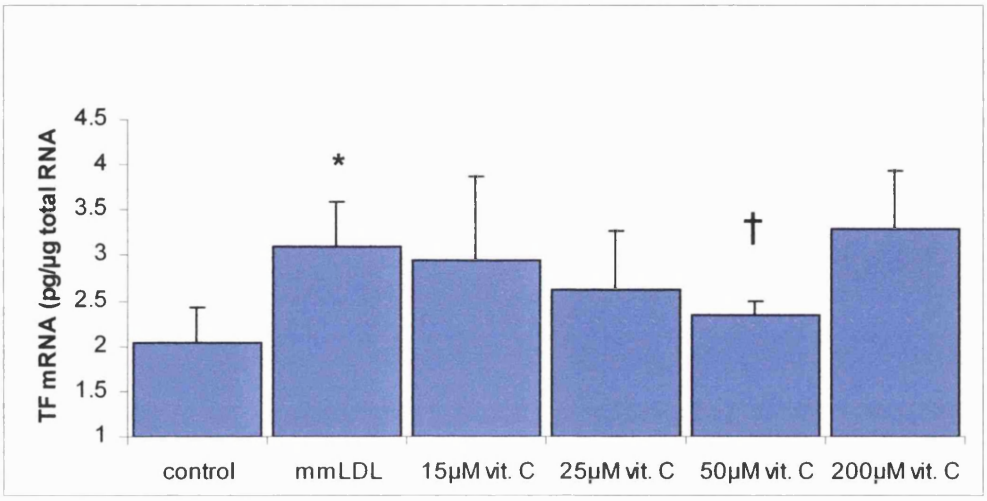


Figure 5.2.2.3a: Dose dependent decrease in the gene expression of TF in THP-1 cells. THP-1 cells were pre-treated with the specified concentration of vitamin C for 18 hours before 1 hour incubation in the presence of 50µg protein/ml mmLDL. (mean ± SD, n=4; * $p<0.05$ compared to control : † $p<0.05$ compared to mmLDL alone)

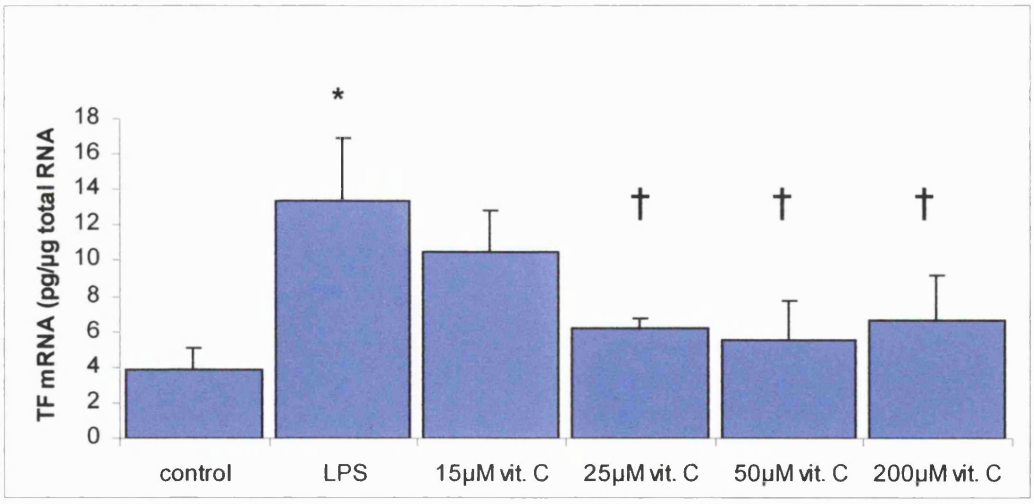


Figure 5.2.2.3b: Effect of increasing concentration of vitamin C on LPS induced tissue factor expression. THP-1 cells were pre-treated with the specified concentration of vitamin C for 18 hours. The cells were then cultured for additional 1 hour in the presence of 10µg / ml LPS. (mean ± SD, n=4; * $p<0.05$ compared to control : † $p<0.05$ compared to LPS alone)

The effect of antioxidant vitamins on SIN-1 was studied since peroxynitrite is capable of initiating lipid peroxidation *in vivo* (Patel *et al*, 1996) and it may play a role in the formation of atherosclerosis (White *et al*, 1994). Figure 5.2.2.4 shows the effect of SIN-1 on the expression of tissue factor mRNA in THP-1 cells. The cells were pre-treated for 18h with either vitamin C (50μM) or vitamin E (10μM) and afterwards incubated for 1h with 50μM SIN-1. Treatment of the cells with SIN-1 caused an up-regulation of tissue factor and this effect was inhibited by vitamin C and vitamin E. The up-regulation of tissue factor gene expression by SIN-1 is in agreement with the studies by Gerlach *et al* (1998) which showed that low doses of SIN-1 (up to 250μM) increase TF mRNA and antigen expression in human monocytes. Studies by Polack *et al* (1997) also showed that endogenous NO and O_2^- increase TF expression in isolated monocytes

5.2.3 An investigation into the effects of vitamin E on the expression of TF and TFPI genes

Lipophilic antioxidants particularly *α*-tocopherol are well recognized inhibitors of lipid peroxidation in biological membranes. The antioxidant vitamins protect cells during oxidative stress and are considered as antiatherogenic.

5.2.3.1 Effect of Vitamin E on TF and TFPI gene expression induced in response to LPS in THP-1 cells

After pre-treating THP1 cells with 10μM or 50μM vitamin E for 18h, the cells were incubated with LPS (10μg/ml) for 1 h. The results showed that LPS caused a 2–5 fold induction in TF mRNA (figure 5.2.3.1a) and suppressed TFPI mRNA expression (figure 5.2.3.1b). Vitamin E significantly decreased the induced TF expression by ~65% at a concentration of 10μM and ~80% inhibition at a concentration of 50μM. Vitamin E (50μM) also reduced the basal TF expression in THP-1 cells. Vitamin E had no significant effect on the expression of TFPI.

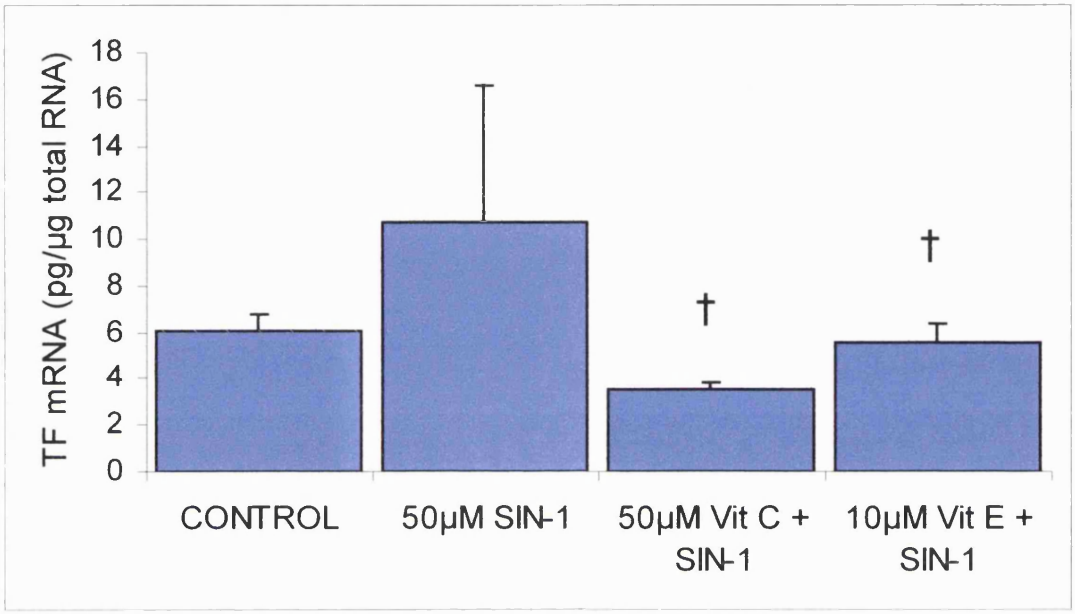


Figure 5.2.2.4: Effect of vitamin C and vitamin E on SIN-1(50µM) induced tissue factor mRNA in THP-1 cells. THP1 cells were pre-treated for 18h with vitamin C (50µM) or 10µM vitamin E prior to 1h incubation with 50µM SIN-1. The data shows that SIN-1 caused an increase in the expression of TF mRNA. The treatment of cells with vitamin C and vitamin inhibited the induced expression of TF mRNA . † $p<0.05$ compared to SIN-1 (± SD, n=4)

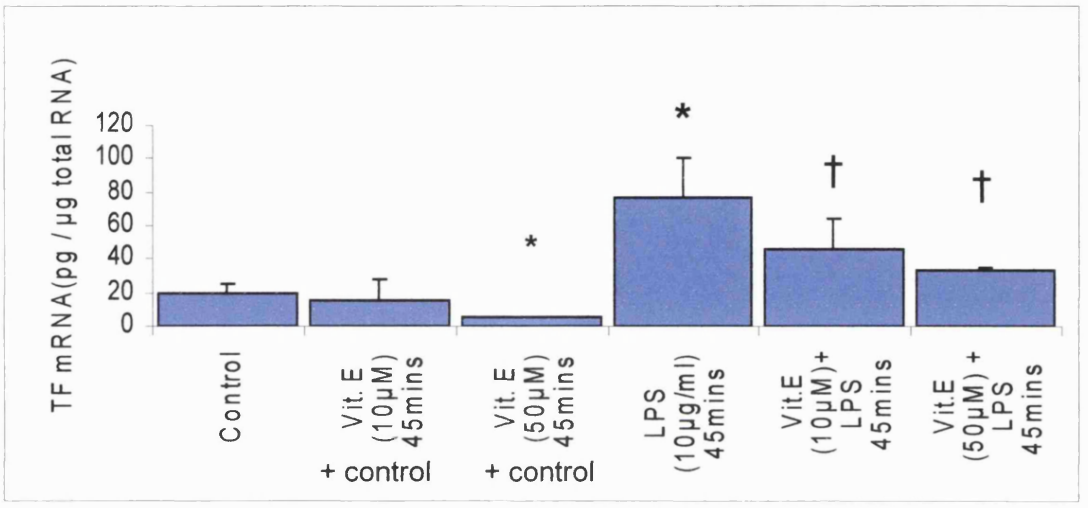


Figure 5.2.3.1a: Effect of vitamin E on LPS induced TF mRNA in THP-1 cells. THP1 cells were treated with either 10µg/ml LPS alone or with vitamin E (10µM and 50µM) for 45mins. * $p<0.05$ compared to control: † $p<0.05$ compared to LPS alone (mean ± SD, n=4)

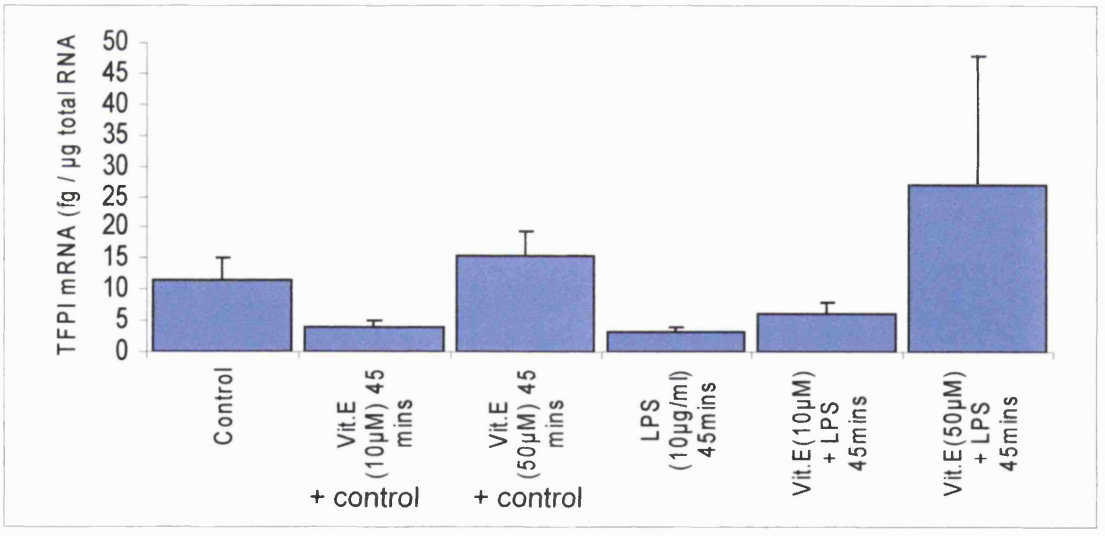


Figure 5.2.3.1b: Effect of vitamin E on LPS induced TFPI gene expression in THP-1 cells. THP1 cells were treated with LPS alone, vitamin E (10µM and 50µM) alone, and LPS together with vitamin E. Vitamin E at a concentration of 10µM had no effect on LPS induced TFPI mRNA. At a concentration of 50µM, vitamin E stimulated the expression of TFPI mRNA in THP1 cells when the cells were treated with LPS (mean ± SD, n=4).

5.2.3.2: Concentration dependent effects of vitamin E on the expression of tissue factor gene.

The dose response effect of the lipophilic antioxidant vitamin E on the expression of TF in THP-1 cells was studied. The cells were pre-treated with vitamin E (2.5μM – 50μM) for 18 h before stimulation with either LPS (10μg/ml) or mmLDL (50μg/ml). In the mmLDL-treated cells, the effect of the vitamin was concentration dependent. The inhibitory effect gradually increased from 5μM with maximum inhibition at a concentration of 25μM (figure 5.2.3.2a). The inhibitory effect of vitamin E on LPS-induced TF gene expression was invariable over the chosen concentration range (figure 5.2.3.2b). The inhibition of LPS-induced TF gene expression was fairly constant when the concentration was raised from 2.5μM through to 30μM.

5.2.4 A study on the effects of lutein on TF and TFPI gene expression

Lutein is more hydrophilic than the other carotenoids and therefore more easily presented to cells. The effect of lutein on the expression of TF was studied in ECV 304 cells. The antioxidant (10μM or 50μM) was added to the cells at the same time as the mmLDL / LPS and incubated for 1 h. In another set of experiments, the cells were pre-treated for 18 h in the presence or absence of 10μM and 50μM lutein and LPS (10μg/ml) or mmLDL (50μg/ml) was added to the pre-treated cells for 1 h. Pre-treatment of the cells with lutein (10-50μM) caused a marked inhibition of basal TF and induced TF expression in mmLDL-treated cells; the greatest inhibition was achieved in the cells pre-treated with 50μM lutein (figure 5.2.4.1a). In LPS-stimulated cells (figure 5.2.4.1b), lutein (10-50μM) caused a 98 - 100% inhibition of TF expression even when the vitamin and LPS were added to the cells at the same time. In the cells pre-treated for 18 h with lutein, 74 - 81% inhibition was observed. Lutein had no effect on TFPI gene expression irrespective of when it was added (data not shown).

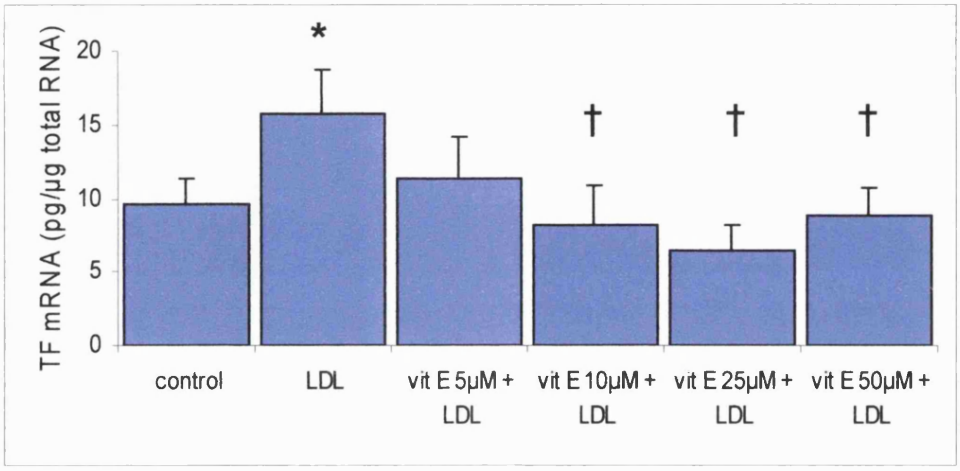


Figure 5.2.3.2a: Increasing concentration of vitamin E (preT) on mmLDL induced TF in THP-1 cells. THP-1 cells pre-treated with the specified concentration of vitamin E for 18 h, were incubated with 50µg protein/ml mmLDL for 1 h. (mean ± SD, n=4; * $p<0.05$ compared to control : † $p<0.05$ compared to mmLDL alone)

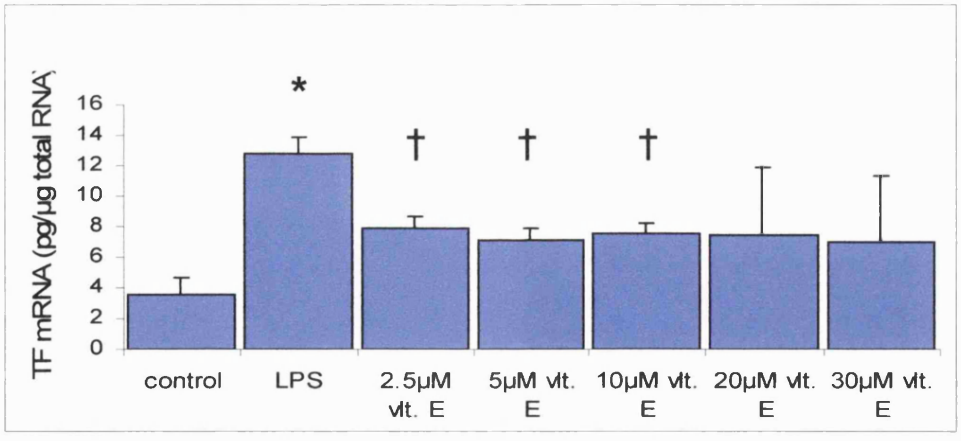


Figure 5.2.3.2b: Dose response of vitamin E (preT) on LPS induced tissue factor gene expression in THP-1 cells. THP-1 cells pre-treated with the specified concentration of vitamin E for 18 h, were incubated with 10µg LPS for 1 h. (mean ± SD, n=4 * $p<0.05$ compared to control * $p<0.05$ compared to control : † $p<0.05$ compared to LPS alone)

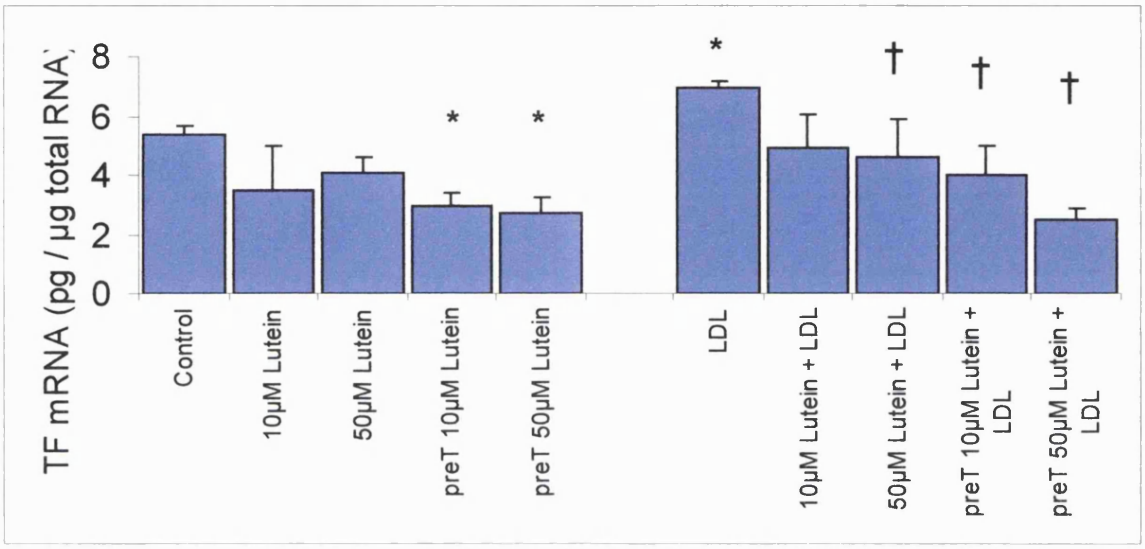


Figure 5.2.4.1a: Effect of lutein on mmLDL induced tissue factor mRNA in ECV 304 cells. ECV 304 cells were incubated with either mmLDL(50µg/ml) or 50µg protein/ml mmLDL and lutein (10µM or 50µM). The lutein was added either simultaneously or 18 hour prior (preT) to the addition of mmLDL.(Mean ± SEM, n=3). * P<0.05 compared to control; † p<0.05 compared to mmLDL.

