University of Reading

Lysosomal oxidation of Low Density Lipoproteins

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Declaration

All of the work reported in this thesis is my own work. No part of this thesis has been submitted for a degree, diploma or other qualification at any other University

Feroz Ahmad

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Posters and Publications

Published abstracts

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Posters

- Initial oxidation of LDL by iron at lysosomal pH is due to tryptophan radicals and is not inhibited by probucol. *17TH International Congress on Atherosclerosis*. Amsterdam. 2015.

Submitted papers

- Wen, Y. Ahmad, F. Mohri, Z. Weinberg, P.D. Leake, D.S. Cysteamine inhibits Lysosomal Oxidation of Low Density Lipoprotein in Human Macrophages and Atherosclerosis in Mice (submitted to *ATVB*)

Full papers to be prepared and submitted

- Ahmad, F. Leake, D.S. Mechanism of low density lipoprotein oxidation by iron at lysosomal pH.
- Ahmad, F. Leake, D.S. Lysosomal oxidation of sphingomyelinase aggregated-LDL affects lysosomal function in human macrophages which is prevented by cysteamine.

Abstract

Oxidation of LDL is widely believed to be a key process in the pathogenesis of atherosclerosis. However, LDL oxidation has been shown to be inhibited by interstitial fluid and also large clinical trials have shown no protection by antioxidant. Recent work has shown that LDL can be oxidised by iron within the lysosomes of macrophages. Here, we have explored the possible mechanism by which iron is able to oxidise LDL under lysosomal conditions, and also how lysosomotropic antioxidant, cysteamine is able to prevent it.

More recently, it has been shown that human macrophages are able to rapidly phagocytose LDL aggregated by enzymes, such as sphingomyelinase (SMase-LDL) and oxidised it by iron inside lysosomes, which have a pH of about 4.5. Here, the chemical characteristics (lipid hydroperoxides and oxysterols) of SMase-LDL oxidised by inorganic iron at lysosomal pH (4.5) have been determined *in vitro* and compared to the native LDL. In the lysosomes of macrophages, SMase-LDL increased the intralysosomal lipid peroxidation and ceroid formation which was greatly inhibited by cysteamine.

There is good evidence which suggests that lysosomal dysfunction plays an important role in the atherosclerotic plaque development. Here, it is shown that lysosomal oxidation of SMase-LDL in human macrophages can cause lysosomal dysfunction, induce ceroid associated cellular senescence, and increase the expression of inflammatory cytokine like TNF-α. The work here also demonstrates that preventing the lysosomal LDL oxidation, with antioxidants like cysteamine, offers protection against the SMase-LDL induced lysosomal dysfunction.

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List of Abbreviations

ACAT	AcylCoA:cholesterol acyltransferase
ABCA1	ATP binding cassette transporter-1
ANOVA	One-way analysis of variance
AP-1	Activator protein-1
ApoB-100	Apolipoprotein B-100
ApoE	Apolipoprotein E
BHT	Butylated hydroxytoluene
BP	Bathophenanthrolinedisulfonic acid
BSA	Bovine serum albumin
CA	Cholesteryl arachidonate
CE	Cholesteryl esters
CHD	Coronary heart disease
CL	Cholesteryl linoleate
CLOOH	Cholesteryl linoleate hydroperoxide
CVD	Cardiovascular disease
DMSO	Dimethyl sulphoxide
DTPA	Diethylenetriaminepentacetate
EDTA	Ethylenediaminetetraacetic acid
EC	Endothelial cells
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FH	Familial hypercholesterolemia
FITC	Fluorescein isothiocyanate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HDL	High density lipoprotein
HPODE	13S-hydroperoxy-9Z,11E-octadecadienoic acid
HPLC	High performance liquid chromatography
IDL	Intermediate density lipoprotein
IFNγ	Interferon-γ
IL	Interleukin

LAL	Lysosomal acid lipase
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low density lipoprotein
LPL	Lipoprotein lipase
LOOH	Lipid hydroperoxide
LOX-1	Lectin-like oxidised LDL receptor-1
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
MES	2-(N-morpholino)ethanesulfonic acid
MI	Myocardial infarction
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor-kappa of activated B cells
oxLDL	Oxidized LDL
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PLA_2	Phospholipase A2
PMA	Phorbol 12-myristate 13-acetate
PUFA	Polyunsaturated fatty acid
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
SR-A	Scavenger receptor A
SR-B1	Scavenger receptor B1
SREBP	Sterol regulatory element binding protein
TNF-α	Tumour necrosis factor -alpha
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell
WHHL	Watanabe Heritable Hyperlipidemic

Chapter 1- Introduction

1.1 Atherosclerosis

1.1.1 Definition and clinical significance

Atherosclerosis is a pathological process characterised by the build-up of lipids and fibrous material in the walls of large and medium-sized arteries. Atherosclerosis is a major risk factor for many cardiovascular diseases (CVDs) including coronary heart disease (CHD), cerebrovascular disease, peripheral arterial disease and diseases of the aorta.

CVD is the major cause of death in the United Kingdom (Bhatnagar et al., 2015) and worldwide (WHO, 2014). The World Health Organisation estimate that in 2012, 17.5 million people died globally from CVD, accounting for approximately one third of all death worldwide. Of these deaths, an estimated 7.4 million were due to coronary heart disease (CHD) and 6.7 million were due to stroke. Over 80% of these CVD deaths take place in low-and middle-income countries and occur almost equally in men and women. WHO estimates that CVD death will increase to reach 22.2 million by 2030, based on current projections (WHO, 2014).

According to the British Heart Foundation, in 2014 alone over 154639 people died from CVD in the UK. One in six male deaths and one in nine female deaths were from coronary heart disease (CHD) - a total of nearly 74,000 deaths. Stroke caused nearly 39,282 deaths in the UK. CVD is the most common cause of premature death in the UK causing 26% of premature death in men and 18% of premature death in women. In total CVD is estimated to cost the UK economy just under £19 billion per year as a result of direct health costs, productivity losses and informal care of people with the disease (Bhatnagar et al., 2015).

1.1.2 Theories of Atherosclerosis

Over the last century, a number of theories have been advanced to explain how atherosclerosis evolves. These hypotheses are not mutually exclusive and numerous experimental and clinical observations have shown how processes highlighted in one theory may be linked to those in another (Fuster et al., 1992, Huff et al., 2016). Much of the controversy over these theories has to do with individual opinion concerning which of the aspects of atherosclerosis is most important.

1.1.2.1 Lipid Insudation Hypothesis

This theory was proposed by the German pathologist Rudolf Virchow in 1856 (Virchow, 1856). He suggested that the critical events in atherosclerosis centre on the focal accumulation of fat in the vessel wall and that the lipid in the atherosclerotic lesion is derived from the lipids in the blood (Mayerl et al., 2006). There is good evidence now that the lipid in the plaque comes from the blood; there is also substantial evidence correlating the severity of hypercholesterolaemia (particularly elevated LDL cholesterol) with the severity of atherosclerosis both in human and in animal models (Gould, 1998; Steinberg, 2002). Although there is still controversy over how the lipid accumulates in the vessel wall, the hypothesis is widely accepted. Whereas this hypothesis explains the source of plaque lipid, it does not provide a complete explanation for the pathogenesis of the atherosclerotic lesion. Since many other clinically important features of the plaque, such as smooth muscle cell (SMC) proliferation and thrombosis, remain unexplained, lipid deposition appears to be necessary but not sufficient to explain plaque development and growth (Brown et al., 1980).

1.1.2.2 The Incrustation Hypothesis

In 1852, Carl Rokitansky, a renowned Austrian pathologist, proposed the 'incrustation theory' of atherosclerosis. This theory asserted that material from blood gets deposited on the inner surface of arteries and leads to thickening of the inner lining (Rokitansky, 1852). A modern version of this idea holds that intimal thickenings present in artery walls result from fibrin deposition from thrombi, with subsequent organization by smooth muscle cells and secondary lipid accumulation (Duguid, 1946). There is however little evidence that thrombosis is a factor in the initiation of spontaneous, or naturally occurring, atherosclerosis, but substantial evidence suggests that thrombosis has an essential role in the progression of spontaneous atherosclerosis (Fuster et al., 1991).

1.1.2.3 The Reaction-to-Injury Hypothesis

This theory proposed that the cells at atherosclerotic sites played an active role in the initiation and progression of the lesions (Ross and Glomset, 1973). According to the hypothesis, endothelial injury or dysfunction (loss of normal homeostatic function) enhances endothelial adhesiveness for leukocytes and platelets. The recruited leucocytes and platelets release cytokines and growth factors which cause smooth muscle cells to migrate to the intima and proliferate. Alternatively, monocytes might be attracted to the zone of injury; the monocyte/macrophage might then be activated and start to elaborate growth-promoting activity (Ross and Glomset, 1976a, 1976b; Ross, 1999). The reaction-to-injury hypothesis suggests a possible mechanism for the accumulation of connective tissue cells and matrix but it fails to provide an explanation for the lipid accumulation or the monoclonal nature of the advanced atherosclerotic plaque (Benditt and Benditt, 1973).

1.1.2.4 The Monoclonal Hypothesis

The monoclonal concept is focused on smooth muscle proliferation and comes from the observation that the smooth muscle cells that form fibrous caps of atherosclerotic plaques appear to migrate from the underlying media and then proliferate. This hypothesis is based on the observation that individual plaques of human females who are heterozygotes for the X-linked marker glucose-6-phosphate dehydrogenase frequently exhibit one, but not both, of the isotypes of this enzyme (Benditt and Benditt, 1973). Single cells might be stimulated to enter the growth cycle and undergo several rounds of division leading to the formation of a monoclonal lesion. The mechanism of cell activation leading to such lesions is not evident as yet; the only other known monoclonal cell masses in humans are neoplasias (e.g., leiomyomas). This would tend to suggest carcinogens or possibly viruses as possible etiologic agents and thereby might explain the link between cigarette smoking and atherosclerosis (Murry et al., 1997).

1.1.2.5 The Lipoprotein Oxidation Theory

The 'oxidation theory' for atherosclerosis proposes that lipid and/or protein oxidation products are responsible for lesion formation/development. It suggests that some pathogenetic stimuli take part in the production of reactive oxidative species in the endothelial microenvironment which target the intimal low-density lipoprotein (LDL). Oxidation of LDL particles represents a crucial process in the development of modified LDL forms. This theory originated from studies demonstrating that LDL oxidised by endothelial cells could be internalized and accumulated quickly by macrophages, leading to foam cell formation (Steinbrecher et al., 1984).

1.1.3 Development of atherosclerotic plaque

1.1.3.1 Normal Arterial Wall

A normal artery wall consists of three layers: the intima, closest to the arterial lumen and therefore most ‘intimate’ with the blood; the media which is the middle layer; and the outer layer, the adventitia (Figure 1.1). The intimal surface consists of a single layer of endothelial cells which acts as a metabolically active barrier between circulating blood and the vessel wall. The internal elastic lamella separates the intima from the media. The media consists predominantly of layers of smooth muscle cells surrounded by an extracellular matrix consisting mainly of elastic fibres and collagen. The external elastic lamina separates this layer from the outermost layer, which is known as the adventitia. The adventitia consists of a loose matrix of elastin, fibroblasts and collagen and contains the nerves, lymphatics and blood vessels (vasa vasorum) that nourish the cells of the arterial wall. The living artery wall is a scene of dynamic interchange between its cellular components – most importantly, endothelial cells, vascular smooth muscle cells, and their surrounding extracellular matrix (Lilly, 2012; Yuan XM, Brunk UT, 2000).

1.1.3.2 Atherosclerotic plaque development

Animal models of atherosclerosis, by virtue of the fact that the disease process happens much more quickly in them than in humans, have provided an insight into how atherosclerosis develops and progresses. The genetically modified apo E-deficient mouse and the Watanabe heritable hypercholesteraemic rabbit, which lacks a functional LDL receptor, have been widely studied. The first observable change in these hypercholesteraemic animals is the accumulation of lipid particles and their aggregates in the intima of the arteries (Williams and Tabas, 1995). This

is followed by the adherence of monocytes to the endothelial layer and their migration into the intima, where they differentiate into macrophages. Macrophages accumulate lipid and become foam cells. As the lesion progresses, an extracellular pool of lipid is formed, probably due to the release of lipids from dying foam cells or direct deposition from lipoproteins. In more advanced lesions, this core of lipid is separated from the lumen by a fibrous cap. The fibrous cap is formed when smooth muscle cells migrate into the intima from the media and begin to secret extracellular matrix. As the lesions advance they can encroach upon the lumen, restricting blood flow through the arteries which in the case of the coronary artery can cause angina. The advanced lesions are vulnerable to rupture and can trigger the formation of a thrombus. The thrombus may occlude the arteries supplying the heart causing a myocardial infarction (MI), or the brain causing a stroke. A diagram of advanced atherosclerotic lesion is shown in Figure 1.1.

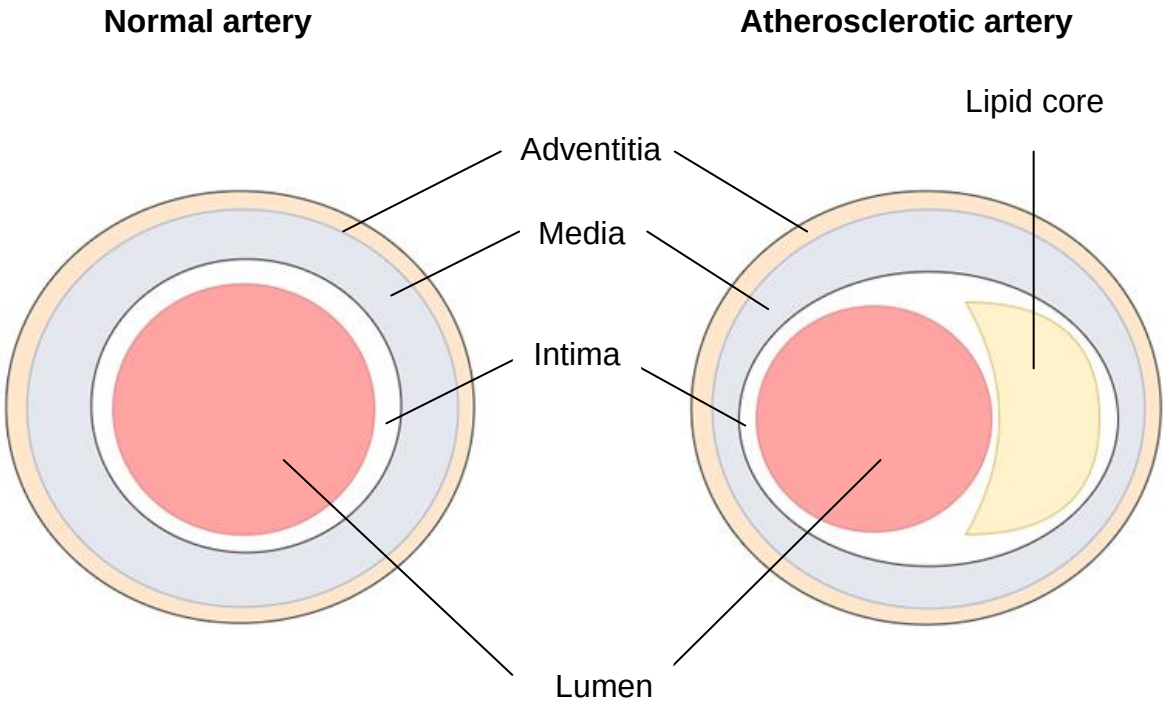


Figure 1.1 A normal and an atherosclerotic artey

The diagram shows a normal artery and an atherosclerotic artery at the ‘atheroma’ stage of the disease. The lumen of the atherosclerotic artery has not been compromised by the plaque, due to the outward compensatory expansion of the artery.

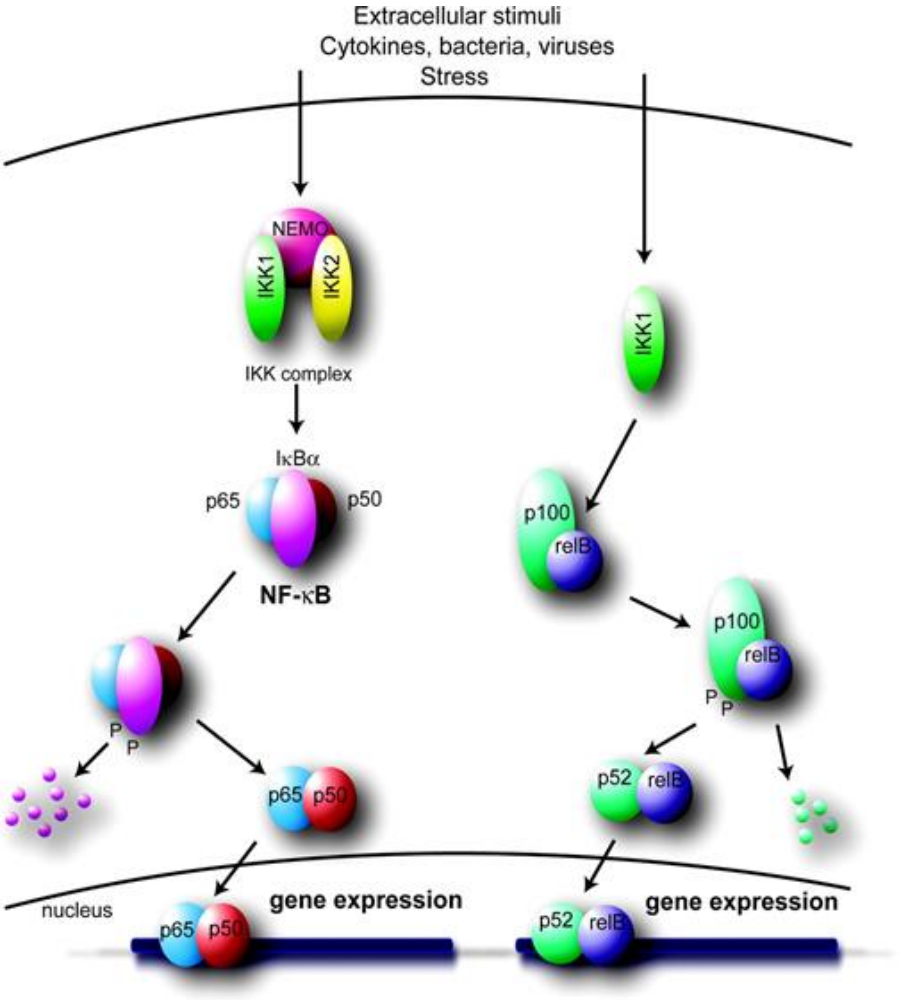
1.1.3.3 Lesion-prone areas

Atherosclerotic lesions form preferentially on the inner curvatures of the arteries or at arterial branch points. The development of lesions at these sites is thought to be due to increased or disturbed shear stress (Davies, 1997). Shear stress is the drag force exerted on the endothelium as a result of blood flow. It modulates endothelial cell structure and function through a mechanotransduction mechanism and low shear stress leads to the activation of transcription factors such as nuclear factor-kappa B (NF-κB) (Tzima et al., 2005).

NF-κB is the collective name for a group of ubiquitous transcription factors that regulate cellular responses to multiple stimuli, primarily by initiating transcription of proinflammatory genes (Li and Verma, 2002). The NF-κB dimer, consisting commonly of the subunits p50 and p65 (RelA), is present in the cytosol in an inactive state, bound to inhibitory proteins that are collectively termed as IκB. Upon cell stimulation, IκB is phosphorylated by IκB kinase complex (IKK) leading to degradation of the inhibitor, leaving NF-κB free to translocate into the nucleus and induce transcription (Ghosh and Karin, 2002, Pomerantz and Baltimore, 2002). The classical activation of the canonical NF-κB pathway (Figure 1.2) can be initiated by a wide range of stimuli. Firstly, cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) acting as paracrine signals lead to NF-kB activation. Additionally, pathogen associated molecular patterns (PAMPs) originating from viruses, bacteria and fungi, function as ligands for different Toll-like receptors (TLRs), upstream of the NF-kB signalling pathway (de Winther et al., 2005).

Atherosclerotic processes involve several mediators including adhesion molecules and chemokines, which play a role in different stages of the disease from the initiation of plaque formation to the plaque rupture (Pamukcu et al., 2011). Activation of NF-κB leads to expression of genes encoding proteins that are thought to be involved in atherogenesis, such as vascular cell adhesion molecule 1 (VCAM-1) which binds and recruits inflammatory cells such as monocytes, aiding their infiltration into the intima (Monaco and Paleolog, 2004, Yu et al., 2015). In areas of high laminar shear stress, the endothelial cells adapt to the flow and the response to the stress is downregulated, however, at areas of low or disturbed stress, the genes are activated in a sustained manner (Mohan et al., 1999). The expression of proteins encoded by NF-κB activated genes is thought to

be the initial event that occurs at lesion-prone sites, before other markers of atherosclerosis are apparent (Yu et al., 2015).



Adapted from (de Winther et al., 2005)

Figure 1.2 NF-κB activation pathway

The NF-κB activation can occur by two ways. The classical NF-κB activation pathway (left) involves the activation of the IKK complex with the subsequent degradation of IκBα and nuclear translocation of the NF-κB dimer. The alternative NF-κB activation cascade (right) is mediated through IKK1 and results in the processing of p100 to p52, resulting in the nuclear transfer of the relB-p52 dimer.

1.1.3.4 Classification of lesions

Pathologists have subdivided the lesions of atherosclerosis into various stages based on their morphology. In 1994-95, the American Heart Association (AHA) through the appointment of a consortium of investigators in the field of atherosclerosis classified the atherosclerotic lesions into 8 categories (Table 1.1) (Stary et al., 1994a). Early lesions, marked by foam cell infiltration (type I lesions), mature into lesions with smooth muscle infiltration and lipid (type II, “fatty streak”) and connective tissue deposition (type III). The early lesions develop within the first three decades of life in areas of localized turbulent flow within the coronary arteries. As these early lesions grow into softer plaques with a high extracellular lipid and cholesteryl ester content and progressively thinner fibrous cap (types IV–Va, “atheroma”), they become more vulnerable to disruption (Loree et al., 1994; Stary et al., 1995). Disruption of plaque exposes thrombogenic substances within the plaque to blood and may result in thrombotic occlusion of the affected vessel (type VI or complicated lesions). When they achieve a significant degree of stenosis without sufficient collateralization (circulation of blood established through enlargement of minor vessels and anastomosis of vessels with those of adjacent parts when a major vein or artery is functionally impaired), these lesions result in acute coronary syndromes. In the period after the acute syndrome, thrombus over the complicated disrupted lesion organizes and the lesion calcifies (type Vb) or fibroses (type Vc) into a chronic stenotic lesion. The complicated lesion may contain organizing thrombus from prior episodes of plaque rupture, cap ulceration or intra-plaque haemorrhage, followed by lysis of the thrombus and organization. The AHA classification is based on the premise that plaque rupture is the only mechanism responsible for coronary thrombosis however, some studies have

described existence of plaque erosion as well (van der Wal et al., 1994; Virmani et al., 2000).

Table 1.1 Atherosclerotic lesion types according to the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association (Stary et al., 1994a, 1994b)

Plaque type	**Characteristics of plaque**	**Associated clinical syndrome**
I, "Initial Lesion"	Intimal thickening, macrophages, isolated foam cells	Asymptomatic
IIa, "Progression–prone type II lesion"	Accumulation of intracellular lipid in infiltrating macrophages and smooth muscle cells	Asymptomatic
IIb, "Progression-resistant type II lesion"	Accumulation of intracellular lipid in infiltrating macrophages and smooth muscle cells	Asymptomatic
III, "Intermediate lesion" (preatheroma)	As above, plus incipient extracellular lipid and connective tissue deposition	Asymptomatic
IV, "Atheroma"	Large extracellular intimal lipid core; inflammatory cell infiltration, including macrophages, foam cells and T-cells	Usually asymptomatic; can also be associated with stable angina
Va, "Fibroatheroma"	Atheroma with fibrous layer or layers	Same as type IV
Vb "Calcific lesion"	Atheroma with extensive calcification in the lipid core or elsewhere in the lesion	Stable angina pectoris; can also be asymptomatic
Vc "Fibratheroma"	Fibrosed atheroma or organized mural thrombus with minimal or absent lipid component	Same as type Vb
VI, "Complicated lesion"	Disrupted type IV or V lesion with intramural haemorrhage and/or overlying thrombosis	MI or thrombotic stroke

1.1.3.5 Plaque vulnerability

It is well established now that the majority of fatal MI arises due to rupture of the fibrous cap of advanced atherosclerotic plaques (Braganza and Bennett, 2001, Davies, 2000, Falk et al., 1995). The plaques that are more likely to rupture are known as 'vulnerable' plaques and are characterised as being rich in macrophages and having a thin, collagen fibrous cap, covering a large necrotic core (Shah, 2003). Plaque rupture is often localised to the regions of the atherosclerotic lesion where macrophages are found in abundance (Bjorkerud and Bjorkerud, 1996). Macrophages express various proteases including matrix metalloproteinases (MMPs) which degrade extracellular matrix material, thereby thinning the fibrous cap and making it more susceptible to rupture (Anderson and Mosser, 2002, Galis et al., 1995, Jones et al., 2003a, Newby, 2007). Macrophages also secrete pro-inflammatory cytokines such as interleukin-1β, TNF-α and nitric oxide which are able to induce expression of MMPs by smooth muscle cells (Galis et al., 1994, Lee et al., 1995, Liang et al., 2007).

Plaque stability may be further disrupted by the death of macrophages and smooth muscle cells, particularly if death occurs near the surface of the atherosclerotic lesion. The morphological changes that occur in apoptosis are due to the action of a family of cysteine proteases known as caspases. Caspases are activated by proteolytic cleavage and activate each other in a cascade that leads to the cleavage of cellular proteins (Hirata et al., 1998). The caspase cascade can be activated by both extrinsic and intrinsic pathways. The major extrinsic pathways occur via membrane bound receptors of the tumour necrosis family (TNF), such as the TNF-receptor 1 and the Fas receptor (reviewed in Kavurma et al., 2008,

Schmitz et al., 2000). These receptors contain death domains within their cytoplasmic tails. When the receptors bind their ligands (such as Fas and TNF-alpha) they aggregate, leading to the recruitment and binding of adaptor proteins, such as Fas-associated death domain (FADD), to the death domain of the receptor (Muzio et al., 1996). The main intrinsic pathway of apoptosis involves cytochrome c release from the mitochondria, a process that is regulated by the Bcl-2 family of proteins. Released cytochrome c binds to apoptotic protease activating factor 1 (Apaf-1) and procaspase 9 (reviewed in Kavurma et al., 2008). It is noteworthy that apoptotic macrophages and SMCs are found in the shoulder region and fibrous cap of plaques (Falk et al., 1995, Kolodgie et al., 2000, Lim and Park, 2014). Moreover, the pro-apoptotic Fas receptor appears to be expressed on up to 66% of all cells in the fibrous cap (Geng et al., 1997). Due to the fact that macrophages play a central role in plaque rupture, one would assume that macrophage apoptosis in advanced plaques would stabilise the plaque, however this is usually not the case (Schrijvers et al., 2007). In advanced plaques the ability of macrophages to remove apoptotic bodies is impaired causing secondary necrosis. Secondary necrosis triggers inflammation and may contribute to the growth of the necrotic core (Clarke et al., 2006, Gautier et al., 2009, Tabas, 2004). Plaque rupture is a structural defect in the fibrous cap which exposes the highly thrombogenic core, including collagen to coagulation factors and platelets present in the bloodstream (Farb et al., 1996). Macrophages within the plaque express tissue factor which triggers the coagulation cascade and plasminogen activator inhibitor-1, which inhibits fibrinolysis and thus stabilises thrombi (Hutter et al., 2004, Lupu et al., 1993, Oikonomopoulou et al., 2012, Wilcox et al., 1989). Superficial erosion of endothelial cells from the surface of the plaque can also

expose the plaque contents to the bloodstream, triggering thrombus formation (Matsuzawa and Lerman, 2014). Activated endothelial cells express MMP-1 that activates MMP-2, which in turn can cleave the collagen in the basement membrane leading to endothelial cell erosion (Rajavashisth et al., 1999). The activated endothelial cells may also stabilise the thrombus through the expression of fibrinolysis inhibitors such as PAI-1 (Schneiderman et al., 1992).

Neovascularisation (the formation of new blood vessels) is another factor that has emerged as a feature of vulnerable plaques (Huang et al., 2010, McCarthy et al., 1999, Mofidi et al., 2001, Simone et al., 2014). Neovessels mainly originate from the adventitia and grow into the base of atherosclerotic lesions, thus providing an alternative entry pathway for monocytes and immune cells (Kumamoto et al., 1995). The plaque neovessels lack supporting cells and are thin and fragile, giving rise to local extravasation of plasma proteins and erythrocytes (Sluimer et al., 2009). Such intraplaque haemorrhages can cause plaque instability, and may expand the necrotic core and promote inflammation (Kolodgie et al., 2003).

1.1.4 Risk factors

Classical risk factors for CVD have been identified from the large prospective cohort studies such as the Framingham Heart Study, the Seven Countries study and the INTERHEART study. It is estimated that 90% of the population attributable cardiovascular events are due to smoking, high blood pressure, obesity, abnormal lipids, sedentary lifestyle and diabetes mellitus. Prevention of cardiovascular disease morbidity and mortality in patients at high risk of developing the disease (primary prevention) and in patients with existing CVD (secondary prevention) is

largely through modification of these risk factors, through lifestyle changes and /or drug therapy.

1.1.4.1 Lipids

Abnormal blood lipids are a major risk factor for CVD. These include elevated total plasma cholesterol, triglyceride and LDL levels and lowered high density lipoprotein (HDL) (Sherpa et al., 2011). Hypertriglyceridemia is often associated with insulin resistance, diabetes mellitus, obesity and hyperinsulinemia. Fasting triglycerides are predictive of future CHD among men independent of age, lipid-lowering medication, diabetes, total cholesterol, in a population in which metabolic syndrome prevails (Onat et al., 2006).

1.1.4.2 High blood pressure

Raised blood pressure is a risk factor for ischaemic and haemorrhagic stroke as well as coronary heart disease (Whitworth, 2003). The risk of death from CVD increases with increasing blood pressure (Kannel, 1996, McMahon et al., 2011, Pei et al., 2011). Randomised clinical trials have indicated that a reduction in blood pressure can reduce stroke by 40% and myocardial infarction by 15% (Chalmers et al., 2003). Following a stroke or transient ischaemic attack (TIA), blood pressure lowering has been shown to reduce subsequent major cardiovascular events (Lawes et al., 2004, Williams et al., 2004).

1.1.4.3 Obesity

Obesity has been shown to be an important long term predictor of CVD incidence among individuals. CVD risk increases as body weight (measured as body mass index, BMI) increases (Jousilahti et al., 1996, Khosravi et al., 2012, Poirier et al., 2006). The distribution of body fat (adipose tissue) is thought to be important, with

an increase in abdominal fat causing an increased CVD risk (Després, 2012, Zhang et al., 2008). Adipose tissue secretes various cytokines and pro-inflammatory factors such as tumour necrosis factor-alpha (TNF-α), IL-6 and IL-1, which are involved in atherosclerosis and insulin resistance (Ahima and Flier, 2000, Fortuno et al., 2003, Hauner, 2005). Weight loss has been shown to affect CVD, for example, in a study conducted by Pascual et al. (2009) metabolic syndrome participants who lost weight during the trial had decreased systolic and diastolic blood pressures as well as LDL cholesterol.

1.1.4.4 Diabetes

Diabetic patients both types 1 and 2 are at a higher risk of CVD compared with non-diabetics, even when taking into account other risk factors (Bierman, 1992, Laakso, 2010). The Framingham Study demonstrated that diabetes mellitus is associated with a two to five fold increase in CVD and related death (Hubert et al., 1983). Diabetics lack the ability to make sufficient insulin or there is insulin resistance and thus glucose becomes abundant in the blood. With glucose build up, arteries become damaged perpetuating CVD (Kalofoutis et al., 2007, Wilcox, 2005). Some of the increased risk for CVD seen in diabetic individuals is attributable to the concurrent presence of other risk factors such as dyslipidaemia, hypertension, and obesity (Buse et al., 2007). Management of these risk factors has been shown to effectively reduce the incidence of major coronary events in persons with diabetes (Gibbons et al., 2002).

1.1.4.5 Smoking

Smoking has been recognized as an increased risk for CVD for over 40 years (Mahan and Krause, 2012). The risk of developing CVD from smoking is related to

the duration of smoking and the amount of tobacco smoked daily (Ambrose and Barua, 2004, Benowitz, 2003, Burns, 2003). A 50 year cohort study of British doctors found that mortality from CHD was around 60% higher in smokers than non-smokers (Doll et al., 2004). Studies have shown that even passive smoking increases the risk of CHD, and it has been seen that there is about a 25% increase in risk in those regularly exposed to passive smoking (He et al., 1999, Law et al., 1997, Metsios et al., 2010, Whincup et al., 2004). Cessation of smoking has been seen to decline the CVD risk (Mons et al., 2015). The risk in people who show no symptoms drops to the level of those that have never smoked within 10 years of stopping (Dobson et al., 1991, Kawachi et al., 1994). Smoking has been shown to increase fibrinogen levels (Kaptoge et al., 2007), levels of tissue factor (Matetzky et al., 2000, Sambola et al., 2003), platelet P-selectin expression (Neubauer et al., 2009) and platelet thrombin generation (Hioki et al., 2001). Smokers have high PAI-1 levels, which impairs the fibrinolytic activity of the plasma (Simpson et al., 1997) and thus delays thrombus dissolution.

1.1.4.6 Sedentary lifestyle

Low level of fitness (i.e. physical inactivity) is an independent risk factor for CVD (Mahan and Krause, 2012). Sedentary lifestyle has been shown to increase the risk of heart disease and stroke by 50% (Warren et al., 2010). Without exercise, atherogenesis can occur rapidly forming plaque in the arterial walls and decrease the vascularity of the myocardium (Okabe et al., 2006, Puffer, 2001). Physical inactivity also has an impact on other risk factors including hypertension, triglycerides, HDL, diabetes and obesity which when combined with lack of exercise can increase the risk of CVD (Braith et al., 1994, Hills et al., 2011, Taylor et al., 2004). The UK government recommends that adults participate in moderate

physical activity for a minimum of thirty min or at least five days of the week, however more men meet these requirements then women and it seems to decline with age in both the genders (BHF, 2015).

1.1.4.7 Non-modifiable risk factors

Non-modifiable risk factors include age, gender and family history. In both men and women, with increase in age, there is an increase in CVD mortality rates, with 96% of death from CHD or stroke occurring in people aged 55 or over (Yazdanyar and Newman, 2009). The incidence of premature CVD in men aged 35 to 44 is about three times that of women of the same age (Mahan & Escott-Stump, 2008). There is a speculation that oestrogen may play a protective role against CVD in young women, however large clinical trials of hormone replacement therapy in postmenopausal women have shown no cardioprotective effect (Yang and Reckelhoff, 2011). Ethnic differences have also been seen to affect the CVD risk, with some ethnic groups, like South Asians, African-Americans and Mexican Americans, exhibiting a higher prevalence of CVD than Caucasians (Forouhi and Sattar, 2006).