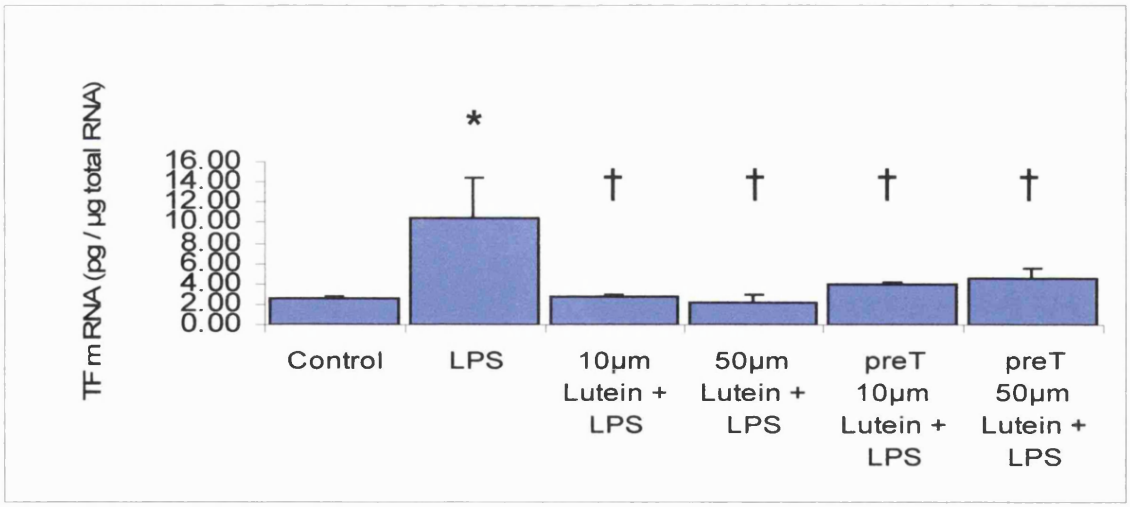


Figure 5.2.4.1b: Effect of lutein on LPS induced tissue factor gene expression in ECV 304 cells. ECV 304 cells were incubated with either LPS(10µg/ml) or 10µg/ml LPS and lutein (10µM or 50µM). The lutein was added either simultaneously or 18 hour prior (preT) to the addition of LPS. (Mean ± SEM, n=3). * p<0.05 compared to control; † p<0.05 compared to LPS.

5.2.5: Folic acid and TF gene expression

Folic acid is essential for the synthesis and repair of DNA (Green *et al*, 1988) and its deficiency may largely contribute to the increased cardiovascular risk associated with hyperhomocysteinemia. Studies were carried out to investigate whether folic acid could also inhibit the expression of TF in mmLDL and LPS stimulated THP-1 cells since thiol dysregulation may also cause oxidative stress. Figure 5.2.5.1 shows that folic acid, at a concentration of 1μM, had no inhibitory effect on LPS-stimulated tissue factor gene expression but effectively inhibited TF expression in mmLDL treated cells.

5.2.6: The effects of antioxidants added after induction of TF in THP-1 cells activated by mmLDL and LPS.

So far, the inhibitory effects of antioxidant vitamins on the gene expression of TF was observed when the vitamins were added at the same time as with the stimulus (mmLDL and LPS) and when the cells were pre-treated for 18 h before the addition of the stimulus. It was therefore of interest to know whether the antioxidants could be effective after addition of an oxidant challenge. This might mimic taking antioxidant supplements by patients who already had cardiovascular disease. In this study, experiments were carried out to measure the effects of the post-treatment with antioxidants on the expression of TF after the cells had been exposed to mmLDL and LPS. THP-1 cells were incubated with either 50μg mmLDL protein/ml or 10μg/ml LPS for 20min before treatment with 50μM vitamin C, 10 μM vitamin E, 10μM lutein or 1μM folic acid. Contrary to the antioxidants pre-treatment and co-treatment data, the post-treatment of LPS-stimulated THP-1 cells with antioxidants resulted in a consistent increase in the expression of TF (figure 5.2.6.1a). In mmLDL-stimulated cells, the post-treatment of the cells with antioxidants had no effect on TF expression (figure 5.2.6.1b).

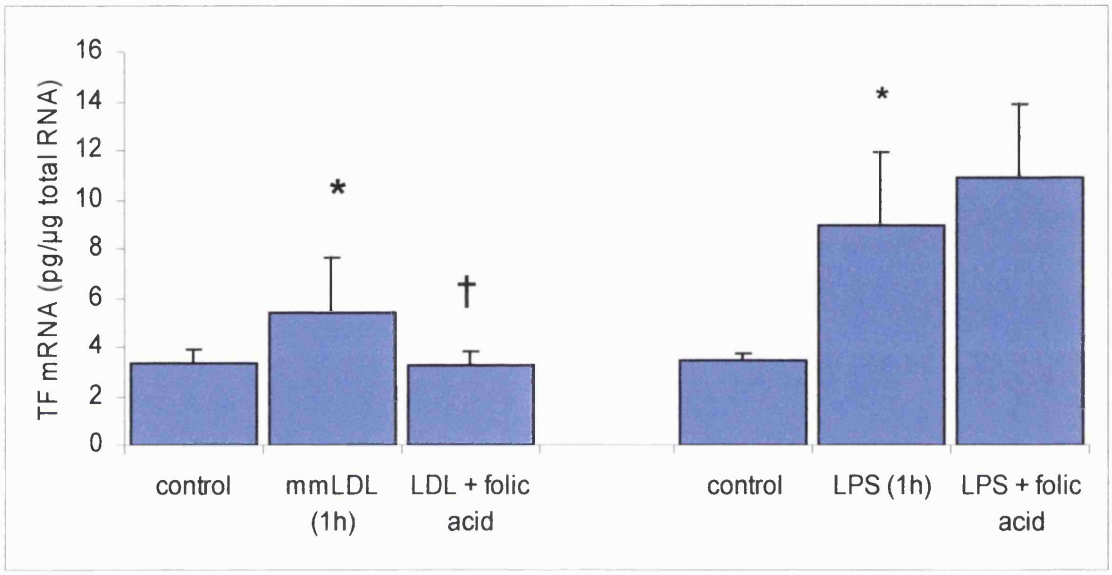
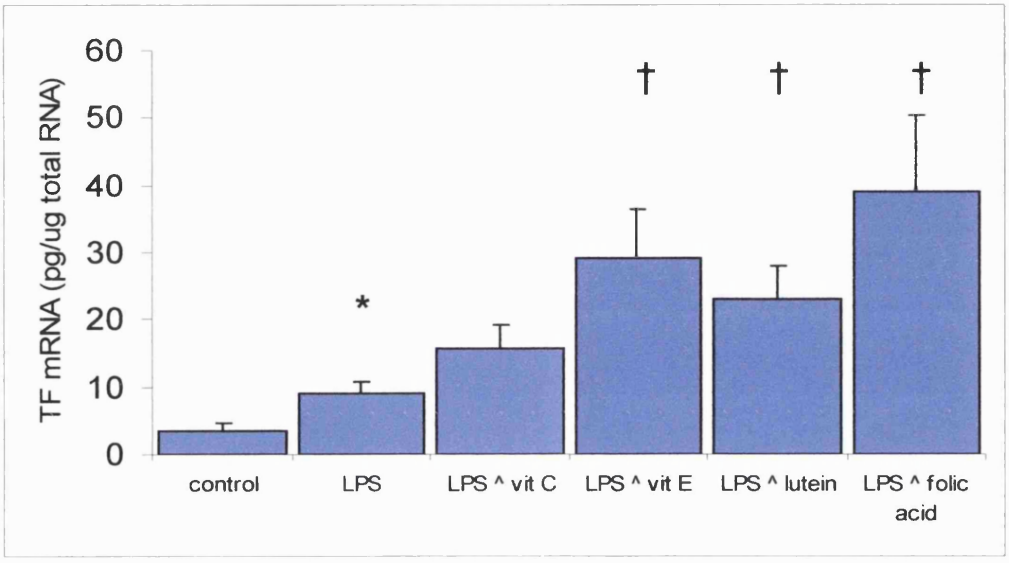
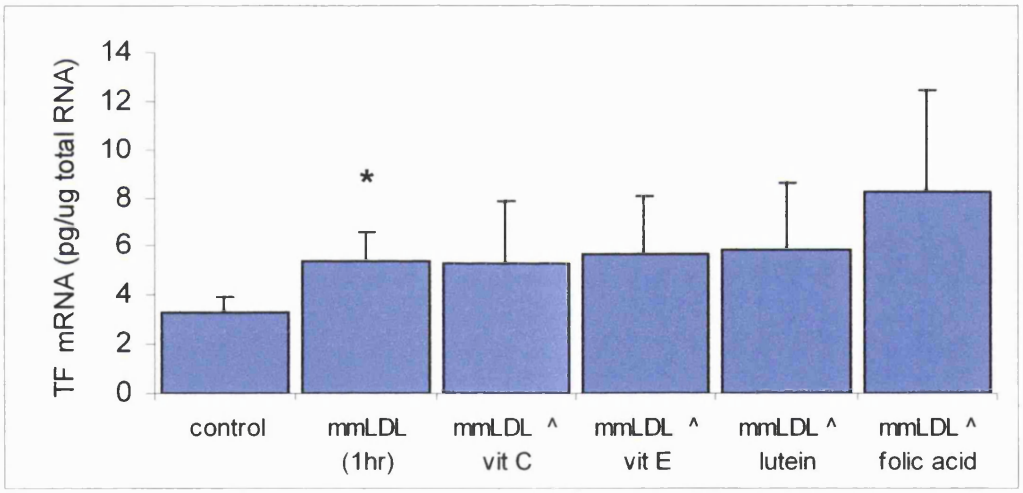


Figure 5.2.5.1: Effect of folic acid on mmLDL and LPS induced TF gene expression. THP1 cells were first pre-incubated with 1µM folic acid for 18h before another 1h incubation with 50µg protein/ml mmLDL or 10µg/ml LPS. Result represent mean ± SD; n=4. * $p<0.05$ compared to control; † $p<0.05$ compared to mmLDL.



A



B

Figure 5.2.6.1: Effect of antioxidant post-treatment (^) in THP-1 cells. THP-1 cells were treated with 50μM vitamin C, 10μM vitamin E, 10μM lutein and 1μM folic acid 20 min after the exposure of the cells to 10μg/ml LPS (A) or 50μg protein/ml mmLDL (B). The cells were incubated for another 40 min in the presence of the antioxidant vitamins. An increase in the expression of TF was noted when the cells were treated with the vitamins after the initial stimulation with LPS (A). The post-treatment of mmLD-stimulated cells with antioxidant vitamins had no effect on the expression of TF (B). (Mean ± SEM * $p<0.05$ compared to control : † $p<0.05$ compared to LPS alone (n=3)

5.2.7: Epigallocatechin gallate and TF gene expression

Epigallocatechin gallate is an antioxidant which acts against peroxynitrite and mitochondrial ROS production at concentrations as low as 2μM (Feng *et al*, 2001). The effect of epigallocatechin gallate (EGCG) on mmLDL and LPS-induced TF gene expression was studied to compare it to the other antioxidants previously used in the study. THP-1 cells were treated simultaneously with a physiological relevant concentration of EGCG (1μM or 10μM) and mmLDL (50μg/ml) or LPS (10μg/ml) for 1 h. Total RNA was prepare from the cells and amplified for TF using QC-RT-PCR. The data from the study showed that EGCG had very little effect on the LPS-induced TF expression (figure 5.2.7.1a). The inhibitory effect of the EGCG appeared to be more pronounced in the mmLDL stimulated cells (figure 5.2.7.1b), although it did not reach statistical significance.

5.2.8 Effect of nitric oxide on the expression of TF

Nitric oxide is also an antioxidant; low levels of nitric oxide generated by endothelial nitric oxide synthase (eNOS) inhibit lipid peroxidation (Volk and Kox, 2000). Graier *et al* (1994) reported that cultured bovine and porcine aortic endothelial cells showed decreased nitric oxide production when treated with LPS; likewise, oxidized LDL are known to inhibit the production of NO by macrophages (Dulak *et al*, 1999). There is also evidence suggesting that nitric oxide may reduce intracellular reactive oxygen species (Clancy *et al*, 1992; Tsao *et al*, 1997). Vitamin C is known to be capable of stimulating or restoring NO activity, hence studies were carried out to examine whether the inhibition of TF expression by vitamin C was a result of NO stimulation by vitamin C. THP-1 cells were stimulated with 50μg protein/ml mmLDL (figure 5.2.8.1a) and 10μg/ml LPS (figure 5.2.8.1b) in the presence of 0.1-10μM DEA NONOate- a nitric oxide donor. The results showed that nitric oxide had no effect on the induced expression of TF.

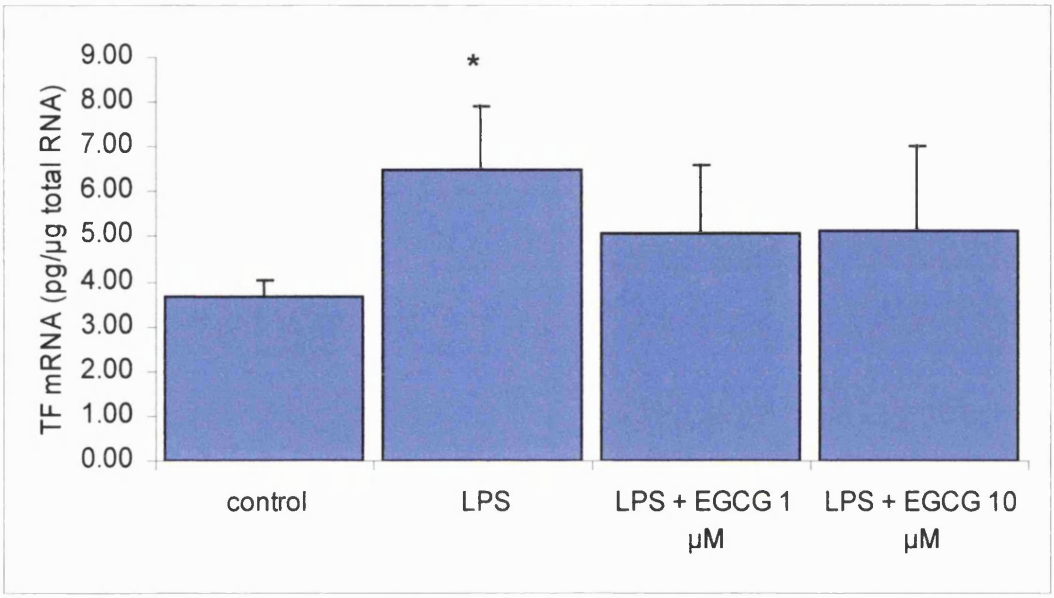


Figure 5.2.7.1a: Effect of epigallocatechin gallate (EGCG) on LPS induced tissue factor expression. THP-1 cells were co-treated with 10µg/ml LPS and EGCG for 1 h. The results represent mean ± SD, n=4. * $p<0.05$ compared to control.

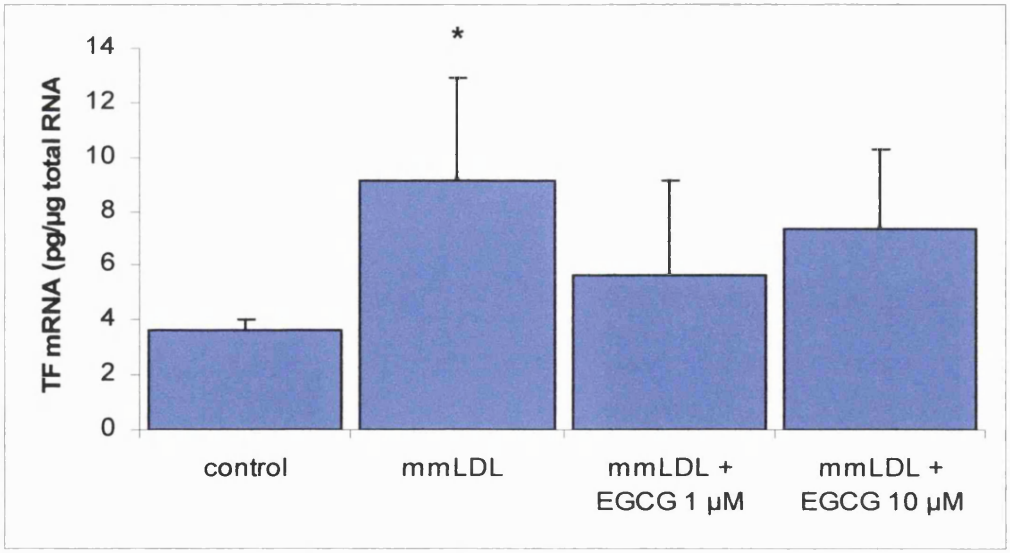


Figure 5.2.7.1b: Effect of epigallocatechin gallate (EGCG) on mmLDL induced tissue factor expression. THP-1 cells were co-treated with 50µg protein/ml mm LDL and EGCG for 1 h. The results represent mean ± SD, n=4. * $p<0.05$ compared to control.

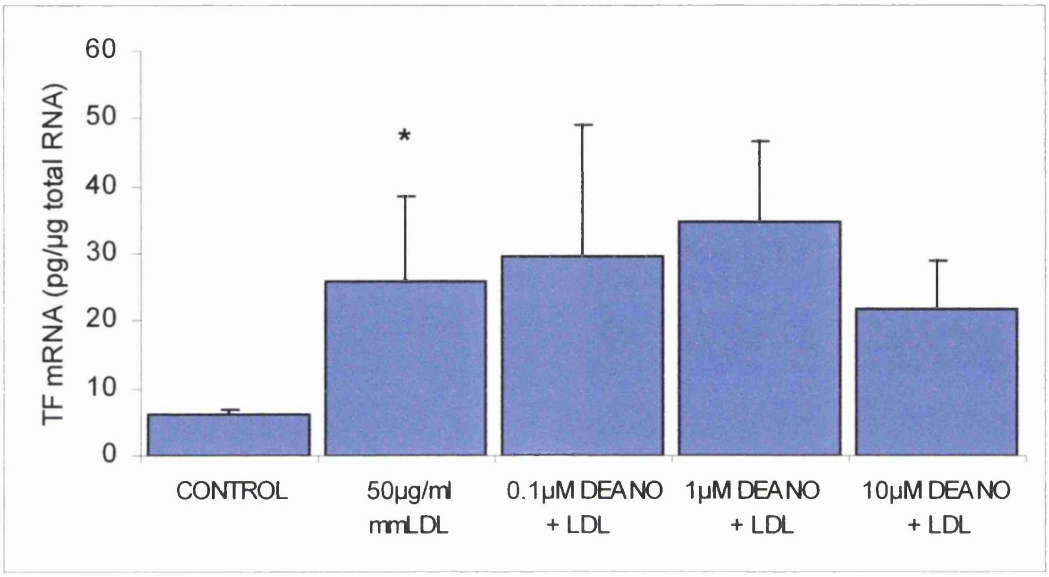


Figure 5.2.8.1a: Effect of DEA NONOate (a compound that releases nitric oxide) on the expression of mmLDL induced tissue factor in THP-1 cells. THP-1 cells were incubated for 1 h in the presence of 50µg protein/ml mmLDL and the indicated concentrations of DEANO. Values denote mean ± SD, n=4; * $p<0.05$ compared to control.

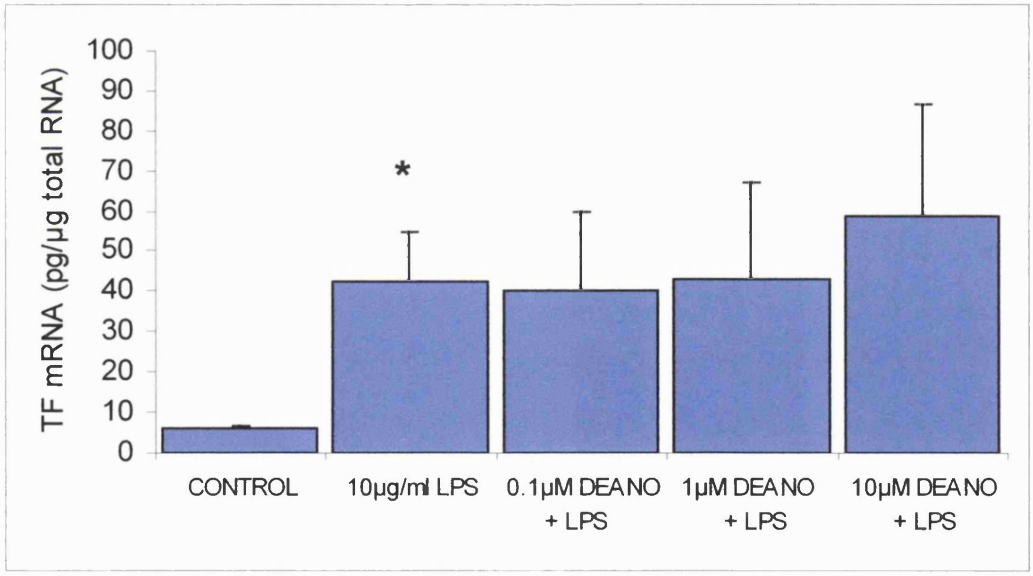


Figure 5.2.8.1b: Effect of DEA NONOate on the expression of LPS induced tissue factor in THP-1 cells. THP-1 cells were incubated for 1 h in the presence of 10µg/ml LPS and the indicated concentrations of DEANO. Values denote mean ± SD, n=4; * $p<0.05$ compared to control.

This is in agreement with the studies by Gerlach *et al* (1998) who found no effect of the NO donor NOC18 on LPS-induced TF expression in human monocytes.

5.2.9 Effects of antioxidant vitamins on tissue factor activity

Tissue factor procoagulant activity was measured by a one-stage prothrombin time assay in a coagulometer (Helena) [see chapter 2]. Cells were harvested by centrifugation, washed and resuspended in PBS ($1x10^6$cells/100µl PBS). The clotting time was converted to arbitrary TF activity units using the standard curve generated from the serial dilution of recombinant TF standard [DADE Innovin, Sysmex UK] (figure 2.3). One unit of TF activity was taken as the equivalent of a 1,000 fold dilution of the recombinant TF standard.

The effect of antioxidant vitamins on the expression of tissue factor activity was studied in both mmLDL and LPS stimulated THP-1 cells. Both cell surface and total cell tissue factor activity were measured. The percentage enhancement of the TF activity by mmLDL or LPS was measured, by comparing the TF activity unit (calculated from the standard curve) of the test sample (mmLDL- or LPS-treated cells) against the control sample in which the mmLDL or LPS had been replaced with an equal volume of PBS, using the equation below:

$$\text{Percentage enhancement} = \frac{(\text{control activity} - \text{test activity}) \times 100}{\text{control activity}}$$

The changes in the procoagulant activity of the test samples due to the presence of antioxidant vitamins (i.e effect of antioxidant vitamins) were calculated using the equation below:

$$\frac{(\text{antioxidant activity} - \text{test activity}) \times 100}{(\text{control activity} - \text{test activity})}$$

Antioxidant activity represents the TF activity unit of the test samples pre-treated with antioxidant vitamins.

THP-1 monocytic cells have a high basal level of tissue factor activity. This may be due to the long-term culture of the cells. The effect of antioxidants on the basal TF activity was measured by pre-treating the cells with 50μM vitamin C, 10μM vitamin E, 10μM lutein or 1μM folic acid for 22 ± 4h. Both the cell surface and total cell TF activities were measured (figure 5.2.9.1); the cell surface TF activity accounts for only ~10-20% of the total cell activity. Treatment of the THP-1 cells with antioxidants had no effect on the basal total cell TF activity but it reduced the basal cell surface TF activity in these cells. This reduction in the basal cell surface TF activity by antioxidant vitamins was not statistically significant.

To study the effect of the antioxidants on mmLDL-induced THP-1 cells, the cells were pre-treated with 50μM vitamin C, 10μM vitamin E, 10μM lutein or 1μM folic acid for 18 h prior to mmLDL (50μg protein/ml) challenge. The antioxidants pre-treated cells were exposed to mmLDL for 2 to 6h. Figures 5.2.9.2 and 5.2.9.3 show the effect of the antioxidant vitamins on mmLDL-induced cell surface and total tissue factor activity respectively. Minimally modified LDL enhanced both the cell surface and total tissue factor activity, which increased gradually up to 6 h. Pre-treatment of the cells with antioxidant vitamins, inhibited the cell surface tissue factor activity. The inhibitory effects of the antioxidants on the procoagulant activity in the mmLDL-induced cells were noted at 2hr, while the maximum inhibition was observed at 4h. In some cases, more than 100% inhibitions were observed where the antioxidants reduced the tissue activity below the basal activity of the control cells.

Vitamin E was most effective in inhibiting the cell surface TF activity in the mmLDL-induced THP-1 cells, but it was the least efficient in inhibiting the total cell TF activity. Pre-treatment of the cells with vitamin C resulted in a prolonged inhibition of the total cell tissue factor activity. Similar effects of vitamin C and vitamin E were noted in LPS-treated cells (figures 5.2.9.4 and 5.2.9.5).

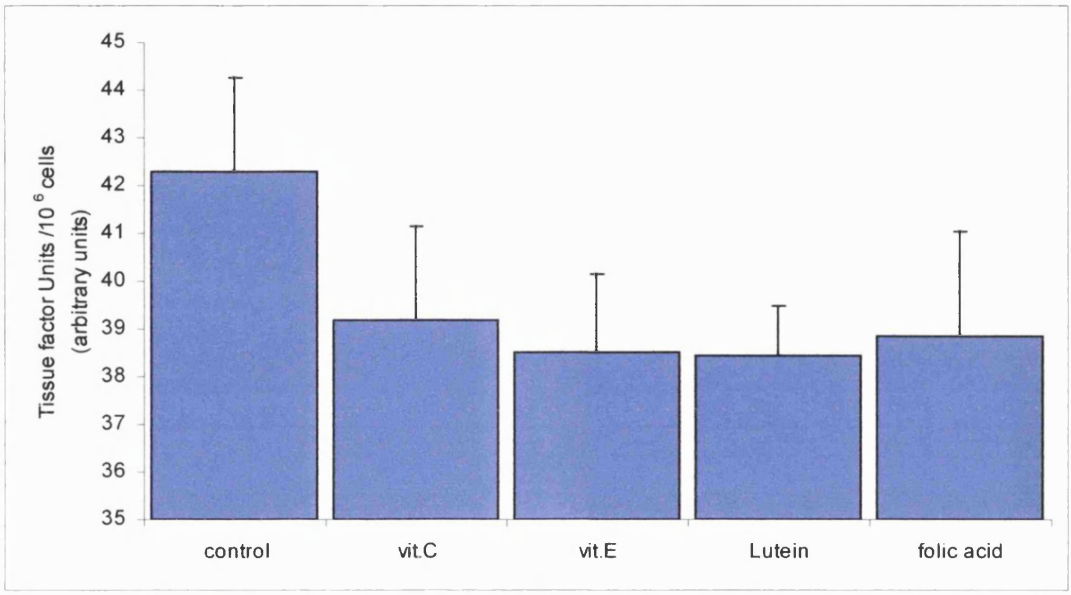


Figure 5.2.9.1a: Effect of antioxidant vitamins on the basal cell surface TF activity In THP-1 cells. THP-1 cells were incubated for 20 ± 4h with 50μM vit.C, 10μM vit.E, 10μM lutein and 1μM folic acid. Cell surface TF activity was determined by the one-stage prothrombin time assay and expressed as TF activity units / 10^6 cells. Result represents mean ± SEM of 6 independent experiments, samples assayed in triplicate.

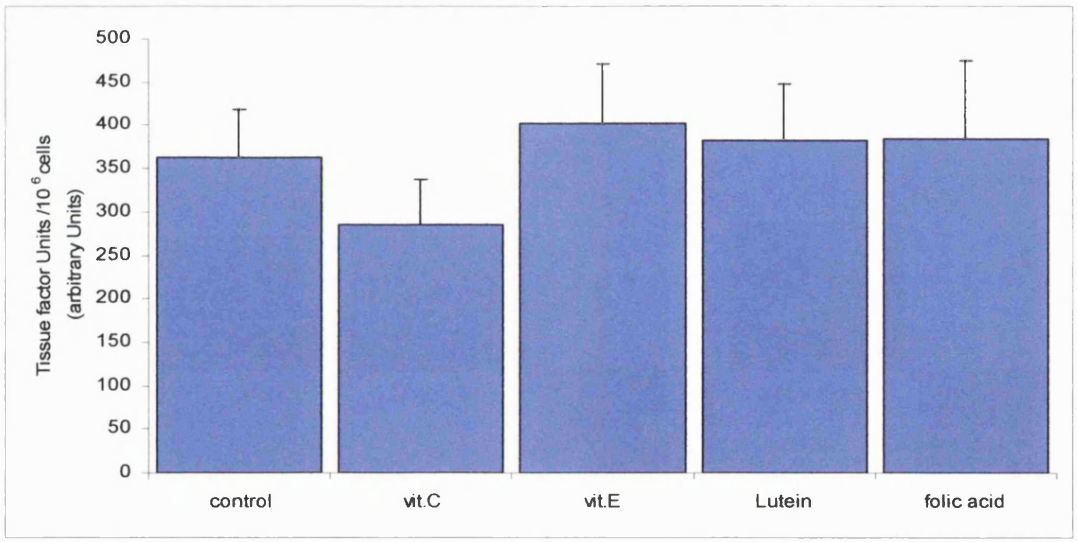


Figure 5.2.9.1b: Effect of antioxidant vitamins on the basal total cell tissue factor activity In THP-1 cells. THP-1 cells were incubated for 20 ± 4h with 50μM vit.C, 10μM vit.E, 10μM lutein and 1μM folic acid. Total cell TF activity was determined by the one-stage prothrombin time assay and expressed as TF activity units / 10^6 cells. Result represents mean ± SEM of 6 independent experiments.

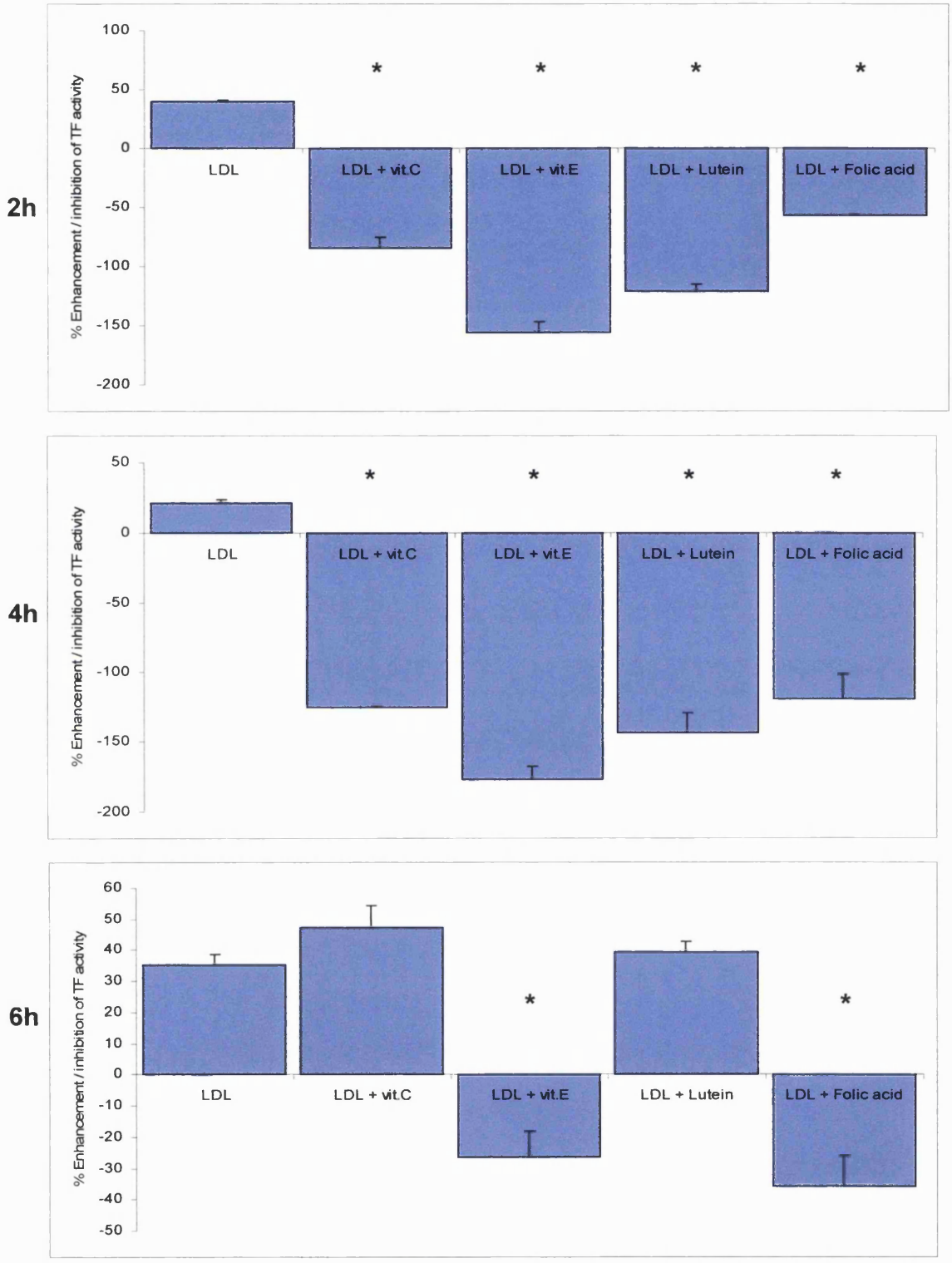


Figure 5.2.9.2: Effect of antioxidant vitamins on mmLDL induced cell surface tissue factor activity in THP-1 cells. THP-1 cells were pre-treated with 50μM vit.C, 10μM vit. E, 10μM lutein and 1μM folic acid for 18 h prior to the addition of 50μg protein/ml mmLDL. The cells were incubated further for 2h, 4h and 6h. The results represent the % enhancement (+ve values) and % inhibition (-ve values) of procoagulant activity compared with control/mmLDL (mean ± SEM, n=3; *P<0.05 compare to mmLDL).

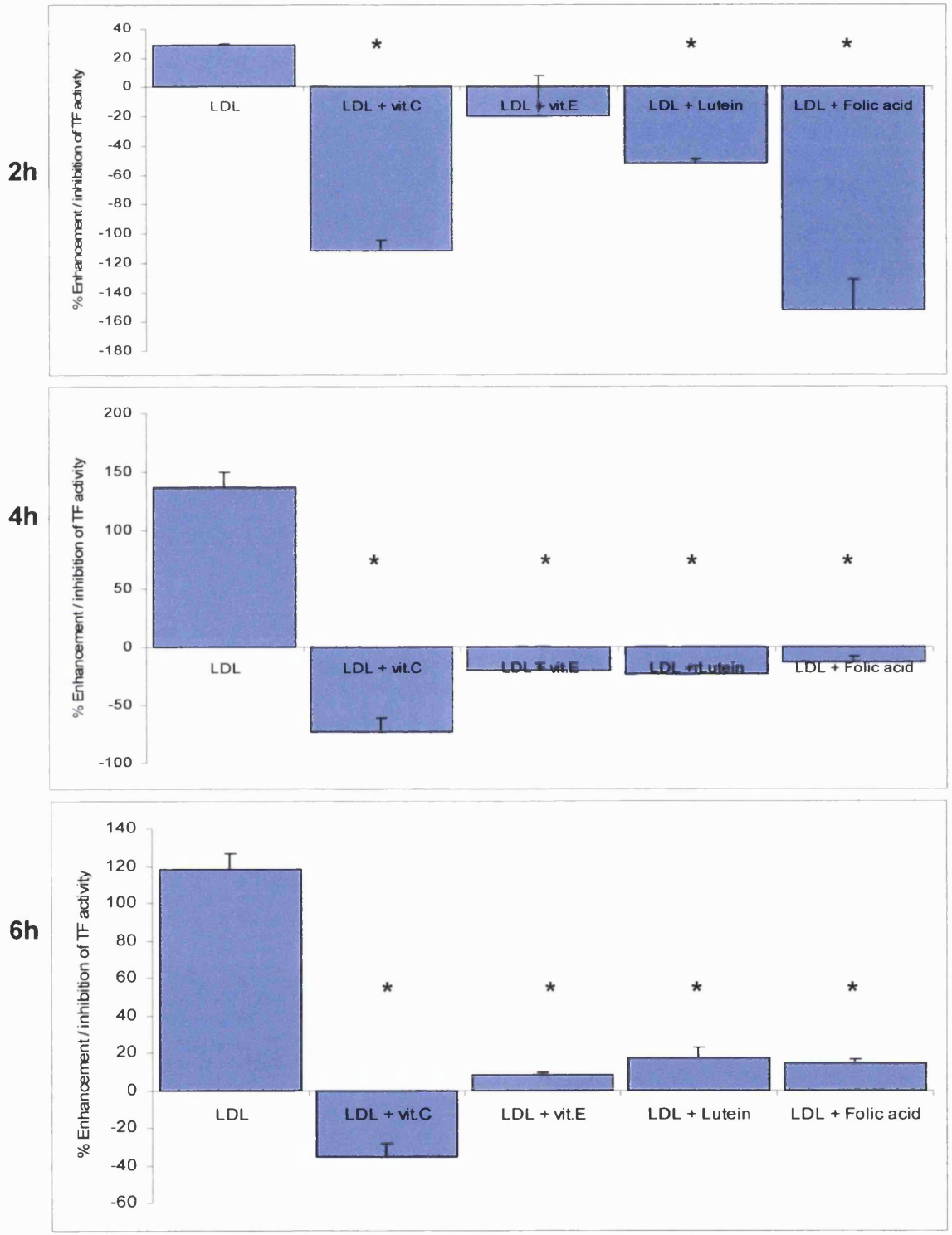


Figure 5.2.9.3: Effect of antioxidant vitamins on mmLDL induced total cell tissue factor activity in THP-1 cells. THP-1 cells were pre-treated with 50μM vit.C,10μM vit. E, 10μM lutein and 1μM folic acid for 18 h prior to the addition of 50μg protein/ml mmLDL. The cells were incubated further for 2h, 4h and 6h. The results represent the % enhancement (+ve values) and % inhibition (-ve values) of procoagulant activity compared with control / mmLDL (mean ± SEM, n=3; * $P<0.05$ compared to mmLDL).

Figures 5.2.9.4 and 5.2.9.5 show the effect of antioxidant vitamins on LPS-induced cell surface and total cell TF activity respectively. The cell surface and total procoagulant activity increased steadly over time up to 6 h. The increase in TF procoagulant activity may have been the result of changes in the amount of TF available on the cell surface due to an increase in the gene expression or the exposure of functional but sequestered TF. The inhibitions by antioxidant vitamin C and vitamin E on the LPS-induced cell surface and total TF activity were similar to that noted in the mmLDL-treated cells. Vitamin E was more effective than vitamin C in inhibiting the cell surface TF activity both in the mmLDL and LPS treated cells (figure 5.2.9.6). The inhibitory effect of vitamin E on the cell surface TF activity was sustained for up to 6h. The pre-treatment of the cells with vitamin C resulted in a prolonged inhibition of the total cell tissue factor activity.