1.1.4.8 Other risk factors

Apart from the above mentioned risk factors, there are other factors which are under investigation. They include elevated levels of C-reactive protein (CRP), lipoprotein (a), fibrinogen and homocysteine. C-reactive protein and fibrinogen are increased during the inflammatory response (Lagrand et al., 1999). Fibrinogen is cleaved during the coagulation cascade to form fibrin, a major component of blood clots (Herrick et al., 1999). Lipoprotein (a) is an LDL-like particle, where an apolipoprotein (a) molecule is attached via a disulphide bond to apolipoprotein B-

100 and its levels are seen to increase during inflammation (Milionis et al., 2000). Studies have also shown that there is an increased relative cardiovascular risk in patients with higher CRP concentrations compared to baseline concentrations in healthy individuals (Ridker, 2003). Homocysteine is a sulphur containing amino acid which is formed as a by-product of methionine metabolism. Large meta-analyses have shown that increased homocysteine levels are associated with increased risk of CHD (Ganguly and Alam, 2015, Wierzbicki, 2007).

1.2 Lipid metabolism

1.2.1 Lipoproteins

Lipids are fats that are either absorbed from food or synthesised by cells. Lipids are hydrophobic and insoluble in water, so the dietary and endogenously produced lipids are transported in the blood plasma bound to proteins, known as apolipoproteins. Lipoproteins are classified by size and density (defined as the ratio of lipid to protein) into five classes, namely chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL and HDL. Apart from different densities, the classes of lipoproteins differ in their lipid composition, size, apolipoprotein composition and electrophoretic mobility. As well as playing a structural role, apolipoproteins are ligands for lipoprotein receptors, facilitating the cellular uptake of lipoproteins (Feingold and Grunfeld, 2000, Zamora and Hidalgo, 2016).

1.2.2 Exogenous metabolism

The vast majority (>95%) of dietary lipids are triglycerides; and the rest are free fatty acids, phospholipids, cholesterol and fat-soluble vitamins (Burdge and Calder, 2015). The dietary triglycerides and cholesterol are emulsified by combination with bile acid and phospholipids to form micelles before being hydrolysed in the intestines by pancreatic enzymes. After absorption, triglycerides and cholesterol are re-esterified in the intestinal mucosal cells and then coupled with various apolipoproteins (e.g. apolipoprotein-B48), phospholipids, and unesterified cholesterol into lipoprotein particles called chylomicrons (Mansbach and Siddiqi, 2010) (Figure 1.3). Chylomicrons are secreted from the intestinal mucosa into the bloodstream via the lymph system and the thoracic duct. In the bloodstream, chylomicrons acquire apolipoprotein C and E from HDL and deliver triglycerides to muscles for use as an energy source or to adipose tissue for storage. The triglycerides are then removed from the chylomicrons by the action of an enzyme called lipoprotein lipase which is attached to the endothelium of capillaries perfusing muscle and adipose tissue (Merkel et al., 2002). HDL transfers cholesteryl esters to the chylomicrons in exchange for triglycerides through the action of cholesteryl ester transfer protein (CETP) (Barter et al., 2003, Chung et al., 2004). The resultant chylomicron remnants richer in cholesterol travels to the liver to be cleared from the blood, through a process mediated by apolipoprotein E (Havel, 1998).

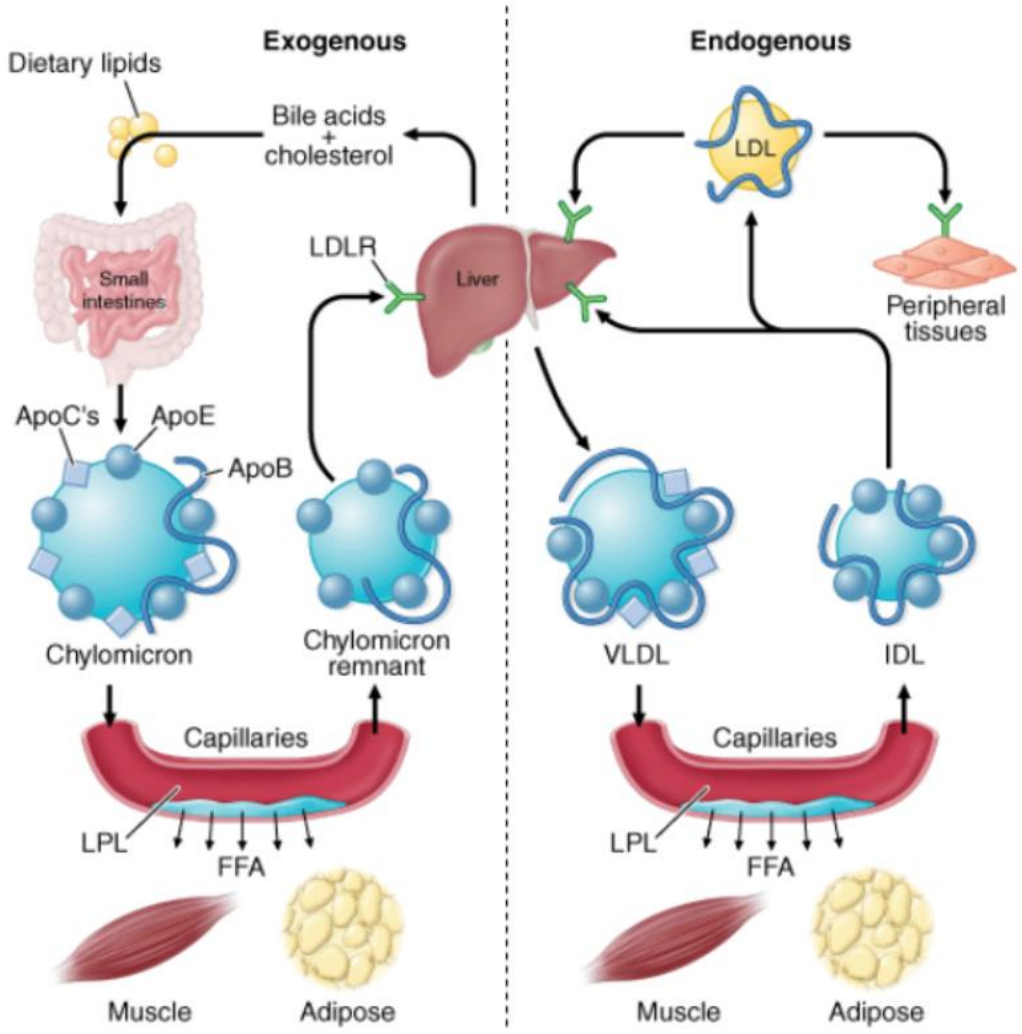


Figure 1.3 Schematic diagram of the exogenous and endogenous lipid metabolism pathways (McLaughlin, 2014)

1.2.3 Endogenous metabolism

The liver constantly synthesises triglycerides from free fatty acids and carbohydrates, which are secreted into the circulation in the core of very-low-density lipoprotein particles (VLDL) (Janero et al., 1984) (Figure 1.3). In the tissue capillaries, lipoprotein lipase causes hydrolysis of the triglyceride-rich core of the VLDL particles producing smaller remnant VLDL particles rich in cholesteryl esters

(intermediate-density lipoproteins, IDL) and liberation of free fatty acids (Merkel et al., 2002). The resultant cholesteryl ester-rich IDL particles can be removed by the liver via LDL receptors or can lose lipoprotein E and further depleted by lipoprotein or hepatic lipases to from LDL particles (Feingold and Grunfeld, 2000). The LDL particles circulate in the bloodstream and can be absorbed by cells in the liver or peripheral tissues. The particles can bind to the target tissue with the LDL receptor (Goldstein and Brown, 2009) through apolipoprotein B-100 and internalised by endocytosis, whereby they are then hydrolysed in lysosomes to release lipids such as cholesterol (Myant, 1990).

1.2.4 Cellular cholesterol metabolism

Cholesterol is delivered to cells via LDL through LDL receptors found on the plasma membrane of most cells (Goldstein and Brown, 1973, 1974). Cholesterol import is initiated by the interaction of the apolipoprotein B-100 (apoB-100) part of the LDL with the LDL receptor (Mahley et al., 1977). The high efficiency of internalization is assured by clustering of the LDL receptor in clathrin-coated pits found on the cell surface (Anderson et al., 1977, Pearse, 1987), which are pinched off to form clathrin-coated vesicles inside the cells (Hirst and Robinson, 1998, Roth and Porter, 1964). These endocytic vesicles fuse with lysosomes, in which the protein moiety of LDL is broken down to free amino acids and the cholesteryl esters are hydrolysed by lysosomal acid lipase, releasing free cholesterol (Brown and Goldstein, 1975). The resulting free cholesterol crosses the lysosomal membrane, with the aid of proteins known as Niemann-Pick C1 (NPC1) and Niemann-Pick C2 (NPC2) (Frolov et al., 2003, Infante et al., 2008), and is thus distributed to other membranes, such as the endoplasmic reticulum, plasma

membrane or mitochondria (Maxfield and Wustner, 2002). Cellular cholesterol synthesis, uptake and processing reactions are regulated by two main nuclear receptor systems, sterol regulatory element binding proteins (SREBPs) (Wang et al., 1994) and liver X receptors (LXRs) (Janowski et al., 1996). SREBP activation increases the transcription of gene products (LDL receptors and 3-hydroxy-3-methylglutaryl coenzyme A reductase) that function to increase cellular cholesterol levels, whereas LXR activation facilitates cholesterol removal from peripheral cells via reverse cholesterol transport and increased biliary sterol secretion (Goldstein et al., 2006, Tontonoz and Mangelsdorf, 2003). Intracellular cholesterol also stimulates cholesterol esterification by the enzyme acyl coenzyme A:cholesterol acyltransferase (ACAT), so excess cholesterol is stored as cholesteryl esters in cytoplasmic droplets (Cheng et al., 1995).

1.2.5 Reverse cholesterol transport

Reverse cholesterol transport is a multi-step process resulting in the net movement of cholesterol from peripheral tissues back to the liver via the plasma (Tall, 1998). Figure 1.4 shows the schematic representation of reverse cholesterol transport process. The first step in reverse cholesterol transport is efflux of free cholesterol from the cell plasma membrane to HDL, either by transporter-independent or transporter-dependent mechanisms. The transporter-independent pathway involves simple diffusion (aqueous diffusion pathway) and facilitated diffusion (SR-BI mediated pathway). The active processes invlove ATP-binding cassette (ABC) family of transmembrane transporters (ABC1 and ABCG1) (Adorni et al., 2007). The free cholesterol is esterified in the HDL particles by lecithin–

cholesterol acyltransferase (LCAT) which is then sequestered into the core of a lipoprotein particle (Glomset et al., 1966).

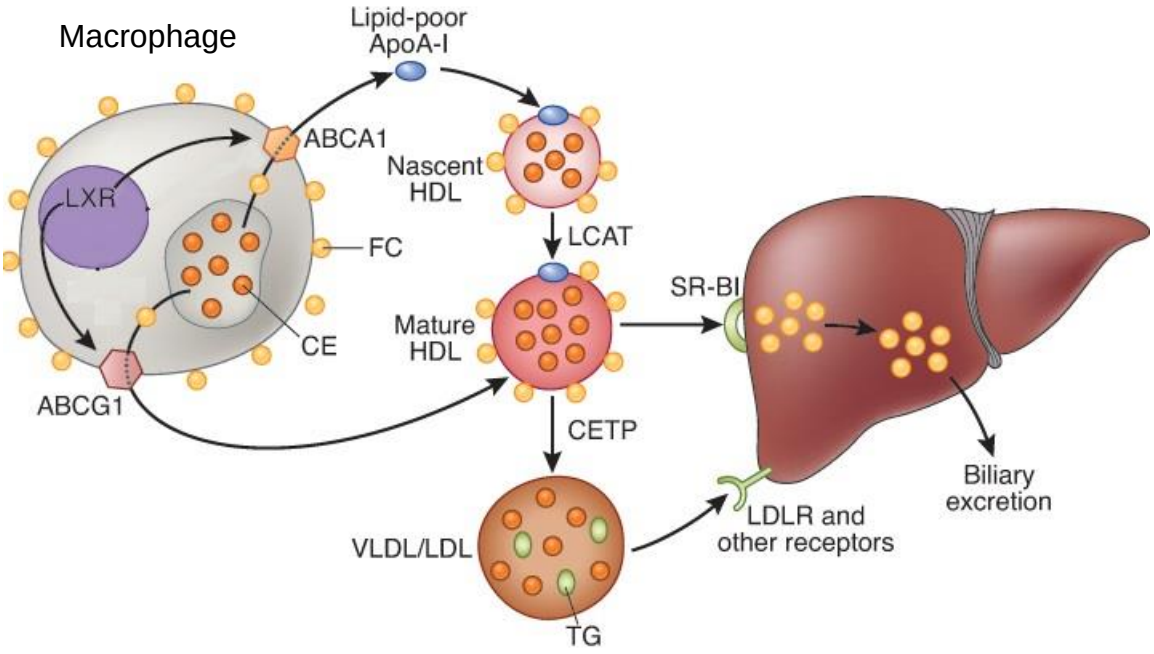


Figure 1.4 Schematic representation of cholesterol efflux

Arrows are indicative of cholesterol movement and particle maturation, (Figure modified from Rader and Tall (2012)

On the other hand, the tissue-derived cholesteryl esters in HDL can also be transferred to the apoB-containing lipoproteins, in exchange for triglycerides, through the action of cholesteryl ester transfer protein (CETP) (Agellon et al., 1991, Lewis and Rader, 2005). As reverse cholesterol transport removes cholesterol from lipid-laden macrophages within atherosclerotic plaques, modifying HDL plasma concentrations and upregulating reverse cholesterol transport are the bases of several therapeutic strategies for the treatment of atherosclerosis that are currently being developed (Ono, 2012).

1.3 Low Density Lipoprotein (LDL)

1.3.1 Structure and characteristics of LDL

Lipoproteins are sub-microscopic particles composed of lipid and protein held together by non-covalent forces. Lipoprotein particles within the density limits of 1.019–1.063 g/ml are referred to as LDL (Atkinson et al., 1977). They have an average diameter of 22 nm and are important for the transport of cholesterol in the blood and around the body, for use by cells (Segrest et al., 2001). The overall structure of an LDL molecule is that of a putative spheroidal micro-emulsion (Figure 1.5) with the core consisting of about 170 triglyceride and 1600 cholesteryl ester molecules and the surface monolayer comprising about 700 phospholipid molecules and a single copy of a protein called apoB-100 (513,000 daltons). In addition, they also contain about 600 molecules of unesterified cholesterol, of which about one-third is located in the core and two-thirds in the surface (Lund-Katz and Phillips, 1986).

The main phospholipid components are phosphatidylcholine (about 450 molecules/LDL particle) and sphingomyelin (about 185 molecules/LDL particle). The LDL particles also contain lysophosphatidylcholine (about 80 molecules/LDL particle), phosphatidylethanolamine (about 10 molecules/LDL particle), diacylglycerol (about 7 molecules/LDL particle), ceramide (about 2 molecules/LDL particle) and some phosphatidylinositol (Hevonoja et al., 2000). In addition to lipids, LDL particles also carry lipophilic antioxidants, such as α-tocopherol (about 6 molecules/LDL particle) and minute amounts of γ-tocopherol, carotenoids, oxycarotenoids and ubiquinol-10 (Esterbauer et al., 1992). The particles are in a dynamic state, their structure and physical properties being dependent on their

lipid composition as well as on the conformation of apoB-100 (Esterbauer et al., 1992).

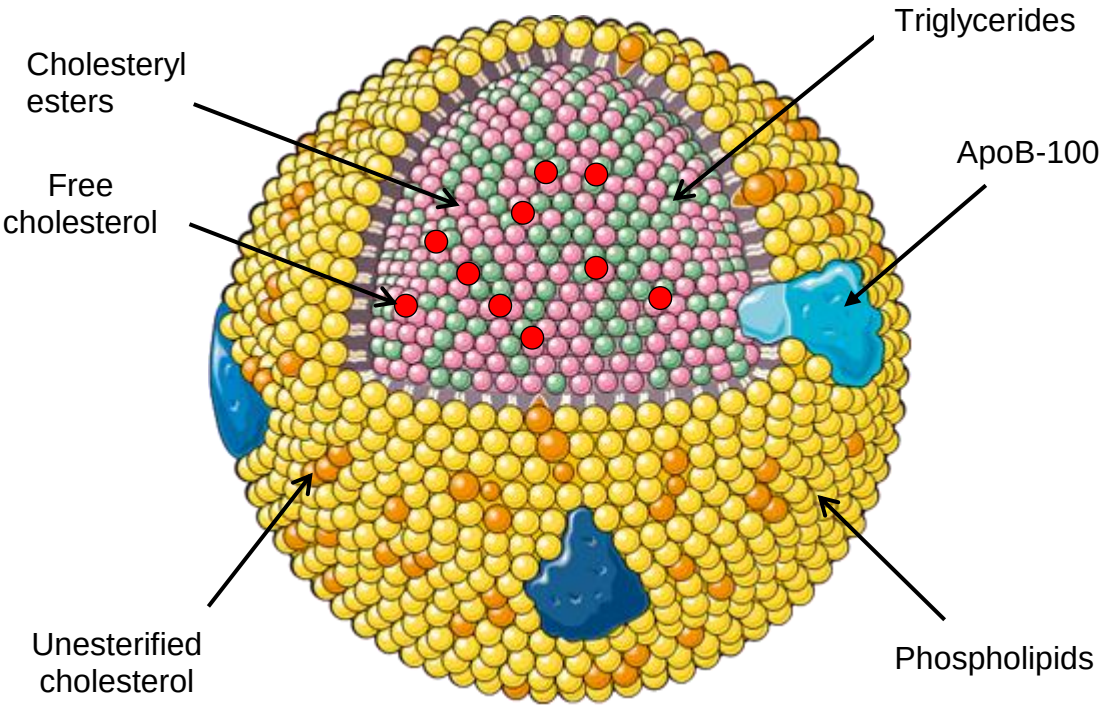


Figure 1.5 A schematic diagram of an LDL particle

The hydrophobic core of LDL constitutes of cholesteryl esters, triglycerides and some free cholesterol while the outer layer constitutes free cholesterol and phospholipids surrounded by apoprotein B-100 molecule.

1.3.2 LDL and atherosclerosis

There is abundant evidence from experimental data, epidemiological studies, therapeutic studies of LDL lowering drugs and genetic studies that links LDL with atherosclerosis. In 1913 Nikolai N. Anitschkow, a Russian pathologist, demonstrated that feeding a high cholesterol diet to rabbits resulted in high plasma cholesterol levels and the formation of lesions resembling atherosclerotic lesions in humans (Steinberg, 2004). In 1950 Gofman and Lingren also observed that feeding rabbits with a high cholesterol diet for 15 weeks increased the plasma LDL levels in them which correlated with the degree of atherosclerosis that they developed. The group also found a correlation between the higher LDL levels in blood and incidences of myocardial infarction in humans (Gofman and Lindgren, 1950).

Further evidence to suggest that high LDL levels were an independent cause of atherosclerosis and CHD came from the observation that patients with familial hypercholesterolaemia (FH) had premature CHD (Khachadurian, 1964). FH is a disease characterised by high plasma LDL cholesterol levels, especially in the homozygous form, which in the majority of cases is due to mutations in the LDL receptor gene (Goldstein and Brown, 1987). FH can lead to the development of atherosclerosis and CHD as early as five years of age occurs in people who are homozygous for the defective LDL receptor gene (Hopkins et al., 2011, Slack, 1969, Stone et al., 1974).

Many epidemiological studies have identified a positive correlation between serum LDL levels and CHD mortality (Epstein, 1996). The Seven Countries study, which began in 1958, examined 12,763 men for CHD risk factors (Gofman, 1958). The

men were aged between 40 and 59 years and formed 16 groups in seven countries: the USA, Finland, the Netherlands, Italy, Greece, the former Yugoslavia and Japan. The men were followed up for 25 years, during which time risk factor surveys were conducted and total mortality data were collected. The study showed that the population average dietary saturated fat and serum cholesterol levels were significantly associated with 25 year mortality from CHD (Kromhout et al., 2002). The Framingham Heart Study began in 1948, originally recruited 5209 men and women between the ages of 30 and 62 (Dawber et al., 1951), but since then have recruited many of their children and grandchildren. Through monitoring of the participants over the last 60 years many CVD risk factors have been identified, including elevated plasma LDL cholesterol levels (Tsao and Vasan, 2015).

Various autopsy studies have also shown a relationship between blood cholesterol and the extent of atherosclerosis (Solberg and Strong, 1983). Several large lipid lowering clinical trials have provided conclusive evidence that lowering the level of plasma LDL causes a decrease in CVD. The first trial to do so was the Lipid Research Clinics Coronary Primary Prevention Trial (Gordon et al., 1986), which followed 3,806 asymptomatic middle aged men with hypercholesterolaemia. They were randomized to receive a bile acid sequestrant (a drug that reduces plasma LDL levels) or a placebo for an average of 7.4 years. Treatment with the drug was associated with a significant 19% reduction in CHD or non-fatal MI. Statins are now the most widely used drugs to lower plasma LDL levels. They work by inhibiting the liver enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG Co-A reductase) (Endo, 1992, Istvan and Deisenhofer, 2001). By inhibiting this enzyme, the conversion of HMG CoA to mevalonic acid is decreased. This step is crucial

for the production of cholesterol by the liver and its inhibition results in the upregulation of the LDL receptors in the liver and thus increased clearance of plasma LDL. Randomised statin trials like the 4S study reported a 30% reduction in mortality (Pedersen et al., 2004).

1.3.3 Origins of the LDL oxidative hypothesis

Lipid oxidation has been implicated in the development of atherosclerosis since 1950's when Glavind and co-workers found that atherosclerotic lesions of the human aorta contained lipid peroxides and that the extent of peroxidation correlated with the extension of the lesions (Glavind et al., 1952). In 1977, Goldstein and Brown observed that FH patients, in spite of defective LDL receptors, contained foam cells, which meant that LDL was able to enter the cells by an alternative mechanism. They proposed that macrophages were taking up LDL after it had been chemically modified. They tested their theory on mouse macrophages, which were incubated with radiolabelled acetylated-LDL. They observed that macrophage uptake of acetylated LDL was 20 times greater than native LDL. It was shown that the acetyl-LDL was being taken up by a separate receptor to the native LDL receptor, as native LDL failed to compete with the acetyl-LDL for binding to the receptor.

In 1979, Goldstein's group observed that prior incubation of the macrophages with unlabelled acetyl-LDL caused a massive deposition of cholesterol and cholesteryl esters in the cells, but this did not inhibit the subsequent binding of radiolabelled acetyl-LDL, indicating that unlike the native receptor, the acetyl-LDL receptor was not regulated by intracellular cholesterol levels (Goldstein et al., 1979). The

acetyl-LDL receptor was later cloned and was named scavenger receptor A (SRA) (Kodama et al., 1990)

After it had been shown that modification of LDL could transform it to a form that was easily taken up by macrophages, the search began to find modifications of LDL that took place *in vivo*. It was shown that incubating LDL overnight with arterial endothelial cells or arterial smooth muscle cells converted it to a form that was rapidly taken up by macrophages, in a saturable manner (Henriksen et al., 1983). It was later shown that when antioxidants or metal chelators were added to the culture medium, the transformation of the LDL was prevented. Further to this, incubating the LDL with copper, in the absence of cells, caused the same transformation of the LDL into the form that was rapidly taken up by macrophages (Steinbrecher et al., 1984). The modification that was taking place was LDL oxidation (Morel et al., 1984). In 1989 Steinberg and co-workers proposed the oxidative modification theory which suggested that LDL, modified by oxidation in the intima of the artery was a key event in the pathogenesis of atherosclerosis (Steinberg et al., 1989).

1.3.4 LDL oxidation

Several modifications of LDL have been described that convert it to a form recognised by macrophage scavenger receptors (Steinberg, 2009). Modifications that can lead to foam cell formation *in vitro* include oxidation, aggregation, enzymatic modification, and immunoglobulin complexing (Oorni et al., 2000, Steinbrecher et al., 1990, Torzewski et al., 2004). However, the best studied and the one for which there are the most *in vivo* data is oxidative modification (Stocker and Keaney, 2004). It was proposed that LDL is oxidised in the arterial wall,

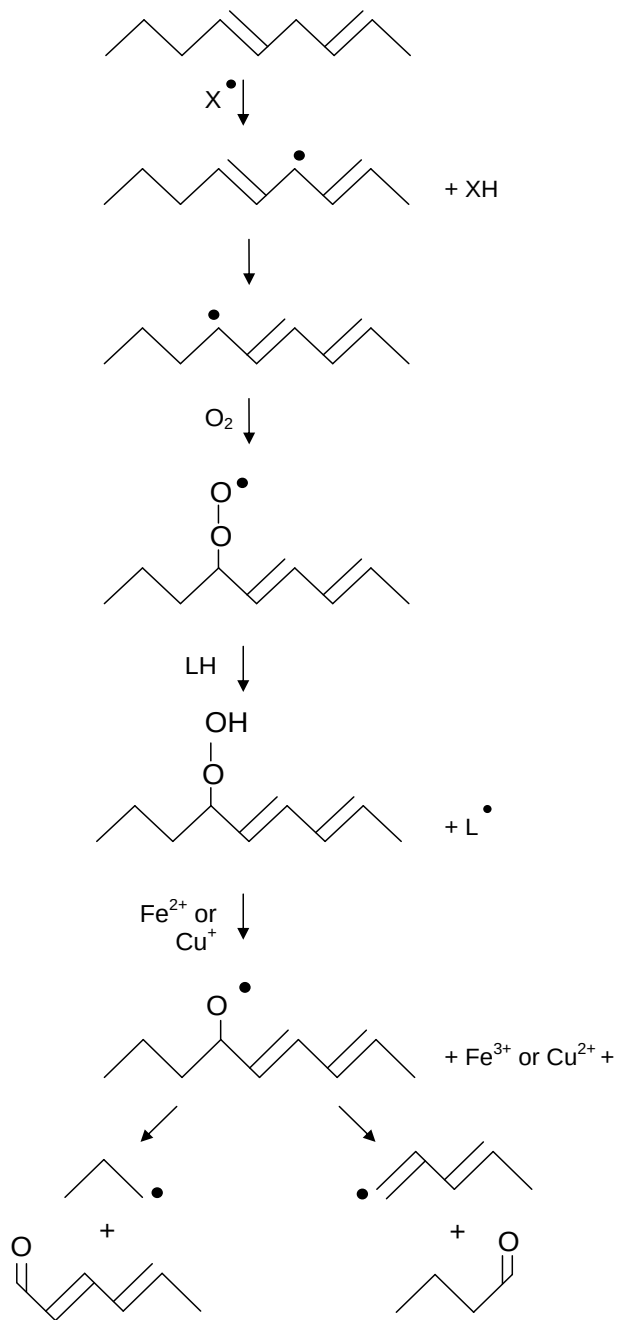
sequestered by proteoglycans and other extracellular matrix constituents, where it is protected from plasma antioxidants. LDL that enters the arterial wall may be oxidized by vascular cells (endothelial cells, smooth muscle cells, macrophages and lymphocytes) with oxidizing enzymes like lipoxygenase and myeloperoxidase in the presence or absence of transition metal ions (iron or copper). Mildly oxidized LDL has a low affinity to macrophage scavenger receptors, and thereby, mildly oxidized LDL can re-enter the blood circulation and can be detected as a serum oxidized LDL (Levitan et al., 2010, Yoshida and Kisugi, 2010). Such mildly oxidized LDL stimulates adhesion molecules and chemokines (Berliner et al., 1990). Extensively oxidized LDL can be taken up by macrophages through the scavenger receptors, leading to the formation of foam cells (Itabe, 2009).

Once initiated, oxidation of LDL is a free radical driven lipid peroxidation chain reaction (Heinecke, 1998). Lipid peroxidation is initiated by free radical attack on a double bond in a polyunsaturated fatty acid (PUFA) (Ayala et al., 2014). Figure 1.6 shows the basic reaction sequence involved in lipid peroxidation. The aldehydes generated, like malonaldehyde, react with amino groups of the lysine residues of apolipoprotein B-100, altering the domain recognised by the classical LDL receptor and causing the LDL to be recognised by scavenger receptors, consequently causing unregulated LDL uptake and the formation of foam cells (Esterbauer et al., 1990). The cholesterol within the LDL particle is also oxidised to form oxysterols. In mildly oxidising conditions it is converted to 7β or 7α-hydroperoxycholesterol (Brown et al., 1997). In more extensively oxidised LDL, the cholesterol is converted to 7-oxygenated sterols such as 7-ketocholesterol (Brown et al., 1996). The degradation of PUFAs by lipid peroxidation is preceded by the loss of the endogenous antioxidants in the LDL particle. The major antioxidant in LDL is α-

tocopherol which is a chain breaking antioxidant, which prevents the propagation of the lipid peroxidation by scavenging the lipid peroxyl radicals, as follows:

$$LOO^{\bullet} + \alpha\text{- tocopherol} \rightarrow LOOH + \alpha\text{ - tocopherol}^{\bullet}$$

In experiments where plasma was supplemented with α-tocopherol prior to LDL isolation, the α-tocopherol content of the LDL was increased and a delay in the onset of the propagation phase of lipid peroxidation, with macrophages (Jessup et al., 1990) or copper ions at pH 7.4, was proportionally increased (Esterbauer et al., 1991).



1. Abstraction of a hydrogen atom from a PUFA give a lipid alkyl radical
2. Intramolecular re-arrangement to form the more stable conjugated diene alkyl radical
3. Reaction with molecular oxygen causes the production of the lipid peroxyl radical
4. Reaction with another PUFA to give a lipid hydroperoxide and a lipid alkyl radical

Figure 1.6 A schematic diagram showing the oxidation of PUFA's by free radical reaction

1.3.5 Atherogenic effects of oxidised LDL (OxLDL)

There is evidence that oxidised LDL exists *in vivo*, within atherosclerotic lesions. Antibodies raised against OxLDL have identified it in atherosclerotic lesions but not in normal arteries (Boyd et al., 1989, Javed et al., 1999, Palinski et al., 1989). Auto-antibodies to oxidised LDL have been identified in human and animal sera (Palinski et al., 1989), indicating that oxidised LDL or something similar must have been present in order to generate an immune response. Oxidised apo B-100 epitopes and increased levels of lipid peroxidation products can be detected in LDL extracted from rabbit and human atherosclerotic lesions (Ylä-Herttuala et al., 1989). Studies in different animal models of atherosclerosis show that progression of the lesions can sometimes be delayed by intervention with antioxidants (reviewed in Feig, 2014). OxLDL exhibits a wide array of biological properties that are proatherogenic: OxLDL can stimulate the release of macrophage colony stimulating factor (MCSF) from the endothelial cells, causing monocytes to differentiate into macrophages (Rajavashisth et al., 1990). Oxidised LDL induces the expression of vascular cell adhesion molecule 1 (VCAM-1) on the surface of the endothelial cells, causing adhesion of monocytes. It may cause endothelial injury or dysfunction as it is cytotoxic for endothelial cells in culture (Hessler et al., 1983) and inhibits nitric oxide-induced vasodilatation by reducing l-arginine availability to endothelial nitric oxide synthase for NO production (Kugiyama et al., 1990). It causes inhibition of lipopolysaccharide-induced expression of NF-κB and increases collagen synthesis by smooth muscle cells (Steinberg, 2009, Young and McEneny, 2001).

1.3.6 Role of transitions metals in LDL oxidation

Various substances have been shown to induce LDL oxidative modification to such a similar form to that observed in atherosclerosis. Transition metals such as iron and copper are the two principle biological redox-active metals that have received the most attention as potential contributors to the oxidation of LDL that is associated with atherosclerosis. Both iron and copper support LDL oxidation *in vitro* in a number of cell types including endothelial cells (Steinbrecher et al., 1984), smooth muscle cells (Heinecke et al., 1986) macrophages (Leake and Rankin, 1990), and lymphocytes (Lamb et al., 1992). There is also evidence for the presence of transition metals in atherosclerotic plaque (Lamb et al., 1995, Stadler et al., 2004, 2008), and it is known that physiologically relevant forms of both iron (e.g. hemin and ferritin) and copper (e.g. ceruloplasmin) can promote LDL oxidation *in vitro*, particularly under conditions related to inflammation (Balla et al., 1991, DiSilvestro and Jones, 1996, Lamb and Leake, 1994, Zhang et al., 2010). Though copper is able to oxidise LDL *in vitro*, the free copper ions are scarce in the blood systems appearing in the form of ceruloplasmin, which is not considered a pro-oxidant (reviewed in detail by Burkitt, 2001). Iron, in contrast, exists in the blood as haemoglobin at extremely high (millimolar) concentrations, as myoglobin and as heme- and iron-sulphur proteins in mammalian cells (Doyle and Hoekstra, 1981, Ponka et al., 1998). In these forms of biological iron, access of redox-active reactants is regulated tightly, which thus limits and directs the catalysis of redox reactions by iron chelates. The intercellular and intracellular transport of iron is regulated by ferritin-transferrin and similar systems (Mackenzie et al., 2008, Ponka et al., 1998). Ferritin, under normal physiological conditions, serves to store much of the excess iron in cells (Theil, 2013), thereby limiting

production of free radical species or related oxidants (Wood et al., 2004). Nevertheless, there is ample data which shows the presence of catalytically active iron in human atherosclerotic plaques (Ahluwalia et al., 2010, Raman et al., 2008, Tasic et al., 2015b).

1.3.7 Antioxidants in the treatment of atherosclerosis

In 1991, the National Heart, Blood and Lung Institute (USA) convened a workshop to review the evidence about the oxidative modification hypothesis. The experts at the workshop concluded that the evidence was sufficiently strong to justify initiating clinical intervention trials. The conferees recommendations of the committee were to start trials with naturally occurring antioxidants like vitamin E, vitamin C or β-carotene (Steinberg, 1992).

Two large primary prevention studies: the α-Tocopherol, β-Carotene Cancer Prevention (ATBC, 1994) and the Primary Prevention Project studies (de Gaetano, 2001) investigated the effect of supplemental vitamin E on myocardial infarction (MI), CVD or stroke at 3–6 years. Neither of them showed any protective effect of supplementation against cardiovascular risk. Two other smaller studies; the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study (Salonen et al., 2000) and Vitamin E Atherosclerosis Prevention Study (VEAPS) (Hodis et al., 2002) used intima-medial thickness progression as a marker of ongoing CVD. Apart from one subgroup, neither showed any effect of vitamin E on progression of disease in healthy subjects at low risk of CVD. Several other prospective double-blind, placebo-controlled studies of the effects of vitamin E supplementation in patients with pre-existing CVD (secondary prevention) showed no benefits of antioxidant supplementation. In 2002, another major study (Heart Protection

Study) concluded that antioxidant vitamins (vitamin E and C and β-Carotene) did not produce any significant reductions in the 5-year mortality from, or incidence of, any type of vascular disease, cancer, or other major outcome (HPS, 2002). However, the Cambridge Heart AntiOxidant Study (Stephens et al., 1996) reported a major reduction in the risk of non-fatal MI (although fatal MI was non-significantly increased in patients with angiographically proven coronary atherosclerosis), whereas a significant decrease in acute MI was reported in a small group of haemodialysis patients with supplemental vitamin E (SPACE) (Boaz et al., 2000).

Looking at the results from various clinical trials, it might seem that the negative results would be conclusive and that additional clinical trials of any antioxidants would be pointless. However, it is important to appreciate that the trials of chronic antioxidant therapies have focused on easily available agents rather than those that are most efficacious. The lack of benefit seen in clinical trials to date does not disprove the central role of oxidative stress in atherosclerosis. Although much effort has been directed at proving the benefits of antioxidants, the findings to date are far from clear mostly because there is no clear understanding on how LDL is oxidised *in vivo*.

1.3.8 LDL aggregation

The molecular mechanisms of foam cell formation in the arterial sub-endothelium is not fully understood, however, it is becoming increasingly clear that aggregation and fusion of LDL particles may prevent their exit from the arterial wall and contribute to atherogenesis (Bancells et al., 2010a, Camejo et al., 1998, Guyton, 1994, Hurt-Camejo et al., 2000, Oorni et al., 2000, Paananen et al., 1995, Parasassi et al., 2008, Pentikainen et al., 1996). Several lines of evidence support

the presence of LDL aggregates in the arterial wall and their involvement in LDL retention by arterial proteoglycans during atherogenesis (Aviram et al., 1995, Hoff and Morton, 1985). For example, the aortic intima in cholesterol-fed and Watanabe Heritable Hyperlipidaemia rabbits has been shown to contain aggregated lipoproteins bound to subendothelial matrix (Frank and Fogelman, 1989). LDL aggregates isolated from atherosclerotic lesions are capable of macrophage foam cell formation in a process which has been seen to be independent of LDL uptake by scavenger receptors (Steinbrecher and Lougheed, 1992). In addition, it has been reported that aggregated LDL can cause cholesterol accumulation in coronary vascular smooth muscle cells and turn them into foam cells (Llorente-Cortes and Badimon, 2005).

Enzymes secreted by the arterial intimal cells, like phospholipase A_2 (PLA2), phospholipase C and sphingomyelinase are capable of causing aggregation of LDL (Oorni et al., 2000). PLA2 hydrolyses fatty acids of phospholipids at the *sn*-2 position to give lysophosphatidylcholine and fatty acids, which in the presence of lipid binding proteins, such as albumin, are released from the LDL particle. This causes rearrangement of the LDL particle structure and causes conformational changes in apo-B100, subsequently causing LDL aggregation (Hakala et al., 1999, Webb, 2005).

Phospholipase C hydrolyses LDL phospholipids to phosphocholine and diacylglycerol. Phosphocholine is released from the LDL particle leaving the hydrophobic domains on the surface of the LDL and thus inducing LDL aggregation via hydrophobic interactions between LDL particles (Liu et al., 1993).

Sphingomyelinase (SMase) is a zinc-dependent acidic metalloenzyme secreted by smooth muscle cells and macrophages of arterial intima (Marathe et al., 1999). The enzyme can act on LDL at pH 7.4 if the lipoprotein is modified by oxidation or phospholipase A_2 or enriched with apo-CIII (Schissel et al., 1998). It hydrolyses sphingomyelin molecules within the LDL particle causing the formation of water-soluble phosphocholine, which is released from the surface, and water-insoluble ceramide which is retained in the core of the LDL (Walters and Wrenn, 2008). This causes an increase in the apolar core lipids at the expense of the polar surface lipids, resulting in a hydrophobic mismatch between the core and surface, which is expected to cause lipoprotein fusion. LDL fusion upon SMase reaction is supported *in vivo* by the observation that aggregated LDL found in atherosclerotic lesions are rich in ceramide (Schissel et al., 1996). Moreover, treatment of isolated LDL with SMase induces lipoprotein aggregation and fusion *in vitro* (Oorni et al., 1998).

LDL retention in the arterial wall by proteoglycans is considered an important event in the development of atherosclerosis. Proteoglycans are a component of the extracellular matrix which can bind to apoB-100, therefore causing LDL to be retained within the arterial intima (Boren et al., 1998, Williams and Tabas, 1995). Aggregated LDL has been seen to bind proteoglycans more readily than native LDL, possibly due to greater number of apoB-100 molecules in LDL aggregates (Paananen et al., 1995). Therefore, as well as causing macrophage lipid uptake, LDL aggregation may enhance the retention of LDL in arterial intima.

1.4 Lysosomes

Lysosomes are spherical cellular organelles, bound by a single membrane. They were discovered in 1955 by Christain De Duve (De Duve et al., 1955). They are involved in the digestion of macromolecules using a range of hydrolytic enzymes which they contain. Lysosomes digest cellular material such as damaged or unwanted organelles, in a process known as autophagy (Eskelinen and Saftig, 2009). They also digest material brought into the cells by endocytosis or phagocytosis such as bacteria and neutrophils. The pH of the lysosomal compartment is around 4.5 which is essential for the hydrolytic activity of its enzymes. The acidic pH is maintained by ATP-dependent proton pumps present within the lysosomal membrane (Ohkuma et al., 1982).

1.4.1 Lysosomes in atherosclerosis

Several studies have demonstrated that incubation of macrophage cell lines with various forms of modified lipids can disrupt lysosomal function resulting in over-accumulation of intralysosomal cholesteryl esters (Jerome, 2010). In 1974 Christian de Duve hypothesised that lysosomal deficiency or lysosomal defects lead to the accumulation of lipids within lysosomes and, eventually, to the formation of foam cells (de Duve, 1974). The hypothesis was based partly on the observation of lipid-engorged lysosomes in post-mortem liver tissue samples from Wolman disease patients, which completely lack lysosomal acid lipase (LAL) (Lake and Patrick, 1970, Patrick and Lake, 1969) and premature atherosclerosis seen in the cholesteryl ester storage disease patients, which have LAL deficiency (Sloan and Fredrickson, 1972). Du et al. (2001) showed that external administration of LAL decreased the amount of atherosclerosis in LDL receptor

knockout mice which were fed an atherogenic diet, indicating that lysosomal accumulation of lipid is important in disease progression. These results were further confirmed by Sun et al. (2014), who demonstrated that LAL treatment is effective in the advanced stages of the disease. Cox et al. (2007) have reported a general loss of lysosomal function including reduction in LAL-dependent cholesteryl ester hydrolysis over time following incubation of human macrophages with mildly oxidised LDL or aggregated LDL. It was suggested that as most lysosomal enzymes require an acidic pH to function properly, neutralisation of the lysosomes could explain the inhibition of cholesteryl ester hydrolysis. Sheedy et al. (2013) demonstrated that inefficient lysosomal hydrolysis and transport of oxLDL-derived cholesteryl esters leads to *in-situ* cholesterol crystal formation. As well as providing a link between oxLDL and cholesterol crystal formation, the study also demonstrated that lysosomal dysfunction can initiate inflammation. Interestingly, Ullery-Ricewick et al. (2009) demonstrated that in THP-1 human macrophages lysosomal cholesterol ester accumulations from aggregated LDL could be reduced by 50% by chase incubation with triglyceride (TG)-rich lipid dispersions or VLDL. They noted that TG treatment re-established the acidic pH by restoring v-ATPases in the lysosomal membrane. Recently (Emanuel et al., 2014), built on these concepts by demonstrating the development of lysosomal dysfunction in primary macrophages exposed to oxLDL and cholesterol crystals. They noticed the effects on morphological changes like lysosomal engorgement and functional changes like increased lysosomal pH and decreased degradation capacity. Moreover, they also noticed that macrophages isolated from atherosclerotic aortas of ApoE-null mice showed signs of lysosomal dysfunction, not found in macrophages from other tissues of the same mice. Moreover, recent large-scale, genome-wide association

studies have identified single nucleotide polymorphisms (SNPs) in lysosomal acid lipase A (LIPA), the gene encoding LAL, as a susceptibility locus for coronary artery disease (C4D, 2011, Consortium, 2011, Wild et al., 2011).

Overall, accumulating evidence suggests that endosomal uptake of modified lipids initiates a condition very similar to lysosomal lipid storage diseases. In fact, in advanced atherosclerosis lesions, foam cells have been seen to accumulate lipids within enlarged lysosomes. Lipids accumulate inside lysosomes causing dysfunction and in cases wherein lipid content overwhelms the transport machinery, cholesterol crystals are formed leading to disruptions in membrane integrity.

1.4.2 Lysosomal iron

Several studies have shown that lysosomes contain redox active iron (Terman and Kurz, 2013, Yu et al., 2003, Yuan et al., 1996, Zdolsek et al., 1993). Lysosomes derive most of their iron from the degradation of ferritin (Kidane et al., 2006, Radisky and Kaplan, 1998, Sibille et al., 1989, Yu et al., 2003), the normal autophagic turn-over of iron-rich mitochondrial proteins (Persson et al., 2001, Yu et al., 2003) and in the case of macrophages the phagocytosis of erythrocytes (Myagkaya et al., 1979, Yuan et al., 1996). However, there is a variation in the concentration of iron in individual lysosomes, probably due to variability in the phagocytic/degradative state of the lysosomes (Kurz et al., 2008). Experiments even suggest that the cytosolic non-ferritin bound iron pool (known as labile iron pool) may actually originate from the lysosomes (Tenopoulou et al., 2005). As the lysosomal compartment is acidic and rich in reducing equivalents, such as cysteine and glutathione, any low mass iron would likely be in the ferrous form

(Fe^{2+}) (Schafer et al., 2000). Fe^{2+} can promote the generation of oxidising species (Graf et al., 1984), which may cause peroxidation of material under degradation, resulting in lipofuscin or, if substantial, damage to and permeabilisation of the lysosomal membrane (Terman et al., 2010).

1.4.3 Lysosomal oxidation theory

The oxidation hypothesis of atherosclerosis fails to address the high antioxidant capacity of extracellular fluids, as even a few percent of serum or interstitial fluid can inhibit the oxidation of LDL (Dabbagh and Frei, 1995, Leake and Rankin, 1990). The conventional theory also fails to explain why the larger antioxidant clinical trials showed no protection against cardiovascular disease (Steinberg, 2002). In 2007, Wen and Leake, in this laboratory showed that aggregated or acetylated LDL is rapidly endocytosed by macrophages and then oxidised within lysosomes. J774 cells (mouse macrophage-like cells) and human monocyte-derived macrophages (HMDM) rapidly took up non-oxidatively aggregated LDL (produced by vortexing) and generated ceroid in their lysosomes after 7 days (Wen and Leake, 2007). Ceroid is the final product of lipid oxidation and it consists of insoluble polymerised lipids and proteins. It has been found within foam cells in atherosclerotic lesions (Mitchinson, 1982).

There was an increased production of oxysterols (e.g. 7-ketocholesterol) in the J774 macrophages when incubated with non-oxidized acetylated LDL (Wen and Leake, 2007). The increase in oxysterols was inhibited by the lipid-soluble antioxidants probucol, α-tocopherol, and butylated hydroxytoluene, which were shown to be taken up by the cells, but not by the water-soluble antioxidant trolox. The water-soluble antioxidant, trolox, may not have been able to enter the

lysosomes efficiently and therefore failed to provide protection against the intralysosomal oxidation of LDL. Chloroquine, a weak base that concentrates in lysosomes and increases their pH, inhibited the oxidation of LDL, showing that the oxidation takes place at acidic pH. Adding ferrous sulphate or ferritin during the chase incubation approximately doubled the oxysterol production and adding desferrioxamine, which is delivered to lysosomes by pinocytosis, significantly inhibited the oxysterol production. It was further established that iron is highly effective in oxidizing LDL in a simple buffer at pH 4.5, the approximate pH of lysosomes, but very poor at doing so at pH 7.4, as iron is more soluble at acidic pH and may precipitate at pH 7.4 (Satchell and Leake, 2012, Wen and Leake, 2007).

Based on these results, this laboratory has proposed that LDL undergoes non-oxidative aggregation in the extracellular space and is then phagocytosed by macrophages and oxidised within lysosomes by catalytically active iron. It has been suggested that the oxidised lipids could then be released from the lysosomes into the rest of the cell or could engorge the lysosomes and disrupt their function. As well as this, the oxidised LDL could be released into the intima upon the death of the cells in which it is formed and affect the function of the neighbouring cells.

More recently this laboratory has shown that LDL aggregated by the enzyme sphingomyelinase (SMase-LDL) is rapidly phagocytosed by human macrophages and through confocal microscopy it was revealed that the lipids and lysosomes co-localised with each other (Wen et al., 2015). Incubating macrophages with SMase-LDL for 7 days produced intralysosomal ceroid in them, while native LDL

treatment produced no ceroid. These results provide further support for the lysosomal LDL oxidation theory.

1.4.4 Lysosomotropic drugs with antioxidant property

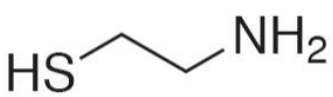
A potential therapy to target lysosomal lipid accumulation in atherosclerosis is to stimulate the transport of cholesterol from lysosomes into the cytoplasm. However, it is a challenging issue as the lysosomal cholesterol derived from oxLDL has been found to be resistant to efflux. Furthermore, studies in both arteries and cells in culture suggest that the cholesterol in lysosomes is trapped partly (in the form of ceroid), and cannot be decreased simply by inhibiting further uptake of lipoproteins or by increasing efflux of extra-lysosomal cholesterol stores (Dhaliwal and Steinbrecher, 2000, Yancey and Jerome, 2001). However, a possible way to prevent the effect of lysosomal cholesterol accumulation is to prevent the intralysosomal oxidation of LDL by using agents which are able to concentrate in the lysosomes and able to sequester intralysosomal oxidising species.

For example, desferrioxamine, which is delivered to lysosomes by pinocytosis, is able to inhibit LDL oxidation process by redox-active iron within lysosomes (Minqin et al., 2005, Wen and Leake, 2007) and have been shown to slow down the early stages of atherosclerosis in mice (Zhang et al., 2010). A study conducted by Yu et al. (2003) found that WR-1065 (thiol metabolite of amifostine) accumulates in lysosomes and interacts with the lysosomal iron thereby minimizing iron-catalysed lysosomal damage and ensuing cell death. Propranolol, a β-receptor blocker has been reported to concentrate up to 1000-fold in lysosomal compartments within cells (Cramb, 1986). Propranolol, 9-amino-acridine-propranolol, 4-hydroxy propranolol and D-propranolol have been reported to exhibit antioxidant activity

attributed to their ability to compartmentalise within lysosomes, providing direct protection against oxidative lysosomal membrane injury and indirect protection by preventing release of iron into the cytosol to perpetuate the oxidative process further (Dickens et al., 2002, Kramer et al., 2012, Mak and Weglicki, 2004). Lipid-soluble antioxidants like probucol, α-tocopherol, and butylated hydroxytoluene have been shown to prevent oxysterol formation in macrophages (Wen and Leake, 2007).

1.4.5 Cysteamine and lysosomal oxidation of LDL

Cysteamine (Figure 1.7) is an aminothiol currently used for the treatment of the lysosomal storage disorder cystinosis, an inherited disease caused by the absence of functional cystinosin, the ubiquitous lysosomal cystine transporter (Kalatzis et al., 2001). The accumulation of cystine in the lysosomes of all the cells in the body leads to progressive dysfunction of multiple organs. Cysteamine reacts with intralysosomal cystine to form the mixed disulfide of cysteamine and cysteine, which can then leave the lysosome via the lysine transport system (Jezegou et al., 2012). The therapy, considered the only effective treatment for cystinosis, prolongs the patients' lives from about 10 years to as much as 40 years (Dohil et al., 2010).



Cysteamine

Figure 1.7 Chemical structure of cysteamine

Cysteamine exhibits strong antioxidant activity and has been proposed for the treatment of neurodegenerative disorders. Recent studies have found that cysteamine exhibits neuroprotective effects in a mouse model of Huntington's disease (HD) through inhibiting the transglutaminase activity and enhancing the brain-derived neurotrophic factor (BDNF) level (Karpuj et al., 2002). Another study has shown that cysteamine, at low dose confers potent neuroprotection against MPTP-induced toxicity of dopaminergic neurones (Sun et al., 2010). Moreover, a phase I study using cysteamine in HD patients showed the drug to be safe and tolerable (Dubinsky and Gray, 2006), suggesting it might be a promising drug for treating neurodegenerative disorders. Cysteamine has also been proposed for the treatment of non-alcoholic fatty liver disease (NAFLD), where it has been seen to improve serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) concentrations by increasing ROS scavenging and glutathione production, as well as by inhibiting transglutaminase activity (2012, Dohil et al., 2011). In addition, the use of cysteamine has been proposed for treating malaria and as an adjunct in cancer chemotherapy (Min-Oo and Gros, 2011, Wan et al., 2011).

This laboratory recently studied the possible protection of cysteamine against LDL oxidation, both *in vitro* and *in vivo* (submitted work). It was found that addition of cysteamine inhibited the oxidation of LDL by iron at pH 4.5 in a concentration-dependent manner. The lag phase, during which endogenous and exogenous antioxidants are consumed, increased from about 1 h to over 30 h on adding 250 µM cysteamine. In human macrophages which were treated with modified LDL (SMase-LDL) cysteamine, at final concentrations of 1, 5, and 10 µM, inhibited the production of intralysosomal ceroid by more than 80%. The effect of cysteamine

was also investigated on atherosclerosis in LDL receptor-deficient mice which were fed on an atherogenic diet. The results showed a significant reduction in the *en face* lesion area in the aortic arch of the mice with the dose of cysteamine equivalent to that received by patients with cystinosis.

Although the laboratory has obtained data demonstrating the anti-atherosclerotic activity of cysteamine, however, there are a substantial number of unanswered questions concerning this activity. The mechanisms by which thiols, such as cysteamine, inhibit oxidation reactions mediated by free radicals are far from clear. Thiols may scavenge free radicals, but whether they scavenge lipid radicals or protein radicals in apolipoprotein B-100 of LDL, which would then go on to oxidise lipids, is unknown.

1.5 Thesis aims and hypothesis

The first aim of this thesis was to explore the mechanism of the oxidation of LDL by iron at lysosomal pH, as this is a novel and yet incompletely characterised method of oxidising LDL that might be relevant *in vivo*. Initial oxidation of LDL by transition metals has been proposed to be due the pre-existing lipid hydroperoxides in LDL. Therefore, it is hypothesised that LDL oxidation by iron at pH 4.5 should take place only in presence of pre-existing lipid hydroperoxides and increasing the initial hydroperoxide load of LDL should increase the rate of LDL oxidation by iron. The scavengers of lipid hydroperoxide radicals, e.g. probucol, should completely prevent the oxidation of LDL by iron at pH 4.5. However, if superoxide ions are involved in the initial oxidation of LDL, compounds, like thiols, which are known superoxide scavengers, should completely protect LDL oxidation by iron.

The second aim of the thesis was to investigate the protective effects of existing lysosomotropic antioxidants in inhibiting the oxidation of LDL at lysosomal pH. Lysosomotropic drugs like propranolol, amiodarone and 7,8-dihydroneopterin have antioxidant properties and are currently in clinical use for treating various diseases. Therefore, it was hypothesised that these lysosomotropic antioxidants should inhibit the oxidation of LDL by iron at pH 4.5. Experiments were conducted to evaluate if they offer better protection than cysteamine in preventing LDL oxidation by iron at pH 4.5.

The third aim of thesis was to investigate in detail the oxidation pattern of sphingomyelinase aggregated LDL (SMase-LDL) under lysosomal conditions. It

was hypothesised that oxidation of SMase-LDL by iron under lysosomal conditions should produce the lipid oxidation products same as native LDL. Characterisation of the oxidation products of SMase-LDL were carried out by high performance liquid chromatography (HPLC). Intralysosomal lipid peroxidation of LDL was studied in human macrophages by using the lysosomotropic dye Foam-LPO and by measuring intralysosomal ceroid formation. The protective effect of cysteamine was also investigated.

The final aim of thesis was to begin to understand how relevant lysosomal oxidation of SMase-LDL might be to induce atherogenic events such as lysosomal dysfunction, cellular senescence and secretion of inflammatory cytokine secretion by macrophages. It was hypothesised that extensive oxidation of LDL in the lysosomes of macrophages would lead to accumulation of LDL oxidation products (e.g. ceroid) in the lysosomes of macrophages which can affect their function and will also induce the secretion of inflammatory cytokines such as TNF-α. Furthermore, inhibition of lysosomal oxidation of LDL by cysteamine should prevent these effects.

Chapter 2- Materials and methods

2.1 General laboratory reagents and solutions

2.1.1 Laboratory reagents

General laboratory reagents and companies from which they were purchased are listed below:

Name	Company of purchase
Acetic acid (glacial)	Sigma-Aldrich Ltd
Acetic anhydride	Fisher Scientific Ltd
Acetonitrile (HPLC grade)	Sigma-Aldrich Ltd
Amphotericin B	Sigma-Aldrich Ltd
Bathophenanthrolinedisulfonic acid	Sigma-Aldrich Ltd
Bovine serum albumin	Invitrogen Ltd
Chelex-100	Sigma-Aldrich Ltd
Cholesterol	Sigma-Aldrich Ltd
Cholesterol arachidonate	Sigma-Aldrich Ltd
Cholesterol linoleate	Sigma-Aldrich Ltd
Cholesterol linoleate hydroperoxide	Cayman chemicals
Cysteine	Sigma-Aldrich Ltd
Cysteamine hydrochloride	Sigma-Aldrich Ltd
Diethylenetriaminepentaacetic acid (DTPA)	Sigma-Aldrich Ltd
Dimethyl sulphoxide	Sigma-Aldrich Ltd
Dipotassium hydrogen orthophosphate	Fisher Scientific Ltd
Disodium hydrogen orthophosphate	Fisher Scientific Ltd
Ethanol (HPLC grade)	Sigma-Aldrich Ltd

Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich Ltd
Ferric chloride	Sigma-Aldrich Ltd
Fetal calf serum (FCS)	Sigma-Aldrich Ltd
Ferrous sulphate	Sigma-Aldrich Ltd
Folin and Ciocalteau phenol reagent	Fisher Scientific Ltd
Hanks' Balanced Salt Solution (HBSS)	Gibco
Hexane (HPLC grade)	Sigma-Aldrich Ltd
Human serum	Sigma-Aldrich Ltd
Hydrochloric acid	Fisher Scientific Ltd
Hydrogen peroxide	Fisher Scientific Ltd
Isopropanol (HPLC grade)	Fisher Scientific Ltd
7-Ketocholesterol	Sigma-Aldrich Ltd
2-Keto-4-thiomethylbutanoic acid	Sigma-Aldrich Ltd
Methanol (HPLC grade)	Fisher Scientific Ltd
N,N,N′,N′-tetraacetylethylenediamine	Sigma-Aldrich Ltd
Potassium acetate	Sigma-Aldrich Ltd
Potassium bromide	Fisher Scientific Ltd
Potassium chloride	Fisher Scientific Ltd
Potassium dihydrogen orthophosphate	Fisher Scientific Ltd
Potassium hydroxide	Fisher Scientific Ltd
Potassium sodium tartrate	Fisher Scientific Ltd
Sodium acetate	Sigma-Aldrich Ltd
Sodium carbonate	Fisher Scientific Ltd
Sodium chloride	Fisher Scientific Ltd
Sodium dihydrogen orthophosphate	Fisher Scientific Ltd
Sodium hydrogen carbonate	Fisher Scientific Ltd
Sodium hydroxide	Fisher Scientific Ltd

Trichloroacetic acid	Sigma-Aldrich Ltd
Triton X-100	Fisher Scientific Ltd
Xylene	Fisher Scientific Ltd

2.1.2 Laboratory equipment

Name	Company of purchase
Optima™ XPN ultracentrifuge	Beckman Coulter
Lambda-2 6-cell spectrophotometer	Perkin Elmer
Lambda Bio 40 8-cell spectrophotometer	Perkin Elmer
Cary Eclipse fluorescence spectrophotometer	Agilent
Libra S22 UV/Vis Spectrophotometer	Biochrom
Agilent 1100 HPLC System	Agilent
BD Accuri™ C6 flow cytometer	BD Biosciences
Axioskop epifluorescent microscope	Carl Zeiss
A1 Inverted epifluorescent Microscope	Carl Zeiss
FLUOstar OPTIMA microplate reader	BMG LABTECH
Countess II FL automated cell counter	Invitrogen
Zetasizer Nano Series particle sizer	Malvern

2.1.3 General Solutions

High density KBr solution (HDS) 1.316 g/ml

KBr (2.97 M), NaCl (2.62 M) and Na_2EDTA (297 µM) made up in ultrapure water, adjusted to pH 7.4 with 0.5M NaOH solution.

Low density KBr solution (LDS) 1.006 g/ml

NaCl (150 mM) and Na_2EDTA (297 µM), made up in ultrapure water, adjusted to pH 7.4 with 0.5M NaOH.

Dialysis buffer

NaCl (140 mM), Na_2HPO_4 (8.1 mM), NaH_2PO_4 (1.9 mM) and Na_2EDTA (100 µM) made up in ultrapure water and adjusted to pH 7.4 with 0.5 M NaOH.

Lowry A reagent

Na_2CO_3 (25 g), NaOH (5 g), potassium sodium tartrate tetrahydrate (0.5 g), dissolved in 230 ml ultrapure water. $CuSO_4.5H_2O$ (0.125 g) dissolved in few millilitres of distilled water and added. Final volume made up to 250 ml with ultrapure water.

Lowry B reagent

Folin and Ciocalteau phenol reagent (6.67%, v/v) in ultrapure water, freshly prepared for each assay.

Lipid hydroperoxide assay colour reagent

KH_2PO_4 (163 mM), KI (120 mM), K_2HPO_4 (37 mM), Triton X-100 (2 g/l), NaN_3 (150 µM), benzalkonium chloride (0.1 g/l), and ammonium molybdate (10 µM) pH adjusted to 6.0.

Sodium chloride/sodium acetate buffer (Chelex-100 treated)

NaCl (150 mM), acetic acid (10 mM), pH 4.5. Chelex-100 (washed in distilled water) added at 0.1 % (w/v), mixed overnight and removed by filtration prior to pH adjustment.

MOPS buffer (Chelex-100 treated)

3-[N-morpholino]propanesulfonic acid (MOPS, 10 mM), NaCl (150 mM), pH 7.4. Chelex-100 (washed in ultrapure water) added at 0.1 % (w/v), mixed overnight and removed by filtration prior to pH adjustment.

Phosphate buffered saline (PBS) -1X

NaCl (137 mM), KCl (2.68 mM), KH_2PO_4 (1.47 mM) and Na_2HPO_4 (8.1 mM) made up in 800 ml of ultrapure water, pH adjusted to 7.4 with 1 M HCl and final volume made up to 1 litre with ultrapure water.

LDL stabilising buffer

HEPES (5 mMl/L), NaCl (150 mM/l) dissolved in ultrapure water and pH adjusted to 7.4 using NaOH.

HPLC oxidation mobile phase

Acetonitrile, isopropanol and water (all HPLC grade): 40/54/2 % by volume.

HPLC ester mobile phase

Acetonitrile and isopropanol (all HPLC grade): 30/70 % by volume.

Cryopreservation medium

RPMI-1640 medium, foetal calf serum (FCS), dimethyl sulphoxide (DMSO), (50/40/10, v/v/v).

Oil Red O Stain

Stock solution: Oil Red O (0.35 g) dissolved in isopropanol (100 ml), filtered and stored at room temperature. Working stain was freshly prepared by mixing Oil Red O stock with ultrapure water 60/40, following by filtering.

FACS buffer

HBSS with 0.6% BSA, 5mM EDTA

Cell fixing solution (4% paraformaldehyde solution)

4 g paraformaldehyde was placed in a 200 ml flask to which 60 ml PBS was added. The mixture was warmed to 60˚C in a fume hood and mixed with a magnetic stirrer. NaOH (0.5 M) was added drop-wise, until a clear solution was produced. The solution was allowed to cool and the pH was checked. Further NaOH was added to titrate to pH 7.4. The volume was made up to 100 ml with PBS.

X-Gal Staining solution

Potassium ferrocyanide (5 mM), potassium ferricyanide (5 mM), sodium chloride (150 mM, magnesium chloride 2 mM, citric acid 40 mM / sodium phosphate 50 mM, (all Sigma) titrated to pH 6.0.

2.2 LDL

2.2.1 LDL isolation

LDL (d=1.019 to 1.063 g/ml) was isolated by sequential ultracentrifugation of plasma (Havel et al., 1955, Wilkins and Leake, 1994). Venous blood was obtained from healthy volunteers (250 ml from each volunteer) who had fasted for approximately 12 h before donation. The blood was drawn into sterile 50 ml syringes, which contained Na_2EDTA (3 mM) as an anticoagulant, before being transferred to 50 ml centrifuge tubes. The blood was centrifuged at 1500 x g for 30 min at 4 °C and the plasma was collected and pooled. High density KBr solution (2.97 M KBr, 2.62 M NaCl and 297 µM EDTA; density =1.316 g/ml) was added to the plasma to adjust its density to approximately 1.019 g/ml, using equation 1 (see below) to calculate the volume of HDS to add.

$$V_{HDS} = V_{Plasma} (D_{Req} - D_{Curr} / D_{Add} - D_{Req}) \qquad (1)$$

Where V_{HDS} is the volume of HDS to be added, D_{Add} is the density of the high density KBr solution, V_{Plasma} is the volume of pooled plasma, D_{Curr} is the current density of the plasma (1.006 g/ml) and D_{Req} is the final required density.

The plasma was then dialysed against 2 litres of 1.019 g/ml density solution for 2 h at 4 °C to adjust the density to exactly 1.019 g/ml. The plasma was transferred to 35 ml Ultracrimp ultracentrifuge tubes (Thermo Fisher Scientific Inc, USA) and was centrifuged at 40,000 rpm for 18 h at 4 °C. The tubes were then sliced through the clear band of solution in their centre, using a tube slicer. The top fraction, containing VLDL and IDL was discarded and the bottom fraction containing LDL, HDL and other plasma proteins was collected and pooled.

The density of the pooled LDL-containing fraction was then adjusted to approximately 1.063 g/ml by the addition of HDS, using equation 1 to determine the volume of HDS required. The density was adjusted to exactly 1.063 g/ml by dialysing the pooled fraction against 2 litres of 1.063 g/ml solution at 4 °C for 2 h. After dialysis, the plasma was transferred to 35 ml Ultracrimp ultracentrifuge tubes and was centrifuged at 40,000 rpm for 18 h at 4 °C. The tubes were then sliced below the upper LDL layer, using a tube slicer. The lower layer was discarded and the upper LDL layer portion collected in a plastic beaker. The LDL portion was then further purified by dialysing with 1 litre of 1.063 g/ml solution at 4 °C for 2 h and centrifuging at 40,000 rpm for further 18 h at 4 °C. The pure LDL portion was then dialysed against NaCl/phosphate/EDTA buffer at 4 °C with several changes overnight with stirring to remove the KBr. Finally the LDL was filter sterilised with 0.45 µm Minisart filter and stored at 4 °C in dark (Wilkins and Leake, 1994).

2.2.2 LDL protein assay

LDL protein concentration was determined using a modified Lowry assay (Markwell et al., 1978). Standard concentrations of bovine serum albumin (BSA) protein were made up from 400 µg/ml stock with ultrapure water. LDL samples were prepared in triplicates as well. Standards and samples (125 µl) were added to assay tubes in triplicates. Lowry A reagent (1.5 ml) was added to each assay tubes and left at room temperature for ten min. Freshly prepared Lowry B reagent (150 µl) was added to each assay tube which were then incubated for 5 min at 55 °C. After allowing the assay tubes to cool to room temperature the absorbance at 650 nm was measured.

The basis for the assay is that peptide bonds in the LDL apolipoprotein B-100 or in the BSA standards form a complex with copper ions in the Lowry A reagent. The complex then reduces the phosphomolybdic-phosphotungstic Folin reagent (Lowry B), giving a blue chromophore which absorbs light at 650 nm. The protein content of the samples is then determined by comparison with the BSA standards.

2.2.3 LDL aggregation by sphingomyelinase

Freshly isolated native LDL was diluted to 2 mg protein/ml with a buffer containing NaCl (150 mM), $MgCl_2$ (10 mM) and HEPES (5 mM), pH 7.4 in 15 ml plastic tubes. Sphingomyelinase from *Bacillus cereus* (Sigma-S9396-25N) was then added, at a final concentration of 10 mU/ml and the sample was then incubated in a water bath maintained at 37 °C to induce aggregation of LDL (Walters and Wrenn, 2008). Aggregation was confirmed by dynamic light scattering in UV grade cuvettes with Zetasizer Nano Series particle sizer (Malvern Instruments, Worcestershire, UK) as well as by light scattering at 680 nm in a spectrophotometer (Khoo et al., 1988) (Figure 2.1). The aggregation was continued till the LDL particle size increased from about 25 nm to 200 nm. The sphingomyelinase aggregated-LDL (SMase-LDL) was then dialysed against phosphate buffer, pH 7.4 (which had been pre-treated with washed Chelex-100 to remove contaminating transition metals) (Van Reyk et al., 1995), and sterilised with 0.45 µm Minisart filter before use.