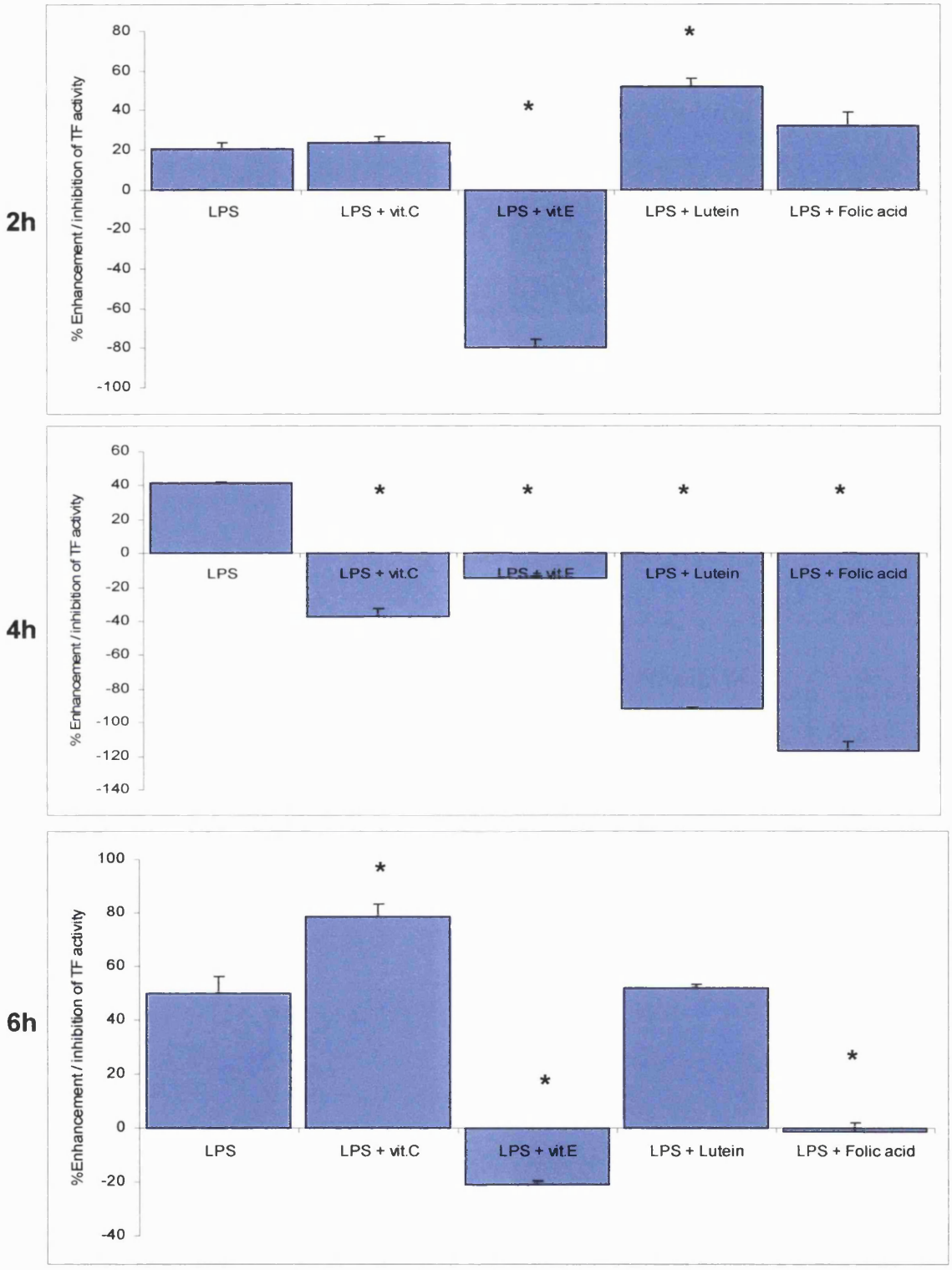


Figure 5.2.9.4: Effect of antioxidant vitamins on LPS induced cell surface tissue factor activity in THP-1 cells. THP-1 cells were pre-treated with 50µM vit.C,10µM vit. E, 10µM lutein and 1µM folic acid18 h prior to the addition of 10µg /ml LPS. The cells were incubated further for 2h, 4h and 6h. The results represent the % enhancement (+ve values) and % inhibition (-ve values) of procoagulant activity compared with control / LPS (mean ± SEM, n=3; *p<0.05 compare to LPS)

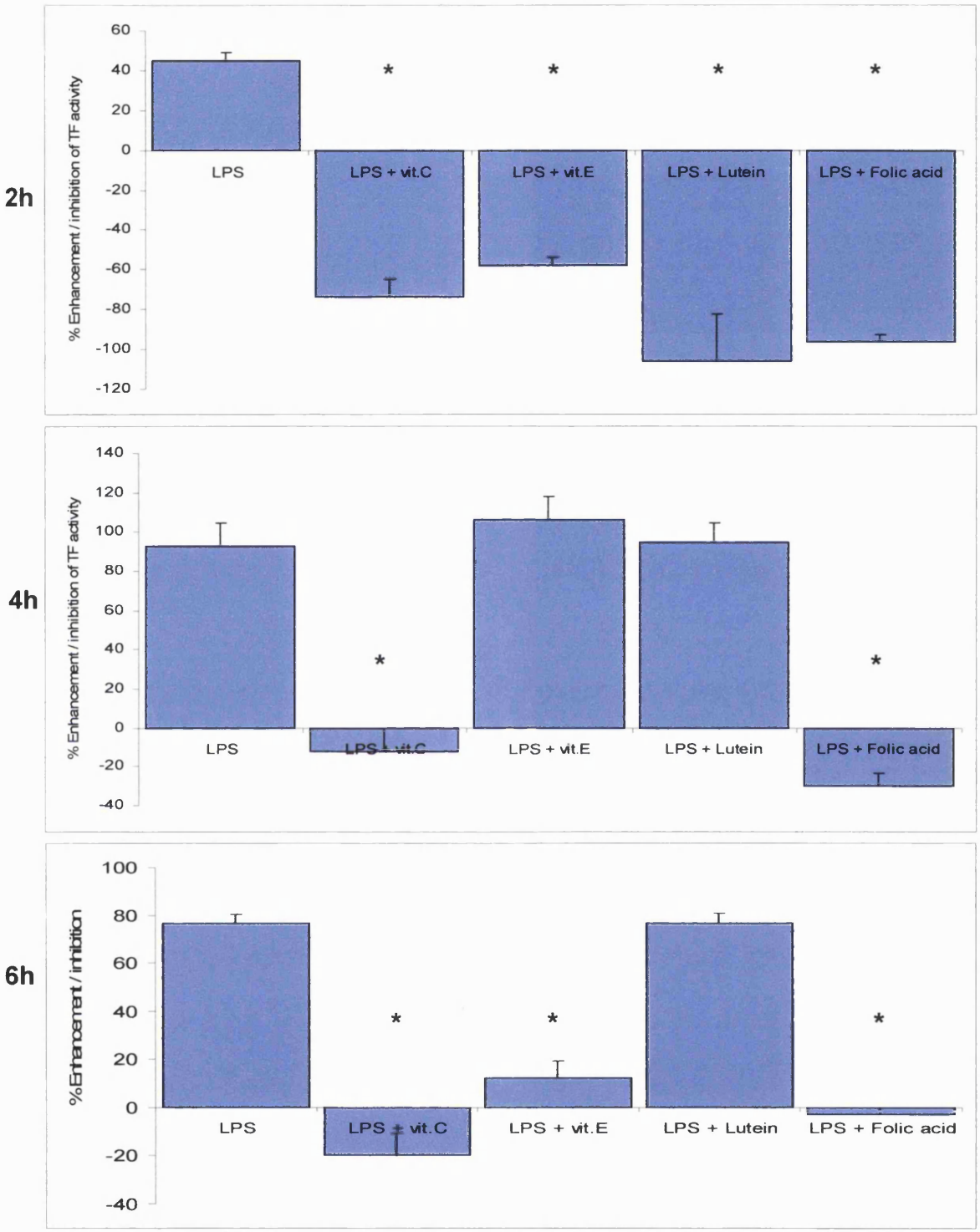


Figure 5.2.9.5: Effect of antioxidant vitamins on LPS induced total cell tissue factor activity in THP-1 cells. THP-1 cells were pre-treated with 50µM vit.C,10µM vit. E, 10µM lutein and 1µM folic acid18 h prior to the addition of 10µg /ml LPS. The cells were incubated further for 2h, 4h and 6h. The results represent the % enhancement (+ve values) and % inhibition (-ve values) of procoagulant activity compared with control / LPS (mean ± SEM, n=3; * $P<0.05$ compared to LPS)

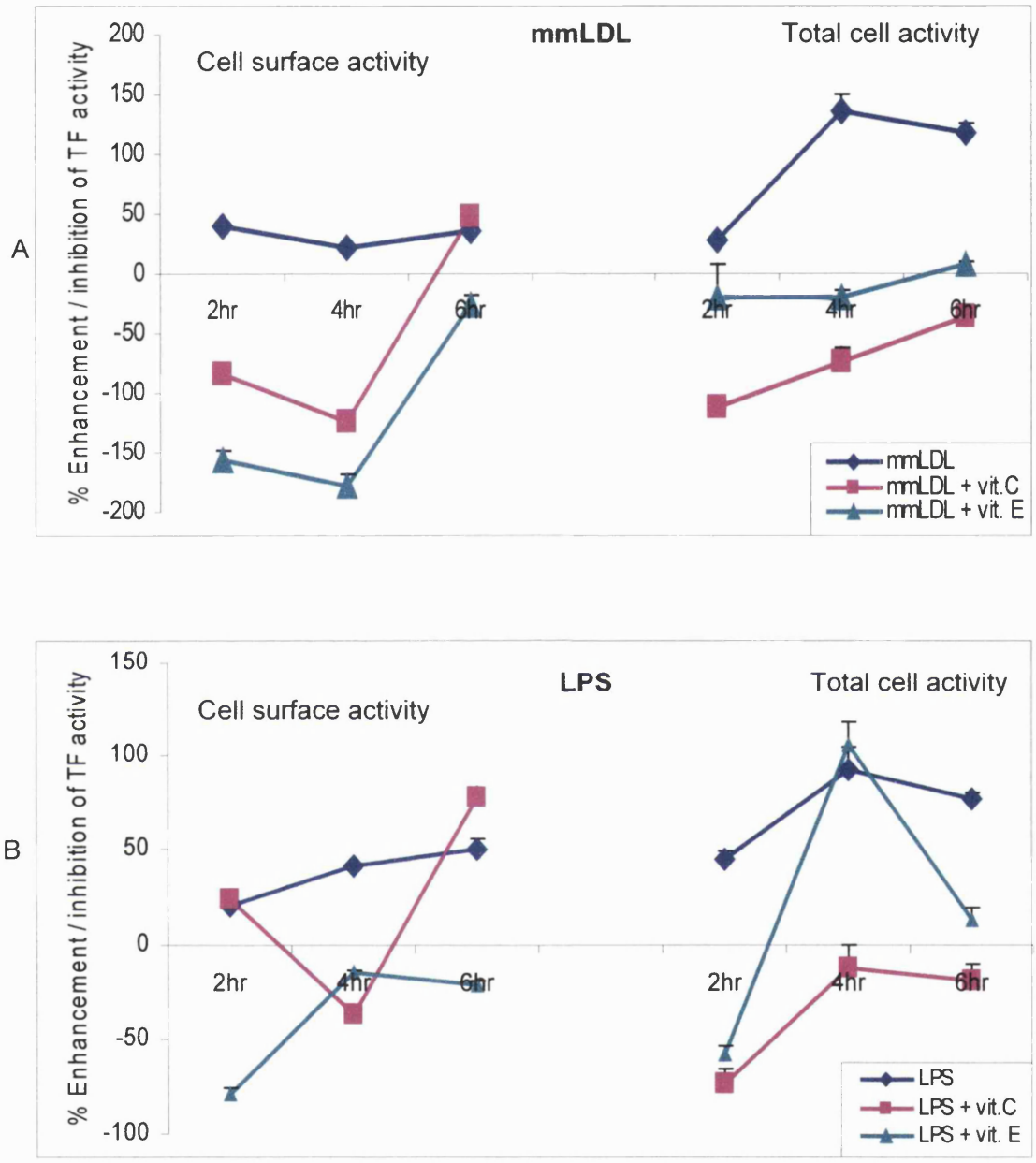


Figure 5.2.9.6: Inhibition of TF activity by vitamin C and vitamin E.
THP-1 cells were pre-treated with 50μM vit.C or 10μM vit. E 18h prior to the addition of 50μg protein/ml mmLDL (A) or 10μg /ml LPS (B). The cells were incubated further for up to 6h. The results represent the % enhancement (+ve values) and % inhibition (-ve values) of procoagulant activity. (mean ± SEM, n=3;)

5.3 Discussion

TF expression by monocyte-derived macrophages and vascular endothelium has been associated with pathological conditions such as cancer, infection, inflammation and atherosclerosis (Edwards *et al*, 1981; Osterud and Flaegstad, 1983; Semararo *et al*, 1985; Landers *et al*, 1994; Taudman *et al*, 1997). TF is also closely tied to the host response in sepsis; it is an important determinant of mortality in the septic shock syndrome (Bohrer *et al*, 1997). TFPI is the physiological inhibitor that regulates TF-induced coagulation. There is substantial evidence for a protective role of dietary antioxidants in the prevention of cardiovascular disease but the mechanisms of action are not fully known. Studies were carried out to investigate the effect of antioxidant vitamins on the expression of TF and TFPI in THP-1 and ECV 304 cells.

In the initial experiments, using Northern blot hybridisation, the protective effect of antioxidant vitamins on the gene expression of tissue factor was evident. Both LPS and mmLDL induced the gene expression of TF in THP-1 and ECV 304 cells, while treatment of both cell lines with ascorbic acid and vitamin E reduced this induction. In ECV 304 cells, LPS and mmLDL both increased the expression of tissue factor pathway inhibitor mRNA while the antioxidant vitamins had little or no effect on the induced transcripts. These findings formed the foundation for the more extensive studies using the QC-RT-PCR assay and a range of antioxidant vitamins. The effects of the antioxidants on the expression of TF activity were also studied. Both LPS and mmLDL increased the cell surface and total cell tissue factor activities. The induction of tissue factor activity involves *de novo* synthesis of tissue factor (Lyberg and Prydz, 1981).

Ascorbate

Ascorbic acid was the most effective in suppressing TF gene expression and total cell TF activity. Vitamin C significantly inhibited TF gene expression induced

by SIN-1, LPS and mmLDL in both ECV 304 and THP1 cells. Exposure of cells to the stimuli caused a significant increase in TF expression while concurrent or pre-treatment of the cells with vitamin C (50 and 100μM) inhibited its expression. The inhibitory effect of vitamin C on the expression of TF was found to be dose dependent. The action of this antioxidant on the expression of TFPI was less pronounced, notwithstanding, the pre-treatment of the cells with the vitamin C consistently up-regulated the expression of TFPI.

Another beneficial effect of vitamin C was demonstrated by Siow *et al* (1998); their studies showed an induction of antioxidant stress protein haem oxygenase-1 (HO-1), in cultured umbilical artery smooth muscle cells, by oxidised LDL and showed that the pre-treatment of the cells with vitamin C attenuates the induction. The European Prospective Investigation into Cancer and Nutrition (EPIC –Norfolk) study found that plasma ascorbic acid concentration was inversely related to mortality from cardiovascular diseases and ischemic heart disease; a 20μmol/L increase in plasma ascorbic acid concentration was associated with 30% reduction in mortality risk (Khaw *et al*, 2001). Despite its protective effects, ascorbic acid has been shown to have a pro-oxidant role *in vitro* (Borg and Schaich, 1989). In the presence of iron, ascorbate can initiate lipid peroxidation, and with copper, it is used to generate hydroxyl radicals, which are known to initiate lipid peroxidation.

α-Tocopherol

Low-density lipoproteins contain endogenous antioxidants particularly α-tocopherol, which confer some protection from oxidation. The pro-oxidant effect of *α*-tocopherol has also been documented. α-tocopherol exhibits anti- and pro-oxidant activity for lipoprotein lipids depending on the degree of radical flux and the reactivity of the oxidant (Thomas and Stocker, 2000). Thomas and co-workers (1998) reported that *in vitro* enrichment of LDL with vitamin E decreased or increased peroxynitrite-mediated lipid peroxidation, depending on the ratio of

the peroxynitrite to LDL particle; at a ratio of <100:1, vitamin E acted as a pro-oxidant while at ratios of >200:1, vitamin E acted as an antioxidant.

In this study, α-tocopherol significantly inhibited the induction of TF mRNA in the endothelial and monocytic cell lines. It was most effective in the inhibition of cell surface TF activity. The dose response effect of the lipophilic antioxidant vitamin E on the expression of TF in THP-1 cells was studied; in mmLDL-treated cells, the inhibitory effect of the vitamin was concentration dependent while the inhibition of TF gene expression in LPS-stimulated cells did not increase with increased concentration of vitamin E. The beneficial effects of vitamin E in atherogenesis have also been documented by others. Azzi *et al* (1995) demonstrated that at physiological concentrations, *α*-tocopherol inhibits SMC proliferation. Keaney *et al* (1996) also showed that *α*-tocopherol prevents oxLDL-induced endothelial dysfunction. In patients with coronary disease; vitamin E supplementation (400-800 IU/day) was found to be protective against cardiovascular disease in the Cambridge Heart Antioxidant Study (Stephens et al, 1996) although other studies by Alpha-Tocopherol and Beta-Carotene Cancer Prevention Study (1994), the Gruppo Italiano per Lo Studio della Sopravvivenza nell'Infarto Miocardico (1999), the Heart Outcomes Prevention Evaluation study (Yusuf *et al*, 2000) and the Secondary Prevention with Antioxidants of Cardiovascular Disease in Endstage (SPACE) renal disease (Boaz *et al*, 2000) showed no effect of vitamin E supplementation on cardiovascular mortality. The Vitamin E Atherosclerosis Prevention Study [VEAPS] (Hodis *et al*, 2002) showed that α-tocopherol supplementation in healthy subjects (mean age 56.2 years; range 40 to 80.2 years) raised plasma vitamin E levels, reduced the circulating oxidised LDL as well as the susceptibility of LDL to oxidation but showed no effect on the progression of atherosclerosis.

The most widely accepted mechanism by which vitamin E might exert a protective effect in atherogenesis is through inhibition of LDL oxidation. The beneficial effects of vitamin E has also been attributed to other properties of the

compound that are not directly related to its antioxidant properties. In SMCs, α-tocopherol is reported to inhibit protein kinase C-α (PKC-α) (Ricciarelli *et al*, 1998) and this inhibition is strongly involved in the proliferative signal transduction pathway and could lead to changes of the gene expression patterns altered in atherosclerosis (Ricciarelli *et al*, 2000). Furthermore Ricciarelli *et al* (2000) found that in cultured human aortic SMCs, CD36 scavenger receptor involved in the uptake of oxidised LDL and the subsequent formation of foam cells, are downregulated by α-tocopherol leading to a reduction in the uptake of oxidised LDL by the cells. Vitamin E also inhibits the production of chemokines and inflammatory cytokines, as well as the adhesion of endothelial cells to monocytes by reducing the expression of adhesion molecules when cells were activated with an inflammatory cytokine (Wu *et al*, 1999).

Carotenoids

Lutein is more hydrophilic than the other carotenoids, which makes it more readily presentable to cells. Pre-treatment of the cells with lutein resulted in a significant inhibition of TF expression in mmLDL-treated cells, but the antioxidant tends to be more effective when added simultaneously with LPS to the cells. Carotenoid antioxidant activity is very effective when the oxygen tension is in the physiological range (<150 Torr). Carotenes interact with peroxyl radical to produce a carotene radical that effectively terminates lipid peroxidation process. As the oxygen tension increases, the carotene radical increasingly reacts with oxygen to generate end products that are capable of propagating lipid peroxidation (Burton, 1989; Handelman *et al*, 1991: Rousseau *et al*, 1992; Lieblar, 1993). In this study the effect of lutein was studied at air oxygen tension and it was effective in inhibiting the expression of TF but had no effect on the expression of TFPI.

Nitric oxide

Antioxidant and pro-oxidant role for nitric oxide (NO) in the development of atherosclerotic plaques have been reported (Buttery *et al* 1996; Leeuwenburgh *et al*, 1997). Nitric oxide could be a better lipid peroxyl scavenger than α-tocopherol because the reaction of nitric oxide with lipid peroxyl radical is about 10,000 times faster than the reaction of α-tocopherol with the same lipid peroxyl radical (O'Donnell *et al*, 1997). Despite the fact that two molecules of nitric oxide are consumed for every lipid peroxyl radical scavenged (Patel *et al*, 2000) while just one molecule of α-tocopherol can scavenge two lipid peroxyl radicals, studies by O'Donnell and co-workers (1997) showed that low concentrations of nitric oxide are very efficient in scavenging lipid peroxyl radical, with 30nM nitric oxide inhibiting lipid peroxidation to the same extent as 20μM α-tocopherol.

The effects of lipoprotein oxidation and its products on monocytes and endothelial cells include a decrease in the release of nitric oxide (Jacobs et al, 1990; Bruckdorfer et al, 1997; Dulak *et al*, 1999). Likewise, Graier *et al* (1994) reported that cultured bovine and porcine aortic endothelial cells showed decreased nitric oxide production when treated with LPS. Nitric oxide may reduce intracellular reactive oxygen species (Clancy *et al*, 1992; Tsao *et al*, 1997) and has also been found to block the activation of NFKB (Lin and Lin, 1997). Vitamin C is known to be capable of stimulating or restoring NO activity; hence studies were carried out to determine if the inhibition of TF expression by vitamin C was a result of NO stimulation by vitamin C. The results showed that DEA NONOate had no effect on the induced expression of TF. This is in agreement with the studies by Gerlach *et al* (1998) who found no effect of the NO donor NOC18 on LPS-induced TF expression but contrary to the findings of Yang and Loscalzo (2000). Studies by Yang and Loscalzo (2000) demonstrated that L-arginine, as a substrate for NO synthase, suppresses endotoxin and cytokine-induced expression of TF in human microvascular endothelial cells suggesting that NO may prevent thrombosis by inhibiting platelet function and

also by suppressing TF-dependent coagulation (Pigazzi, 1999; Yang and Loscalzo, 2000).

Folate

Folic acid is essential for the synthesis and repair of DNA (Green *et al*, 1988) and its deficiency may largely contribute to the increased cardiovascular risk associated with hyperhomocysteinemia. The antioxidant effects of folates have been described (Verhaar et al, 1998). There are increasing data in support of the importance of an adequate intake of folate; low serum levels of folate are associated with an increased risk of cardiovascular disease (Morrison et al, 1996; Rimm et al, 1998). It has been reported that folate may also restore endothelial dysfunction and this effect is independent of the homocysteine-lowering capacity of folate (Verhaar *et al*, 1999; Wilmink *et al*, 2000; Title *et al*, 2000). Folic acid can prevent the oxidative degradation of NO by reducing superoxide generation from NO synthase or xanthine oxidase (Verhaar et al, 1998). It can also increase NO formation by stimulating the recycling of tetrahydrobiopterin, an essential cofactor of endothelial NO synthase (Kaufman, 1991).