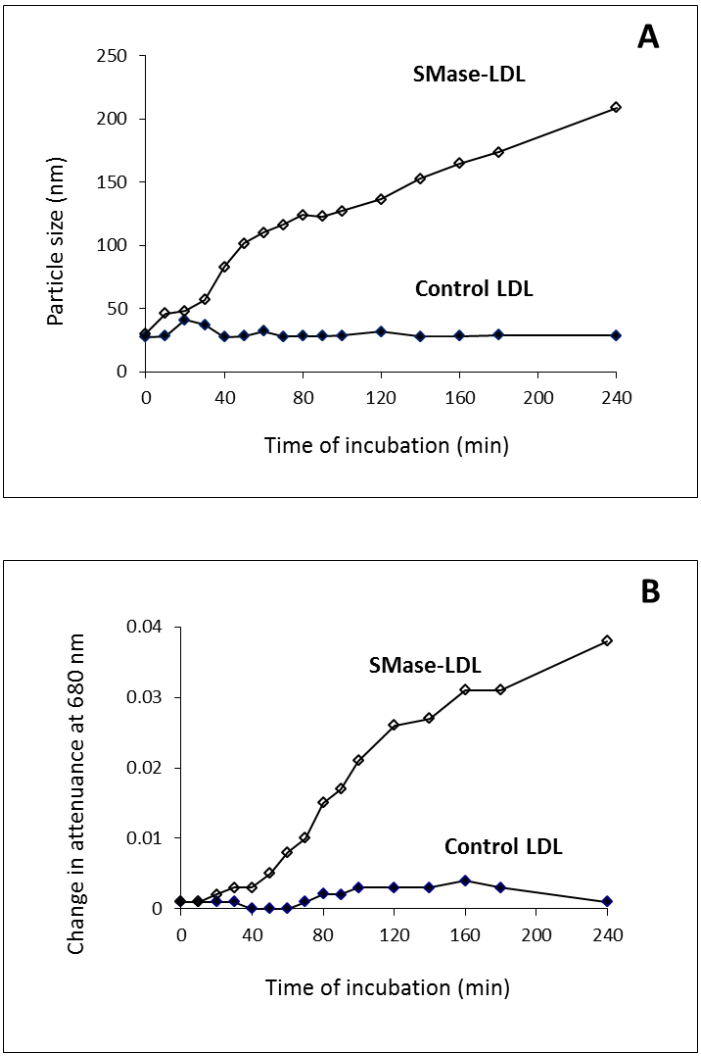


Figure 2.1 Aggregation of LDL by sphingomyelinase

LDL (2 mg protein/mL) was incubated at 37° C with sphingomyelinase (10 mU/mL). (A) LDL particle size was measured by dynamic light scattering. (B) The increase in LDL particle size caused an increase in light scattering at 680 nm (measured after dilution to 100 µg LDL protein/mL) monitored in a spectrophotometer, which is representative of four experiments.

2.2.4 Enrichment of LDL with lipid hydroperoxides

The hydroperoxide content of freshly isolated LDL was increased by adding lipid hydroperoxide in the form of 13(S)-hydroperoxyoctadeca-9Z,11E-dienoic acid (13-HPODE). The 13-HPODE treated samples were incubated for 30 min at 37 °C to allow the hydroperoxide enrichment of LDL particles before oxidation with iron. LDL was enriched to contain 30 nmol/mg or 60 nmol/mg LDL protein of 13-HPODE (final levels), corresponding to about 4-8% of the maximum content of lipid hydroperoxides in oxidised LDL (Patterson et al., 2003a).

2.2.5 LDL oxidation with iron

Native LDL or SMase-LDL (1.6 ml at a concentration of 50 µg protein/ml) was oxidised with freshly dissolved $FeSO_4$ (5 µM) at 37 °C in Chelex-100 treated NaCl/sodium acetate buffer (pH 4.5) in 15 ml polypropylene tubes (Triple Red Ltd, Bucks, UK) in the presence or absence of antioxidants. At various time points over 24 h, the oxidations in one of the tubes was stopped by addition of BHT (final concentration 80 µM, from a stock of 2 mM in ethanol) and EDTA (final concentration 4 mM). Samples were then stored in the dark at 4 °C, until use.

2.2.6 Lipoprotein-deficient serum

Lipoprotein-deficient serum (LPDS) was made according to a protocol adapted from Renaud et al. (1982). FBS (of original density 1.006g/mL) was adjusted to a final density of 1.21 g/mL by adding solid KBr. The serum was centrifuged for over 48 h at 22,000 x g, at 10 °C in Quick-Seal tubes (Beckman) using a 60Ti rotor (Beckman ultracentrifuge), with no brake. After centrifugation, the top layer of lipoproteins was carefully removed and the lower parts were recovered on a plastic cylinder on ice. The lipoprotein-free serum was dialysed in 4 changes of 1x

PBS pH 7.4 at 4 °C over the course of 24 h. The dialyzed LPDS was recovered, adjusted to the original volume of serum using dialysis buffer, and sterile-filtered using a 0.22 µm filter, divided into a number of tubes, and stored at −20 °C until use.

2.3 Assessment of LDL oxidation

2.3.1 Measurement of conjugated diene formation

Free radical attack on polyunsaturated lipids within the LDL particle leads to the formation of lipid species, known as conjugated dienes, which contain two or more conjugated double bonds. Conjugated dienes can be measured directly using a spectrophotometer as they absorb UV strongly at 234 nm (Esterbauer et al., 1989).

LDL (native or SMase-LDL, 50 µg LDL protein/ml) was oxidised with freshly dissolved $FeSO_4$, $FeCl_3$ or $CuSO_4$ (all at 5 µM final concentration) at 37 °C in a Chelex-100 treated NaCl/sodium acetate buffer (pH 4.5) or with $CuSO_4$ in Chelex-100 treated MOPS buffer (pH 7.4), in capped quartz cuvettes. The effect of antioxidants was studied by adding the antioxidants directly to the cuvettes containing LDL prior to the incubation at 37 °C. The change in absorbance at 234 nm was measured at 37 °C against reference cuvettes containing all components except LDL. Measurements were taken at one minute intervals in a double beam Lambda-2 6-cell or a Lambda Bio 40 8-cell spectrophotometer with UV winlab software (Perkin Elmer). The data were exported in excel and normalised for interpretation.

2.3.2 LDL-tryptophan fluorescence measurements

ApoB-100 contains 37 tryptophan residues that give LDL a strong fluorescence at 331 nm (E_x 282 nm). On oxidation, the LDL-tryptophan fluorescence decreases continuously indicating the LDL is being oxidised (Giessauf et al., 1995).

The decrease in lipoprotein-bound tryptophan fluorescence was measured by a Cary Eclipse fluorescence spectrophotometer using the time-drive method at an emission wavelength of 331 nm, with excitation set at 282 nm (Giessauf et al., 1995). The emission and excitation slits were set at 10 nm to obtain optimal fluorescence output. All kinetics experiments were performed as follows: LDL (native or SMase-LDL, 50 µg LDL protein/ml) was oxidised with freshly dissolved $FeSO_4$ (5 µM) at 37 °C in a NaCl/sodium acetate buffer (150 mM NaCl, 10 mM acetic acid; pH 4.5) in capped quartz cuvettes and kinetics of decrease in tryptophan fluorescence was measured every 10 min for 1200 min. The experiments were performed in the presence or absence of different concentrations of antioxidants.

2.3.3 Monitoring of ferrous iron levels

The ferrous iron chelator bathophenanthrolinedisulfonic acid (BP) was used to measure ferrous iron (Fe^{2+}) levels. BP is used to detect iron levels in blood and other biological fluids as it forms a red colour complex with Fe^{2+} at pH 2-9, with a molar absorption coefficient of 22,140 $M^{-1}cm^{-1}$, which absorbs light at 535 nm (Pieroni et al., 2001). Native LDL (50 µg protein/ml) was oxidised with freshly prepared $FeSO_4$ (5 µM) in a NaCl/sodium acetate buffer (150 mM NaCl, 10 mM acetic acid; pH 4.5) in 15 ml polypropylene tubes in the presence or absence of cysteamine in a water bath maintained at 37 °C. Aliquots of 1 ml were taken at

different time points in the new tubes, BP (3 µl of 100 mM) was added to them and absorbance was measured at 535 nm with a spectrophotometer.

2.3.4 Measurement of lipid hydroperoxides

Lipid hydroperoxides (LOOH) were measured using a method adapted from that described by el-Saadani et al. (1989). Standard concentrations of H_2O_2 were made up using ultrapure water. Samples and standards (250 µl) were added to assay tubes in triplicate and 1 ml of colour reagent (0.2 M potassium phosphate, 0.12 M potassium iodide, 0.15 mM sodium azide, 2 g/l polyethyleneglycol mono[p-(1,1',3,3'-tetramethyl-butyl)-phenyl]ether, 0.1 g/l alkylbenzyldimethylammonium chloride, 10 µM of ammonium molybdate; pH 6.0-6.2) was added to each tube. After leaving the tubes in the dark for 1 h, the absorbance at 365 nm was measured.

The basis for the assay is that peroxides in the samples or standards oxidise the iodide ions in the colour reagent to give molecular iodine. This then reacts with excess iodide ions in the colour reagent to from the tri-iodide chromophore (I_3^-) which absorbs ultraviolet radiation at 365 nm. The hydroperoxide content of the samples is then determined by comparison with the H_2O_2 standards.

2.4 HPLC analysis of lipid species

Reverse phase high performance liquid chromatography (RP-HPLC) analysis was performed on the oxidised LDL samples (prepared as described in section 2.2.5) using a method based on that described by Kritharides et al. (1993). The HPLC was performed on Agilent 1200 system and the data was analysed using ChemStation software.

2.4.1 Preparing oxidised LDL samples for HPLC analysis

Methanol (1 ml) was added to each oxidised LDL sample (1.6 ml) and the samples were vortexed for 10 seconds. Hexane (3 ml) was then added to each sample and the samples were vortexed for 30 seconds to transfer the hydrophobic lipid fraction (cholesterol, cholesteryl esters and triglycerides) of LDL into the hexane. After centrifuging the samples for 15 min at room temperature and 1500 x g, 2 ml of the upper hexane layer was transferred to a 5 ml polypropylene tube and dried in a SpeedVac. The dried samples were dissolved in 200 µl of a relevant mobile phase and stored at -20 °C until analysis.

2.4.2 HPLC analysis

Each sample (20 µl) was injected into a reverse-phase Spherisorb ODS2 column (250 x 4.6 mm, 5 µm guard column, Waters, Hertfordshire, UK). The reverse-phase columns contain hydrophobic stationary phase through which the more hydrophobic molecules are eluted first (Aguilar, 2003). Cholesterol, cholesteryl esters and their oxidation products were detected at 210 nm or 234 nm using the appropriate mobile phase and conditions as described in Table 2.1. Example chromatograms are shown in Figure 2.2 and Figure 2.3. Cholesterol, cholesteryl

esters and their oxidation products were quantified by comparison of peak area measurements with those of known concentrations of commercially available standards.

Table 2.1 Target lipid species and analysis conditions for HPLC

Mobile phase	Wavelength	Flow rate	Time	Lipid detected
(A) - 44% Acetonitrile, 54% Isopropanol, 2% water by volume	234 nm	1.2 ml/min	15 min	7-ketocholesterol, cholesteryl linoleate hydroperoxide (CLOOH)
(B) - 30% Acetonitrile, 70% Isopropanol	210 nm	1 ml/ml	15 min	Cholesterol, Cholesteryl arachidonate, Cholesteryl linoleate, Cholesteryl oleate

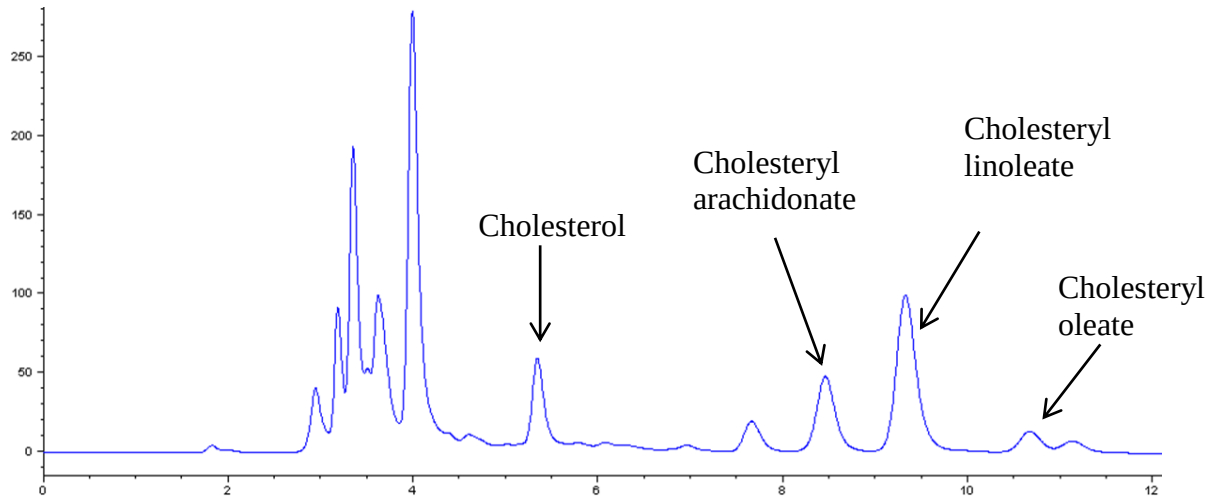


Figure 2.2 Example chromatogram showing cholesterol, cholesteryl oleate, cholesteryl arachidonate and cholesteryl arachidonate in SMase-LDL

Mobile phase 'B', a flow rate of 1 ml/min and a detection wavelength of 210 nm were used.

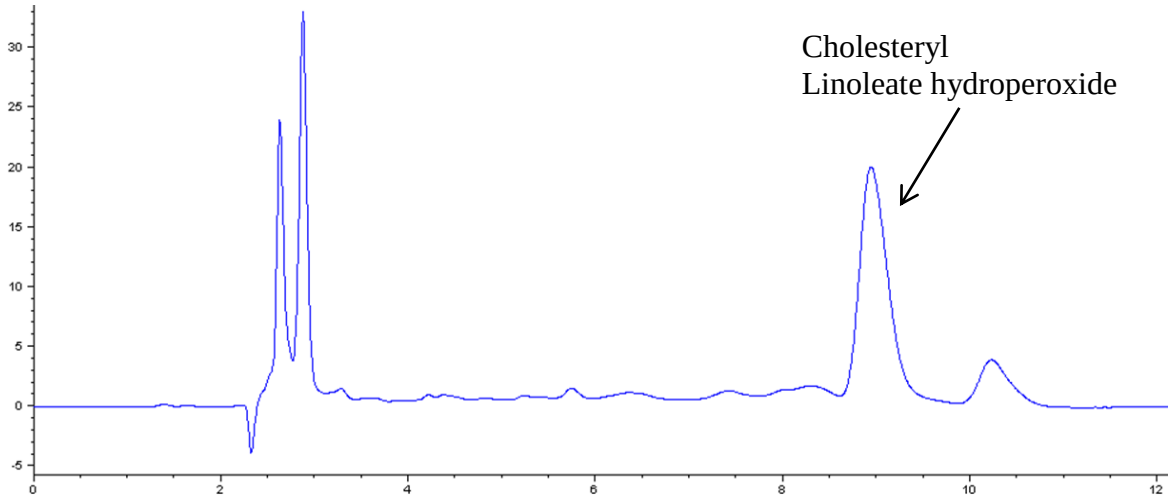


Figure 2.3 Example chromatogram showing cholesteryl linoleate hydroperoxide in SMase-LDL

Mobile phase 'B', a flow rate of 1.2 ml/min and a detection wavelength of 234 nm were used.

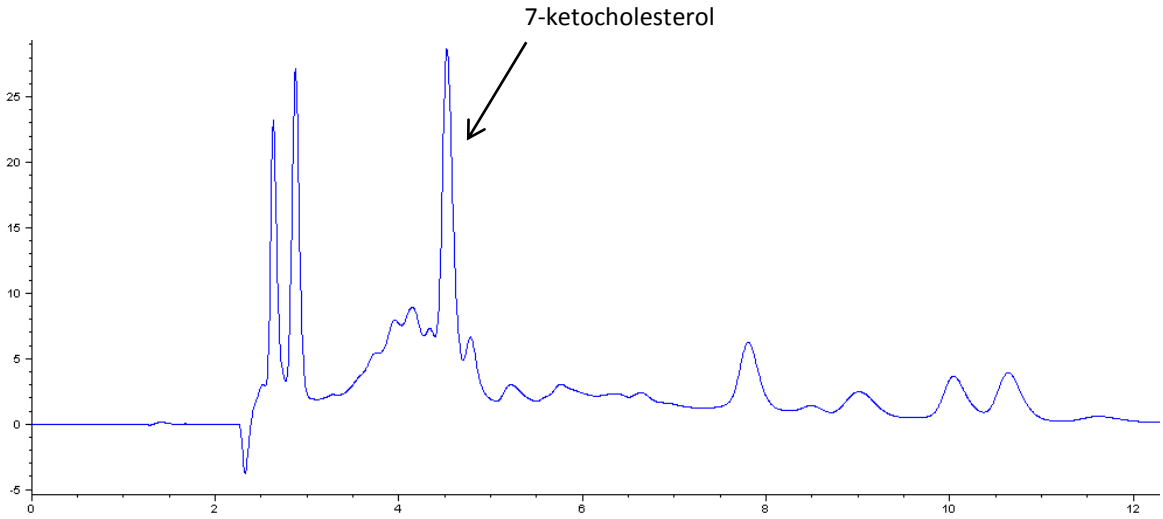


Figure 2.4 Example chromatogram showing 7-ketocholesterol in SMase-LDL

Mobile phase 'B', a flow rate of 1.2 ml/min and a detection wavelength of 234 nm were used.

2.5 Cell culture

2.5.1 Human THP-1 cells

Human THP-1 cells, a monocytic cell line derived from a monocytic leukaemia patient, were purchased from the European Collection of Cell Cultures (Salisbury, UK). Cells were counted with a Countess II FL automated cell counter (Invitrogen) and seeded at 3 x 10^5 cells/ml in RPMI-1640 medium [containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, glucose (4.5 g/l), and sodium bicarbonate (1.5 g/l)], supplemented with 10% v/v fetal calf serum (FCS), penicillin (20 IU/ml), streptomycin (20 µg/ml) and amphotericin B (0.95 µg/ml), in Corning® T75 or T150 flat bottomed flasks with filter caps. Cells were cultured at 37 °C in a humidified 95% air and 5% CO_2 atmosphere until confluent. Culture medium was changed every 3-4 days to maintain the cells at desired confluency of 3 x 10^5 cells/ml.

Cells were subcultured by transferring the cell suspension under sterile conditions into 50 ml centrifuge tubes and centrifuging at 500 X g for 5 min at room temperature. The culture medium was then removed and the pellet was resuspended in fresh pre-warmed culture medium and seeded into new flasks as above.

2.5.2 Cryopreservation of THP-1 cells

THP-1 cells were frozen in cryopreservation media. Cells were centrifuged at 500 x g for 5 min at room temperature. The culture medium was removed and the pellet was resuspended in cryopreservation media (2 ml per confluent T150). The cells were transferred to sterile cryopreservation vials and were frozen in

polystyrene containers (in order to allow them to freeze slowly) at -80 °C overnight. Vials were transferred to liquid nitrogen for long-term storage. When required, vials were thawed rapidly in a 37 °C water bath and their contents were transferred into 50 ml centrifuge tubes containing RPMI-1640 medium, in order to dilute the DMSO in the cryopreservation media. They were centrifuged at 500 x g for 5 min at room temperature, resuspended in fresh culture medium and seeded as described above.

2.5.3 Differentiation of THP-1 cells into macrophages

The THP-1 cells were subcultured in a T75 flask and allowed to reach the density of 8 x 10^5 cells per ml. To obtain large cell numbers, the cells were subcultured in T150 flasks containing a maximum of 80 ml of culture medium. To maintain the viability of the cells, the cell density was not allowed to exceed 1 x 10^6 cells per ml. Differentiation of THP-1 monocytes into macrophages was triggered by incubating the cells with phorbol 12-myristate 13-acetate (PMA) (McSwine-Kennick et al., 1991, Park et al., 2007). PMA differentiates THP-1 cells into macrophage-like cells by activating protein kinase C, ultimately resulting in cells with increased adherence and loss of proliferative activity (Richter et al., 2016). PMA inhibits THP-1 cells at G1-phase of the cell cycle, via a complex mechanism associated with the modulation of the expression of several cell cycle regulators (Traore et al., 2005). The differentiation was done in 6-, 12- or 96-well plates depending on the type of experiment by incubating THP-1 monocytes for 72 h in the culture medium containing PMA (25 ng/ml). After 72 h, the non-adherent cells were removed by washing with sterile PBS and the adherent macrophages were allowed to rest in PMA-free culture media for another 24 h before conducting experiments on them.

2.5.4 Detection of intralysosomal ceroid

To detect intralysosomal ceroid formation, the monocytes were grown on sterile glass coverslips. Prior to starting the experiments sterile coverslips (18 x 18 mm) were carefully placed in 6-well tissue culture plates. THP-1 cells were adjusted to a density of 30,000 cells per ml in fresh culture media and treated with PMA at a final concentration of 25 ng/ml. With the help of a sterile micropipette, 200 µl of the cell suspension was carefully placed on the coverslip and incubated for 72 h at 37 °C in a humidified 95% air and 5% CO_2 to allow differentiation into macrophages. On differentiation the THP-1 cells attach to the glass coverslip and the undifferentiated cells were removed by washing the cells twice with sterile warm PBS. To each well of the culture plates was added 2 ml of fresh culture medium (enough to cover the cells) and rested for 24 h before LDL treatment. Pre-warmed culture media (2 ml per well) either alone or containing native LDL (200 µg LDL protein/ml) or SMase-LDL (200 µg LDL protein/ml) was then added to the cells. After 24 h incubation, cells were washed three times with warm PBS to remove the LDL and the incubation was continued for 7 days with culture media containing lipoprotein deficient-serum with media changed every two days. To study the effects of antioxidants, freshly prepared cysteamine or probucol were added to the cells every day after the LDL was washed off.

To demonstrate ceroid, cells on the coverslips were fixed with 4% (w/v) paraformaldehyde in PBS (2 ml per well) for 10 min. After washing the wells with PBS, coverslips were transferred to a wire staining rack and were submerged in 60% (v/v) ethanol, 90% (v/v) ethanol, pure ethanol for 3 min each, in succession and then in xylene for five min to dissolve the soluble non-ceroid lipids within the cells. Xylene was replaced with ethanol, 90% ethanol (v/v) and 60% ethanol (v/v)

for 3 min each, in succession. The coverslips were then rinsed briefly in 60% (v/v) isopropanol before being stained for 10 min with Oil Red O (0.5%, w/v) in 60% (v/v) isopropanol. Coverslips were then rinsed with 60% (v/v) isopropanol and washed four times with distilled water. They were mounted onto slides using Vectashield® mounting medium (Vector Laboratories, UK), sealed with nail varnish and stored at 4 °C. Ceroid was visualised using light microscopy (Axioskop 2, Carl Zeiss Ltd) and images were captured with Axiovision software. The quantification of ceroid was done using ImageJ (National Institute of Mental Health, Bethesda, Maryland, USA) by calculating the integrated density in five randomly positioned digital images containing a total of at least 100 cells in each slide.

2.5.5 Lysosomal lipid peroxidation

The process of lipid peroxidation in the lysosomes of macrophages was studied by employing a fluorescent probe called Foam-LPO, recently synthesised by Zhang et al. (2015). Foam-LPO is a BODIPY derivative containing a conjugated diene group within its fluorophoric structure which behaves as a lipid peroxidation signalling unit and a weakly alkaline tertiary amino group which enables the probe to be protonated and hence trapped and accumulated in the lysosomes. The conjugated diene group degrades in response to lipid peroxidation causing a fluorescent spectral shift from 586 nm to 512 nm, which can be measured by flow cytometry.

THP-1 cells were incubated in RPMI-1640 (2 ml per well) (containing 10% v/v FCS) with PMA (25 ng/ml) in 12-well tissue culture plates at 1 x 10^6 cells per well for 72 h, under 5% CO_2 and 95% humidified air conditions, to differentiate into

macrophages. The macrophages were then washed and rested for another 24 h before treatment with LDL. After 24 h, they were incubated with pre-warmed culture media (2 ml per well) either alone or containing native LDL (200 µg protein/ml) or SMase-LDL (200 µg protein/ml) in the presence or absence of cysteamine for 24 h at 37 °C. The adherent macrophages were washed three times with pre-warmed PBS and then scraped into culture media using a plastic cell scraper. The culture medium was collected into 15 ml sterile polypropylene tubes and centrifuged at 500 x g for 5 min at room temperature. The cells were resuspended into 200 µl RPMI-1640 media each and transferred into a clear 96-well round bottom microplate (Greiner CellStar®) and treated with Foam-LPO (2 µM, a kind gift from Dr Xinfu Zhang, Dalian University of Technology, PRC) in RPMI-1640 for 15 min. Cells were pelleted at 500 x g for 5 min, and washed twice with HBSS and resuspended in FACS buffer. The cells were then analysed using a BD Biosciences C6 flow cytometer and the data was analysed using FlowJo software by determining mean fluorescence intensity for each histogram using untreated cells as a control. The fluorescence intensity ratio of the green channel (FL1) to red channel (FL2) (ratiometry) was taken as a measure of lysosomal lipid peroxidation.

2.5.6 Assessment of lysosomal function

The lysosomal function of cells was measured using a lysosomotropic tracking dye called LysoTracker® Red DND-99 (Life Technologies) (Pierzynska-Mach et al., 2014). THP-1 cells were incubated in RPMI-1640 (2 ml per well) tissue culture media (containing 10% v/v FCS) with PMA (25 ng/ml) in 12-well tissue culture plates at 1 x 10^6 cells per well for 72 h, under 5% CO_2 and 95% humidified air

conditions, to differentiate into macrophages. The macrophages were then washed and rested for further 24 h before treatment with LDL. The macrophages were then incubated with pre-warmed culture media (2 ml per well) either alone or containing native LDL (100 µg protein/ml) or SMase-LDL (100 µg protein/ml) in the presence or absence of cysteamine for 72 h at 37 °C, with a change of media every 24 h. After 72 h, the macrophages were washed three times with pre-warmed PBS to remove any residual LDL or cysteamine. The adherent macrophages were scraped into culture media using a plastic cell scraper, collected into 15 ml sterile polypropylene tubes and centrifuged at 500 x g for 5 min at room temperature to remove cell debris. The cells were resuspended into 200 µl RPMI-1640 media each and transferred into a clear 96-well round bottom microplate (Greiner CellStar®) and treated with LysoTracker Red (500 nM) in RPMI-1640 for 30 min at 37 °C. Cells were washed twice with HBSS, resuspended in FACS buffer and analysed using a BD Biosciences C6 flow cytometer. The data analysis was done using FlowJo software by determining mean fluorescence intensity for each histogram using untreated cells as a control.

2.5.7 Measurement of lysosomal pH in macrophages

Measurement of lysosomal pH in THP-1 cells was performed using a ratiometric lysosomal pH indicator dye called LysoSensor® Yellow/Blue DND-160 (Invitrogen) according to the protocol established by Lee et al. (2010). THP-1 cells in RPMI medium (containing 10% v/v FCS) were treated with PMA (25 ng/ml) and plated in black 96-well microplate (Corning) at 1 x10^5 cells per well for 72 h to differentiate into macrophages. The macrophages stuck to the plate were then washed with warm PBS and allowed to rest for a further 24 h in fresh culture media without

PMA. The macrophages were then treated with either no LDL or native LDL (100 µg protein/ml) or SMase-LDL (100 µg protein/ml) every 24 h for 72 h in the presence or absence of cysteamine. After 72 h, the media containing LDL and cysteamine was washed off with PBS and the macrophages were then incubated with 5 µM Lysosensor Yellow/Blue for 30 min at 37 °C with 5% CO_2.

A separate set of THP-1 macrophages were used to generate the pH calibration curve according to the protocol established by Diwu et al. (1999). THP-1 cells were differentiated in a black 96-well microplate with PMA (25 ng/ml) at a density of 1 $x10^5$ cells per well. The cells were washed three times with pre-warmed PBS and after resting for 24 h, the macrophages were incubated in MES buffer (5 mM NaCl, 115 mM KCl, 1.3 mM $MgSO_4$, 25 mM MES), with the pH adjusted to a range from 4 - 6.0. Ten min prior to the LysoSensor addition, the H^+/Na^+ ionophore, monensin and H^+/K^+ ionophore, nigericin were added to a final concentration of 10 µM each. This allowed lysosomal pH to equilibrate with the MES/HEPES buffer and facilitated the creation of a standard curve correlating lysosomal pH with the magnitude of fluorescence emission.

Both the plates were read in a Fluo Star Optima fluorometer (BMG Labtech), with excitation at 355 nm. The ratio of emission 440 nm/535 nm was then calculated for each sample and the pH values were determined from the standard plot generated (Figure 2.5).

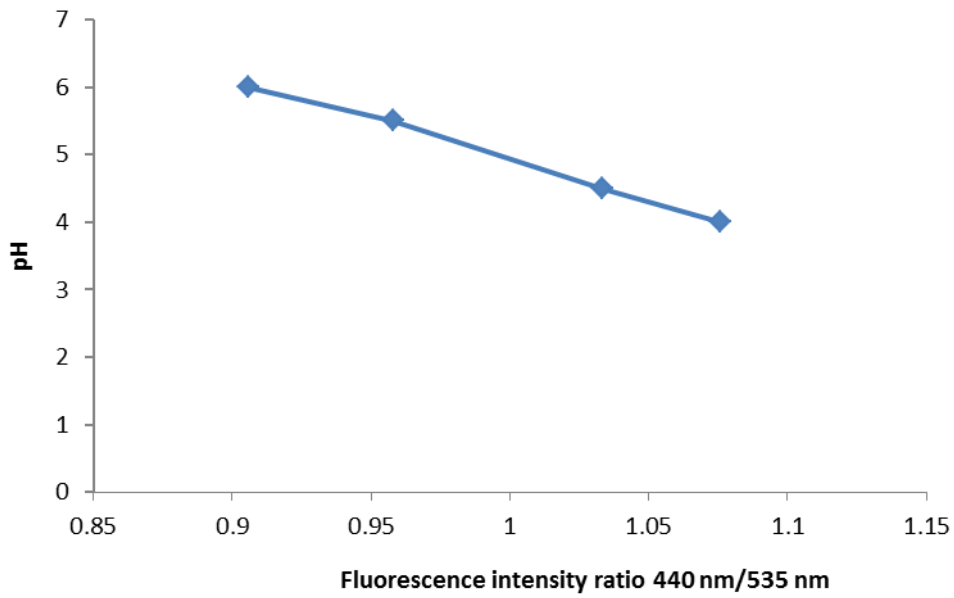


Figure 2.5 Lysosomal pH calibration curve

The ratio of emission at 440 nm and 535 nm plotted against the pH of the lysosomes.

2.5.8 Senescence associated β-galactosidase staining

Detection of senescent cells was done by using the senescence associated β-galactosidase staining procedure described by Dimri et al. (1995). THP-1 cells (4 x 10^3 per well) were differentiated into macrophages in a 12-well tissue culture plate (Corning®), with PMA as described above. The adherent macrophages were washed three times with pre-warmed PBS and rested for 24 h. The cells were then incubated in fresh culture media containing either no LDL, native LDL (100 µg protein/ml) or SMase-LDL (100 µg protein/ml) every 24 h for 72 h in the presence or absence of cysteamine. After 72 h, the medium was removed from the wells and cells were washed twice in PBS (2 ml) at room temperature. PBS was removed and cells were fixed for 3 min with 500 µl paraformaldehyde (4%) per well at room temperature. The fixative was removed and the cells were washed

with PBS. Cells were then exposed to the prepared X-gal staining solution (600 µl per well) and samples were incubated at 37 °C without CO_2 for 18 h. The staining solution was then removed and the plates were washed once with deionised water at room temperature. The cells were then washed through grading ethanol solutions of 75% (v/v), 95% (v/v), and 99.7% (v/v) using 600 µl per well for 1 min each. The plates were allowed to air dry after the last ethanol passage and the blue stained, senescent, cells were visualised using a Nikon inverted phase contrast light microscope, with images taken at ×10 magnification. Quantification of the blue stained cells was done manually (i.e. a cell was either blue or not) from 5 distinct fields of view from each well. After the wells were analysed, the staining solution was removed and 1 ml of 70% (v/v) glycerol was added to each well, allowing long-term storage of the plates at 4 °C.

2.5.9 TNF-α detection and quantification by ELISA

TNF-α expression of THP-1 cells was analysed using the Human TNF alpha ELISA Ready-SET-Go!® reagent kit (eBioscience Cheshire, UK).

THP-1 cells were incubated in RPMI-1640 (500 µl per well) tissue culture medium (containing 10% v/v FCS) with PMA 25 (ng/ml) in 24-well tissue culture plates at 3.5 x 10^5 cells per well for 72 h, under 5% CO_2 and 95% humidified air conditions, to differentiate into macrophages. The macrophages were then washed three times with warm PBS and rested for a further 24 h in fresh culture media. The rested adherent cells were then incubated in fresh culture media either alone or with native LDL or SMase-LDL (both at 50 µg protein/ml) for either 12 h or 24 h. To study the effect of cysteamine, macrophages were pre-treated with different concentrations of cysteamine for 24 h prior to LDL addition. After incubation with

LDL, the media was removed and the wells were washed three times with warm PBS. The washed cells were then treated with fresh culture medium containing ultrapure LPS (10 ng/ml) for 4 h to trigger the production of TNF-α. The medium from each well were collected and assayed immediately using the manufacturer's instructions.

Prior to carrying out the assay all components were brought to room temperature. Firstly, the capture/coating antibody (anti-human TNF-α) was diluted 1:1000 in coating buffer and 100µl was added to each well in a 96-well polystyrene plate (Nunc Maxisorp®). The plate was sealed and incubated overnight at 4 °C. The supernatant was aspirated, wells were washed three times with ELISA wash buffer and the residual buffer was removed by blotting the plate on absorbent paper. The wells were then blocked with 200 µl/well ELISA diluent and incubated at room temperature for 1h. Assay diluent was aspirated and the wells were washed once with ELISA wash buffer. Test samples of cell medium and standards of recombinant human TNF-α, were added in 100 µl to designated wells and were then incubated overnight at 4 °C for maximal sensitivity. Then samples and standard were aspirated and wells were washed three times with ELISA wash buffer. Detection antibody (100 µl; 1:1250 in ELISA assay buffer) was added to each well and the plate was incubated at room temperature for 1 h. Wells were then washed three times, and 100 µl of streptavidin-horseradish peroxidase (HRP) solution was added to each well and incubated at room temperature for 30 min. Wells were washed three times and 50 µl TMB substrate was added and the plate was incubated in the dark for 15 min or until the colour developed. Finally 50 µl of 1M H_3PO_4 stop solution was added and the colour intensity was measured at 450 nm using an LT-4000 micro plate reader and Manta software.

2.6 Statistical analysis

Unless stated otherwise, all results are expressed as means ± the standard error of the mean (S.E.M) of pooled data from 3 to 5 experiments as specified in figure legends. Comparison of two means was done using a 2-tailed unpaired Student's t test. For comparing more than two means one-way ANOVA was used followed by Turkey's post hoc analysis to measure the level of statistical significance between groups. Two-way ANOVA followed by Bonferroni post-tests were used to compare results of different HPLC time points of an LDL sample. The level of significance of difference is indicated in the graph as follows: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. ANOVA and post hoc analyses were carried out with GraphPad Prism 4 software (La Jolla, CA). A *p* value of <0.05 was taken to be a statistically significant difference.