Studies were carried out to investigate whether folic acid could also inhibit the expression of TF in mmLDL and LPS-stimulated THP-1 cells. The results showed that folic acid had no inhibitory effects on LPS-stimulated tissue factor gene expression, but effectively inhibited TF expression in mmLDL-treated cells. This data is in support of a role of folate in the prevention of mmLDL-implicated vascular diseases.

Epigallocatechin gallate

Epigallocatechin gallate is an inhibitor against peroxynitrite and mitochondrial ROS production (Feng *et al*, 2001). The effect of epigallocatechin gallate (EGCG) on mmLDL and LPS-induced TF gene expression was studied to see if it would have the same effect as the other antioxidants used in the study; thereby

raising the possibility that the antioxidants acted against peroxynitrite and/or, the generation of ROS in the mmLDL and LPS-stimulated cells. The data from the study showed that EGCG had little or no effect on the mmLDL and LPS-induced TF expression; suggesting that the inhibitory effects of antioxidants on the expression of TF is not wholly dependable on their ability to prevent the generation of reactive oxygen species and peroxynitrite.

Antioxidant treatment after TF induction

The inhibitory effect of antioxidant vitamins on TF gene expression was evident when the cells were pre-treated with the antioxidants and when the antioxidant and the stimuli were added simultaneously to the cells. Studies were carried out to look at the effects of the antioxidants on the expression of TF after the cells had been exposed to mmLDL or LPS. A consistent increase in the expression of TF was observed when cells were treated with antioxidant after an induction of TF mRNA had set in. This result suggest that not everyone will benefit from antioxidant supplementation; there is a possibility that only the healthy individuals with no trace of cardiovascular disease will be protected by antioxidants against the development of cardiovascular diseases in later life. A lifelong intake of antioxidant vitamins in food or as supplements is likely to protective against future development of cardiovascular disease but giving antioxidants to patients with pre-existing disease may have no beneficial effect or even make things worse. This thought is complementary to the observation made in the Cambridge Heart Antioxidant Study (CHAOS).

In CHAOS, there was a significant benefit associated with vitamin E and this benefit was entirely due to a reduction in non-fatal myocardial infarction. There was a large decrease in the non-fatal myocardial infarction (77%) but an increase in fatal myocardial infarction (18%) after treatment with RRR α-tocopherol. Fatal myocardial infarction is often associated with rupture of advanced unstable plaques where in TF expression as been implicated. Similar

observations were made in the Alpha-Tocopherol, Beta Carotene study; in which there was a reduction in non-fatal myocardial infarction by 38% (vit.E) and 23% (β-carotene). But the risk of fatal myocardial infarction increased by 33% (vit E) and 75% (β-carotene) in individuals with a history of myocardial infarction (Giuliano, 2000). However, in the Heart Outcomes Prevention Evaluation (HOPE) study (Yusuf *et al*, 2000) no clinical benefit or adverse events were noted in the vitamin E treated group. In the Secondary Prevention with Antioxidants of Cardiovascular Disease in Endstage (SPACE) renal disease (Boaz *et al*, 2000), the effect of vitamin E supplementation on cardiovascular disease outcomes in haemodialysis patients with pre-existing heart disease was investigated and no effect on total or cardiovascular mortality was observed. The study by Gruppo Italiano per Lo Studio della Sopravvivenza nell'Infarto Miocardico (1999) also showed no effect of vitamin E supplementation on cardiovascular mortality.

Comment

These data support the concept of a protective role of antioxidant vitamins against cardiovascular diseases in which oxidative stress has been implicated. The antioxidant vitamins used in this study are at physiologically relevant concentrations. Although the concentrations of lutein used may be higher than that found in plasma and since its cellular concentration is uncertain, this concentration was in a reasonable relationship to that of the other lipid soluble antioxidant (α-tocopherol) that were also used in this study. It is clear from this study that antioxidants are effective in modifying the response of endothelial and monocytic cells to mmLDL and LPS at the gene level by inhibiting the induction of TF expression. The antioxidants had no marked effect on the expression of TFPI which would also be considered to be protective. The inhibitory effects of the antioxidant vitamins on the gene expression of tissue factor were observed when the vitamins were added at the same time as with the stimulus and when the cells were pre-treated for 18 h before the addition of the stimulus indicating

that a long-term antioxidant intake may play a role in primary prevention of cardiovascular disease in healthy individuals while treatment may be ineffective in patients with pre-existing cardiovascular disease.

The overall beneficial effect of antioxidants to human being is questionable since antioxidant vitamins can also suppress protective genes such as haemoxygenase-1. Haemoxygenase-1 contributes to resistance to various types of free radical damage and it has been demonstrated that pre-treatment of umbilical artery smooth muscle cells with vitamin C attenuates the induction of this gene (Siow *et al*, 1998; Siow *et al*, 1999b). Also, the suppression of TF gene expression by antioxidant vitamins may not, in all cases, be advantageous considering the role of TF in blood vessel development (Carmeliet *et al*, 1996). By and large, antioxidant vitamins that suppress the expression of TF may be vital in the prevention or inhibition of atherosclerosis by scavenging the reactive oxygen species generated from the pro-oxidants and the inflammatory stimulus. The antioxidants, therefore, block the induction of gene expression by reactive oxygen species e.g. by exposure to LPS as well as prevent further oxidation of LDL. Antioxidant vitamins may also act in the cell by activating signal transduction pathways that can lead to the increase in the synthesis of endogenous antioxidants or by down-regulating the genes that are induced in response to oxidants and inflammatory agents. In conclusion, the results of this study show that mmLDL and LPS induce the expression of TF in monocytes and endothelial cells and this induction is inhibited by treating the cells with antioxidants, supporting the notion that antioxidant vitamins have a protective effects against cardiovascular diseases specially by reducing the risk of thrombogenesis.

CHAPTER 6

Modulation of transcription factors by oxidative stress and antioxidant vitamins

6.1 Introduction

It has been described in the previous chapter that oxidative stress and antioxidant vitamins can modulate the expression and activity of tissue factor and the expression of tissue factor pathway inhibitor. Importantly, the antioxidants were able to have an effect, on the gene expression, relatively quickly even when the vitamins and the mmLDL / LPS were added together at the same time. The antioxidant vitamins may be acting to prevent the oxidation of biological molecules and to prevent the generation of reactive oxygen species. The other possible mechanism of action of the antioxidants is the regulation of signal transduction pathway by the modulation of transcription factors. The human TF promoter contains binding sites for the transcription factors such as AP-1, NFκB/Rel, Egr-1, Sp1 and HIF-1. The relative balance of the activating and inhibiting actions of the transcription factors could be the key factor in the regulation of gene transcription in a specific cell type and by a particular stimulus (Latchman, 2001).

Gene expression is regulated by the binding of *trans*-acting transcription factors and other cooperative proteins like RNA polymerase to *cis*-acting consensus recognition sequences that lie in the promoter region that contains multiple sequences recognized by a variety of transcription factors. Transcription factors can translocate to the nucleus in response to specific stimuli; they are modulated by upstream signalling events. They determine the magnitude and specificity of gene expression by their interaction with specific DNA sequences in the regulatory region of the specific gene.

Activation of transcription factors stimulates gene transcription in response to specific stimuli. When a cell is stimulated, the stimuli activate or induce the specific transcription factor, which in turn stimulates specific gene expression by binding to the responsive binding sequence of the gene. Transcription factors may also act by inhibiting gene expression through a negatively acting factor interfering with the action of a positively acting factor or by direct interaction with the basal transcriptional complex causing a reduction in the activity of the complex (Latchman, 2001). The effect of reactive oxygen species on the expression and activity of transcription factors is complex and occurs at multiple levels (Allen and Tresini, 2000). Reactive oxygen species generally cause an increase in AP-1 and NFκB, but oxidant stress can, at the same time, reduce the transcriptional activity of these molecules through the direct oxidation of critical cysteine residues contained within the DNA-binding domain (Allen and Tresini, 2000).

Nuclear Factor Kappa B (NFκB)

NFκB is a transcription factor that controls the expression of a multitude of genes involved in inflammation and proliferation. Its activation often reflects the activation of more than one transcription factor. The NFκB/Rel family of transcription factors includes NFκB1 (p50), NFκB2 (p52), RelA (p65), RelB and cRel (Cogswell *et al*, 2000). They can form homodimers or heterodimers consisting of two proteins and regulate expression of many different genes. The cRel/p65 heterodimer has been implicated in the inducible expression of TF in monocytes and endothelial cells (Mackman, 1995). NFκB can be activated by a multiple of stimuli including the inflammatory cytokines and their receptors, cell adhesion molecules, growth factors tumor necrosis factor -α, interleukin-1, lipopolysaccharide, oxidant stress, advanced glycosylation endproducts and trauma (Thurberg and Collins, 1998; Pahl, 1999; Murley *et al*, 2001). These stimuli induce the dissociation of the inhibitory protein IκBα from pre-existing cytoplasmic complexes (e.g. cRel/p65), allowing the transcription factor to

translocate to the nucleus and initiate expression of target genes (Beg and Baldwin, 1993; Baeuerle *et al*, 1996; Bowie and O'Neill, 2000).

Cyclic AMP-responsive element binding protein (CREB)

cAMP induces the expression of numerous genes through the protein kinase A (PKA) mediated phosphorylation of CRE- binding factors, including CRE-binding protein (CREB) (Lalli and Sassone-Corsi, 1994). Ollivier *et al* (1996) showed that activation of PKA by agents that elevate cAMP in human monocytic and endothelial cells inhibits the expression of TF. Elevated cAMP may also regulate the activity of other transcription factors that are required for the expression of the TF gene (Ollivier *et al* 1996).

Elk-1

Elk-1, also known as p62 ternary complex factor, is an Ets-related transcription factor that mediates growth factor stimulation of the c-fos promoter (Price *et al*, 1996). It is a member of the Ets family, which also include Sap-1a and Fli-1 (Watson *et al*, 1997). Elk-1 binds to DNA in part via interaction with serum response factor (SRF). The Egr-1 promoter contains several serum response elements (SRE) and Ets binding sites that mediate induction. Serum response factor and ternary complex factors form a ternary complex at these SRE and Ets sites. These proteins are phosphorylated and activated by upstream mitogen-activated protein kinase (MAPKs) in a cell-type and stimulus-specific manner (Janknecht *et al*, 1993). Phosphorylation of Elk-1 in its C-terminal domain induces a conformational change that promotes binding of Elk-1 to Ets sites via its N-terminal Ets domain and binding to SRE via its B box domain (Yang *et al*, 1999). Growth hormone stimulation of cells leads to the phosphorylation of Elk-1 and formation of an Elk-1/SRE complex that mediates induction of Egr-1 gene expression (Clarkson *et al*, 1999).

Hypoxia-induced factor-1 (HIF-1)

Hypoxia-induced factor-1 is involved in the regulation of gene expression in response to changes in cellular oxygen tension (Semenza, 2001). It has been shown recently that HIF-1 can be activated by reactive oxygen species (Chandel *et al*, 2000; Richard *et al*, 2000; Tacchini *et al*, 2002) and it can transcriptionally regulate genes containing the recognition sequence in their regulatory region. It is a heterodimeric transcription factor that consists of a constitutively expressed HIF-1β and an inducible protein HIF-1α (Wenger, 2000). Several genes that play key roles in cell survival, vascular tone, angiogenesis and cell proliferation are regulated by HIF-1. These genes include vascular endothelial growth factor (Ryan *et al*, 1998; Liu *et al*, 1995), nitric oxide synthase 2 (Palmer *et al*, 1998; Jung *et al*, 2000) transforming growth factor β3 (Caniggia *et al*, 2000), plasminogen activator inhibitor-1 (Kietzmann *et al*, 1999) and haem oxygenase-1 (Lee *et al*, 1997). Since there are 3 HIF-1 recognition sites in the 5' promoter region of TF gene, it is likely that HIF-1 is involved in the expression of TF gene during oxidative stress.

The aim of this study was to investigate the effect of LPS, mmLDL, SIN-1 and antioxidant vitamins on transcription factors NFκB, HIF-1, and the signalling molecules that lead to the activation of transcription factors Elk-1 and CREB in human monocytic cell line (THP 1). The transcription activators Elk-1 and CREB are phosphorylated and are activated by mitogen-activated protein kinase and cyclic-AMP- dependent kinase respectively. The PathDetect *in vivo* signal transduction pathway *Cis*-reporting (NFκB) and *trans*-reporting systems (pFA Elk-1 and pFA CREB) were used as described in section 2.27. The electophoretic mobility shift assays (NFκB and HIF-1) were carried out as described in section 2.29.

6.2 Results

6.2.1 An investigation into effect of oxidative stress and antioxidant vitamins on the transcriptionai regulation of ELK-1

THP-1 cells were transfected as described in section 2.27 with the plasmid pFA2-Elk-1, which expresses the GAL4 DNA-binding domain fused with the transactivation domain of Elk-1 (Stratagene Cloning Systems) and the reporter plasmid pFR-LUC, which contains 5 copies of the GAL4 binding site upstream of a minimal promoter that drives expression of the firefly luciferase reporter gene. The empty plasmid containing only the DNA binding domain (dbd) was used as a negative control. After transfection, cells were incubated in serum free medium for 24 h before stimulating with LPS (10μg/ml), mmLDL (50μg/ml) or SIN-1 (10μM), in the presence or absence of the antioxidant vitamins for 5 h. The antioxidants used in the studies were 50μM ascorbic acid (vitamin C), 10μM α-tocopherol (vitamin E), 1μM lutein and 1μM folic acid. Cell lysates were assayed for luciferase activity as described in section 2.28 using the TR717 microplate luminometer.

The luciferase activity was slightly, but not significantly, increased (1.3 fold) in the mmLDL stimulated cells that contained pFA2-Elk-1 (figure 6.2.1.1A). The treatment of the cells with antioxidant vitamins had little effect on the activity of Elk-1, except in the folic acid treated cells where a 52% inhibition was observed. A 2.5 fold increase in ELK-1 activity was noted in the SIN-1 stimulated cells (figure 6.2.1.1B) while a larger effect was noted in the LPS stimulated cells (figure 6.2.1.1C) with a 3.2 fold increase in ELK-1 activity, although the increase may merely reflect the activation of MAP Kinases. Ascorbic acid and folic acid did not inhibit the activition of ELK-1 or the MAP Kinases in THP-1 cells treated with LPS. Lutein and vitamin E on the other hand inhibited the activation by 100% and 51% respectively.

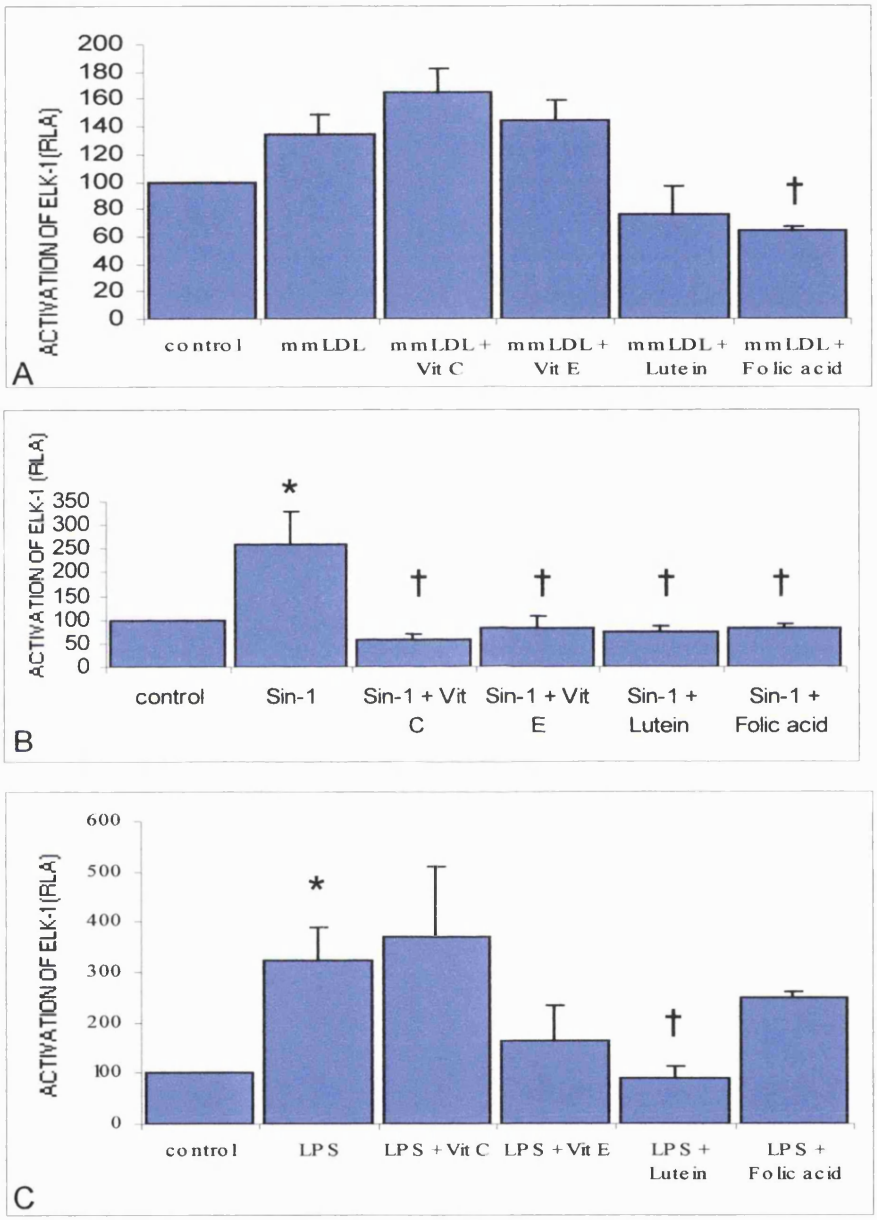


Figure 6.2.1.1: Relative luciferase activity (RLA) of extracts from cells transfected with ELK-1. THP-1 cells were co-transfected with pElk-1 and the reporter plasmid-pLuc. The transfected cells were incubated with 50µg protein/ml mmLDL (A), 50µM SIN-1 (B) or 10µg/ml LPS (C) in the presence or absence of antioxidant vitamins for 5 hours. After the incubation, the relative luciferase activity was determined using promega's luciferase assay system (each result is mean ± SEM of 3 independent experiments, * $p< 0.050$ compared to control : † $p<0.05$ compared to LPS / Sin-1 / mmLDL).

These studied indicate that ascorbic acid did not reduce LPS and mmLDL induction of TF expression through LPS and mmLDL-induced ELK-1 or MAP kinase activities. The inhibition of ELK-1 or MAP Kinase activition by vitamin E (in LPS stimulated cells), folic acid and lutein (in mmLDL stimulated cells) could account, in part, for the inhibition of LPS and mmLDL-induced TF expression by the antioxidants in THP-1 cells.

All the antioxidants used in the study (folic acid, lutein, ascorbic acid and α-tocopherol) strongly inhibited the SIN-1-induced ELK-1 or MAP Kinase activation in THP-1 cells. This inhibition could account for the inhibition, by the vitamins, of SIN-1-induced TF expression.

6.2.2 The effects of LDL, LPS, SIN-1 and antioxidant vitamins on the transcriptional regulation of CREB

THP-1 cells were transfected with the pFA2-CREB and the reporter plasmid pFR-LUC using the Superfect reagent (see section 2.27.2). About 24 h post-transfection, the cells were exposed to LPS, mmLDL, or SIN-1 in the presence or absence of antioxidants for 5 h. Cell lysates were prepared and assayed for luciferase activity as described in section 2.28. CREB transcription factor activity was not affected by mmLDL (figure 6.2.2.1A), SIN-1 (figure 6.2.2.1B), nor by LPS (figure 6.2.2.1C), in pCREB transiently transfected THP-1. Treatment of the transfected cells with lutein and α-tocopherol did not have any effect either. When the transfected cells were treated with mmLDL in the presence of ascorbic acid, there was a 7.3 fold induction of CREB transcription activity. This increase in CREB activities could be a reflection of the activation of cellular cyclic AMP (cAMP). Ollivier *et al* (1993 and 1996) showed that agents that elevate cAMP inhibit the functional expression of TF by reducing the rate of TF gene transcription in LPS stimulated monocytes.