Chapter 3-

Mechanism of LDL oxidation by iron at lysosomal pH

3.1 Introduction

Many lines of evidence suggest that oxidation of LDL plays an important role in the pathogenesis of atherosclerosis, particularly in animal models (Maiolino et al., 2013). The process of LDL oxidation is thought to occur in two main stages that are associated with a number of potentially proatherogenic events. For example, during the initial stages of LDL oxidation *in vitro*, oxidative modification of LDL cholesterol can occur in absence of any changes to apoB-100. Such LDL, now called minimally oxidised LDL, retains the affinity to the LDL receptor and induces inflammatory changes that increase local amounts of chemokines and cytokines (Cushing et al., 1990, Lee et al., 2000). The recruitment of inflammatory cells results in a variety of increased cytokines and thus continued oxidation of LDL during which the LDL protein is also modified (Parthasarathy et al., 2010). This highly oxidised LDL, now termed as oxidised LDL (OxLDL), loses recognition by the LDL receptor and shifts to recognition by scavenger receptors (oxidized LDL receptors). Hence results in the development of macrophage foam cells, the hallmark of the artery lesion (Levitan et al., 2010).

It is believed that LDL oxidation does not take place in the circulation, as serum lipoprotein lipids are well protected from oxidation by the robust antioxidant defences (Leake and Rankin, 1990) and also because LDL itself contains a number of endogenous antioxidants (Stocker and Keaney, 2004). All the four major cells types present in early atherosclerosis lesions (endothelial cells, smooth muscle cells, monocyte-macrophages and lymphocytes) have been shown to oxidise LDL *in vitro* but its relevance *in vivo* has been questionable (Reviewed in detail by Steinberg and Witztum, 2010).

Despite these extensive data suggesting an important role for oxidation modification of LDL in atherosclerosis, both the location and *the* mechanisms of LDL oxidation have remained the subject of debate (Steinberg and Witztum, 2010, Yoshida and Kisugi, 2010).

As mentioned previously, iron has been implicated as an obvious candidate for catalysing the oxidation of LDL *in vivo*. Animal studies have shown a positive correlation between iron-deposition within plaques and the severity of atherosclerosis (Lee et al., 1999). In addition, the rate of LDL oxidation by iron has been seen to increase at acidic pH. For example, under lower pH conditions, iron sequestering proteins release catalytically active free iron (Ji et al., 2012, Lee and Goodfellow, 1998) with the potential to catalyse the oxidation of LDL (Lamb and Leake, 1994, Morgan and Leake, 1995). One of the reasons for the increased rate of LDL oxidation by iron at low pH is the ability of the iron to redox cycle between the ferrous and ferric state and also the increased solubility at such lower pH (Leake, 1997, Morgan and Leake, 1993). It is also possible that if superoxide ions are involved, they would be converted to more powerful hydroperoxyl radicals at lower pH (Bedwell et al., 1989).

Redox-active iron is also present within lysosomes (Yu et al., 2003). The source of elevated lysosomal iron is likely of a combination of processes including degradation of ferritin, the turnover of iron-containing organelles and the phagocytosis of erythrocyte (Zhang et al., 2010). To this end, Kidane et al. (2006) demonstrated that the release of free iron from ferritin occurs through lysosomal proteolysis. Furthermore, iron-catalysed free radical producing reactions within lysosomes lead to peroxidative damage to the lysosomal membrane and can

cause lysosomal rupture (Persson et al., 2001, Persson et al., 2003, Yu et al., 2003). Whether iron in lysosomes exists in the ferric or ferrous state has been a matter of discussion (Collins et al., 1991), but because of the acidic and reducing environment within lysosomes the ferrous oxidation state of iron is likely to be favoured (Terman and Kurz, 2013). However, this is not to say that the lower oxidation state predominates completely. In fact, the phagolysosomes of macrophages are known to contain ferric as well as ferrous iron (Meguro et al., 2005).

Strong evidence also exist that human macrophages, when incubated with modified LDL (e.g. aggregated LDL), internalise the lipoproteins via scavenger receptors, LDL receptors or lipoprotein receptor-related proteins (LRP) and deliver it to the lysosomes for degradation (Griffin et al., 2005, Jerome, 2006). In further support of this, Wen and Leake (2007) showed that nonoxidatively aggregated or acetylated LDL is endocytosed by macrophages and then is oxidised by the iron present in lysosomes (Wen and Leake, 2007). They further established that iron is highly effective in oxidizing LDL at pH 4.5, the approximate pH of lysosomes, but very poor at doing so at pH 7.4 (Wen and Leake, 2007).

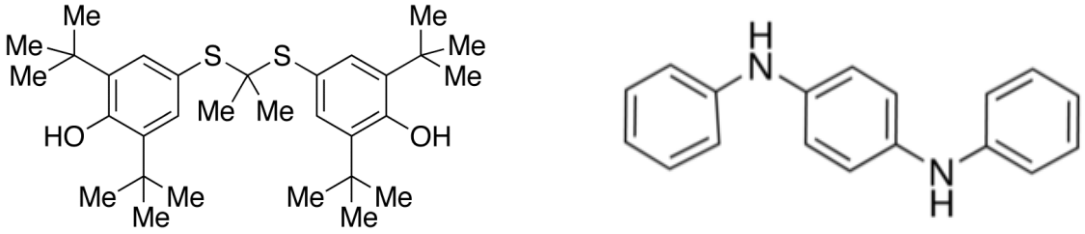
The mechanisms underlying LDL oxidation by iron *in vitro* and *in vivo* are wide ranging and complex. Various theories have been proposed for the mechanism of LDL oxidation by iron. It is also believed that iron may be able to initiate the oxidation of LDL by reacting with pre-existing lipid hydroperoxides (LOOH) in the LDL particles (Esterbauer et al., 1990). Some suggest that iron may be able to oxidise LDL via the production of hydroxyl radicals from hydrogen peroxide (Halliwell and Gutteridge, 1986, Lieu et al., 2001), while others believe that

superoxide ion is required for the initiation of LDL oxidation (Hiramatsu et al., 1987, Lynch and Frei, 1993). The generation of highly reactive hydroxyl radicals through the Fenton reaction of ferrous iron with hydrogen peroxide has also been suggested to be the mechanism by which iron may be involved in LDL oxidation (Agil, Fuller et al. 1995) however, addition of hydrogen peroxide has been shown to inhibit the oxidation of LDL by iron at lysosomal pH (Satchell, 2007).

A product of superoxide and nitric oxide, peroxynitrate, has also been suggested to oxidise lipoproteins in the artery wall and thus cause atherosclerosis (Darley-Usmar et al., 1992). However, these mechanisms are based on reactions occurring at physiological pH (Lynch and Frei, 1993, Tang et al., 2000). Under such conditions, iron is prone to olation and ultimately precipitation (Dorey et al., 1993). Hence, under more basic conditions the availability of free iron would be limited leaving the majority oxidised and unable to partake in the redox cycle. For the purpose of studying any antioxidant treatment for atherosclerosis, it is therefore essential to establish the exact mechanism by which iron oxidises LDL under conditions that are both amenable to the reactions of interest and representative of the conditions found within macrophage cells.

Probucol is a lipid-soluble, bis-tertiary butyl phenol (Figure 3.1) capable of easily donating one of the hydrogens from its phenolic-OH groups and thus acting as an antioxidant. During lipid oxidation the lipid radical ($L^{\bullet}$), lipid alkoxyl radical ($LO^{\bullet}$) or lipid peroxyl radical ($LOO^{\bullet}$) easily abstract a hydrogen atom from probucol, hence inhibiting the lipid peroxidation chain reaction (Jackson et al., 1991). Therefore if the oxidation of LDL is mediated by any of these radicals (as is believed) probucol should be able to completely inhibit the oxidation of LDL by iron (Coffey et al.,

1995). N,N'-Diphenyl-1,4-phenylenediamine (DPPD) is another strong antioxidant which is capable of donating a hydrogen atom from each of its secondary amine groups and hence prevents lipid peroxidation (Pryor et al., 1988, Takahashi et al., 1989). Both probucol and DPPD are able to inhibit copper-catalysed oxidation of LDL at physiological pH and prevent atherosclerosis in animals (Braesen et al., 1995, Niimi et al., 2013, Sparrow et al., 1992, Tangirala et al., 1995).



Probucol DPPD

Figure 3.1 Chemical structures of probucol and DPPD

Superoxide anion ($O_2^{\bullet -}$) has been proposed as a primary cell-derived oxidant with a role in LDL oxidation (Steinbrecher, 1988). A relationship between LDL oxidation by cells and superoxide production was first proposed in 1984 (Morel et al., 1984) and since then various groups have shown its role in LDL oxidation in smooth muscle cells (Heinecke et al., 1986), monocytes (Cathcart et al., 1989, Hiramatsu et al., 1987), and macrophages (Wilkins and Leake, 1990). Thiols are present in human cells at millimolar concentrations and are an important component of the cell's antioxidant defence and are known to scavenge superoxide ions in protic as well as aprotic media (Cardey et al., 2007, Feroci and Fini, 2007). Therefore, if

LDL oxidation by iron at lysosomal pH is mediated by superoxide ions, addition of thiols like cysteamine should completely inhibit this process.

Thus, the aim of this chapter is to establish the mechanisms which may be involved in oxidation of LDL by iron under lysosomal conditions.

3.2 Methods

Presence of lipid hydroperoxides in fresh LDL were measured by a tri-iodide assay as described in detail in section 2.3.4. The course of oxidation was followed by measuring the formation of conjugate dienes, kinetics of LDL-tryptophan fluorescence, specified oxidised lipids by HPLC or monitoring Fe^{2+} levels, as described in sections 2.3.1, 2.3.2 and 2.4 respectively.

3.3 Results

3.3.1 Oxidation of LDL with ferrous and ferric iron

As mentioned before, the oxidation of LDL by iron is supposed to take place only in the presence of pre-existing lipid hydroperoxides and hence LDL which has no detectable pre-existing hydroperoxides should resist oxidation by iron. Also, lysosomes of macrophages have been shown to contain catalytically active iron which redox cycles between ferrous (Fe^{2+}) and ferric (Fe^{3+}) states. Therefore, the oxidation of LDL can occur by both ferrous and ferric forms of iron and hence it is important to examine the nature of LDL oxidation with both ferrous and ferric iron.

Freshly isolated LDL was first checked for the presence of pre-existing lipid hydroperoxides before conducting any oxidation experiments. There were no lipid hydroperoxides detected in the freshly isolated LDL samples at all times. The same LDL (50 µg LDL protein/ml) was oxidised in NaCl/sodium acetate buffer (pH 4.5) at 37 °C in the presence of ferrous iron ($FeSO_4$, 5 µM) or ferric iron ($FeCl_3$, 5 µM). The course of oxidation was followed by measuring the formation of conjugated dienes and loss of LDL-tryptophan fluorescence.

Both ferrous sulphate and ferric chloride were able to oxidise fresh LDL at pH 4.5, even though no pre-existing lipid hydroperoxides were detected. The oxidation reaction proceeded at a significantly slower rate with ferric iron compared with ferrous iron and this was confirmed by both the conjugated diene measurement and loss of LDL-tryptophan fluorescence.

There was continuous formation of conjugated dienes during LDL oxidation with both ferrous and ferric iron (Figure 3.2). The time taken to reach an attenuance of 0.1 was taken as the measure of oxidation as it represents the rapid oxidation phase during LDL oxidation with iron at pH 4.5 (Satchell and Leake, 2012).

In the case of conjugated diene experiments, the time taken to reach an attenuance of 0.1 was 58 ± 1.4 min in the presence of ferrous iron compared with 85 ± 9 min with ferric iron (p<0.05), mean±SEM of three independent experiments (Figure 3.2) indicating that the lipid peroxidation occurs faster with ferrous iron.

There was a continuous loss of LDL-tryptophan fluorescence during oxidation of LDL with both ferric and ferrous iron (Figure 3.3). There was a sharp initial decrease in LDL-tryptophan fluorescence by ferrous iron oxidation. The percentage loss of tryptophan fluorescence after 150 min was 51 ± 1.3% in case of the ferrous-catalysed reaction while the loss was 25 ± 2% in case of the ferric-catalysed oxidation reaction (Figure 3.3), indicating that apoB-100 of LDL was oxidised faster with ferrous iron. These results suggest that iron is able to oxidise LDL at pH 4.5 in absence of hydroperoxides and ferrous iron is a stronger oxidising agent than ferric iron.

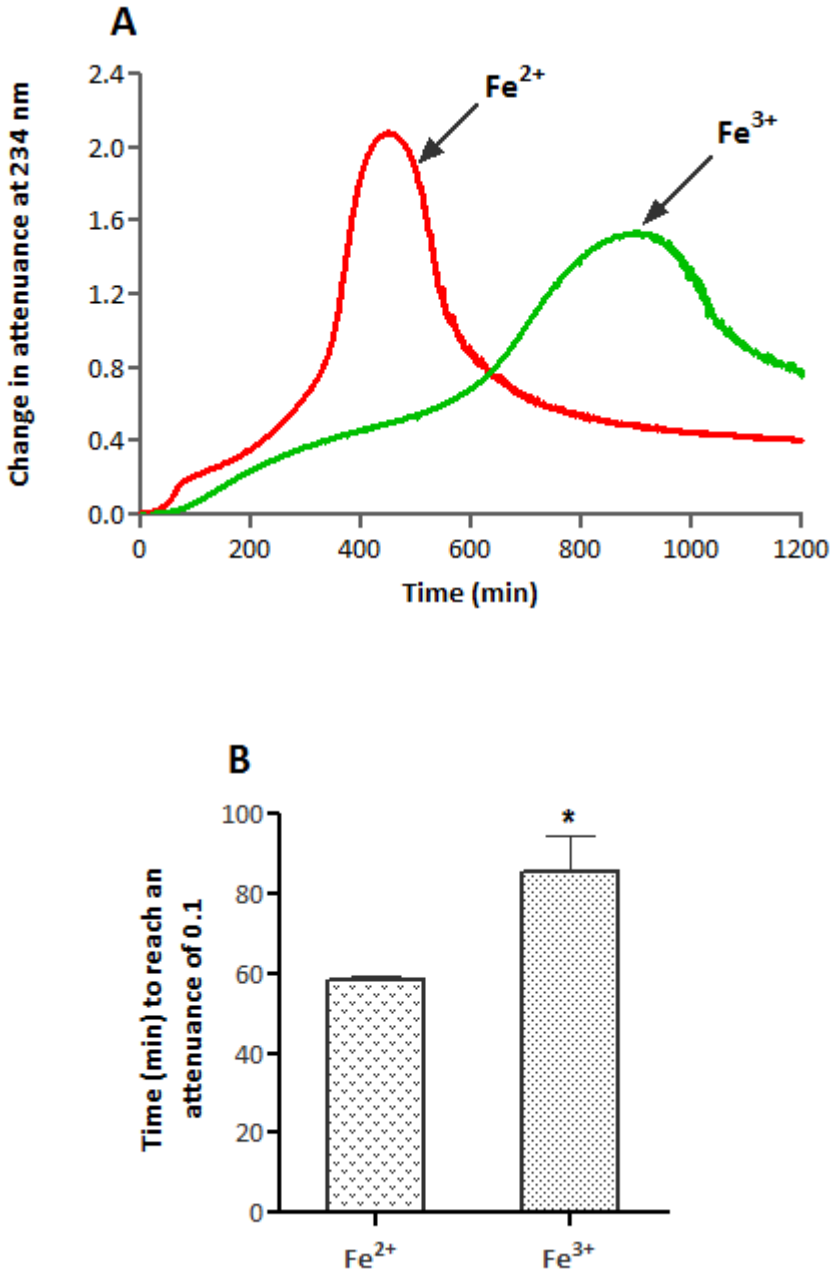


Figure 3.2 Comparison of the oxidation of LDL by ferrous and ferric iron at pH 4.5

LDL (50 µg LDL protein/ml) in sodium chloride/sodium acetate buffer (pH 4.5) was incubated with 5 µM $FeSO_4$ or $FeCl_3$ at 37 °C, in capped quartz cuvettes. (A) Oxidation of LDL cholesterol was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of 3 independent experiments. (B) Time taken to reach an attenuance of 0.1 during the oxidation process (* indicates $p<0.05$, 2-tailed unpaired Student's t test, n = 3 independent experiments).

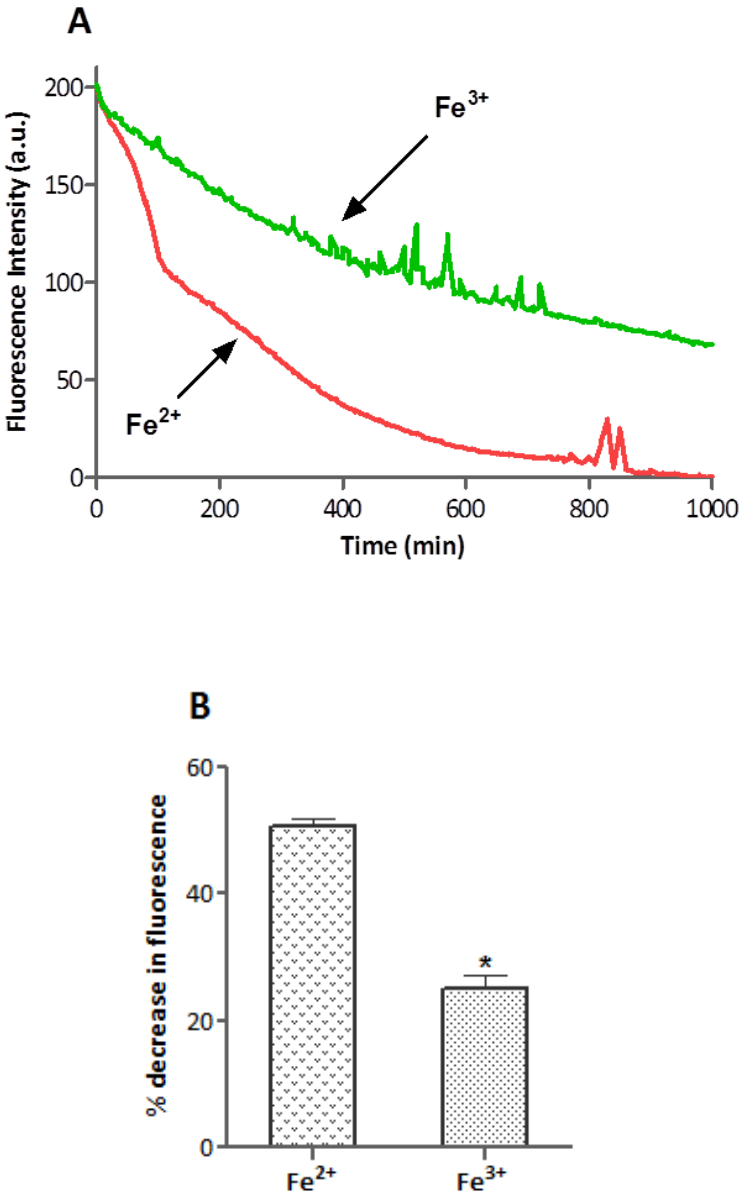


Figure 3.3 Kinetics of decrease of LDL-tryptophan fluorescence during Fe^{2+} and Fe^{3+} mediated oxidation.

LDL (50 µg protein/ml) in sodium chloride/sodium acetate buffer (pH 4.5) was incubated with either 5 µM $FeSO_4$ or $FeCl_3$ at 37 °C in quartz cuvettes. (A) The kinetics of decrease in LDL-tryptophan fluorescence was measured every 10 min at excitation wavelength of 282 nm and emission wavelength 331 nm in a spectrofluorometer. This is a representative example of at least three independent experiments. (B) Shows decrease in LDL-tryptophan fluorescence after 150 min of oxidation (* indicates $p<0.05$, 2-tailed unpaired Student's t test, n = 3 independent experiments).

3.3.2 Measurement of ferrous ion levels

Under lysosomal conditions ferrous iron would be expected to be the main species and since LDL oxidation was seen to be faster with ferrous iron, it is therefore of interest to monitor the kinetics of ferrous iron during the LDL oxidation process. The levels of ferrous iron were monitored during LDL oxidation using the ferrous iron chelator bathophenanthrolinedisulfonic acid (BP). LDL (50 µg LDL protein/ml) was oxidised in NaCl/sodium buffer (pH 4.5) at 37 °C in the presence of ferrous iron ($FeSO_4$, 5 µM) in plastic tubes and during the oxidation process samples were taken and checked for the ferrous iron levels using BP (10 mM).

During the course of LDL oxidation, ferrous iron (Fe^{2+}) was consumed in the first 120 min (Figure 3.4). There was a sharp decrease in the Fe^{2+} level during the first 60 min of oxidation where the concentration of Fe^{2+} decreased from 4.57 ± 0.6 µM to 0.40 ± 0.2 µM. This sharp decrease corresponds to the rapid oxidation phase as seen in the conjugated diene measurement and LDL-tryptophan fluorescence during LDL oxidation with iron (section 3.1.1 above). These results confirm that ferrous iron is consumed during the rapid phase of LDL oxidation and would be converted by oxidation presumably to ferric state. This probably explains why the rate of LDL oxidation slowed down afterwards.

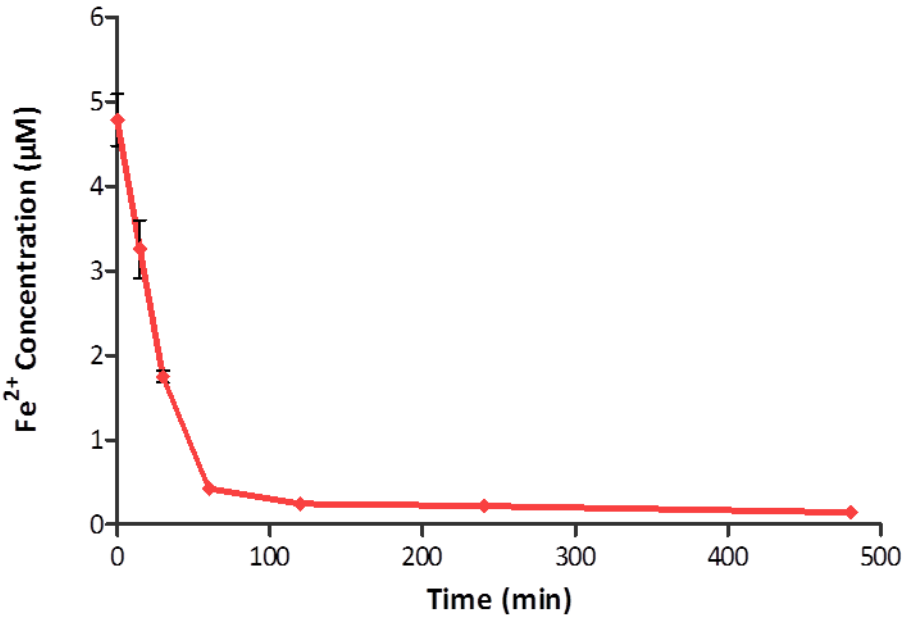


Figure 3.4 Kinetics of ferrous ion levels during LDL oxidation by $FeSO_4$ at pH 4.5

LDL (50 µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated with 5 µM $FeSO_4$ at plastic tubes maintained 37 °C. At various time points, aliquots were taken and ferrous ion concentration determined by measuring absorbance at 535 nm after treating with the iron chelator bathophenanthrolinedisulfonic acid (10 mM). This is a representative of 3 independent experiments.

3.3.3 Oxidation of hydroperoxide-rich LDL

The results above show that iron is able to oxidise freshly isolated LDL even when it contains no detectable pre-existing lipid hydroperoxides. It could be that the levels of lipid hydroperoxides required to initiate the LDL oxidation are undetectable by the current methods. Therefore, to investigate if lipid hydroperoxides play any role in LDL oxidation by iron under lysosomal pH, fresh native LDL was externally supplemented with the lipid hydroperoxide 13(S)-hydroperoxyoctadeca-9Z,11E-dienoic acid (13-HPODE). 13-HPODE is a significant component of oxidized LDL and has been shown to be present in atherosclerotic lesions (Folcik and Cathcart, 1994). It is the lipoxygenase product of linoleic acid. 13-HPODE has been shown to increase the rate of LDL oxidation with copper at pH 7.4 (Patterson et al., 2003a).

LDL (50 µg LDL protein/ml) was enriched to contain 30 nmol/mg or 60 nmol/mg LDL protein of 13-HPODE and then oxidised in NaCl/sodium buffer (pH 4.5) at 37 °C, in the presence of ferrous iron (5 µM $FeSO_4$). The oxidation process was followed by measuring the formation of conjugated dienes and loss of LDL-tryptophan fluorescence.

Addition of lipid hydroperoxide to native LDL did not increase oxidation with ferrous iron at pH 4.5 (Figure 3.5). Native LDL (no HPODE added) reached an attenuance of 0.1 in 71 ± 6.1 min which was almost similar to the LDL that was spiked with 30 nmol HPODE/mg protein 77 ± 4 min, ($P>0.05$). There was also no significant difference in the rate of oxidation between the native LDL (no HPODE) and the LDL that was spiked with 60 nmol/mg protein HPODE (84.33 ± 2.03 min). Unexpectedly, it was seen that after about 400 min, oxidation slowed down in

HPODE spiked samples indicating that oxidation of LDL with iron at pH 4.5 was decreased by lipid hydroperoxides.

The conjugated diene results were reflected in the LDL-tryptophan fluorescence experiments (Figure 3.6). LDL-tryptophan fluorescence decreased by 20.0 ± 0.36% after 150 min when the native LDL (no HPODE added) was oxidised with 5 μM $FeSO_4$ and the fluorescence decreased by 19.0 ± 0.8% ($P>0.05$) in the case of LDL to which was added HPODE at 30 nmol/mg LDL protein (Figure 3.6B). In the case of LDL which was spiked with 60 nmol/mg HPODE the LDL-tryptophan fluorescence decreased by 19.0 ± 1% ($P>0.05$) after 150 min when compared with control LDL. These results suggest that pre-existing lipid hydroperoxides and hence lipid radicals do not initiate the iron mediated LDL oxidation at lysosomal pH.

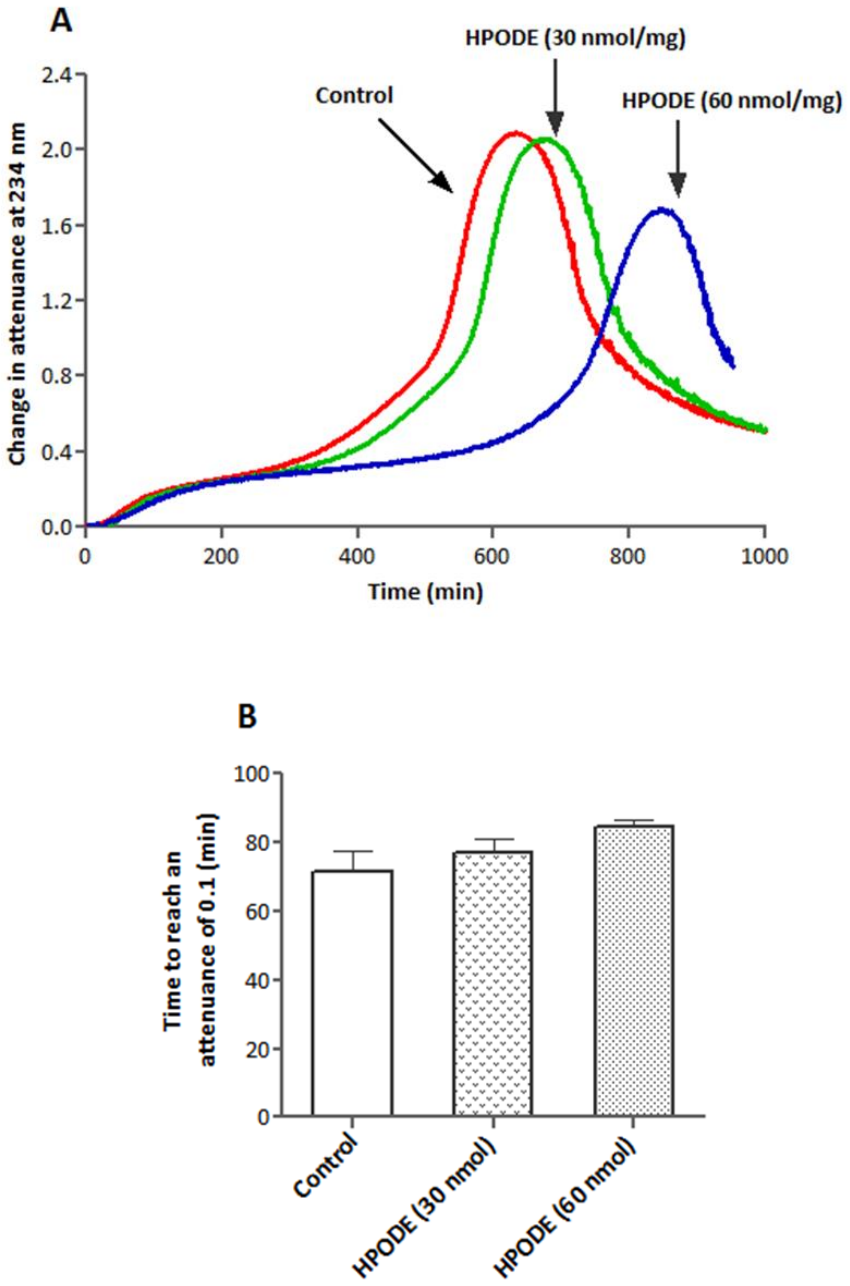


Figure 3.5 Effect of 13-HPODE on LDL oxidation with $FeSO_4$ at pH 4.5

Native LDL and HPODE-rich native LDL (30 nmol/mg or 60 nmol/mg LDL protein) were oxidised by 5 µM $FeSO_4$ at pH 4.5 in NaCl/sodium acetate buffer (pH 4.5) at 37 °C in quartz cuvettes. (A) Oxidation was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of at least three independent experiments. (B) Shows the time taken to reach an attenuance of 0.1 during LDL oxidation ($p>0.05$, ANOVA followed by Tukeys post-test, n = 3 independent experiments).

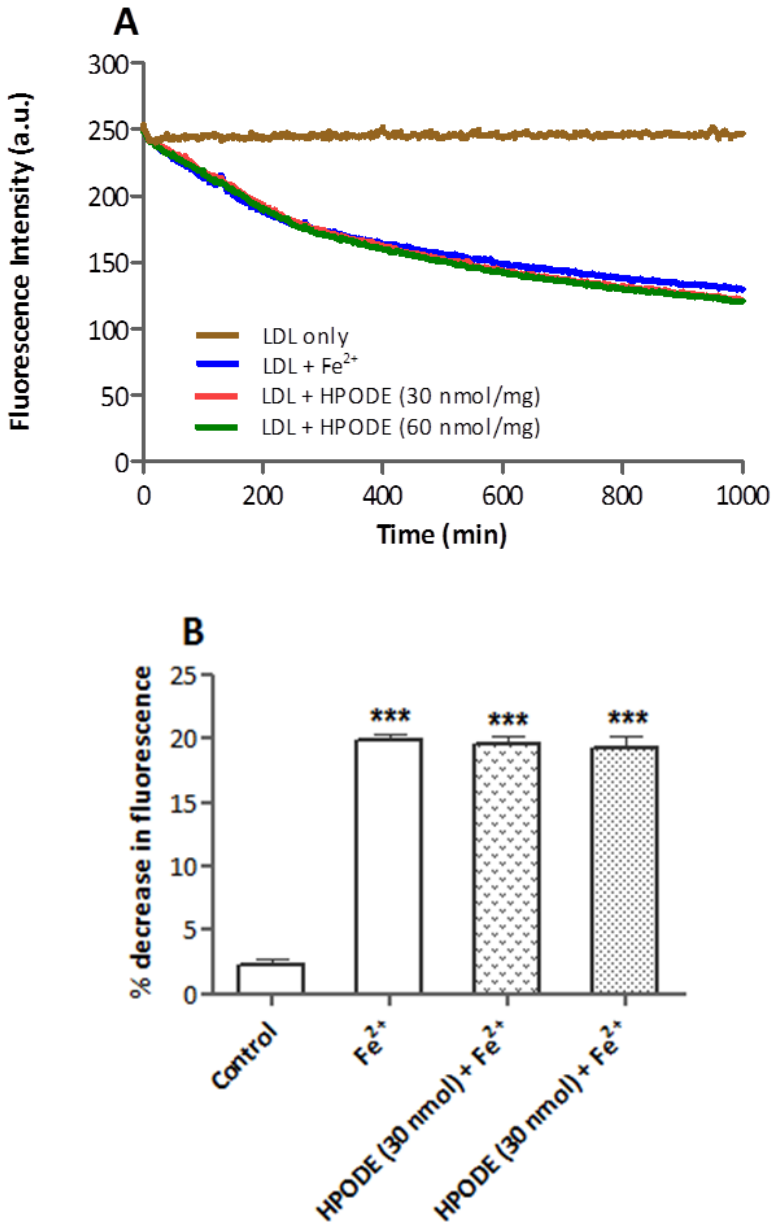


Figure 3.6 Effect of 13-HPODE on LDL-tryptophan fluorescence during Fe^{2+} mediated oxidation.

Native LDL (50 µg protein/ml) or HPODE enriched LDL (30 or 60 nmol/mg LDL protein) in NaCl/sodium acetate buffer (pH 4.5) was incubated with either 5 µM $FeSO_4$ at 37 °C in quartz cuvettes. (A) Loss of LDL-tryptophan fluorescence was measured every 10 min at excitation wavelength of 282 nm and emission wavelength 331nm in a spectrofluorometer. This is a representative example of at least three independent experiments. (B) Shows decrease in LDL-tryptophan fluorescence after 150 min of oxidation (*** indicates $p<0.001$ compared to untreated control LDL, student t-test, n = 3 independent experiments).

3.3.4 Oxidation of LDL by iron in presence of probucol

As mentioned above, oxidation of LDL at physiological pH is believed to take place by the breakdown of pre-existing lipid hydroperoxides present in LDL and probucol is able to prevent this oxidation. It was therefore of interest to examine if probucol will prevent the oxidation of LDL by iron at lysosomal pH.

LDL (50 µg LDL protein/ml) was oxidised in NaCl/sodium buffer (pH 4.5) at 37 °C in the presence of probucol (2 µM) with ferrous iron (5 µM). The course of the oxidation was followed by measuring the formation of conjugated dienes at 234 nm and loss of LDL-tryptophan fluorescence (Ex/Em = 282/331 nm).

Interestingly, probucol (2 µM) was unable to prevent initial oxidation of LDL by iron at pH 4.5. Probucol showed no antioxidant effect for about first 80 min, however the rate of oxidation slowed down later (Figure 3.7). The time required for the attenuance to reach 0.1 was 102 ± 6 min when LDL was incubated alone with iron and 116 ± 11 min when incubated in presence of probucol (p>0.05, n = 6 independent experiments).