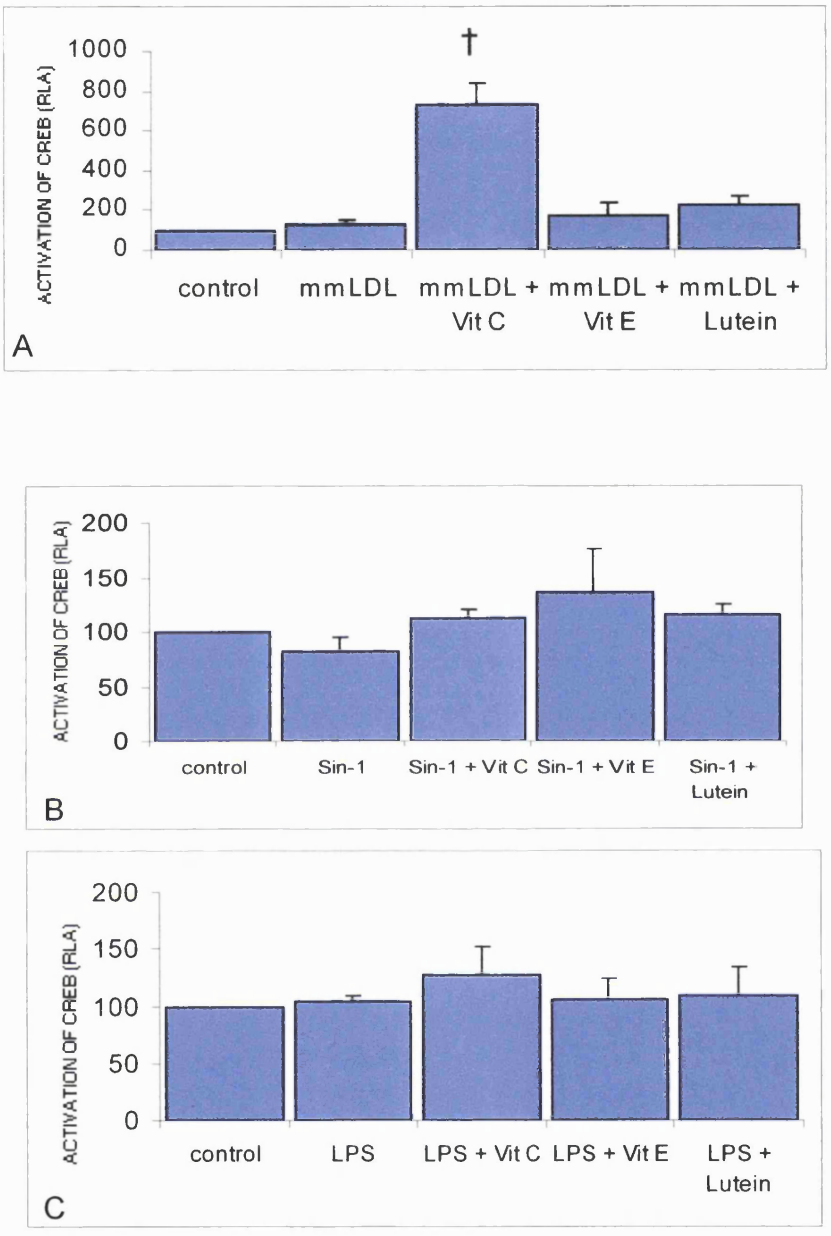


Figure 6.2.2.1: Relative luciferase activity (RLA) of extracts from cells transfected with pCREB. THP-1 cells were co-transfected with pCREB and the reporter plasmid-pLuc. 24 hour after transfection, the cells were incubated with 50µg protein/ml mmLDL (A), 50µM SIN-1 (B) or 10µg/ml LPS (C) in the presence or absence of antioxidant vitamins for 5 hours. After the incubation, the relative luciferase activity was determined using promega's luciferase assay system (each result is mean ± SEM of 3 independent experiments, † $p<0.05$ compared to mmLDL).

Hence, the elevation of cAMP by ascorbic acid in mmLDL-treated cells could account for the inhibition of TF gene expression by ascorbic acid in mmLDL-induced THP-1 monocytic cell line.

6.2.3 The effects of LDL, LPS, SIN-1 and antioxidant vitamins on the nuclear transcription factor NFκB

The effect of oxidative stress and antioxidant vitamins on the activation of NFκB was determined by the luciferase assay and gel mobility shift assay. THP-1 cells were transiently transfected, with the pNFκB-Luc construct containing 5 copies of NFκB linked to a firefly luciferase reporter gene, using the gene pulser 11 electroporation system (see section 2.27.1). The cells were exposed 24h after transfection to mmLDL, SIN-1 or LPS in the presence or absence of antioxidant vitamins; cell extracts were prepared 5h after treatment. Using the luciferase reporter gene assay (see section 2.28), 1.4 fold induction of promoter activity was observed following the treatment of the transfected cells with mmLDL (figure 6.2.3.1A). Co- treatment of the pNFκB transfected cells with mmLDL and the antioxidant vitamins had no effect the transcription factor. The pNFκB transfection experimemts result indicated that the antioxidants used in the study had little or no effect on NFκB and the inhibition of TF gene by the antioxidants was not dependent on this transcription factor.

Treatment of cells with SIN-1 did not increase the luciferase response as shown in figure 6.2.3.1B. A 1.7 fold induction of promoter activity was observed following the treatment of the transfected cells with 10 μg/ml LPS (figure 6.2.3.1C). The simultaneous treatment of the cells with LPS and vitamin C enhanced the luciferase activity; hence the inhibition of TF expression by the antioxidant vitamins used in these studies does not appear to be due to a reduction in the activity of NFκB/Rel proteins, because the vitamins had no inhibitory effect on mmLDL and LPS induction of the reporter plasmids.

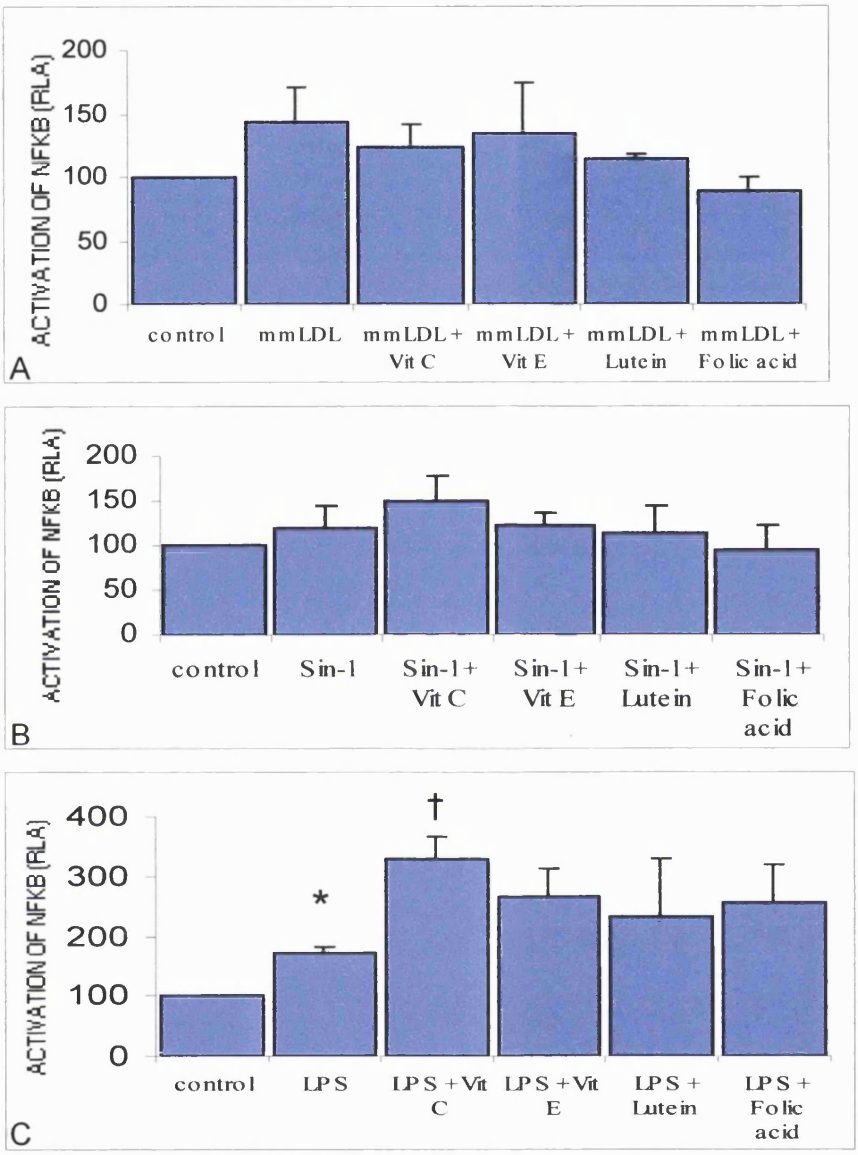


Figure 6.2.3.1: Relative luciferase activity (RLA) of extracts from cells transfected with pNFkB. THP-1 cell transfected with pNFkB was incubated for 5 hour with 50µg protein/ml mmLDL (A), 50µM SIN-1 (B) or 10µg/ml LPS (C) in the presence or absence of antioxidant vitamins. After the incubation, NFkB dependent transcription was determined using the promega's luciferase assay system. The results are expressed as % activation relative to control (mean ± SEM of 3 independent experiments, * $p< 0.050$ compared to control : † $p<0.05$ compared to LPS).

In order to confirm the transfection result, the possible role of the activation of NFκB in the regulation of TF expression by oxidative stress and antioxidant vitamins was investigated by electrophoretic mobility shift assay (EMSA) as described in section 2.29. THP-1 cells were incubated with LPS or mmLDL, in the presence or absence of ascorbic acid and vitamin E, for 1 h. Nuclear extracts were prepared (see section 2.29.1) and the electrophoretic mobility shift assay carried out using a radiolabeled oligonucleotide containing the NFκB binding sequence. Binding of nuclear proteins from THP-1 cells was examined 1h after stimulation because transcription of the TF gene is significantly increased at this time. The double stranded DNA probe for the consensus sequence of NFκB (5'AGTTGAGGGGACTTTCCCAGGC3') was used for the gel shift analysis after end-labeling of the probe with (γ-^{32}P) ATP and T_4 polynucleotide kinase (see section 2.29).

The electrophoretic mobility shift assay revealed that NFκB activities were increased after 1 h incubation with LPS and mmLDL (figure 6.2.3.2). To confirm the specificity of the protein-DNA interaction, a gel supershift assay with antibody to the p65 subunit of NFκB was carried out. The antibody for p65 was used because it is the cRel/p65 heterodimers that are involved in the inducible expression of TF in monocytes and endothelial cells (Mackman, 1995). The gel supershift assay with antibody for p65 subunit of NFκB revealed that a specific DNA binding complex of NFκB interacted with the p65 antibody (figure 6.2.3.3a). The p65 supershifted band was absent in the control cells, but was induced by LPS and mmLDL. To verify the identity of the bands observed in the electrophoretic mobility shift assay, competition reactions with unlabeled NFκB oligonucleotide were carried out. The unlabeled NFκB oligonucleotide inhibited the binding of the radiolabeled probe resulting in the disappearance of the bands established by mmLDL and LPS (figure 6.2.3.3b).

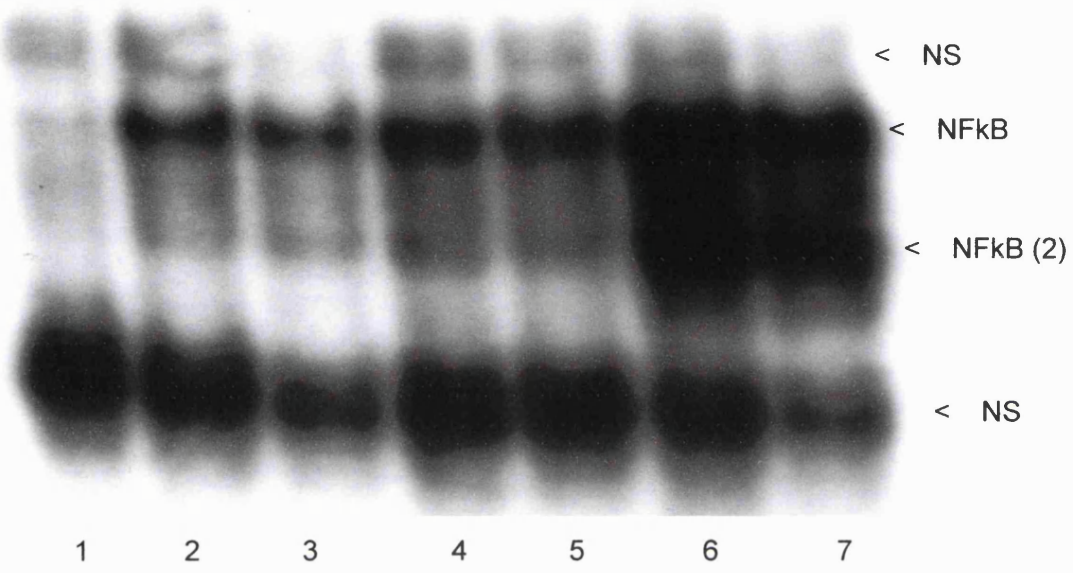


Figure 6.2.3.2: DNA binding activity of nuclear factor kappa B.
Electrophoretic mobility shift assay was carried out to determine the NFkB binding activity in THP-1 cells incubated for 1 hour. Lane (1) the control cells; lane (2) 50µg protein/ml mmLDL; lane (3) mmLDL and 50µM vit.C; lane (4) mmLDL and 10µM vit. E; lane (5) 10µg/ml LPS; lane (6) LPS and 50µM vit. C; lane (7) LPS and 10µM vit. E. N.S= non-specific binding

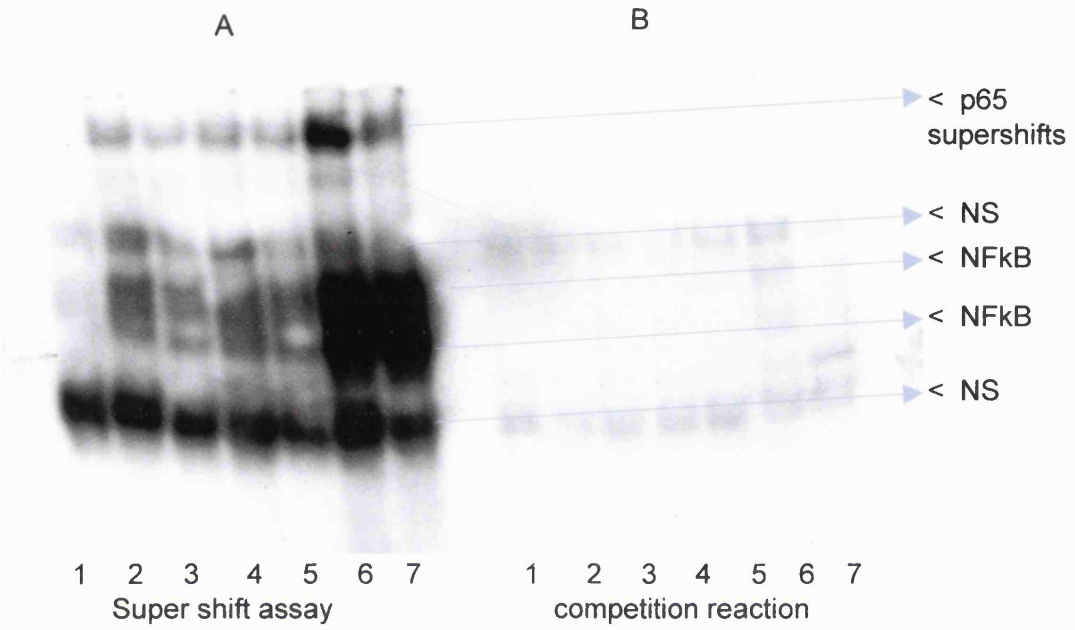


Figure 6.2.3.3: Super shift assay of the activity of p65 subunit of NFkB. THP-1 cells were incubated for 1 hour as follow; Lane (1) the control cells; lane (2) 50µg protein/ml mmLDL; lane (3) mmLDL and 50µM vit.C; lane (4) mmLDL and 10µM vit. E; lane (5) 10µg/ml LPS; lane (6) LPS and 50µM vit. C; lane (7) LPS and 10µM vit. E. Super shift assay with antibody for p65 subunit of NFkB and competition reaction with cold unlabelled oligonucleotides were carried out using the nuclear extracts prepared from the cells. N.S= non-specific binding

The protein binding activity of the p65 subunit of NFκB was induced by mmLDL, but the induction was partially inhibited by ascorbic acid and vitamin E treatment, suggesting that NFκB could be involved, in part, in the modulation of TF gene expression by the antioxidants. The discrepancy seen in the transfection experiments and the electrophoretic mobility shift assay is likely to be due to the duration of mmLDL stimulation. In the electrophoretic mobility shift assay, the cells were incubated with mmLDL for 1 h while in the transfection experiments, the cells were incubated for 5 h which is optimal for the expression of luciferase protein.

In the electrophoretic mobility shift assay, the protein binding activity of the p65 subunit of NFκB was induced by LPS (figure 6.2.3.3a). In agreement with the transfection experiments, co-treatment of the cells with LPS and ascorbic acid or vitamin E activates the p65 subunit of NFκB more than when the cells were treated with LPS alone (lanes 6 & 7). The increase in the DNA binding activity of NFκB by ascorbic acid and vitamin E was unexpected, but it has been reported before that antioxidants can also activate NFκB (Das *et al*, 1995; Antras-Ferry *et al*, 1997).

6.2.4 An investigation into effects of LDL, LPS, SIN-1 and antioxidant vitamins on the transcriptionai regulation of HIF-1

The 5' promoter region of the TF gene contains 3 HIF-1 binding sites. Thus the activation or inhibition of HIF-1 would lead to the suppression or enhancement in the transcription of TF. The possible role of the activation of HIF-1 in the regulation of TF expression was investigated by electrophoretic mobility shift assay. Nuclear extracts were prepared from THP-1 cells treated for 1 h with LPS or mmLDL in the presence or absence of ascorbic acid and α-tocopherol. Figure 6.2.4.1 represents the binding shift analysis showing the effect of ascorbic acid

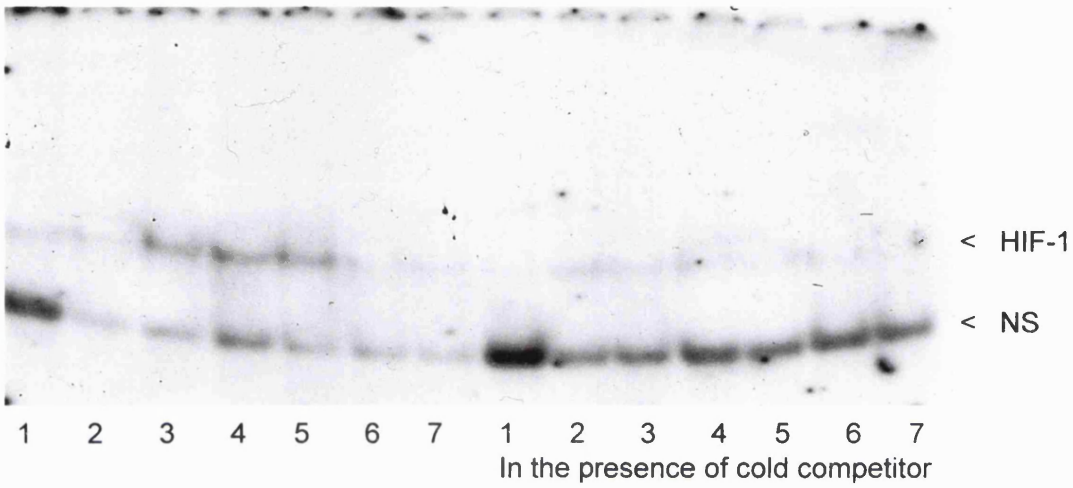


Figure 6.2.4.1: DNA binding activity of hypoxia induced factor-1.
THP-1 cells were treated for 1 hour as follow; Lane (1) the control cells; lane (2) 50µg protein/ml mmLDL; lane (3) mmLDL and 50µM vit.C; lane (4) mmLDL and 10µM vit. E; lane (5) 10µg/ml LPS; lane (6) LPS and 50µM vit. C; lane (7) LPS and 10µM vit. E. Nuclear extracts were prepared from the cells and HIF-1 DNA binding activity was determined by electrophoretic mobility shift assay using the radiolabelled consensus oligonucleotide. The specificity of the two observed bands was determined by incubating the nuclear extracts with cold unlabelled oligonucleotide before adding the radiolabelled oligonucleotide. The unlabelled oligonucleotide reduced the HIF-1 binding, while the intensity of the non-specific (NS) band was not reduced.

and vitamin E on the activity of HIF-1 in nuclear extracts of cells treated with mmLDL and LPS The electrophoretic mobility shift assay was carried out, using the double stranded DNA probe for the consensus sequence of HIF-1 (5'CGGTCACACCGTGGCTGA3'), as described in section 2.29. The electrophoretic mobility shift assay reaction specificity was verified by competition assays with a 50-fold excess of unlabeled oligonucleotide, which resulted in the disappearance of the bands established by LPS and mmLDL. The results for electrophoretic mobility shift assay for HIF-1 was quite the opposite of what was observed with the NFκB. The treatment of THP-1 cells with mmLDL had no effect on the DNA binding activity of HIF-1 but vitamin C and vitamin E treatment resulted in an increase in the level of HIF-1 nuclear transcription complex. LPS on the other hand increased the binding activity of HIF-1 which was inhibited by antioxidant treatment.