There was a continuous loss of LDL-tryptophan fluorescence when LDL was incubated with iron and probucol (2 µM) appeared to moderately decrease the loss of LDL-tryptophan fluorescence, but the effect was not statistically significant, while the fluorescence intensity remained intact when the LDL was incubated alone at pH 4.5 (Figure 3.8A). After 80 min of LDL oxidation with iron the fluorescence intensity decreased by 24 ± 3% which was not significantly different (p>0.05) from that in presence of probucol where it decreased by 19 ± 2 % mean± SEM, unpaired t-test (Figure 3.8B).

Based on the above results it can be interpreted that the lipid radical species which are usually thought to initiate lipid peroxidation are not involved in initiating LDL oxidation at lysosomal pH. These results also suggest that perhaps the lipid peroxidation first occurs in the cholesterol ester rich hydrophobic core of LDL to which probucol has limited access. Probucol inhibits oxidation later on because the oxidation would spread from the core to the phospholipid monolayer where probucol resides.

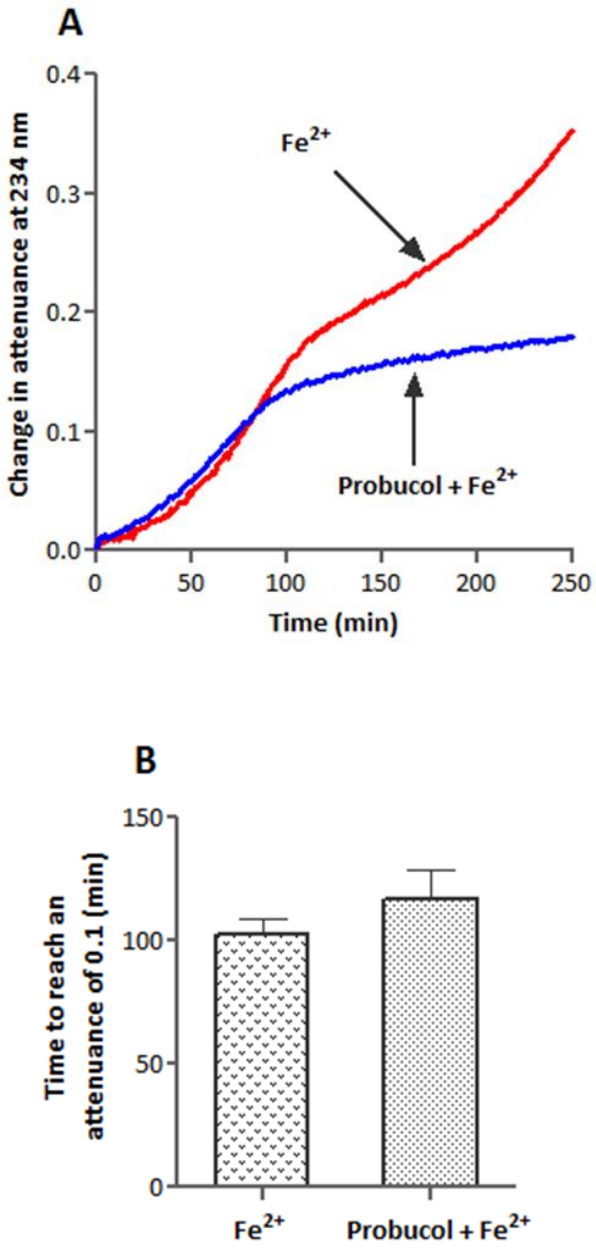


Figure 3.7 Effect of probucol on LDL oxidation by iron at pH 4.5

Native LDL (50µg protein/ml) was oxidised in presence or absence of 2 µM probucol by $FeSO_4$ at pH 4.5 in NaCl/sodium acetate buffer at 37 °C in quartz cuvettes. (A) Oxidation was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of three independent experiments. (B) Shows the time taken to reach an attenuance of 0.1 during the oxidation process (p>0.05, unpaired student t-test, n = 6).

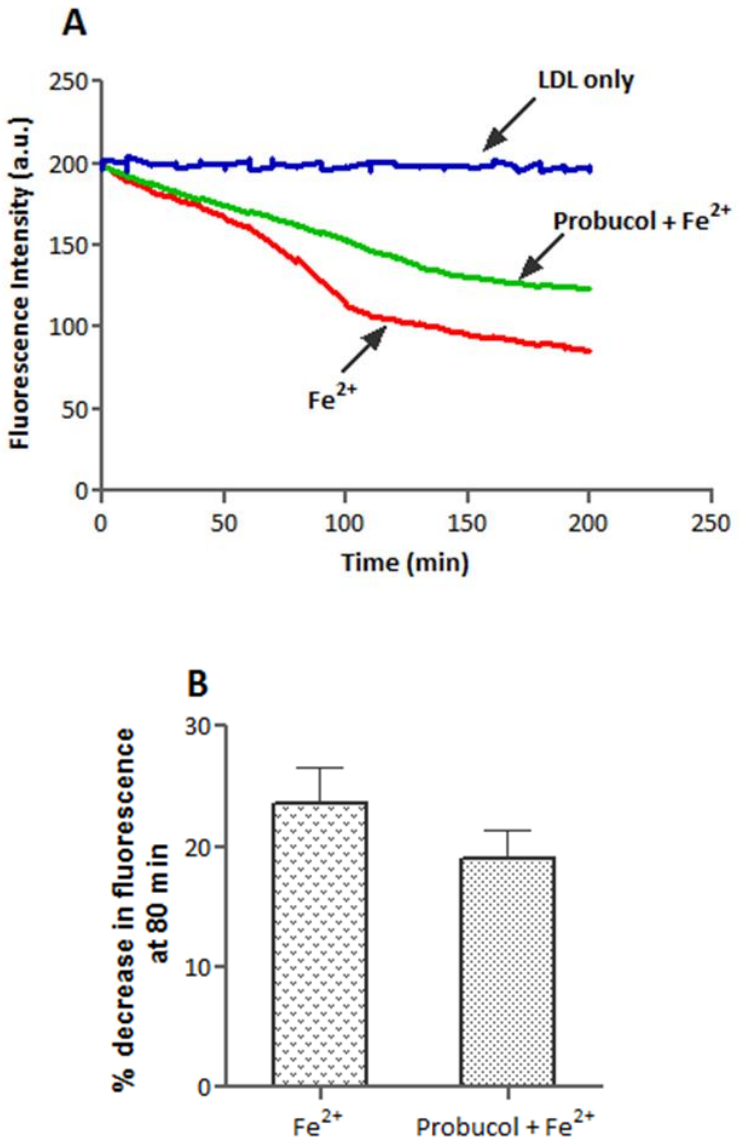


Figure 3.8 Effect of probucol on LDL-tryptophan fluorescence during LDL oxidation by iron

LDL (50 µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated alone or with 5 µM $FeSO_4$ at 37 °C in presence of absence of 2 µM probucol in quartz cuvettes. (A) Kinetics of decrease in tryptophan fluorescence was measured every 10 min at excitation wavelength of 282 nm and emission wavelength 331nm in a spectrofluorometer. This is a representative example of at least three independent experiments. (B) Shows the percentage loss of LDL-tryptophan fluorescence after 80 min of oxidation (p>0.05, t-test, n=6).

3.3.5 Oxidation of LDL by iron in presence of DPPD

DPPD is a potent antioxidant used to study the mechanisms of lipid peroxidation processes; however, it has limited application in living systems because of its mutagenic effects (Sparrow et al., 1992).

Furthermore, DPPD has a polar surface area of 24.06 (PubChem) and thus has a greater access to the hydrophobic core of LDL than probucol which has a polar surface area of 91.1 (PubChem2). Therefore, if the initial lipid peroxidation of LDL which still occurs in the presence of probucol takes place in the hydrophobic core, DPPD should completely inhibit this oxidation.

LDL (50 µg LDL protein/ml) was oxidised in NaCl/sodium buffer (pH 4.5) at 37 °C, in presence of DPPD (2 µM) with ferrous iron (5 µM). The course of the oxidation was followed by measuring the formation of conjugated dienes at 234 nm and loss of LDL-tryptophan fluorescence (Ex/Em = 282/331 nm). DPPD (2 µM) completely prevented the initial lipid peroxidation of LDL as measured by the formation of conjugated dienes (Figure 3.9). In presence of DPPD there was almost complete inhibition of conjugated diene formation initially and even after 250 min of oxidation attenuance did not reach the value of 0.1.

As expected LDL oxidation by iron caused continuous loss of LDL-tryptophan fluorescence, interestingly, in the presence of DPPD (2 µM) LDL-tryptophan lost the fluorescence from the start of the experiment (Figure 3.10). After 100 min of the oxidation process, ferrous iron decreased the LDL-tryptophan fluorescence by 49 ± 5 %, whereas in the presence of DPPD the fluorescence decreased by 61 ± 3 % compared to the control to which no iron was added.

Therefore, DPPD is able to prevent the oxidation of the lipid portion of the LDL but is unable to protect the apoB-100 part of the LDL further indicating that the lipid radicals are not involved in initiating LDL oxidation by iron. These results also suggest that the initial lipid peroxidation (as seen in the presence of probucol) probably occurs in the hydrophobic core of the LDL molecule which can be inhibited by DPPD as it has access to the hydrophobic core.

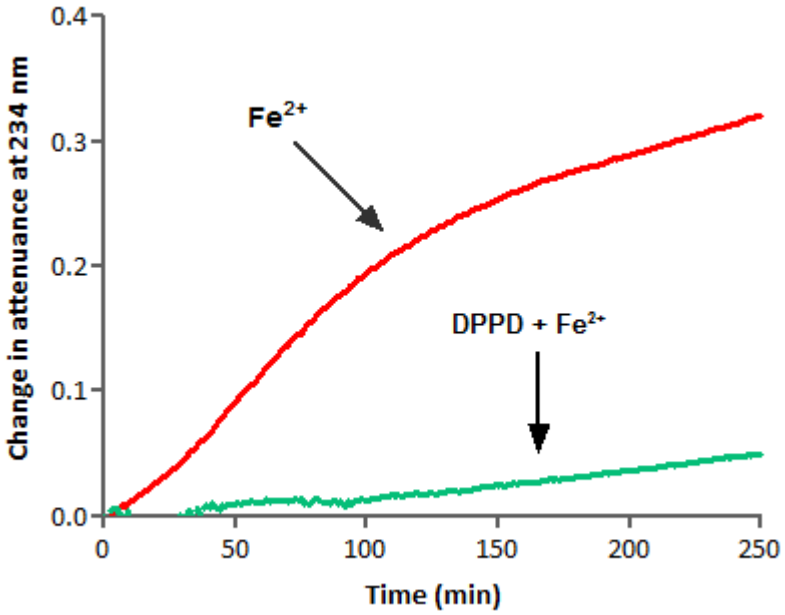


Figure 3.9 Effect of DPPD on the oxidation of LDL by iron at pH 4.5

LDL (50µg protein/ml) was oxidised in presence or absence of 2 µM DPPD by $FeSO_4$ at pH 4.5 in sodium chloride/sodium acetate buffer at 37 °C in quartz cuvettes. Oxidation was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of three independent experiments.

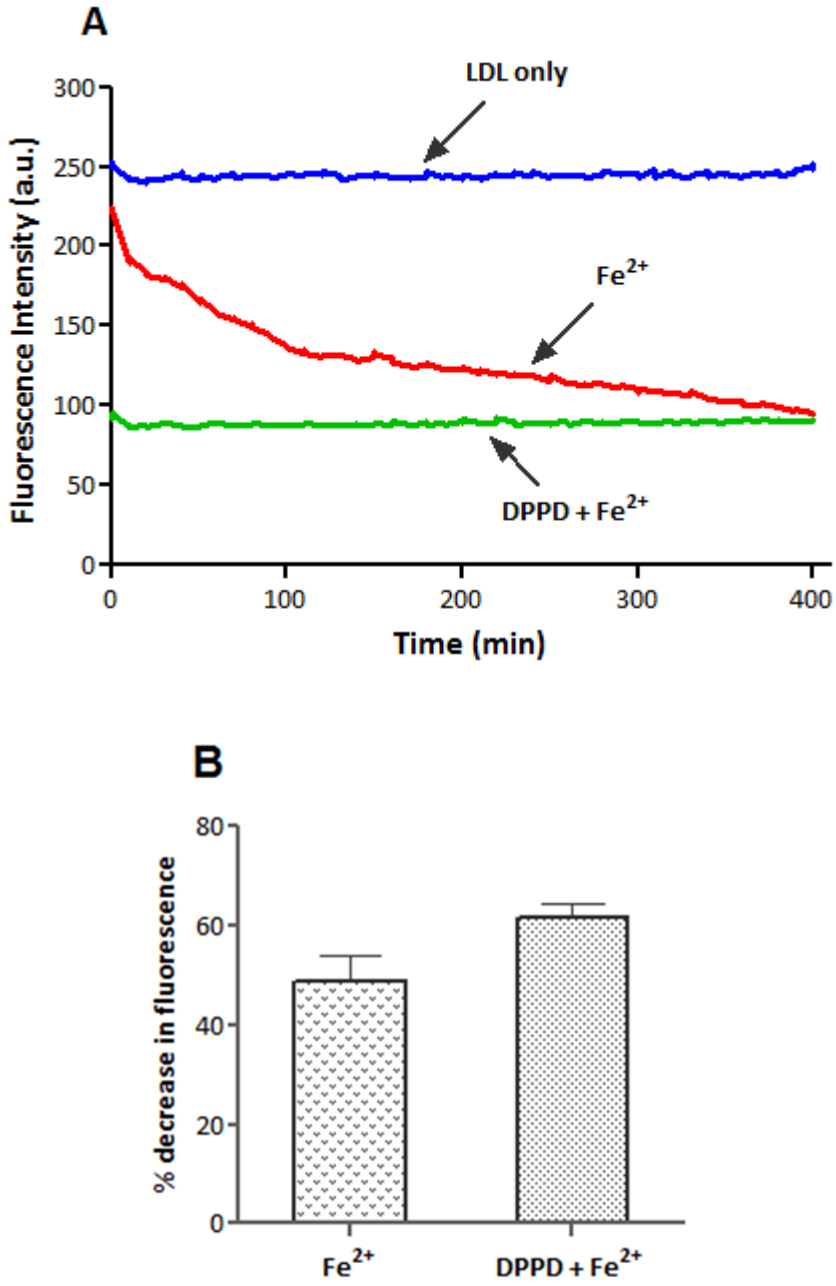


Figure 3.10 Effect of DPPD on LDL-tryptophan fluorescence during LDL oxidation by iron

LDL (50 µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated alone or with 5 µM $FeSO_4$ at 37 °C in the presence of absence of 2 µM DPPD in quartz cuvettes. (A) Kinetics of decrease in tryptophan fluorescence was measured every 10 min at excitation wavelength of 282 nm and emission wavelength 331nm in a spectrofluorometer. This is a representative example of at least three independent experiments. (B) Shows the percentage loss of LDL-tryptophan fluorescence after 100 min of oxidation (p>0.05, t-test, n=3).

3.3.6 HPLC analysis of LDL oxidation by iron in presence of probucol and DPPD

Reverse phase high performance liquid chromatography (HPLC) is an important technique which enables the quantification of the lipid peroxidation process during LDL oxidation. HPLC measures the oxidation of the hydrophobic core of LDL by measuring the decrease in cholesterol ester content of LDL particles which absorb UV light at 210nm and the formation of lipid hydroperoxides, most importantly cholesterol linoleate hydroperoxide (CLOOH), which absorbs UV light at 234nm.

Probucol has been found to concentrate more in the phospholipid monolayer surrounding the hydrophobic lipid core (Bard et al., 1994) while DPPD has been shown to penetrate the hydrophobic zones (Butterfield and McGraw, 1978, Demopoulos, 1973). Therefore, if the initial oxidation of LDL (seen in the presence of probucol) occurs in the hydrophobic core, the lipophilic antioxidant DPPD should be able to protect the oxidation of cholesterol esters in the hydrophobic core completely while probucol should have no effect on this oxidation.

LDL (50 µg LDL protein/ml) was oxidised in sodium chloride/sodium buffer (pH 4.5) at 37 °C with ferrous iron (5 µM), in the presence or absence of either probucol (2 µM) or DPPD (2 µM). Aliquots were taken at different times and were processed for HPLC analysis as mentioned in detail in the methods section in chapter 2.

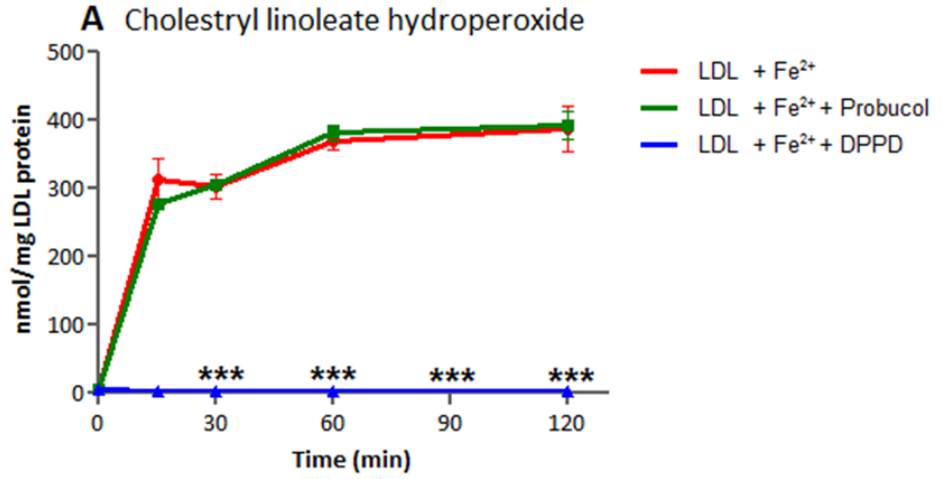
During LDL oxidation by iron there was continuous formation of CLOOH which was completely prevented by DPPD while probucol was unable to prevent it (Figure 3.11). CLOOH levels increased to 386 ± 32 nmol/mg LDL protein in control samples compared to 392 ± 21 nmol/mg LDL protein in the presence of probucol

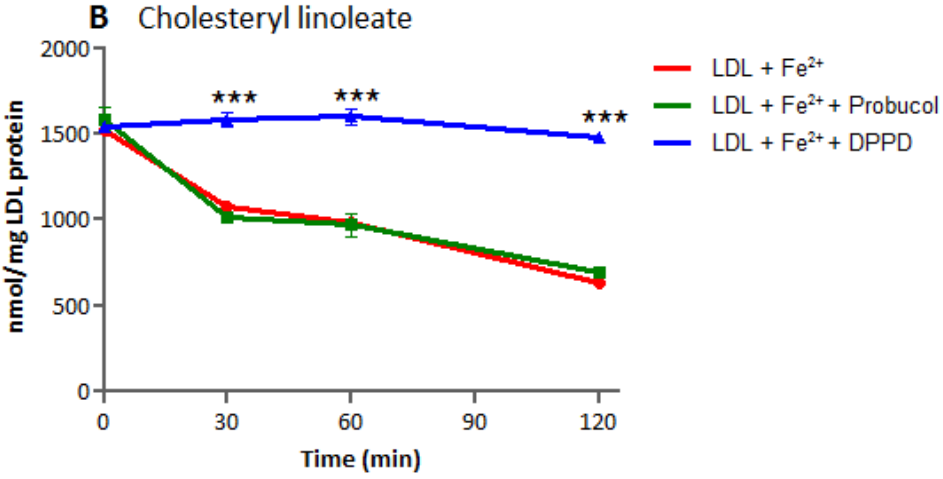
(2 μM) ($p>0.05$), whereas there was no CLOOH detected in the samples treated with DPPD (2 μM). These results agreed with the decreasing levels of cholesterol linoleate (CL). CL levels decreased from 1520 ± 20 to 635 ± 20 nmol/mg LDL protein in the first 120 min of oxidation in the control samples and in presence of probucol the levels decreased to 690 ± 0.4 nmol/mg LDL protein ($p<0.05$) after 120 min, whereas DPPD greatly prevented the oxidation and levels only decreased to 1480 ± 22 nmol/mg LDL protein (Figure 3.11)

The levels of the other major cholesteryl ester present in the core of LDL, cholesteryl arachidonate (CA), also decreased greatly both in control samples and in those treated with probucol while DPPD prevented it (Figure 3.11). As expected (Esterbauer et al., 1987), the levels of CA decreased faster than those of CL, as it contains four double bonds in its fatty acid moiety compared to two in CL. After 120 min CA levels decreased from 150 ± 0.3 to 36.0 ± 1.2 nmol/mg LDL protein in control samples and it dropped to 38.7 ± 0.04 nmol/mg LDL protein in presence of probucol ($p>0.05$) while in presence of DPPD the levels only decreased to 148 ± 3 nmol/mg LDL protein.

Figure 3.11 HPLC profile of LDL oxidation by iron in presence of probucol or DPPD

Native LDL (50 µg LDL protein/ml) was oxidised with freshly dissolved $FeSO_4$ (5 µM) in the presence or absence of either probucol (2 µM) or DPPD (2 µM) at 37 °C in a sodium chloride/sodium acetate buffer, pH 4.5. At various time points up to 2 h, oxidation was stopped by addition of EDTA (final concentration 2mM) and BHT (2mM). The samples were then assayed for cholesteryl linoleate hydroperoxide (A), cholesteryl linoleate (B) and cholesteryl arachidonate (C) by reverse phase HPLC. Graph represent mean (± SEM) of at least 3 independent experiments (*** indicates $p<0.001$ compared with the control sample to which no antioxidant was added, Two-way ANOVA followed by Bonferroni post-test).


A Cholestryl linoleate hydroperoxide
nmol/mg LDL protein
500
400
300
200
100
0
***
***
***
***
0
30
60
90
120
Time (min)
LDL + Fe2+
LDL + Fe2+ + Probucol
LDL + Fe2+ + DPPD


B Cholesteryl linoleate
nmol/mg LDL protein
2000
1500
1000
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0
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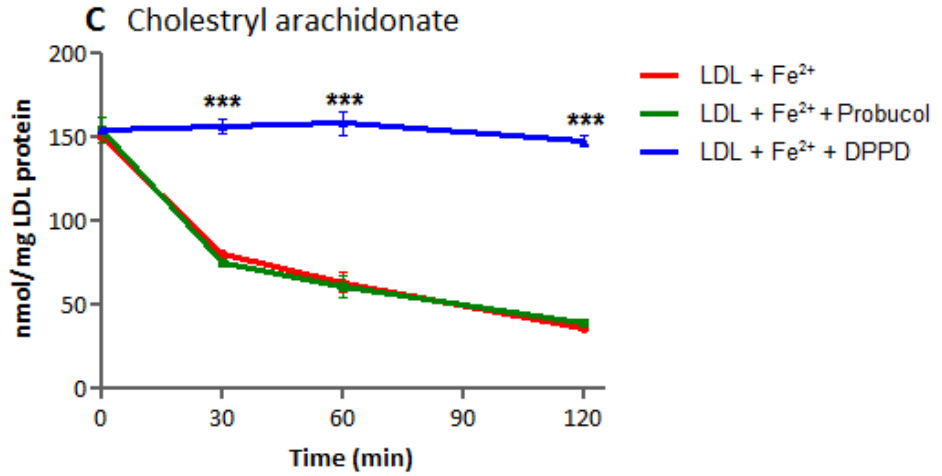
LDL + Fe2+ + DPPD


C Cholestryl arachidonate
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200
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LDL + Fe2+ + Probucol
LDL + Fe2+ + DPPD

3.3.7 Oxidation of LDL by iron in presence of cysteamine

Cysteamine belongs to a group of antioxidants called thiols. Thiol-type antioxidants constituting a class of organic sulfur derivatives (mercaptans) having the sulfhydryl functional groups (–SH) play a crucial role in protecting cells from oxidative damage (Deneke, 2000). Thiols are known to react with superoxide radical in both anionic form ($O_2^{\bullet-}$) and protonated form (the hydroperoxyl radical, $HO_2^{\bullet}$) (Cardey et al., 2007) and therefore prevent the oxidation process. To explore the hypothesis that LDL oxidation by iron at lysosomal pH is mediated by superoxide radicals, LDL oxidation was carried out in the presence of cysteamine. Cysteamine (pK_a 8.60 for the thiol group, 10.75 for the amine group) is the simplest stable amino-thiol and during the antioxidant process is converted to its disulfide dimer, cystamine (Jocelyn, 1967, Mezyk, 1995).

LDL (50 µg LDL protein/ml) was oxidised in NaCl/sodium buffer (pH 4.5) with ferrous iron (5 µM) at 37 °C, in the presence of different doses of cysteamine. The course of the oxidation was followed by measuring the formation of conjugated dienes at 234 nm and loss of LDL-tryptophan fluorescence (Ex/Em = 282/331 nm).

As expected LDL was effectively oxidised when incubated with iron alone and followed the usual pattern of conjugated diene formation, aggregation and sedimentation (Figure 3.12). However, cysteamine showed a concentration-dependent inhibition of LDL cholesterol oxidation by iron with 250 µM offering complete protection over at least 2000 min and the lower concentration of 25 µM preventing oxidation for about 500 min. The lower dose of 25 µM was chosen for statistical analysis and to conduct further experiments. In control samples to which no cysteamine was added it took 64 ± 4 min to reach an attenuance of 0.1 while in

the presence of cysteamine (25 µM) the conjugated dienes reached an attenuance of 0.1 after 526 ± 74 min ($p<0.001$), mean±SEM of 4 independent experiments (Figure 3.12).

As discussed above, control samples which were incubated without iron showed no decrease in the loss of LDL-tryptophan fluorescence and incubation with iron led to continuous loss of this fluorescence indicating oxidation of apoB-100 of LDL (Figure 3.13). Cysteamine (25 µM) was able to completely prevent the oxidation of the apoB-100 part of LDL by iron for about first 500 min. After 150 min of LDL oxidation by iron the fluorescence of LDL decreased by 61 ± 3 %, whereas in the presence of cysteamine there was only 8 ± 1 % decrease in the fluorescence ($p<0.001$), mean±SEM of 4 independent experiments.

These results show that superoxide scavengers like cysteamine are able to greatly prevent the initial oxidation of LDL cholesterol as well as apoB-100 by iron at lysosomal pH. The results so far indicate that during initial LDL oxidation by iron at pH 4.5 the oxidising agent not only attacks the protein part of LDL but is also able to penetrate into the cholesteryl ester-rich LDL core.

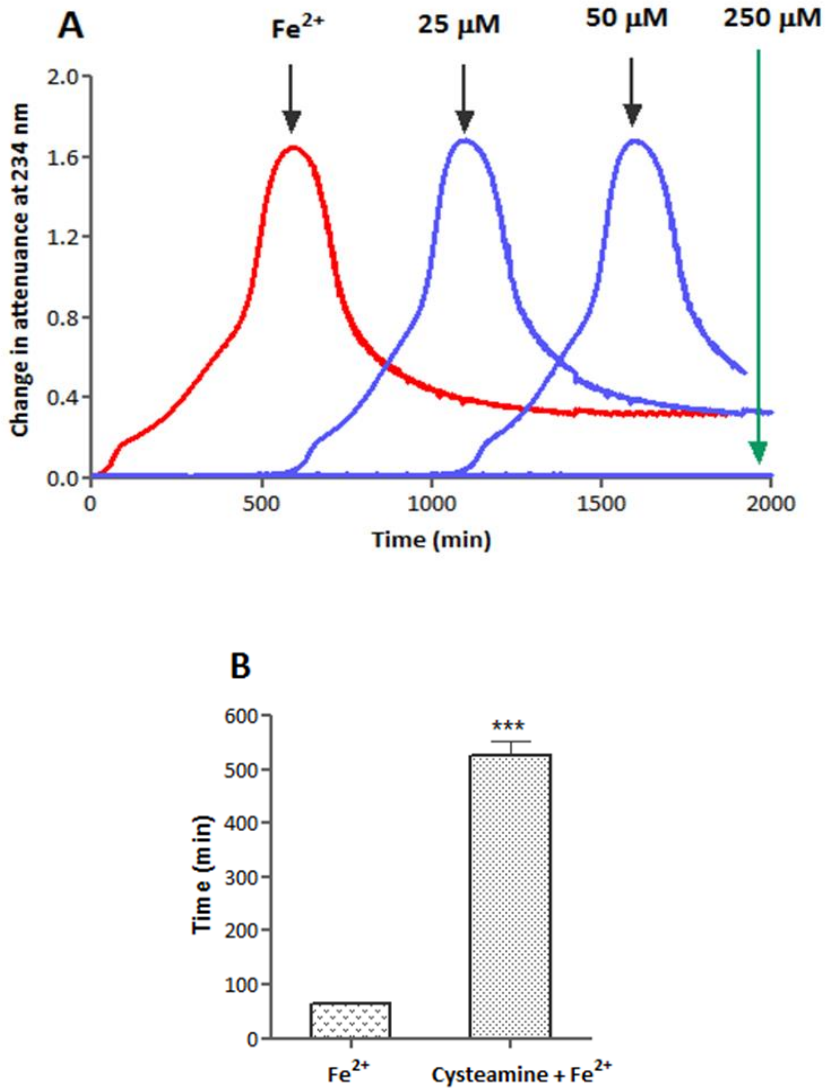


Figure 3.12 Effect of cysteamine on LDL oxidation by iron at pH 4.5

LDL (50 µg protein/ml) was oxidised in the absence or presence of cysteamine (25 µM, 50 µM or 250 µM) by $FeSO_4$ at pH 4.5 in sodium chloride/sodium acetate buffer at 37 °C in quartz cuvettes. (A) Oxidation was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of three independent experiments. (B) Shows the time taken to reach an attenuance of 0.1 during LDL oxidation in presence and absence of 25 µM cysteamine (******* indicates $p<0.001$, t-test, n=4 independent experiments).

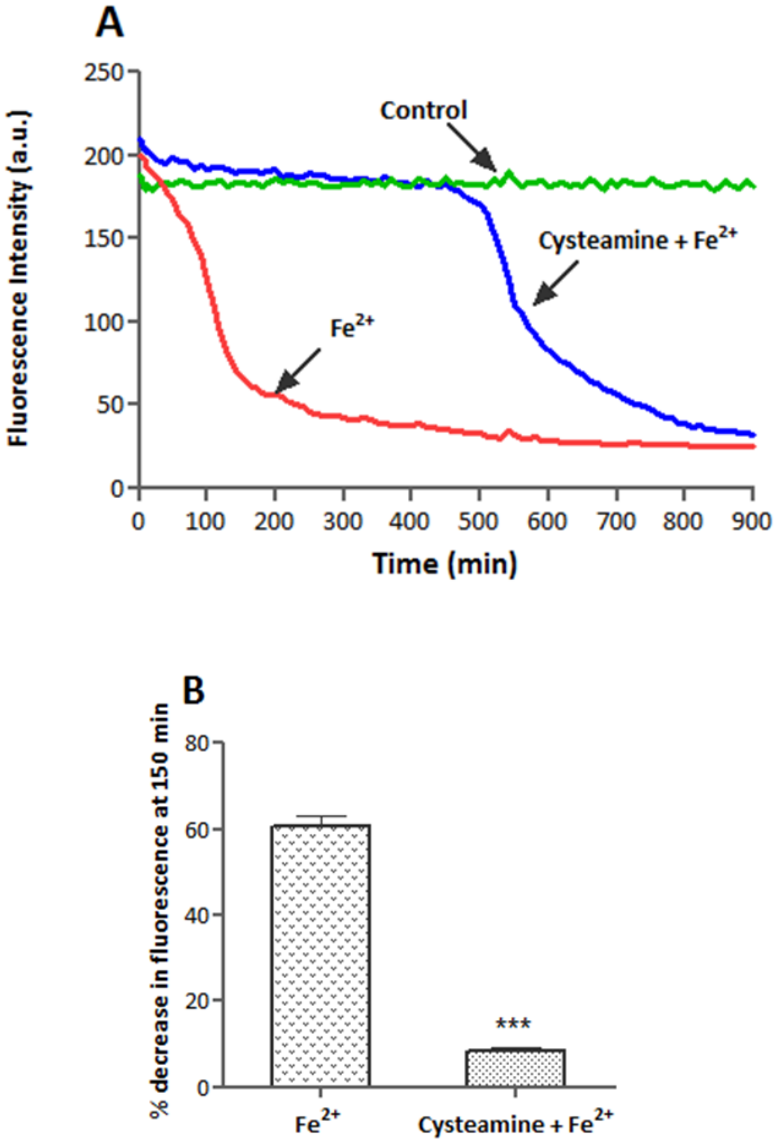


Figure 3.13 Effect of cysteamine on LDL-tryptophan fluorescence during oxidation by iron at pH 4.5

LDL (50 µg protein/ml) was oxidised in the absence or presence of cysteamine (25 µM) by $FeSO_4$ at pH 4.5 in sodium chloride/sodium acetate buffer at 37 °C in quartz cuvettes. (A) Loss of LDL- tryptophan fluorescence was measured every 10 min at excitation wavelength of 282 nm and emission wavelength 331nm in a spectrofluorometer. This is a representative example of four independent experiments. (B) Shows the percentage loss of LDL-tryptophan fluorescence after 150 min of oxidation (*** indicates p<0.001, t-test, n=4).

3.3.8 HPLC analysis of LDL oxidation by iron in presence of cysteamine

Cysteamine completely prevented the initial oxidation of LDL cholesterol by iron at pH 4.5. To further investigate the protective mechanism of cysteamine in preventing oxidation of LDL by iron, HPLC analysis was performed on the LDL samples.

LDL (50 µg LDL protein/ml) was oxidised in NaCl/sodium buffer (pH 4.5) at 37 °C with ferrous iron (5 µM), in the presence or absence of cysteamine (25 µM). Samples were taken at different times and were processed for HPLC analysis as mentioned in detail in the methods section in chapter 2.

During the oxidation of LDL with iron there was a continuous decrease in the levels of cholesterol arachidonate (CA) with time. However, cysteamine (25 µM) prevented the loss of CA levels for 6 h during LDL oxidation by iron (Figure 3.14). After 6 h of LDL oxidation CA levels in control samples decreased from 318 ± 31 to 70 ± 27, whereas in the presence of cysteamine the levels only dropped to 301 ± 30 nmol/mg LDL protein ($p<0.001$), mean±SEM of 3 independent experiments.

Similar trend was seen in the cholesterol linoleate (CL) levels. CL levels decreased continuously when LDL was oxidised by iron, however cysteamine (25 µM) completely prevented the decrease in CL levels during initial 6 h of oxidation. After 6 h CL levels only decreased from 2034 ± 139 to 2027 ± 138 nmol/mg LDL protein when LDL was oxidised in the presence of cysteamine, whereas the levels dropped to 1200 ± 100 nmol/mg LDL protein in the absence of cysteamine ($p<0.05$), mean±SEM of 3 independent experiments.

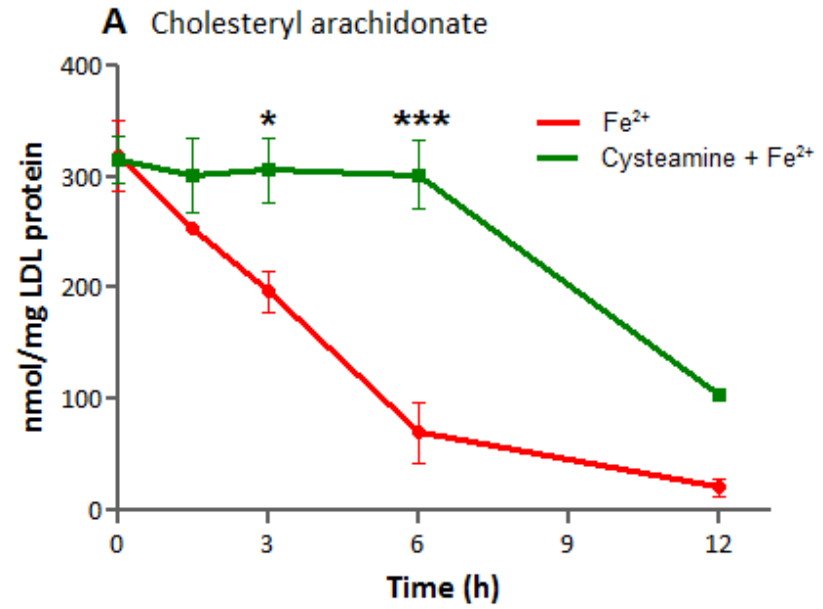
The hydroperoxide product of CL was also measured by HPLC at 234 nm. There was continuous formation of cholesterol linoleate hydroperoxide (CLOOH) during oxidation of LDL with iron and cysteamine (25 μM) significantly prevented its formation during the course of LDL oxidation. In presence of cysteamine (25 μM) no CLOOH was detected for first 6 h during LDL oxidation, and after 12 h the CLOOH levels reached 21 ± 4 nmol/mg LDL protein (Figure 3.15). However, in control samples to which no cysteamine was added the CLOOH levels detected were 134 ± 16 nmol/mg LDL protein after 3 h ($p<0.001$), 170 ± 11 after 6 h ($p< 0.001$) and 206 ± 41 after 12 h ($p<0.001$), mean±SEM of 3 independent experiments.

The levels of the cholesterol oxidation product, 7-ketocholesterol, increased slowly and then rapidly from 1.15 ± 0.5 to 14 ± 1 nmol/mg LDL protein after 12 h of LDL oxidation by iron, in agreement with previous findings (Satchell and Leake, 2012), as the cholesterol is more difficult to oxidise than the fatty acyl groups of cholesteryl ester, whereas in the presence of cysteamine the levels detected after 12 h were 3.2 ± 0.2 nmol/mg LDL protein ($p<0.001$ compared to control), mean±SEM of 3 independent experiments.

These results show that cysteamine is able to prevent the oxidation of the cholesteryl ester-rich hydrophobic core of LDL as well as the oxidation of free cholesterol, which is mainly in the surface monolayer of LDL. Together with the earlier results, where cysteamine prevented iron-mediated oxidation of LDL-tryptophan, it can be concluded that cysteamine completely prevents the initial oxidation of LDL by iron at lysosomal pH.

Figure 3.14 Effect of cysteamine on LDL cholesteryl esters during oxidation by iron at pH 4.5

Native LDL (50 µg LDL protein/ml) was oxidised with freshly dissolved $FeSO_4$ (5 µM) in the presence or absence of cysteamine (25 µM) at 37 °C in a sodium chloride/sodium acetate buffer of pH 4.5. At various time points up to 12 h, oxidation was stopped by addition of EDTA (final concentration 2mM) and BHT (2mM). The samples were then assayed for cholesteryl arachidonate (A) and cholesteryl linoleate (B) by reverse phase HPLC. Graph represent mean (± SEM) of at least 3 independent experiments (* indicates $p<0.05$, *** indicates $p<0.001$ compared with the control sample to which no antioxidant was added, Two-way ANOVA followed by Bonferroni post-test).


A Cholesteryl arachidonate
400
300
200
100
0
nmol/mg LDL protein
*
***
Fe2+
Cysteamine + Fe2+
0
3
6
9
12
Time (h)

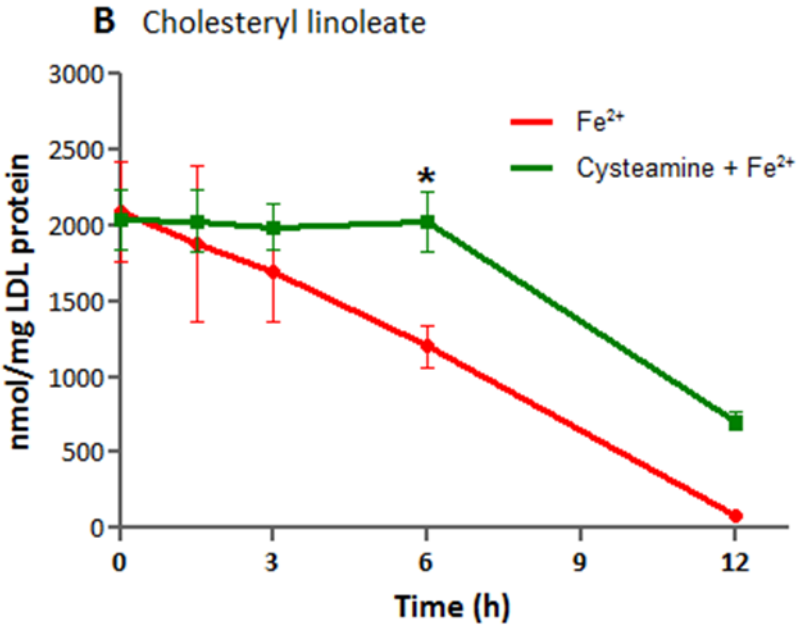
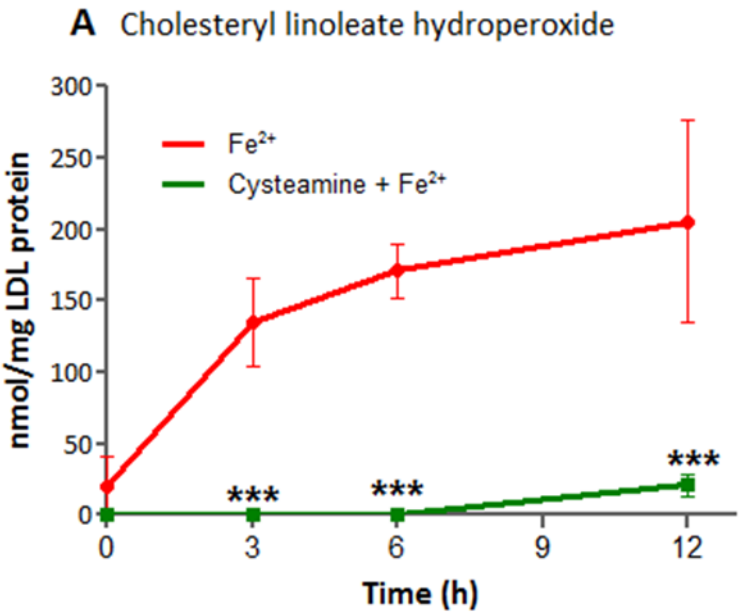
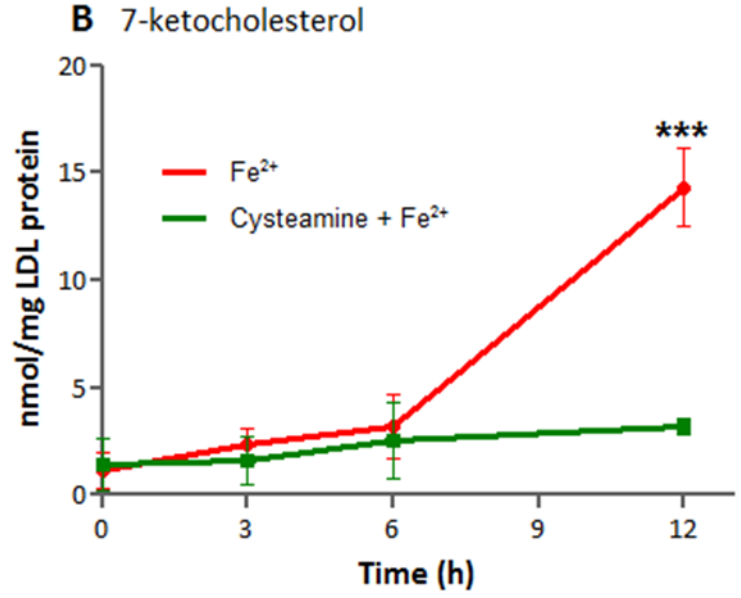

B Cholesteryl linoleate
3000
2500
2000
1500
1000
500
0
nmol/mg LDL protein
*
Fe2+
Cysteamine + Fe2+
0
3
6
9
12
Time (h)

Figure 3.15 Effect of cysteamine on LDL oxidation products during oxidation by iron at pH 4.5

Native LDL (50 µg LDL protein/ml) was oxidised with freshly dissolved $FeSO_4$ (5 µM) in presence or absence of cysteamine (25 µM) at 37 °C in a sodium chloride/sodium acetate buffer of pH 4.5. At various time points up to 12 h, oxidation was stopped by addition of EDTA (final concentration 2mM) and BHT (2mM). The samples were then assayed for cholesteryl linoleate hydroperoxides (A) and 7-ketocholesterol (B) by reverse phase HPLC. Graph represent mean (± SEM) of at least 3 independent experiments (*** indicates $p<0.001$ compared with the control sample to which no antioxidant was added, Two-way ANOVA followed by Bonferroni post-test).


A Cholesteryl linoleate hydroperoxide
300
250
200
150
100
50
0
nmol/mg LDL protein
Fe2+
Cysteamine + Fe2+
***
***
***
0
3
6
9
12
Time (h)


B 7-ketocholesterol
20
15
10
5
0
nmol/mg LDL protein
Fe2+
Cysteamine + Fe2+
***
0
3
6
9
12
Time (h)

3.4 Discussion

Iron is a transition metal capable of redox cycling between oxidation states of Fe^{2+} and Fe^{3+}. It is believed that iron is able to initiate the oxidation of LDL by reacting with pre-existing lipid hydroperoxides in the LDL particle (Esterbauer et al., 1990). Ferrous iron oxidizes LDL faster than ferric iron (Kuzuya et al., 1991, Satchell and Leake, 2012) and it is believed to be due to the faster reaction of Fe^{2+} with lipid hydroperoxides (reaction 2) than Fe^{3+} (reaction 3) (Satchell and Leake, 2012).

$$\mathbf{Fe^{2+} + LOOH \longrightarrow Fe^{3+} + LO^{\bullet} + OH^{-} \text{ (fast)}} \quad (2)$$

$$\mathbf{Fe^{3+} + LOOH \longrightarrow Fe^{2+} + LOO^{\bullet} + H^{+} \text{ (slow)}} \quad (3)$$

The problem with the above reactions is that they have been proposed on the basis of rate constants for the analogous reactions of Fe^{2+} and Fe^{3+} with H_2O_2 and not the actual lipid hydroperoxides (Dunford, 1987, Minotti and Aust, 1992). In addition, these reactions were calculated at physiological pH (Lynch and Frei, 1993, Tang et al., 2000), at which ferrous and ferric iron are insoluble and precipitate out (Dorey et al., 1993). The generation of highly reactive hydroxyl radicals through the Fenton reaction of ferrous iron with hydrogen peroxide has also been suggested to be the mechanism by which iron may be involved in LDL oxidation (Agil et al., 1995) however, addition of hydrogen peroxide has been shown to inhibit the oxidation of LDL by iron at lysosomal pH, maybe because it converts ferrous ions to the less reactive ferric ions (Satchell, 2007).

Interestingly, the results of the current study show that oxidation of freshly isolated LDL by iron at lysosomal pH takes place even when there are no pre-existing lipid hydroperoxides detected in the LDL samples. Surprisingly, enrichment of fresh

LDL with lipid hydroperoxides like 13-HPODE does not increase the rate of LDL oxidation by iron, in fact addition of higher amounts of hydroperoxide 13-HPODE slightly decreases the oxidation of LDL. Probucol efficiently prevents copper catalysed oxidation of LDL at physiological pH (Tikhaze et al., 1999) by scavenging various lipid radicals ($L^{\bullet}$, $LO^{\bullet}$, $LOO^{\bullet}$) and hence inhibiting the lipid peroxidation chain reaction. However, in the present experimental conditions this strong antioxidant was unable to protect the initial oxidation of LDL by Fe^{2+} while the more hydrophobic antioxidant DPPD completely inhibited it.

The HPLC data from this study have for the first time shown that initial oxidation of LDL by iron occurs mostly in the core of the LDL particles and probucol is unable to inhibit this oxidation but N,N′-diphenyl-p-phenylenediamine (DPPD) completely prevents it. During the course of LDL oxidation with $FeSO_4$, Fe^{2+} levels decrease sharply during the first 60 min of the reaction corresponding to the rapid phase of LDL oxidation. This would suggest that during this rapid phase of LDL oxidation, ferrous iron is almost completely consumed. Previous work carried out in this laboratory have also shown that addition of higher concentrations of Fe^{2+} shortened the lag phase and increased the rates of the rapid, slow, and aggregation phases, whereas addition of higher concentrations of Fe^{3+} had little effect (Satchell and Leake 2012). Here it was noted that the rate at which conjugated dienes form during LDL oxidation occurs faster with Fe^{2+} than with Fe^{3+}, therefore providing some further explanation of the role of Fe^{2+} in the observed results.

Tryptophan loss is one of the most significant oxidative changes in oxidised proteins due to the high susceptibility of this amino acid to reactive oxygen species

(Ronsein et al., 2011). ApoB-100 of LDL contains 37 tryptophan residues and these have been proposed to be responsible for the initiation of LDL oxidation by copper ions at pH 7.4 (Giessauf et al., 1995). The results here show that there is a loss of LDL-tryptophan fluorescence during LDL oxidation with iron at pH 4.5. Furthermore, this loss of fluorescence is unaffected by the addition of hydroperoxides and is not prevented by antioxidants like probucol and DPPD. It was also noted that the loss of LDL-tryptophan fluorescence occurs faster when LDL is oxidised with Fe^{2+} than Fe^{3+}, which implies that the presence of existing lipid hydroperoxides are not essential for the oxidation of LDL apoB-100 by iron at lysosomal pH.

Lynch and Frei (1993) suggested that reduction of Fe^{3+} to Fe^{2+} in the presence of an exogenous reductant like superoxide ($O_2^{\bullet-}$) is essential for iron-mediated LDL oxidation at pH 7.4. As mentioned before, at pH 4.5 Fe^{2+} levels dominate which during LDL oxidation is consumed and converted preferably to ferric iron with the generation of superoxide radicals (O_2^-) (4) (Grady and Chasteen, 1991, Morgan and Lahav, 2007).

$$\mathbf{Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{\bullet-}} \qquad (4)$$

Superoxide anions have been found to be weak oxidants and therefore fail to initiate LDL oxidation (Jessup et al., 1993), possibly as $O_2^{\bullet-}$ is unable to penetrate into the LDL core due to its negative charge and also because it is non-reactive in aprotic environments and for amino acids (Abreu and Cabelli, 2010, Bielski, 1985, Frimer et al., 1996, Mao and Poznansky, 1992). Around 0.3% of superoxide anion ($O_2^{\bullet-}$) present in the cell cytosol is found in its protonated form ($HO_2^{\bullet}$) (5) which is a stronger oxidant than the superoxide anion (De Grey, 2002). However, at pH 4.5

about 90% of the superoxide anion will be present as hydroperoxyl radical ($HO_2^{\bullet}$) which has a pK_a value of 4.8, closer to lysosomal pH (Bedwell et al., 1989, Bielski et al., 1985, De Grey, 2002, Kehrer et al., 2010, Liochev and Fridovich, 2001).

$$\mathbf{O_2^{\bullet-} + H^+ \longrightarrow HO_2^{\bullet}} \quad (pK_a\ 4.8) \qquad (5)$$

The hydroperoxyl radical ($HO_2^{\bullet}$) is highly reactive at acidic pH and is capable of abstracting a hydrogen atom from a polyunsaturated fatty acid (LH) (6) (Bedwell et al., 1989, Bielski et al., 1983, Liochev and Fridovich, 2001) as well as tryptophan molecules (TrpH) (7) (Armstrong and Swallow, 1969, Dubinina et al., 2002). The uncharged condition of $HO_2^{\bullet}$, unlike $O_2^{\bullet-}$, allows it to permeate into the hydrophobic core where it could initiate lipid peroxidation (Cordeiro, 2014, Gebicki and Bielski, 1981). Therefore, at lysosomal pH, $HO_2^{\bullet}$ is proposed to be the main species responsible for the oxidation of LDL and the uncharged nature of $HO_2^{\bullet}$, unlike O_2^-, will allow it to permeate into the hydrophobic core and thus initiate lipid peroxidation (Cordeiro, 2014, Gebicki and Bielski, 1981).

$$\mathbf{HO_2^{\bullet} + LH \longrightarrow L^{\bullet} + H_2O_2} \qquad (6)$$

$$\mathbf{HO_2^{\bullet} + TrpH \longrightarrow Trp^{\bullet} + H_2O_2} \qquad (7)$$

Lipid radicals and tryptophan radicals are unstable and would react with oxygen to form respective peroxyl radicals (8, 9). The lipid peroxyl radicals would then ultimately lead to lipid peroxidation chain reaction (Yin et al., 2011).

$$\mathbf{L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}}\ \text{(followed by a chain reaction)} \qquad (8)$$

$$\mathbf{Trp^{\bullet} + O_2 \longrightarrow TrpOO^{\bullet}} \qquad (9)$$

Thiol-type antioxidants play a crucial role in protecting cells from oxidative damage (Deneke, 2000). The –SH group makes thiols potent nucleophiles and ready to interact with electrophilic groups of reactive oxygen species (ROS) (Güngör et al., 2011). Thiols, like cysteamine, can scavenge superoxide radical in both its anionic form ($O_2^{\bullet-}$) and protonated form ($HO_2^{\bullet}$) (Cardey et al., 2007, Feroci and Fini, 2007, Jones et al., 2003b) and therefore prevent the oxidation reactions. The thiol antioxidant, cysteamine, in the present experimental setup completely prevents the initial oxidation of LDL by iron through inhibition of LDL lipid and protein oxidation. Cysteamine, unlike probucol, greatly protected the oxidation of the cholesterol ester-rich hydrophobic LDL core as well preventing the loss of LDL tryptophan fluorescence during oxidation with iron. During such antioxidant reactions, thiols undergo one-electron oxidation with the formation of thiyl radicals (10) and ultimately form a disulphide (11).

$$\mathbf{NH_2\text{-}CH_2\text{-}CH_2\text{-}SH + HO_2^{\bullet} \longrightarrow NH_2\text{-}CH_2\text{-}CH_2\text{-}S^{\bullet} + H_2O_2} \quad (10)$$

Cysteamine thiyl radical

$$\mathbf{2NH_2\text{-}CH_2\text{-}CH_2\text{-}S^{\bullet} \longrightarrow NH_2\text{-}CH_2\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}CH_2\text{-}NH_2} \quad (11)$$

Cysteamine thiyl radical Cystamine

Work presented in this chapter therefore suggests that the oxidation of LDL via hydroperoxyl radicals ($HO_2^{\bullet}$) is a plausible avenue at which this process may be

initiated under lysosomal conditions and also the compounds which scavenge these radicals could be potential therapeutic agents in preventing the development of atherosclerosis.

Chapter 4-

Antioxidant potential of cysteamine in preventing LDL oxidation

4.1 Introduction

Cysteamine is currently used for the treatment of the lysosomal storage disorder cystinosis, an inherited disease caused by the absence of functional cystinosin, the ubiquitous lysosomal cystine transporter (Kalatzis et al., 2001). Cysteamine reacts with intralysosomal cysteine to form the mixed disulfide of cysteamine and cysteine, which can in turn leave the lysosome via the lysine transport system (Jezegou et al., 2012). Cysteamine is also a potential drug for the treatment of various neurodegenerative disorders such as neuronal ceroid lipofuscinoses (Zhang et al., 2001) and Huntington's disease (Dubinsky and Gray, 2006).

Cysteamine belongs to a group of antioxidants called thiols. Thiols are organosulfur compounds that contain a carbon-bonded sulfhydryl group (–SH). Thiol compounds are known for general antioxidant properties, such as radical quenching, and thus play a crucial role in protecting cells from oxidative damage (Deneke, 2000). The –SH group makes thiols potent nucleophiles and ready to interact with electrophilic groups of ROS and therefore is an initial and major member of the physiological antioxidant defence system (Güngör et al., 2011). Thiols in general are mild acids although the presence of a positively charged residue, such as lysine or arginine, as well as the formation of a hydrogen bond may increase their acidity by 3-4 orders of magnitude (Copley et al., 2004). As such, the reactivity of thiols depends on their pKa values (Brandes et al., 2009, Nagy, 2013). Biologically derived thiols such as glutathione, cysteine, and homocysteine are often called biothiols. The side chain functional group: CH_2–SH

of cysteinyl residues serves as an active site for most biologically important thiols (–SH type-antioxidants) (Konarkowska et al., 2005, Luo et al., 2005).

Cysteamine is a lysosomotropic drug which greatly inhibits oxidation of LDL by iron at lysosomal pH (discussed in the previous chapter) and unpublished animal work from this laboratory has shown that, with cysteamine, there was 33% reduction in the atherosclerotic lesion area in the aortic arch of LDL receptor deficient mice which were fed an atherogenic diet. This work is based on the theory that LDL is mostly oxidised by iron in lysosomes of macrophages and because cysteamine concentrates in lysosomes by several orders of magnitude due to the protonation of its amine group, it is able to inhibit oxidation of LDL. Some other lysosomotropic antioxidants have been seen to inhibit oxidation of LDL. From the literature it was found that propranolol, amiodarone and 7,8-dihydroneopterin were promising antioxidants for LDL (Figure 4.1). Propranolol and its metabolites concentrate into the lysosomes and have been seen to prevent LDL oxidation at pH 7.4 and confer endothelial cytoprotection (Mak et al., 2006, Mak and Weglicki, 2004). The antioxidant activity of propranolol and its metabolites has been attributed to its antiperoxidative activity and by preventing release of iron into the cytosol (Dickens et al., 2002).

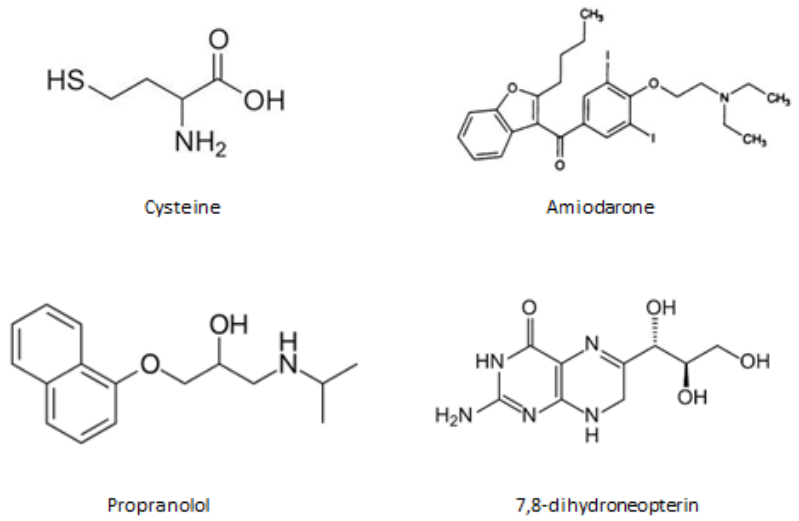


Figure 4.1 Chemical structures of cysteine, amiodarone, propranolol and 7,8-dihydroneopterin.

Amiodarone is a lipophilic antiarrhythmic/antianginal drug which is able to influence the physicochemical status of biological lipid components by binding to lipoproteins. Amiodarone and its primary metabolite, desethylamiodarone, have been reported to prevent copper-catalysed oxidation of lipoproteins at pH 7.4 in a dose-dependent manner. The activity has been suggested to be due to stabilisation of the lipid element of lipoprotein (Lapenna et al., 2001, Ribeiro et al., 1997). 7,8-Dihydroneopterin is a pteridine secreted by human macrophages when stimulated with interferon-gamma during inflammation, as a protective mechanism (Gieseg et al., 2009). 7,8-Dihydroneopterin has been shown to prevent copper mediated oxidation of native LDL (Greilberger et al., 2004) and to protect human macrophages by inhibiting oxidised LDL-dependent generation of lipid peroxyl radicals (Baird et al., 2005). A cysteine-specific lysosomal transport system enables the delivery of thiol into lysosomes (Pisoni et al., 1990). Cysteine has also

been shown to prevent LDL oxidation by copper at pH 7.4 (Patterson et al., 2003b) and lipid peroxidation in animals (Saravanan et al., 1995).

Cysteine, because of its thiol group is an effective scavenger of free radicals as well as a major contributor to maintenance of the cellular glutathione status in skeletal muscle cells (Kerksick and Willoughby, 2005). Most of the *in vitro* studies on these antioxidants were carried out at pH 7.4 and given the lysosomal LDL oxidation theory, it is therefore of interest to study the effect of these antioxidants on LDL oxidation by iron at lysosomal pH.

Copper is the most commonly used transition metal to study the susceptibility of LDL towards oxidation and LDL oxidised by copper at pH 7.4 is the most widely used oxidised LDL in many *in vitro* and *in vivo* experiments (Proudfoot et al., 1997, Roland et al., 2001). Higher copper levels in serum are considered as one of the risk factor for atherosclerosis (Tasic et al., 2015a). Ceruloplasmin, a copper-carrying protein in plasma, has been shown to oxidise LDL *in vitro* at pH 7.4 and in smooth muscle and endothelial cells by a superoxide-dependent mechanism (Mukhopadhyay et al., 1996). Furthermore, the rate of LDL oxidation by ceruloplasmin in macrophages has been seen to increase significantly at acidic pH (Lamb and Leake, 1994, Leake, 1997). Recently copper has been detected in lysosomes as well (Ren et al., 2015). Hence, we have examined whether cysteamine would be able to prevent the oxidation of LDL by copper at interstitial pH (pH 7.4) and lysosomal pH (pH 4.5).

LDL oxidation and aggregation are known to increase the atherogenic potential of LDL (Panasenko et al., 2007) and both these forms of modified LDL have been reported to be present in the plasma of atherosclerotic patients (Bancells et al.,

2010b, Pawlak et al., 2013). Oxidation of LDL by iron at lysosomal pH has been seen to cause spontaneous aggregation of LDL (Satchell and Leake, 2012). Therefore, an effective, antioxidant in addition to inhibiting the oxidation of LDL, should be able to prevent the aggregation of LDL.

Data are presented in this chapter that show that cysteamine is much better at preventing LDL oxidation by iron at lysosomal pH than other lysosomotropic antioxidants like cysteine, propranolol, amiodarone and 7,8-dihydroneopterin. Also, cysteamine is able to prevent the oxidation LDL by copper and the aggregation of LDL during oxidation by iron.

4.2 Methods

LDL oxidation was studied by measuring the conjugated diene formation as described in section 2.3.

4.3 Results

4.3.1 Comparison between antioxidant activity of cysteamine and cysteine on LDL oxidation by iron at pH 4.5

Cysteamine is the simplest stable amino-thiol and a degradation product of coenzyme A (Besouw et al., 2013). Both cysteine and cysteamine are known antioxidants because of the presence of a thiol group in them. The aim of this experiment was to compare the effects of these two thiols in inhibiting LDL oxidation by iron at lysosomal pH. In order to compare the antioxidant activity of cysteine with cysteamine, native LDL (50 µg protein/ml) in sodium chloride/sodium acetate buffer (pH 4.5) was incubated with 5 µM $FeSO_4$ at 37°C in quartz cuvettes, in the presence or absence of either cysteamine or cysteine (final concentration 25µM). The course of oxidation was followed by measuring the formation of conjugated dienes at 234 nm.

Both cysteine and cysteamine inhibited the initial oxidation of LDL (Figure 4.2). However, in the presence of cysteine, the time taken during LDL oxidation by iron to reach an attenuance of 0.1 was 225 ± 22 min which was not statistically significant when compared to the control (78 ± 11 min) to which no antioxidant was added. On the other hand, it took 525 ± 57 min for the attenuance to reach 0.1 when LDL oxidation was conducted in the presence of cysteamine (mean±SEM, ANOVA followed by tukey's test, $p<0.01$).

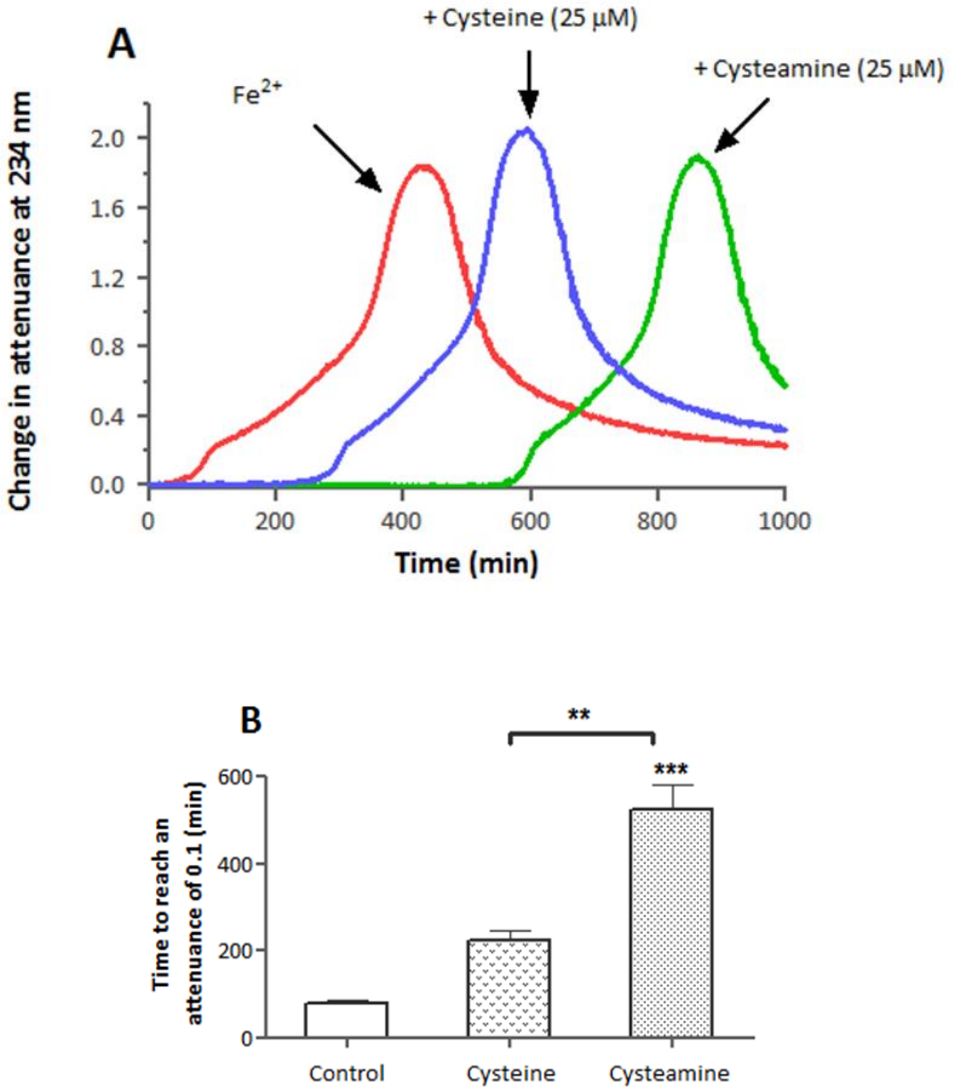


Figure 4.2 Comparison of antioxidant effect of cysteine and cysteamine on LDL oxidation by iron at pH 4.5

LDL (50 µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated with 5 µM $FeSO_4$ at 37°C in quartz cuvettes in the presence or absence of either cysteamine or cysteine (final concentration 25 µM). (A) Oxidation was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of at least three independent experiments. (B) The time taken to reach an attenuance of 0.1 is shown. (** indicates $p<0.01$ between two antioxidants, *** indicates $p<0.001$ compared to control, ANOVA followed by tukey's test).

4.3.2 Comparison of antioxidant activity of cysteamine with other known lysosomotropic antioxidants

In order to compare the antioxidant effect between cysteamine and lysosomotropic agents like propranolol, amiodarone and 7,8-dihydroneopterin on LDL oxidation by iron at lysosomal pH, native LDL (50 µg protein/ml) in sodium chloride/sodium acetate buffer (pH 4.5) was incubated with 5 µM $FeSO_4$ at 37°C, in presence or absence of either cysteamine or propranolol or amiodarone or 7,8-dihydroneopterin (final concentration 25 µM). The course of oxidation was followed by measuring the formation of conjugated dienes at 234 nm.

The oxidation of native LDL by iron was inhibited significantly by cysteamine (Figure 4.3). During oxidation of LDL by iron, the time taken to reach an attenuance of 0.1 was 63 ± 4 min when incubated alone whereas addition of cysteamine (25 µM) increased the time to 590 ± 17 min (p<0.001, n=3), which is about a 9 fold increase. Addition of propranolol (25 µM) slowed down the oxidation LDL at the later stages of the oxidative process, but there was no significant effect on the initial oxidation of LDL by iron at pH 4.5 (Figure 4.3). In the presence of propranolol, the attenuance of 0.1 was reached in 89 ± 6 min, about a 1.4 fold increase, but was not statistically significantly different than the controls.

The presence 25 µM of 7,8-dihydoneopterin (7,8-DNP) had absolutely no effect on the oxidation of LDL by iron at lysosomal pH (Figure 4.4). The time taken to reach an attenuance of 0.1 was 61 ± 0.4 min, which was similar to that of the controls, 63 ± 3 min, to which no antioxidant was added. However, as expected cysteamine completely inhibited the initial oxidation of LDL and increased the time to reach an

attenuance of 0.1 to 582 ± 16 min (mean±SEM, ANOVA followed by tukey's test, $p<0.001$, n=3).

Addition of amiodarone (25 µM) did not prevent the oxidation, but gave an initial pro-oxidant effect for the first 450 min (Figure 4.5). In the presence of amiodarone, the attenuance of 0.1 was reached after 30 ± 8 min, whereas in the controls to which no antioxidant was added, the attenuance reached 0.1 after 63 ± 3 min. However, cysteamine completely inhibited the initial oxidation and increased the time to reach an attenuance of 0.1 to 576 ± 19 min (mean±SEM, ANOVA followed by tukey's test, $p<0.001$, n=3).