6.3 Discussion

The TF promoter contains quite a number of transcription factor binding sites that may contribute to the induction and/or repression of the gene. The antioxidant vitamins may reduce TF gene transcription by modulating protein binding to the transcription factor sites or by inhibiting the activities of the transcription factors. LPS initiates multiple intracellular signalling events, including the stimulation of pathways that lead to the activation of NFκB and three mitogen-activated protein (MAP) kinases, the ERK, p38 and JNK protein (Sweet and Hume, 1996). LPS activation of monocytes stimulates protein tyrosine kinase, protein kinase C and protein kinase A (Geng *et al*, 1993) and the signal through the protein kinase C activate Raf-1 and mitogen activated protein kinase (Kharbanda *et al*, 1994). Studies of Moll *et al* (1995) and Bierhaus *et al* (1995) demonstrated that LPS induce TF gene expression in monocytic cells and endothelial cells through activation of AP-1, Sp 1 and NFκB transcription factors. Oeth *et al* (1997) found that c-Rel/p65 and Sp 1 were not sufficient to mediate LPS induction of TF in THP-1 cells, but that LPS-induced transcriptional activation of the TF gene required functional interaction between c-Fos/c-Jun and c-Rel/p65 heterodimers bound to the AP-1 and NFκB sites respectively.

Cui *et al* (1999) conducted a series of transfection studies to examine transcription factors involved in the regulation of TF gene expression in SMC. The effect of native and oxLDL on the transcription factors that binds the promoter region of TF gene was investigated by transfecting rat aortic SMC with deleted TF promoter constructs and subsequently treating the cells with the oxLDL. They demonstrated that neither the AP1 sites nor NFκB site but the three Sp 1 sites and one Egr-1 site in the TF promoter mediated lipoprotein induced TF mRNA expression in SMC. They also found that the transcription factor EGR-1 contributes, in part, to the regulation of TF gene expression in response to oxLDL.

Although the most widely accepted mechanism by which antioxidant vitamins might exert a protective effect in atherogenesis is through inhibition of LDL oxidation, some of the beneficial effects of antioxidants have been attributed to other properties of these compounds that are not directly related to their antioxidant properties. The inhibition of smooth muscle cell proliferation and endothelial dysfunction by alpha tocopherol appears to be through the inhibition of protein kinase C (PKC) rather than by its antioxidant mechanism (Chatelain *et al*, 1993; Tasinato *et al*, 1995; Azzi *et al*, 1995; Keaney *et al*, 1996; Ricciarelli *et al*, 1998). In cultured human aortic SMCs, CD36 scavenger receptors are involved in the uptake of oxidised LDL and the subsequent formation of foam cells, and they are downregulated by α-tocopherol leading to a reduction in the uptake of oxidised LDL by the cells (Ricciarelli *et al*, 2000).

The possible role of antioxidant vitamins in the modulation of TF gene expression via activation and repression of transcription factors was investigated.

Nuclear Factor Kappa B (NFκB)

These studies indicated that LPS and mmLDL increased the transcriptional and binding activities of NFκB. While vitamin C and vitamin E reduced the binding activities of NFκB in mmLDL stimulated cells, the vitamins tend to increase the binding activities in LPS-stimulated cells. Reactive oxygen species generally cause an increase in NFκB, but oxidant stress can at the same time reduce the transcriptional activity of these molecules through the direct oxidation of critical cysteine residues contained within the DNA-binding domain (Allen and Tresini, 2000). There is no compelling evidence for a central role for oxidative stress in diverse pathways to NFκB (Bowie and O'Neill, 2000; Li and Karin, 1999). In fact many authors have found many pathways to NFκB to be insensitive to antioxidants (Harakeh and Jariwalla, 1997; Bowie *et al*, 1997; Moynagh *et al*, 1994; Brennan and O'Neill, 1995). While some studies indicate that oxLDL

activates the transcription factor NFκB (Han *et al*, 2000), some other studies indicate otherwise (Ohlsson *et al*, 1996). Munoz *et al*, 1997 reported that vitamin C (0.2mM) enhanced TNF mediated NFκB activation in Jurkat T cells. Lack of inhibition of NFκB by vitamin C (1.5mM) has also been reported by Harakeh and Jariwalla (1997).

The results of the transfection experiments did not show any significant increase in the activation of NFκB in the mmLDL treated cells compared to the control cells. In contrast to this observation, the electophoretic mobility shift assay showed an increase in the binding activities of the p65 subunit of NFκB in the mmLDL treated cells compared to the control cells. While this discrepancy could be accounted for as being due to the differences in the length of time that the cells were exposed to mmLDL, there is also the possibility of an induction of NFκB DNA-binding activity without transcriptional activation. Campbell *et al* (2001) demonstrated that the exposure of osteosarcoma cell line (U2OS) to UV light triggered the NFκB DNA-binding, but the DNA-bound complex was unable to activate transcription from standard NFκB reporter plasmids, suggesting that the presence of nuclear, DNA-binding NFκB does not necessarily indicate that it is capable of stimulating the transcription of known target genes. Studies by True and colleagues (2000) also demonstrated that although H_2O_2 induced the DNA-binding activity of NFκB, the binding was transcriptionally 'silent' as determined by its inability to activate the NFκB driven reporter gene.

Both the transfection and electrophoretic mobility shift assay indicated that vitamin C and vitamin E increased the activation and binding activities of NFκB in LPS stimulated cells. The activation of NFκB by vitamin E was unexpected, but it is known that NFκB can be activated by both oxidative stress-inducing and antioxidant agents and the resultant gene expression and protein products can be different (Das *et al*, 1995; Antras-Ferry *et al*, 1997; Murley *et al*, 2001). The

activation of NFκB may therefore be determined by the overall redox balance within the cell.

ELK-1

Treatment of pELK-1 transfected cells with LPS resulted in a pronounced activation of ELK-1 in the luciferase assays, which is in agreement with the studies by Guha *et al* (2001). Guha *et al* (2001) found that LPS induces the phosphorylation and functional activity of Elk-1 via the extracellular signal-regulated kinase (MEK-ERK I/2) pathway. They also showed that LPS induces the expression of Egr-1 and this induction of Egr-1 preceded the induction of TF expression. The LPS induction of Egr-1 promoter was mediated by 3 SRE sites, which bound an LPS –inducible complex containing serum response factor and Elk-1. The induction of TF gene expression in response to a variety of stimuli such as oxidized low density lipoprotein (Cui *et al*, 1999), shear stress (Houston *et al*, 1999) and hypoxia (Yan *et al*, 1998) is mediated by Egr-1. Guha *et al* (2001) proposed that LPS stimulation of monocytic cells leads to a rapid MEK 1/2 dependent phosphorylation of Elk-1 and that an LPS-inducible complex consisting of Elk-1 and SRF mediates the induction of Egr-1 gene expression which in turn binds to the TF promoter and contributes to maximal induction of TF expression in LPS stimulated cells.

Cyclic AMP-responsive element binding protein (CREB)

While neither LPS nor mmLDL have any effect on the activation of cyclic AMP-responsive element binding protein (CREB), the co-treatment of pCREB transfected cells with mmLDL and ascorbic acid greatly enhanced its activation. The inhibition of mmLDL-induced TF gene expression by ascorbic acid could largely depend on the activation of CREB. This is in agreement with the studies of Lyberg (1983) and Ollivier *et al* (1993) that show that agents that increase the intracellular levels of cyclic AMP will inhibit the induction of TF expression in human monocytes by decreasing the rate of TF gene transcription. Elevated

cAMP may also regulate the activity of other transcription factors that are required for the expression of TF. In THP-1 cells, Bt_2cAMP abolished LPS induction of TF mRNA expression and also partially inhibited NFκB mediated transcription in transfected cells, suggesting that cAMP may inhibit other transcription factors (Ollivier *et al*, 1996).

Hypoxia-induced factor-1 (HIF-1)

Mackman and co-workers (1989) published the complete sequence of the human tissue factor gene and pointed out the existence of two direct repeats of 11 nucleotides –CACCGTGGCTG (DR-1) upstream of the TATA box. They postulated that these repeats could be cis-acting regulatory elements representing the binding sites for transcription factors. These repeats and the nearby sequences show similarity to sequences flanking the genes for interleukin 1 and plasminogen activator inhibitor 1 (Mackman *et al*, 1989). It was very fascinating to observe that within each of these two repeats lie the binding site for HIF-1 (5'-RCGTG-3') while a third binding site is found 445bp upstream of the TATA box. Endothelial cells when stimulated with LPS express TF as well as interleukin 1 (Libby *et al*, 1986), and plasminogen activator inhibitor 1 (Crutchley and Conanan, 1986), and all these genes contain the HIF-1 recognition sequence in their 5' promoter region. It has been shown that plasminogen activator inhibitor-1 is regulated by HIF-1 (Kietzmann *et al*, 1999) suggesting that HIF-1 is likely to be involved in the regulation of tissue factor mRNA. HIF-1 has also been shown to be activated by reactive oxygen species (Chandel *et al*, 2000; Richard *et al*, 2000).

The partial oxygen pressure in the arterial wall range from about 4-10% O_2 (Crawford and Blankenhorn, 1991), but after the onset of atherogenesis, the partial oxygen pressure in the subendothelial space may drop to about 2.5% O_2 (Hajjar *et al*, 1988). Macrophages exposed to hypoxia have increased angiogenic activity (Knighton *et al*, 1983). Gene expression of angiogenic growth

factors such as vascular endothelial growth factor, platelet-derived growth factor β and transforming growth factor β are all induced by hypoxia (Gleadle *et al*, 1995). In pulmonary endothelial cell cultures, hypoxia can promote the formation of capillary-like networks (Phillips *et al*, 1995).

These studies indicated that mmLDL had no effect on the binding activities of HIF-1, but the co-treatment of THP-1 cells with mmLDL and vitamin C or vitamin E increased the binding activity of the transcription factor. On the other hand, while LPS increased the binding activity of HIF-1, treatment of the cells with vitamin C or vitamin E inhibited the DNA-binding activity of HIF-1.

In conclusion, the promoter and enhancer regions of TF gene contain response elements for several transcription factors, indicating that multiple transcription factors may be required to induce its transcription. The antioxidant vitamins may evoke one or more of the different types of signal transduction pathways depending on the stimuli and cell type. Antioxidants may influence cellular signaling by modifying redox-sensitive sites on molecules involved in the regulation of gene expression. The mmLDL or LPS and the antioxidant vitamins could exert their effects via the same transcription factor, with one acting as an agonist and the other an antagonist. Studies by Ozer and Azzi (2000) showed that α-tocopherol, at a concentration of 50μM, inhibited rat A705 smooth muscle cell proliferation. It also fully prevented cholesterol-induced atherosclerotic lesions and the induction of protein kinase C activity. While α-tocopherol inhibited the smooth muscle cell proliferation and the activity of protein kinase C, β-tocopherol was ineffective. When β-tocopherol and α-tocopherol were added together there was neither an inhibition of cell growth nor of protein kinase C activity. The study supports the existence of a 'binding protein' in the signal transduction pathways that reacts with α-tocopherol as an agonist and β-tocopherol as an antagonist.

CHAPTER 7: FINAL CONCLUSIONS

Atherosclerosis is one of the most prevalent fatal diseases in Western societies and one major disease in which oxidative stress has been implicated. The detrimental effect of LDL in atherosclerosis is mostly attributed to the oxidatively modified form. TF is the cellular initiator of blood coagulation. It plays an essential role in haemostasis by limiting haemorrhage in the event of vascular injury. TF also plays non-haemostatic roles in blood vessel development, tumour metastasis, tumour angiogenesis, cell migration and inflammation (Luther and Mackman, 2001). TF expression has been implicated in the pathogenesis of atherosclerosis. Leatham *et al* (1995) reported the expression of TF by circulating monocytes in patients with coronary artery disease.

LPS is an endotoxin involved in septic shock syndrome and inflammatory responses (Hewett and Roth, 1993). It is capable of enhancing the production of free radicals thus leading to oxidative stress (Landmann *et al*, 1995; Rosen *et al*, 1995; Schreck *et al*, 1992). It is also known to alter vascular functions and impairs contractile responses in human and animals arteries (Gunnett et al, 1998). Tissue Factor is closely tied to the host response and it is an important determinant of mortality in sepsis (Bohrer et al, 1997).

In this study, the effect of mmLDL and LPS on the expression of TF and TFPI mRNAs in monocyte and endothelial cells was studied. THP-1 monocytic cell line was chosen for this study because it behaves more like native peripheral blood monocytes. The only disadvantage is that it has a high basal TF mRNA and activity compared with peripheral blood monocytes. The ECV 304 cell line was used because it was originally characterized as a good model for endothelial cells activation by cytokines (Bowie *et al*, 1995), although its endothelial characteristics have now been disproven and has also been

described as a cellular model to study TF gene expression (Lopez-Pedrera *et al* 1997).

The method used for the LDL oxidation is known to be able to oxidise LDL slightly so that only the lipids were oxidised without any modification to the proteins. The protective role of antioxidant vitamins in atherosclerosis was pursued by observing the effects of a range of antioxidant vitamins on the expression of TF and TFPI. Also the transcription factors mediating the induction of TF expression were investigated.

Initial studies were done using Northern blotting technique but with this, only moderately abundant mRNAs could be detected; TFPI transcripts in THP-1 cells were not detectable by this technique. Another technique that could have been used, but not common at the beginning of the research project, is the DNA microarray analysis. The microarray analysis is a comparative gene expression profiling making use of the immobilized DNA sequences in a girded array on the surface of a solid support (DNA chips). It has the ability to monitor the expression levels of thousands of genes simultaneously. In this study, the absolute levels of TF and TFPI mRNA were measured using quantitative competitive RT-PCR based on competitive co-amplification of a specific target sequence together with known concentrations of a competitor in one reaction tube. Quantitative competitive RT-PCR had been used by different authors to measure the abundances of specific mRNAs (Zhang and Byrne, 1997; Zhang et al, 1997; Gattel et al, 1997). Potgens et al (1994) described a quantitative RT-PCR for measurement of TF in human endothelial cells (HUVEC); the levels of TF measured both in their stimulated and non-stimulated cells are very comparable to the levels found in this study.

Induction of TF and TFPI gene expression

The time courses of induction of TF mRNA by LPS and mmLDL are very similar. An increase in TF gene expression in the endothelial and monocyte cells was noted by 30 min with peak levels of mRNA occurring at 45-90 minutes. The concentrations of LPS and mmLDL capable of inducing the expression of TF in non-toxic doses were used in the study. These studies confirmed both minimally modified LDL and LPS as potent inducers of TF expression in monocytes and endothelial cells; inducing the cell surface procoagulant activity as well. The biological active component of the mmLDL, which may be responsible for the induction of TF, was investigated by looking at the direct effect of lysophosphatidylcholine on the expression of TF. It was found that the effect of mmLDL was not due to its lysophosphatidylcholine component and it is consistent with the study by Engelmann *et al* (1999) that showed an inhibition of TF in human monocytes by lysophosphatidylcholine.

Oxidatively modified LDL is present in atherosclerotic lesions, but little is known about the degree of LDL modification in the arterial wall. It has been suggested that the cells in early atherosclerotic lesions are mainly exposed to minimally oxidized LDL (Napoli *et al*, 2000). The studies show that native LDL had little effect on the expression of tissue factor. Some preparations of the native LDL used in the experiment actually suppressed the expression of tissue factor, while others had little or no effect at all on its expression. In contrast, minimally oxidised LDL significantly increased the expression of tissue factor while highly oxidized LDL only moderately induced the expression of tissue factor. This is in agreement with studies by Drake *et al* (1991), which showed that the expression of tissue factor in endothelial cells was induced by mmLDL but not by highly oxidized LDL

These studies revealed that low-density lipoprotein isolated from different individuals differ in their susceptibility to oxidation as well as in their capacity to

induce tissue factor gene expression. Hence, LDL samples from each individual should be taken as an entity because the composition of LDL varies from individual to individual. Reaven *et al* (1991) found that LDL rich in polyunsaturated fatty acid are more readily oxidized than LDL rich in saturated fatty acids or monounsaturated fatty acid. Intervention trials should therefore look at the composition of individuals LDL (i.e. polyunsaturated, monounsaturated or saturated), oxidizability of individuals LDL, measure the oxidants in the LDL and the effect of the LDL on genes related to atherosclerosis. All these could be predictive of future cardiovascular events.

Minimally oxidised LDL is capable of initiating an intracellular oxidative stress by means of its lipid peroxidation products. Meziere *et al* (2000) demonstrated that oxidized LDL at a concentration of 25-75 µg protein /ml induces a dose-dependent increase in the intracellular levels of reactive oxygen species (ROS) and lipid peroxidation end products (TBARS) in cultured human fibroblasts. It has found that peroxynitrite is capable of initiating lipid peroxidation *in vivo* (Patel *et al*, 1996) and may also play a role in the formation of atherosclerosis (White *et al*, 1994). It can cause the generation of other reactive radical species resulting in cellular damage; it can also react with proteins resulting in nitration of tyrosine residues and formation of 3-nitrotyrosine (Beckman et al, 1994). Nitrotyrosine residues had been detected in the LDL of normal plasma implying that plasma proteins including lipoproteins are constantly exposed to some level of oxidant (Khan et al, 1998) The results of this study show that peroxynitrite is also capable of inducing the expression of TF.

Protective effect of antioxidants

Antioxidants have been shown to inhibit the development of atherosclerotic plaques in experimental animals (Steinberg, 1997). It has also been reported that antioxidants may retard the progression of atherosclerosis (Hodis *et al*, 1995) and also reduce the risk of coronary heart disease (Losonczy *et al*, 1996).

Randomised trials, observational as well as epidemiological data have provided mixed results on the protective role of antioxidant vitamins against cardiovascular diseases. While some epidemiological studies are supportive of the beneficial effects of antioxidant in reducing incidence of cardiovascular disease and cancer (Albanes, 1999; Kushi, 1999; Rimm and Stampfer, 2000), a number of large intervention trials have reported otherwise. Epidemiologic data presumably represent correlations with long-term or even lifetime exposure to a given diet which can not be duplicated by an intervention starting late and lasting for a short period of time (Witztum and Steinberg, 2001).

Data from this study shows that antioxidant vitamins could be of benefit in cardiovascular diseases by inhibiting TF expression and activity. The inhibitory effects of the antioxidant vitamins on the gene expression of tissue factor were observed when the vitamins were added at the same time as with the stimulus and when the cells were pre-treated with antioxidant before the addition of the stimulus. However, when the cells were stimulated with LPS before the addition of antioxidant vitamins, i.e. after the onset of TF induction, an increase in the expression of tissue factor was noted; suggesting that the antioxidant can have a deleterious effect if administered after the onset of cardiovascular disease and this findings could explain the results of some of the antioxidant trials.

In the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group, wherein there was a reduction in non-fatal myocardial infarction by 38% (vit.E) and 23% (β-carotene) but an increase by 33% (vit E) and 75% (β-carotene) of fatal myocardial infarction, the study was done among individuals with a history of myocardial infarction. All participants were smokers, smoked an average of 20.4 cigarettes daily and smoked for an average of 35.9 years. The protective effect of the vitamin E was entirely due to a reduction (77%) in non-fatal myocardial infarction for alongside there was an increase (18%) in fatal myocardial infarction (Buring and Hennekens, 1997). A 28% increase in lung cancer was also observed

among male and female smokers given both β-carotene and retinyl palmitate in the U.S. carotene and retinol efficacy trials (Omenn *et al*, 1996). Smoking is associated with free radical-induced damage to tissues and organs (Pryor and Stone, 1993). The plasma concentrations of *α*- and *β*-carotene and ascorbic acid, measured in Scottish male smokers and non-smokers, were significantly lower in smokers as compared to non-smokers, though the levels of lycopene, lutein, and vitamin E were similar among the two groups (Ross *et al*, 1995)

In contrast to the above trials, there are studies that are supportive of the beneficial effects of antioxidants in reducing incidence of cardiovascular disease. A recent report by Fang *et al* (2002) examined the effect of vitamin C and vitamin E on the progression of transplant-associated arteriosclerosis. Their studies showed that the supplementation with the antioxidants retards the early progression of transplant-associated coronary arteriosclerosis. An intervention study in Linxian, China, showed that supplementation with β-carotene and α-tocopherol reduced the risk of cancer by 13% (Blot *et al*, 1993). In the EPIC-Norfolk prospective study (Khaw *et al*, 2001); it was found that plasma ascorbic acid concentration was inversely related to mortality from cardiovascular diseases and ischemic heart disease.

The discrepancies between the results of the trials may be due to the type, the combination and the concentration of antioxidant used, as well as the population studied. It may depend on whether the trials were done in the general population, a low-risk population or in a high-risk population. If intervention trials are carried out on subjects that already have adequate tissue and body fluid levels of antioxidant vitamins, then additional supplementation with vitamins may not be effective. Hence the discrepancies may also be due to the oxidative status of the population studied. In a recent study, the mean *α*-tocopherol and vitamin C plasma concentration in healthy women with an average age of 66 years was found to be 44.7 and 67 μM respectively, whereas among smokers

there was a significant difference, alpha tocopherol was found to be 19.8 and vitamin C was 44.2 μM (Polidori *et al*, 2001).

The discrepancies seen in antioxidant trials may also depend on the lipoprotein-profiles of the populations tested. The protective effect of antioxidants against cardiovascular disease could be related to decreased susceptibility of LDL to oxidation. Chopra et al, (2000) found that an increase in the vegetable intake of 300-400g /day increased the plasma and LDL concentrations of carotenoids and also increased the resistance of LDL to oxidation. However, another study by Hininger et al (2001) showed no significant effect of lutein, lycopene or β-carotene supplementation on biological markers of oxidative stress and LDL oxidizability in healthy adult males, despite an increase in the carotenoid plasma levels. The results presented in this thesis highlight the importance of individual donor specific factors in the induction of tissue factor gene by mmLDL. If the lipoproteins of the subjects tested are less susceptible to oxidation or are incapable of inducing the genes involved in cardiovascular diseases, then changes in cardiovascular disease may not be observed. The evidence for the influence of LDL donor-specific factors on the effects of LDL on gene expression comes from the observation that mmLDL from some donors did not induce tissue factor mRNA after minimal oxidative modification. Also, there was a variation in the susceptibility of LDL isolated from different donors, to oxidation. LDL from certain donors required a long exposure to air before becoming minimally oxidised while LDL preparation from other donors oxidised rapidly. This may be due to the different amounts of antioxidant present in the LDL samples or the detailed size profile of the lipoprotein fractions. Low levels of antioxidant vitamins have been associated with increased risk of cardiovascular diseases and increased intake has been shown to be protective (Gey et al, 1987; Kinlay et al, 1999).

Esterbauer *et al* (1987) reported that α-tocopherol-depleted LDL undergo rapid lipid peroxidation while LDL isolated from α-tocopherol-supplemented subjects have increased resistance to ex-vivo copper-induced oxidation (Dieber-Rotheneder *et al*, 1991). However, there are recent reports that suggest that the resistance of LDL to oxidation has little to do with its vitamin E content (Diaz *et al*, 1997; Neuzil *et al*, 1997). LDL resistance to oxidation ex vivo might not even accurately predict the ability of antioxidant to inhibit atherosclerosis. A study by Pratico et al (1998) showed that vitamin E treatment blocked lipid peroxidation in the artery wall and inhibited atherosclerosis in apoE-/- mice. In another study, it was demonstrated that the probucol metabolite bisphenol appeared to completely block lipid peroxidation in the artery wall of hypercholesterolemic rabbits without affecting the extent of atherosclerosis (Witting et al, 1999).

Activation of transcription factors

The most widely accepted mechanism by which antioxidants may exert a protective effect in atherogenesis is through inhibition of LDL oxidation. The results presented in this thesis showed that antioxidant vitamins modulate the expression and activity of TF. The treatment of cells with antioxidants significantly suppressed the LPS, SIN-1 and mmLDL-induced tissue factor gene as well as the induced cell surface pro-coagulant activity. Importantly, the antioxidants were able to have an effect relatively quickly even when the vitamins and the mmLDL or LPS were added together at the same time. Their ability to function is, therefore, not only due to the prevention of the oxidation of biological molecules or the generation of reactive oxygen species, suggesting that they regulate the signal transduction pathway directly via the modulation of transcription factors that are activated in response to oxidative stress.

Antioxidants may act in the cells by activating signal transduction pathways that can lead to the increase in the synthesis of endogenous antioxidants or by down-regulating the expression of tissue factor gene and other genes that are

induced in response to oxidants and inflammatory agents. The possible role of antioxidant vitamins in the modulation of TF gene expression via activation and repression of transcription factors was investigated. Both the transfection and electrophoretic mobility shift assay showed that NFκB may play a central role in stress responses induced by LPS and mmLDL. LPS and mmLDL increased the transcriptional and binding activities of NFκB; vitamin C and vitamin E reduced the binding activities of the NFκB in mmLDL stimulated cells.

Another transcription factor that may play a role in the oxidative stress responses is ELK-1. LPS rapidly increased the transcriptional activation of ELK-1 in the transfection experiments, which is in agreement with the studies by Guha *et al* (2001). Likewise, SIN-1 increased the transcriptional activation of ELK-1 and this was completely blocked by antioxidants. LPS and mmLDL had no effect on the activation of cyclic AMP-responsive element binding protein (CREB), but the co-treatment of pCREB-transfected cells with mmLDL and ascorbic acid largely enhanced its activation. In these studies, mmLDL has also been shown to have no effect on the binding activities of HIF-1, but the co-treatment of THP-1 cells with mmLDL and vitamin C or vitamin E increases the binding activity of transcription factor. On the other hand, while LPS increases the binding activity of HIF-1, treatment of the cells with vitamin C or vitamin E inhibits the DNA binding activity of HIF-1.

Taken together, these data indicate that the transcriptional mechanisms mediating the elevation and inhibition of TF in mmLDL and LPS treated THP-1 cells are very complex. The data is supportive of NFκB as an important transcription factor governing TF promoter response to LPS and mmLDL in THP-1 cells. This study also revealed a novel role for ELK-1, HIF-1 and CREB in mediating the effect of mmLDL, LPS, and antioxidants on the expression of TF. It is widely accepted that ROS acts as a signalling molecule facilitating gene expression by activation of NFKB. The activation of NFKB by mmLDL was

blocked by antioxidants and this could be responsible for the inhibition of TF gene transcription by the antioxidant. Also the transcriptional activities of CREB and the binding activities of HIF-1 were found to increase when the mmLDL-induced cells were treated with antioxidant suggesting that these transcription factors could be the mediator of the inhibition of TF, in mmLDL-induced cells, by antioxidants. The induction of TF by SIN-1 and the inhibition by antioxidant could be wholly attributed to ELK-1.

Effect of antioxidant on oxidative stress: The third face of the coin

In conclusion, the results of this study show that mmLDL and LPS induce the expression of TF in monocytes and endothelial cells and this induction can be inhibited by treating the cells with antioxidants, supporting the notion that antioxidant vitamins have a protective effects against cardiovascular diseases. The series of events leading to the effect of antioxidant vitamins on the expression of tissue factor in human monocytic and endothelial cells can be summarised as shown in figure 7.1. LPS and mmLDL stimulate vascular cells to generate reactive oxygen species. These reactive oxygen species in turn oxidise native low-density lipoprotein to form more minimally oxidised LDL (mmLDL). The oxidation of low-density lipoproteins initiates a complex sequence of events, which includes the induction of tissue factor. On the other hand, the stimuli could directly modulate the gene expression of tissue factor via the activation of redox-sensitive transcription factors or signal transduction pathways. The induced expression of tissue factor causes the thrombotic episode associated with cardiovascular diseases while the expression of tissue factor pathway inhibitor inhibits the tissue factor pathway.

The roles of antioxidant vitamins in this model may be to protect vascular cells against oxidative stress by:

(1) scavenging the reactive oxygen species generated from the pro-oxidants and the inflammatory stimulus;

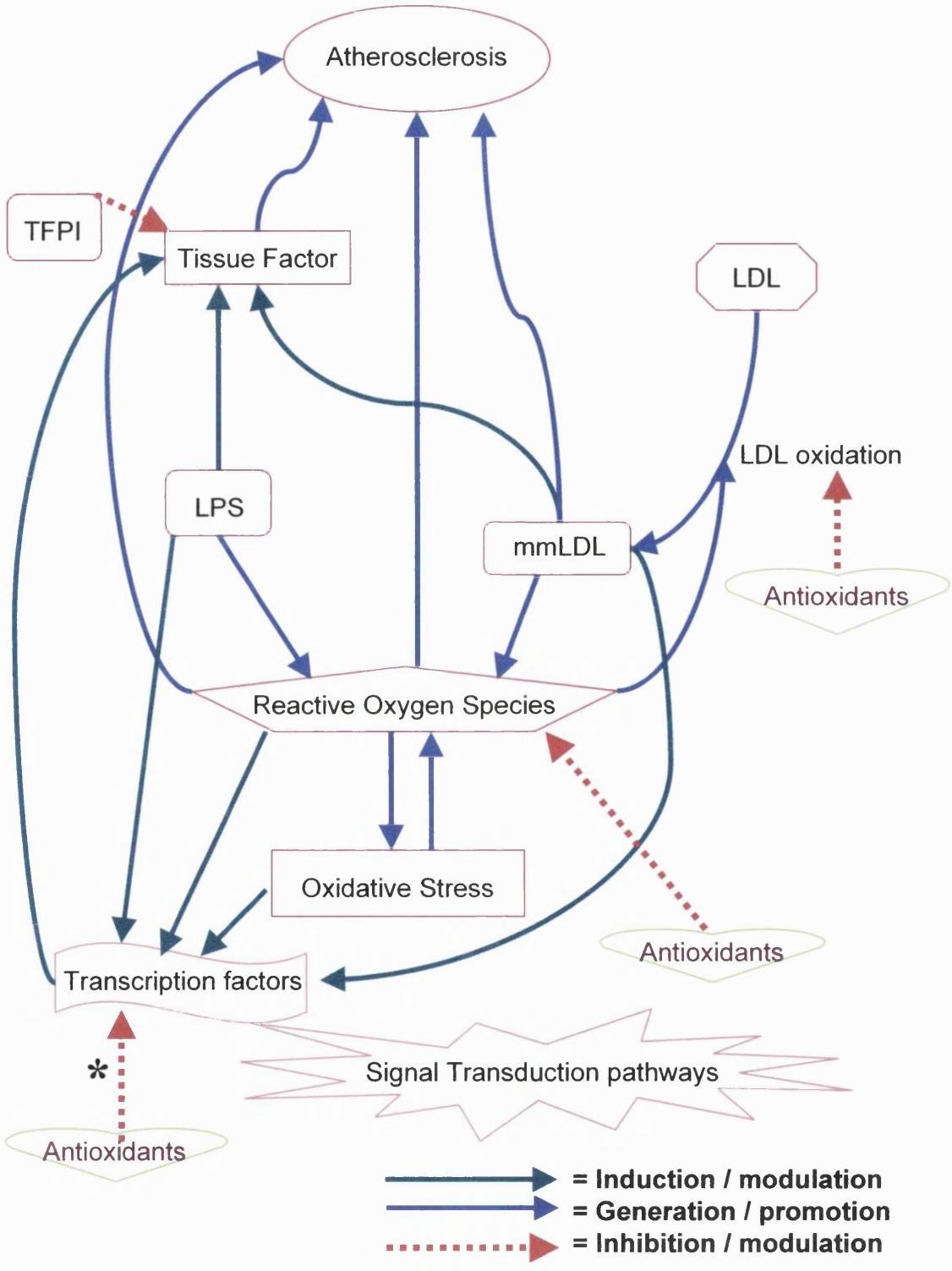


Figure 7.1 The possible sites for the effect of antioxidant vitamins on the expression of tissue factor gene in endothelial and monocyte cells

(2) preventing cellular oxidation of LDL; and

(3) directly modulating redox-sensitive signalling pathways via transcription factors thereby blocking the expression of tissue factor and other proteins implicated in cardiovascular disease.

The protective effect of antioxidants on the expression of tissue factor and tissue factor pathway inhibitor in endothelial cells and monocytic cells, when induced by LPS and mmLDL may be due to the first, the second, the third or even the combination of the three mechanisms.

Future Work:

(1) In order to fully comprehend the benefits of antioxidants in prevention of cardiovascular disease, there is a need for the evaluation of the effects of the vitamins on the expression of wide selection key genes implicated in the development of the disease using primary cell lines from both healthy and cardiovascular disease patients.

(2) It will be very informative to measure all the genes that are switched on or off when cells are under oxidative stress and when cells are treated with antioxidants. This can be done by the use of the microarray techniques. This technique enables parallel assessment of the relative expression of thousands of mRNAs in response to experimental conditions, identifying the transcripts that are altered among RNA samples and determining the magnitude of the differences observed (Yuen et al, 2002).

(3) Since little is known about the accuracy of microarrays in identifying regulated transcripts and also about the relationship of the relative changes in mRNA levels obtained using microarrays to the actual relative levels of these mRNAs in the samples assayed, the QC-RT-PCR assay could be indispensable for measuring the abundance of specific mRNA.

(4) In this study, the antioxidant contents of the individual LDL samples used was not measured, in future; it will be more informative to measure the antioxidant content of the LDL samples.

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Appendix A: Abbreviations

ApoB-100	-Apolipoprotein B-100
ACD	-Acid citrate dextrose
BSA	-Bovine Serum Albumin
CERB	-Cyclic AMP-responsive element binding protein
CHAOS	-Cambridge Heart Antioxidant Study
Cu,Zn-SOD	-Copper- and zinc- containing SOD
DEPC	-Diethyl pyrocarbonate
DMSO	-Dimethyl sulphoxide
DNA	-Deoxyribonucleic acid
DTPA	-Diethylenetriaminepentaacetic acid
DTT	-1,4-Dithio-DL-threitol solution
E-coli	-Escherichia coli
EDTA	-Ethylenediamine-tetraacetic acid
EGCG	-Epigallocatechin gallate
EMSA	-Electrophoretic mobility shift assay
eNOS	-Endothelial nitric oxide synthase
EPIC	-European Prospective Investigation into Cancer and Nutrition
FBS	-Fetal Bovine Serum
GAPDG	-Glyceraldehyde-3-phosphate Dehydrogenase
GITC	-2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate
HDL	-High density lipoprotein
HEPES	-4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIF-1	-Hypoxia-induced factor-1
HUVECS	-Human Umbilical Vein Endothelial Cells
IDL	-Intermediate density lipoprotein
iNOS	-Inducible nitric oxide synthase
IPTG	-Isopropyl β-D-thiogalactoside
JNK	-c-Jun N-Terminal Kinase
LB	-Luria - Bertani
LDL	-Low Density Lipoprotein
LOX-1	-Lectin-like ox-LDL receptor-1
LPS	-Lipopolysaccharide
MAPK	-Mitogen activated protein kinase
MCP-1	-Monocyte Chemotactic Protein 1
mmLDL	-Minimally modified low-density lipoprotein

Mn-SOD	-Manganese containing SOD
MOPS	-3-Morpholinopropanesulfonic acid
mRNA	-Messenger ribonucleic acid
NFκB	-Nuclear Factor Kappa B
nLDL	-Native low-density lipoprotein
oxLDL	-Oxidised low-density lipoprotein
PBS	-Phosphate buffered saline
PKA	-cAMP-Dependent Protein Kinase
PNK	-Polynucleotide kinase
QC RT-PCR	-Quantitative Competitive RT-PCR
RLA	-Relative light unit
RNA	-Ribonucleic acid
RNase	-Ribonucleases
ROS	-Reactive oxygen species
RT-PCR	-Reverse Transcription Polymerase Chain Reaction
SIN-1	-3-Morpholinosydnonimine
SMC	-Smooth muscle cells
SOD	-Superoxide dismutases
SSC	-Saline sodium citrate
TBE	-TRIS borate – EDTA buffer solution
TEMED	-N,N,N′,N′-Tetramethylethylenediamine
TF	-Tissue factor
TFPI	-Tissue factor pathway inhibitor
VLDL	-Very low density lipoproteins
X-GAL	-5-Bromo-4-chloro-3-indolyl β-D-galactoside

Appendix B: Components of the culture media

Dulbecco's Modifled Eagle Medium (DMEM)(1X).

COMPONENTS	Mole. Weight	Conc. (mg/L)	Molarity (mM)
INORGANIC SALTS:			
Calcium Chloride (CaCl2) (anhyd.)	111	200.00	1.80
Ferric Nitrate (Fe(NO3)3-9H2O)	404	0.10	0.000248
Potassium Chloride (KCl)	75	400.00	5.30
Magnesium Sulfate (MgSO4)	120	97.67	0.813
Sodium Chloride (NaCl)	58	6400.00	110.34
Sodium Bicarbonate (NaHCO3)	84	3700.00	44.10
Sodium Phosphate (NaH2PO4-H2O)	138	125.00	0.906
OTHER COMPONENTS:			
D-Glucose	180	4500.00	25.00
Phenol red	398	15.00	0.0346
AMINO ACIDS:			
L-Arginine-HCl	211	84.00	0.398
L-Cystine 2HCl	313	63.00	0.200
L-Glutamine	146	584.00	4.00
Glycine	75	30.00	0.399
L-Histidine HCl-H2O	210	42.00	0.20
L-Isoleucine	131	105.00	0.802
L-Leucine	131	105.00	0.802
L-Lysine-HCl	183	146.00	0.798
L-Methionine	149	30.00	0.201
L-Phenylalanine	165	66.00	0.400
L-Serine	105	42.00	0.400
L-Threonine	119	95.00	0.078
L-Tryptophan	204	16.00	0.078
L-Tyrosine 2Na 2H20	261	104.00	0.398
L-Valine	117	94.00	0.803

VITAMINS:			
D-Ca pantothenate	477	4.00	0.0083
Choline Chloride	140	4.00	0.0285
Folic Acid	441	4.00	0.00906
i-Inositol	180	7.20	0.04
Niacinamide	122	4.00	0.0328
Pyridoxine HCl	204	4.00	0.0196
Riboflavin	376	0.40	0.00106
Thiamine HCl	337	4.00	0.0118

RPMI Medium 1640 (1X)

COMPONENTS	Mole. Weight	Concentration (mg/L)	Molarity (mM)
INORGANIC SALTS:			
Calcium nitrate (Ca(NO3)2 4H2O)	236	100.00	0.424
Potassium chloride (KCl)	75	400.00	5.30
Magnesium sulfate (MgSO4 7H20)	246	100.00	0.407
Sodium chloride (NaCl)	58	5500.00	103.44
Sodium bicarbonate (NaHCO3)	84	2000.00	23.800
Sodium phosphate (Na2HPO4)	142	800.00	5.63
OTHER COMPONENTS:			
Glucose	180	2000.00	11.10
Glutathione Reduced	307	1.00	0.0032
HEPES	238	5958.00	25.00
Phenol red	398	5.00	0.0125
AMINO ACIDS:			
L-Arginine	174	200.00	1.10
L-Asparagine	132	50.00	0.379
L-Aspartic Acid	133	20.00	0.150
L-Cystine	240	50.00	0.208
L-Glutamic Acid	147	20.00	0.136
L-Alanyl-Glutamine	217	446.00	2.05
Glycine	75	10.00	0.133
L-Histidine	155	15.00	0.0967

L-Hydroxyproline	131	20.00	0.153
L-Isoleucine	131	50.00	0.382
L-Leucine	131	50.00	0.382
L-Lysine hydrochloride	146	40.00	0.219
L-Phenylalanine	165	15.00	0.0909
L-Proline	115	20.00	0.174
L-Serine	105	30.00	0.286
L-Threonine	119	20.00	0.168
L-Tryptophan	204	5.00	0.0245
L-Tyrosine	181	20.00	0.110
L-Valine	117	20.00	0.171
VITAMINS:			
Biotin	244	0.2	0.008
D-Ca Pantothenate	477	0.25	0.0005
Choline Chloride	140	3.00	0.0214
Folic Acid	441	1.00	0.0022
i-Inositol	180	35.00	0.194
Niacinamide	122	1.00	0.0081
p-Aminobenzoic Acid (PABA)	137	1.00	0.0072
Pyridoxine HCl	206	1.00	0.0048
Riboflavin	376	0.20	0.0005
Thiamine HCl	337	1.00	0.0029
Vitamin B12	1355	0.005	0.00000369

Appendix C: Characteristics of ECV 304 cells.

Typical endothelial morphology in the absence of specific growth factors	Takahashi et al, 1990
They exhibit angiogenic behaviour when cultured on matrigel	Hughes, 1996
They express epithelial cell markers cytokeratin 6, 8, 10, 17, 18 and 19	Hughes, 1996
They express calcitonin receptors	Howl et al, 1998
They do not have the endothelial form of NOS	Sowa et al, 1999
They express a relatively high basal activity of NOS	Sowa et al, 1999