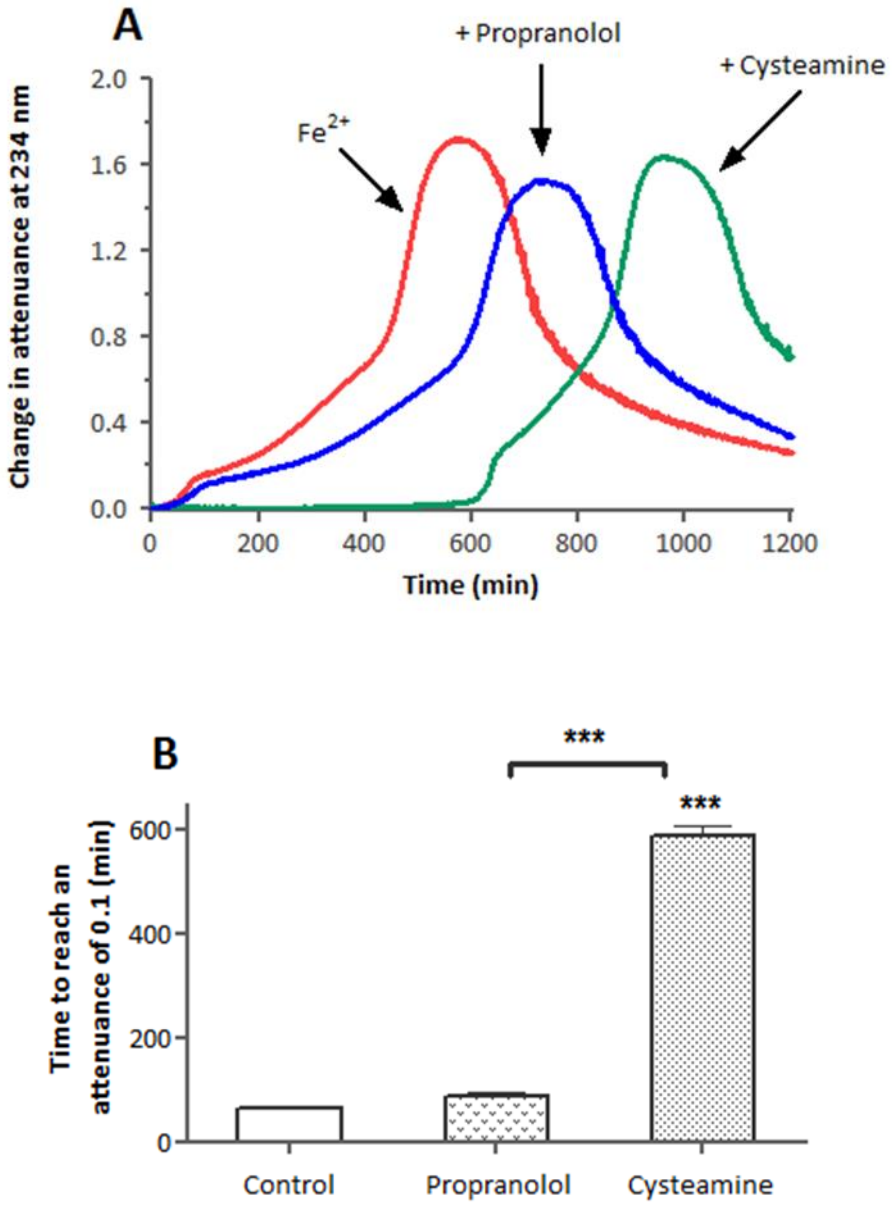


Figure 4.3 Comparison of antioxidant effect of propranolol and cysteamine on LDL oxidation by iron at pH 4.5

LDL (50 µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated with 5 µM $FeSO_4$ at 37°C in quartz cuvettes in the presence or absence of either cysteamine or propranolol (final concentration 25 µM). (A) Oxidation was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of at least three independent experiments. (B) The time taken to reach an attenuance of 0.1 is shown. (*** indicates $p<0.01$, ANOVA followed by tukey's test, n=3).

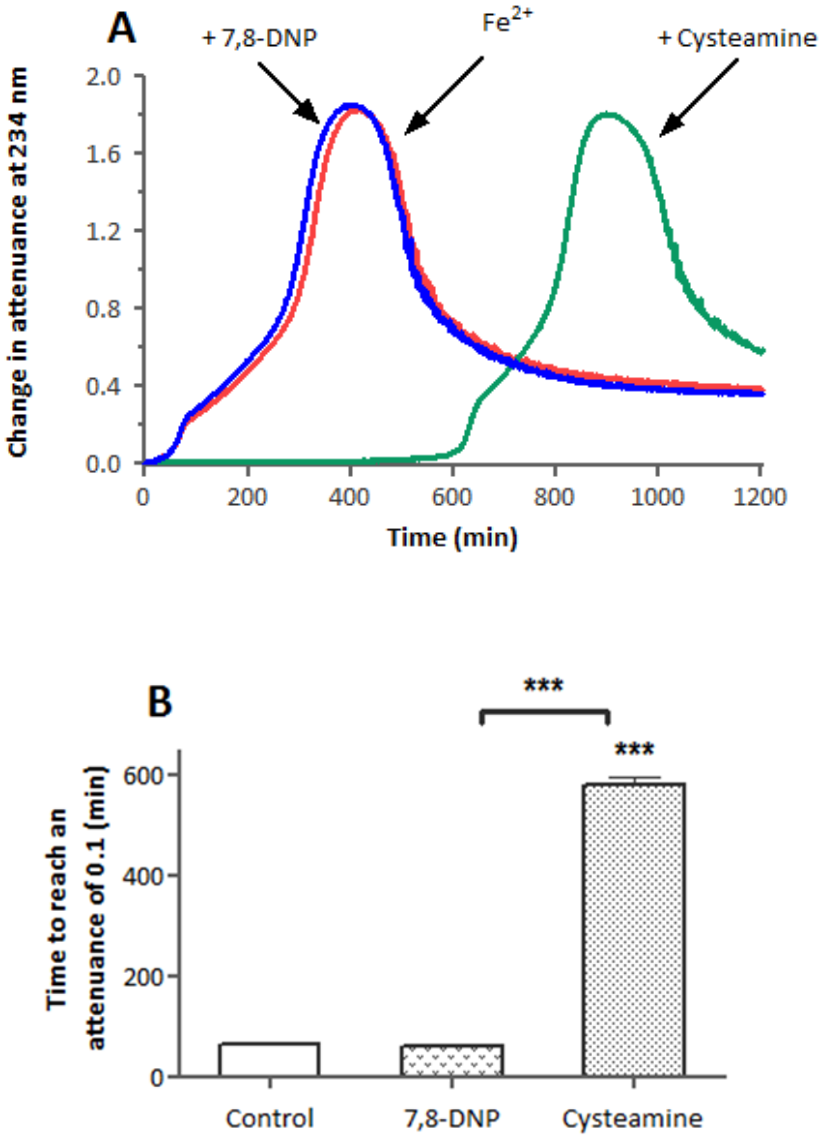


Figure 4.4 Comparison of antioxidant effect of 7,8-dihydroneopterin and cysteamine on LDL oxidation by iron at pH 4.5

LDL (50 µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated with 5 µM $FeSO_4$ at 37°C in quartz cuvettes in the presence or absence of either cysteamine or 7,8-dihydroneopterin (7,8-DNP) at a final concentration 25 µM. (A) Oxidation was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of at least three independent experiments. (B) The time taken to reach an attenuance of 0.1 is shown. (*** indicates $p<0.01$, ANOVA followed by tukey's test, n=3).

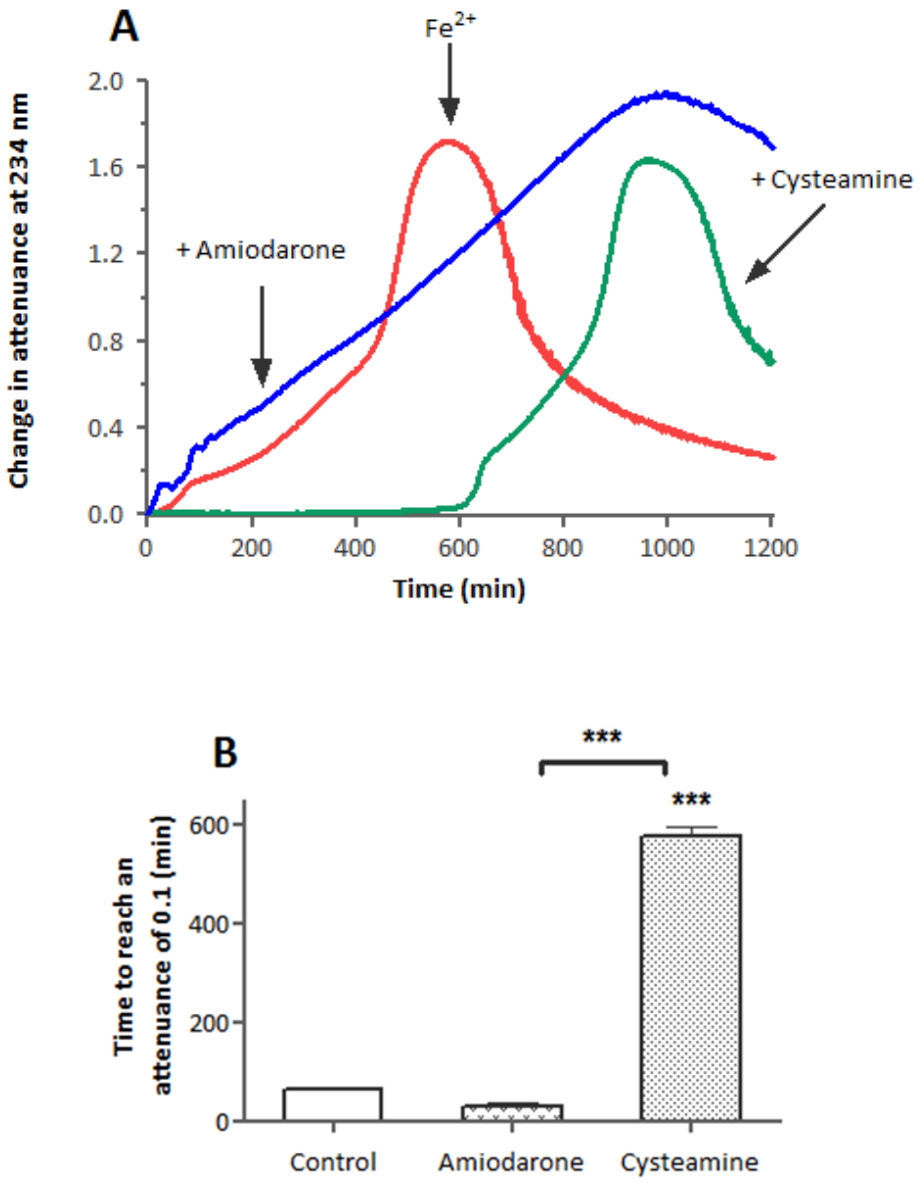


Figure 4.5 Comparison of antioxidant effect of amiodarone and cysteamine on LDL oxidation by iron at pH 4.5

LDL (50 µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated with 5 µM $FeSO_4$ at 37°C in quartz cuvettes in the presence or absence of either cysteamine or amiodarone (final concentration 25 µM). (A) Oxidation was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of at least three independent experiments. (B) The time taken to reach an attenuance of 0.1 is shown. (*** indicates $p<0.01$, ANOVA followed by tukey's test n=3).

4.3.3 Effect of cysteamine on LDL aggregation during oxidation with iron at pH 4.5

Cysteamine is able to prevent the oxidation of LDL by iron at lysosomal pH. In order to study the effect of cysteamine on LDL aggregation by ion at pH 4.5, native LDL (50 µg protein/ml) in sodium chloride/sodium acetate buffer (pH 4.5) was incubated either alone or with 5 µM $FeSO_4$ at 37°C in quartz cuvettes, in the presence or absence of cysteamine (25 µM). The aggregation was monitored by measuring light scattering at 680 nm in a spectrophotometer.

Light scattering at 680 nm showed no change in the samples containing LDL alone, and the scattering increased rapidly at around 200 min in the LDL samples which were treated with iron. After 500 min the scattering decreased indicating sedimentation of large aggregates of LDL below the beam of light (Figure 4.6). Cysteamine greatly prevented the aggregation of LDL for about 500 min and the sedimentation of the aggregates started after around 800 min of oxidation process. The traces became ‘noisier’ at later time points as the aggregates moved into and out of the beam of light. The attenuance reached 0.1 after 274 ± 6 when no cysteamine was added to LDL, whereas addition of cysteamine increased the time to reach the attenuance of 0.1 to 552 ± 10 min (P<0.001, n=3).

These data collectively show that cysteamine at pH 4.5 is able to delay the oxidation of LDL and this in turn delays the aggregation of LDL. Once the aggregation of LDL starts it proceeds at the same rate as in the absence of cysteamine.

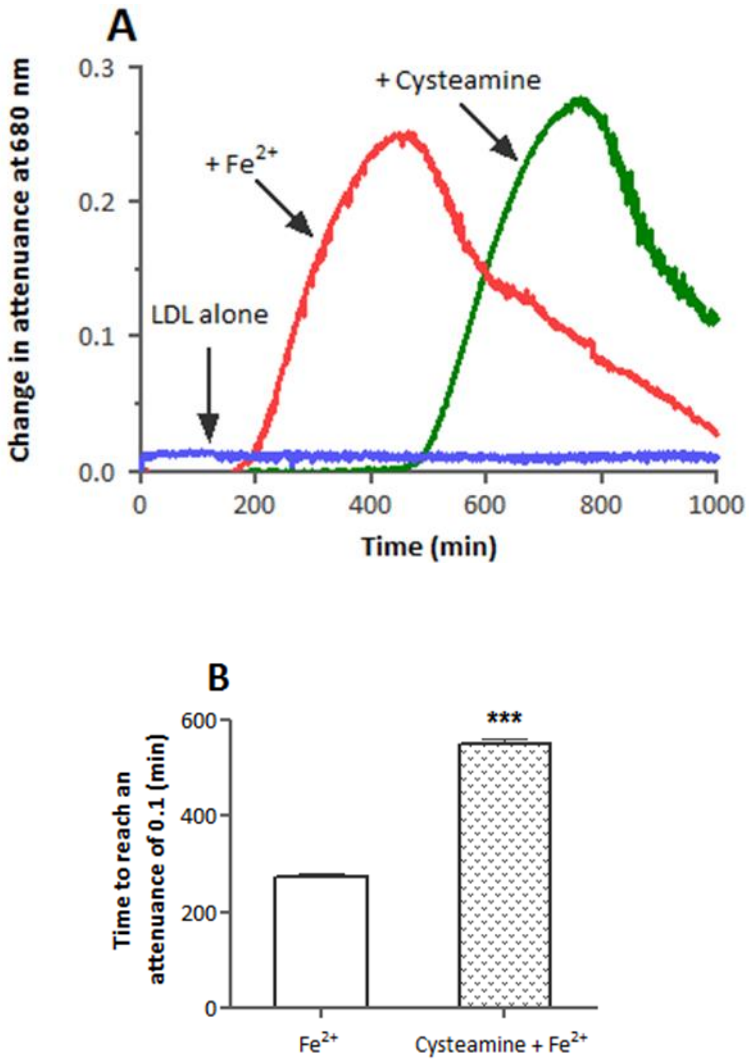


Figure 4.6 Effect of cysteamine on aggregation of LDL during oxidation with iron at pH 4.5

LDL (50 µg protein/ml) in sodium acetate buffer (pH 4.5) was incubated with 5 µM $FeSO_4$ in presence or absence of cysteamine (25 µM) at 37°C in quartz cuvettes. (A) Aggregation was monitored by measuring attenuance at 680 nm against appropriate cuvettes. This is a representative example of three independent experiments. (B) The time taken to reach an attenuance of 0.1 is shown (******* indicates $p<0.001$, t-test, n=3).

4.3.4 Effect of cysteamine on LDL oxidation by copper at pH 4.5

In order to study the effect of cysteamine on LDL oxidation by copper at lysosomal pH, native LDL (50 µg protein/ml) in sodium acetate buffer (pH 4.5) was incubated with 5 µM $CuSO_4$ at 37°C in quartz cuvettes, in the absence (control) or presence of cysteamine (final concentration (25 µM). The course of oxidation was followed by measuring the formation of conjugated dienes at 234 nm.

The oxidation of LDL by copper showed an initial short lag phase followed by a faster oxidation phase, a slower oxidation phase, an aggregation phase and finally a sedimentation phase during which the large aggregates sediment in a similar manner to iron (Figure 4.7). The time taken to reach an attenuance of 0.1 during oxidation with copper was 51 ± 2 min. The effect of 25 µM cysteamine on LDL oxidation by copper at pH 4.5 was not as strong as that seen with iron catalysed LDL oxidation; however, cysteamine inhibited the initial oxidation of LDL by copper for an extra 50 min or so. In presence of cysteamine, it took 91 ± 3 min for the attenuance to reach the value of 0.1 which was found to be statistically significant (P<0.01) compared to the control to which no cysteamine was added.

These data suggest that cysteamine would be able to inhibit the oxidation of LDL by copper in the lysosomes of cells.

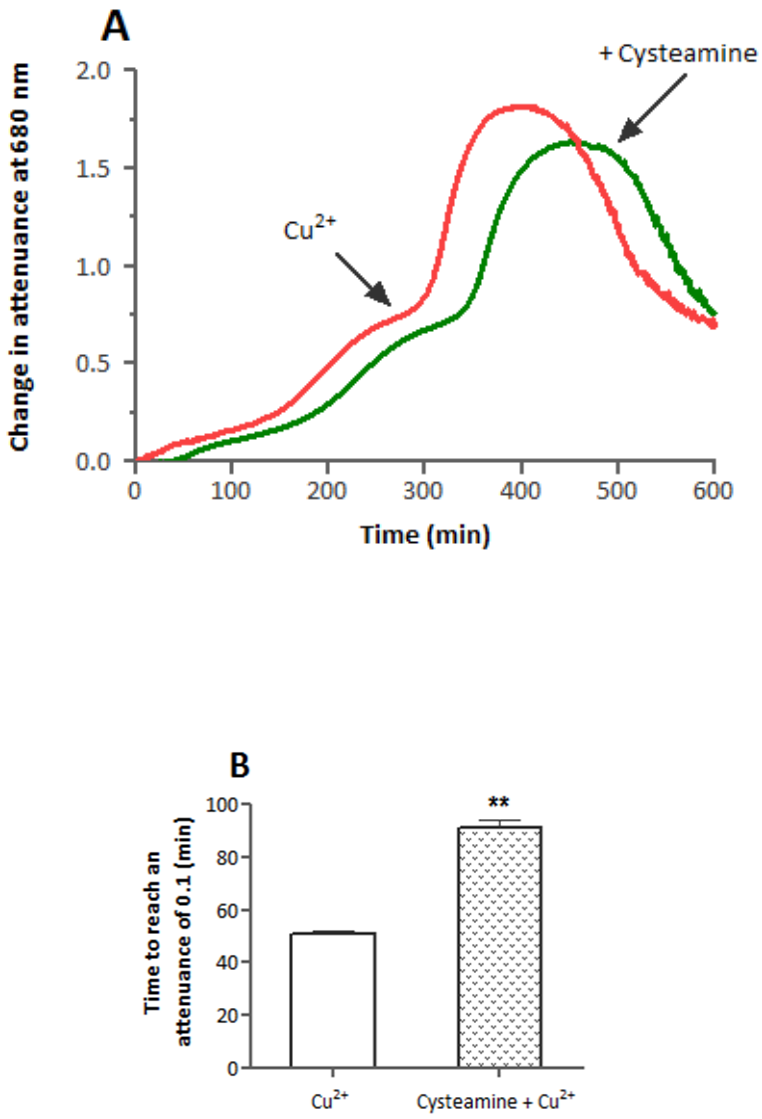


Figure 4.7 Effect of cysteamine on LDL oxidation by copper at pH 4.5

LDL (50 µg protein/ml) in sodium acetate buffer (pH 4.5) was incubated with 5 µM $CuSO_4$ at 37°C in quartz cuvettes, in the presence or absence of cysteamine (25 µM). (A) Conjugated diene formation was monitored by measuring the change in attenuance at 234. This is a representative example of 3 independent experiments. (B) The time taken to reach an attenuance of 0.1 is shown. (** indicates $p<0.01$ compared to control, t-test, n =3).

4.3.5 Effect of cysteamine on LDL oxidation by copper at pH 7.4

Copper at physiological pH has been shown to oxidise LDL effectively and involves an initial lag phase followed by a rapid oxidation phase, decomposition phase and a slow oxidation phase (Esterbauer et al., 1989, Rodríguez-Malaver et al., 1997).

To study the effect of cysteamine on LDL oxidation by copper at pH 7.4, native LDL (50 µg protein/ml) in MOPS buffer (pH 7.4) was incubated with 5 µM $CuSO_4$ at 37°C in quartz cuvettes, in the absence or presence of cysteamine (final concentration 10 µM or 25 µM). The course of oxidation was followed by measuring the formation of conjugated dienes at 234 nm.

The oxidation was much faster with copper at pH 7.4 than pH 4.5 (Figure 4.8). Cysteamine, at both the concentrations, inhibited the oxidation of LDL by copper by greatly extending the lag phase (Figure 4.8). The antioxidant effect was seen to be concentration dependent. In the absence of cysteamine (control), LDL oxidation proceeded normally and reached an absorbance of 0.1 after 35 ± 1 minute. The term absorbance is used for LDL oxidation at pH 7.4 because LDL does not aggregate and scatter ultraviolet radiation at this pH for the incubation periods studied here.

Cysteamine (10 µM) extended the time to reach an absorbance of 0.1 to 130 ± 6 min which is about a 3.5 fold increase compared to control (p<0.01), while 25 µM showed a 6.5 fold increase in this time and reached an absorbance of 0.1 after 232 ± 18 min (p<0.001). The two concentrations of cysteamine differed significantly (p<0.01) in delaying the oxidation of LDL by copper when evaluated in terms of reaching an absorbance of 0.1.

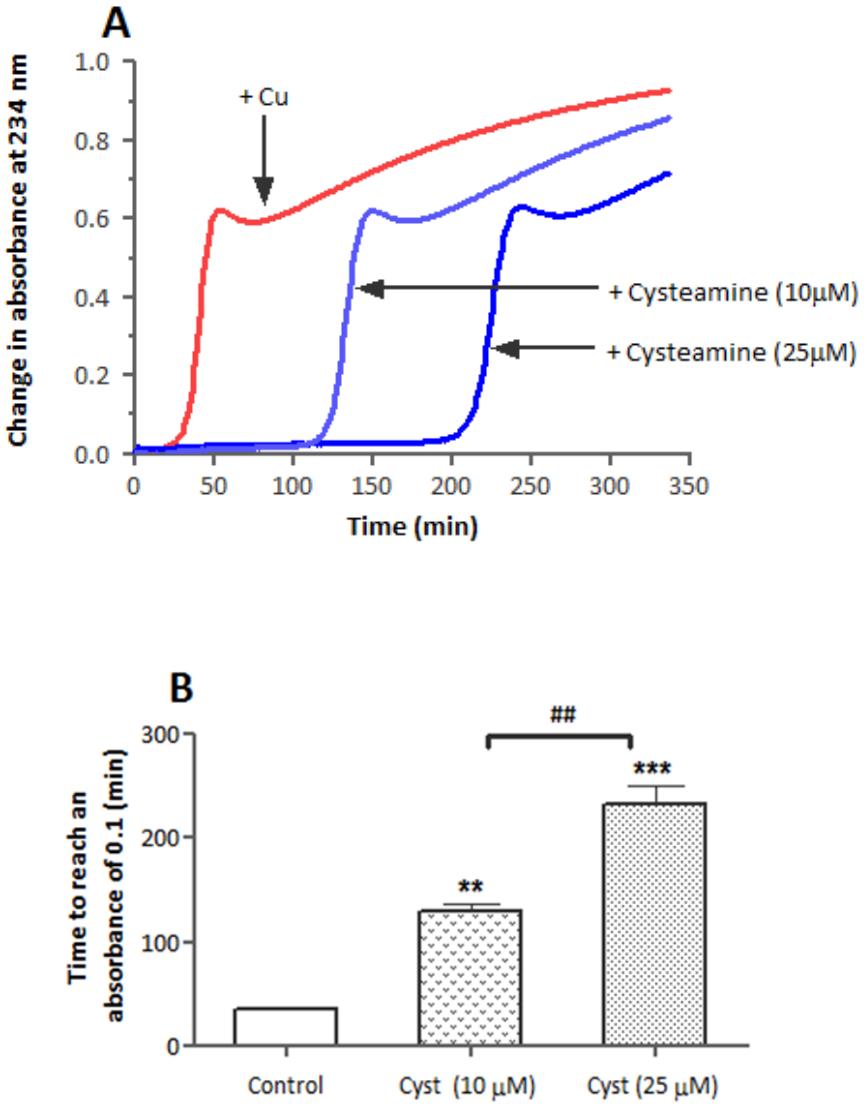


Figure 4.8 The effect of cysteamine on LDL oxidation by copper at pH 7.4

LDL (50 µg protein/ml) in MOPS buffer (pH 7.4) was incubated with 5 µM $CuSO_4$ at 37°C in quartz cuvettes, in the presence or absence of cysteamine (10 µM or 25 µM). (A) Conjugated diene formation was monitored by measuring the change in absorbance at 234. This is a representative example of 3 independent experiments. (B) The time taken to reach an absorbance of 0.1 is shown. (** indicates $p<0.01$, *** indicates $p<0.001$ compared to control, $^{\#\#}$ indicates $p<0.01$, ANOVA followed by tukey's test, n =3).

4.4 Discussion

Thiol compounds are known for general antioxidant properties, such as radical quenching and thus play a crucial role in protecting cells from oxidative damage (Deneke, 2000). The –SH group makes thiols potent nucleophiles and ready to interact with electrophilic groups of reactive oxygen species (ROS) and therefore thiols are the initial and major members of the physiological antioxidant defence system (Gungor et al., 2011). In addition, cysteamine is known to exert some of its antioxidant effect by increasing cellular glutathione levels in the cells (Revesz and Modig, 1965). In general, thiols are mild acids but the presence of a positively charged residue, such as lysine or arginine, as well as the formation of a hydrogen bond may increase their acidity by 3-4 orders of magnitude (Copley et al., 2004). Their reactivity therefore depends on their dissociation constant (pK_a) values (Brandes et al., 2009, Nagy, 2013).

In the previous chapter, it was shown that cysteamine, because of its thiol nature, is able to prevent oxidation of LDL by iron at lysosomal pH. Here, we have shown that another thiol, cysteine, is also able to inhibit the oxidation of LDL by iron at pH 4.5. However, the antioxidant effect is much lower than cysteamine. This could be due to the difference in the pK_a values between the two thiols. The pK_a values of cysteamine are 8.19 for the thiol group and 10.75 for the amine group (Mezyk, 1995, Serjeant and Dempsey, 1979), while the corresponding value is higher for the thiol group of cysteine, 8.33, slightly higher for the amine group, 10.78 (Jocelyn, 1967, Mezyk, 1995); in addition the carboxylic group in cysteine gives it an additional pK_a of 1.71 compared to cysteamine. Thus the thiol group of cysteine is a weaker acid and would ionise less to produce the thiolate ion (—S^-) (12) and

hence would be a weaker antioxidant in preventing LDL oxidation than cysteamine.

$$\underset{\text{Thiol}}{\text{—SH}} \longleftrightarrow \underset{\text{Thiolate}}{\text{—S}^-} + \text{H}^+ \qquad (12)$$

Cysteamine is a lysosomotropic drug and recent unpublished work from this laboratory has shown that cysteamine is able to reduce atherosclerosis in LDL receptor-deficient mice. Other known lysosomotropic drugs like propranolol, amiodarone and 7,8-dihydroneopterin have been shown to inhibit LDL oxidation, however they do so by different mechanisms. At a given concentration and under similar conditions, none of these drugs were better at preventing oxidation of LDL by iron at lysosomal pH compared to cysteamine. Although, propranolol was able to slow down the oxidation, the inhibition was far less compared to cysteamine and it did not produce a prolonged lag phase (Figure 4.3). Amiodarone initially showed a significant pro-oxidant like effect and overall the fold inhibition was much lower than cysteamine (Figure 4.5). However, 7,8-dihydoneopterin showed no effect on LDL oxidation under the current conditions. Therefore, cysteamine is a better antioxidant at preventing oxidation of LDL under lysosomal conditions than propranolol, amiodarone and 7,8-dihydoneopterin.

Aggregation is one of the characteristics of extensively oxidised LDL. Oxidation of LDL is characterised by several structural and chemical changes to the lipoprotein particle that lead to numerous modifications in its functional properties and hence is responsible for enhanced cellular recognition and uptake (Vanderyse et al., 1992). Oxidation of LDL can reduce the LDL surface charge density and therefore result in the fusion of LDL particles and the formation of vesicles and membranous

structures (Pentikainen et al., 1996). In addition, atherosclerotic plaque formation is believed to be initiated by the extracellular aggregation and deposition of lipoproteins trapped in the subendothelial matrix. Furthermore, acidic pH has been shown to promote LDL and oxidised LDL aggregation (Satchell and Leake, 2012, Sneck et al., 2012) and lysosomal pH conditions are considered optimal for lipoprotein aggregation (Xu and Lin, 2001). The presence of oxidized and highly aggregated lipids within lysosomes has the potential to perturb the function of these organelles and to promote atherosclerosis. In agreement with the work carried out previously (Satchell and Leake, 2012), oxidation of LDL with ferrous iron induced aggregation of LDL after around 200 min and no aggregation was seen in the control LDL, to which no oxidising agent was added. The antioxidant cysteamine greatly delayed the oxidation and subsequent aggregation of LDL by iron at lysosomal pH (pH 4.5). Cysteamine (25 µM) offered more than a 2-fold increased delay against LDL oxidation at pH 4.5. This can be explained on the basis that cysteamine is able to scavenge superoxide ions (both protonated and non-protonated forms) produced during iron-catalysed oxidation (13, 14), thus protecting the oxidation induced chemical changes in the lipid and the protein portions of LDL.

$$^{+}NH_3\text{-}CH_2\text{-}CH_2\text{-}S^{-} + HO_2^{\bullet} \xrightarrow{+H^+} {}^{+}NH_3\text{-}CH_2\text{-}CH_2\text{-}S^{\bullet} + H_2O_2 \quad (13)$$

$$^{+}NH_3\text{-}CH_2\text{-}CH_2\text{-}S^{-} + O_2^{\bullet -} \xrightarrow{+2H^+} {}^{+}NH_3\text{-}CH_2\text{-}CH_2\text{-}S^{\bullet} + H_2O_2 \quad (14)$$

LDL oxidised by copper at pH 7.4 has been extensively studied and is the major model used to study the role of oxidised LDL in atherosclerosis, possibly because it shares properties with the LDL oxidised by cells. Here we have shown that cysteamine is able to inhibit oxidation of LDL by copper at pH of the interstitial fluid (pH 7.4). Cysteamine also showed a concentration-dependent increase in the lag phase extension during LDL oxidation by copper at pH 7.4. This means that if copper is able to cause some oxidation of LDL extracellularly at pH 7.4, as suggested by some reports to be important in the pathogenesis of atherosclerosis, then treatment with cysteamine would be beneficial as treatment of atherosclerosis.

Recent reports have found that it is unlikely that oxidative modification of LDL can occur in extracellular spaces because of the strong antioxidant nature of interstitial fluid and plasma (Dabbagh and Frei, 1995, Leake and Rankin, 1990, Patterson and Leake, 1998). *In vivo* it is thought that LDL might be trapped in the arterial wall and then oxidised by metal ions under lower pH conditions (Leake, 1997, Lodge et al., 2000). LDL oxidation occurred significantly by copper at pH 4.5, however, at a slower rate than at pH 7.4, in marked contrast to iron, which may be because copper has been shown to bind LDL particles less at low pH (Leake et al., unpublished data). Cysteamine is able to inhibit the oxidation by copper at lysosomal pH and slows down the overall oxidation process although the antioxidant effect was more prominent at pH 7.4. This might be because cysteamine acts as an antioxidant by donating electrons (15) rather than hydrogen atoms (electron plus proton) (16) to free radicals.

$$^{+}NH_3\text{-}CH_2\text{-}CH_2\text{-}S^{-} + X^{\bullet} \xrightarrow{+H} {}^{+}NH_3\text{-}CH_2\text{-}CH_2\text{-}S^{\bullet} + XH \text{ (faster)} \quad (15)$$

$$NH_2\text{-}CH_2\text{-}CH_2\text{-}SH + X^{\bullet} \xrightarrow{+H} {}^{+}NH_3\text{-}CH_2\text{-}CH_2\text{-}S^{\bullet} + XH \text{ (slower)} \quad (16)$$

Nevertheless, there was also a significant reduction of LDL oxidation by cysteamine at lysosomal pH. Therefore, in addition to preventing the oxidation of LDL by iron under lysosomal conditions, cysteamine would be able to prevent the possible copper-catalysed oxidation, hence proving to be a potentially effective treatment in inhibiting LDL oxidation at pH 4.5 or 7.4.

Chapter 5-

Oxidation of sphingomyelinase aggregated LDL by iron under lysosomal pH conditions

5.1 Introduction

Studies which examine foam cell formation by the incubation of macrophages with modified monomeric LDL (e.g. oxidised LDL) do not fully reflect the *in vivo* environment, as the majority of the LDL in atherosclerotic plaques is found in an aggregated state and bound to subendothelial matrix (Boren et al., 2000, Tabas, 1999). LDL entrapped by extracellular matrix, particularly by proteoglycans, is vulnerable to hydrolysis by lipases and proteases. It has been shown that lipoprotein lipase (LPL), secretory sphingomyelinase (S-SMase), secretory phospholipase A_2 ($sPLA_2$), cholesteryl ester hydrolase (CEH), matrix metalloproteinases (MMP), lysosomal proteases and plasmin are capable of aggregating LDL to produce atherogenic LDL forms that are rapidly internalized by macrophages (Bhakdi et al., 1995, Boyanovsky et al., 2009, Fenske et al., 2008, Hakala et al., 2003, Leake and Rankin, 1990, Maor et al., 2000, Torzewski et al., 2004). Wen and Leake (2007) have also shown that aggregated LDL produced by vortexing is rapidly engulfed by macrophages and then oxidised within the lysosomes by iron leading to the formation of ceroid (Wen and Leake, 2007). These mechanisms of foam cell formation, based on ingestion of aggregated, rather than monomeric LDL, are probably more physiologically relevant *in vivo*.

Sphingomyelinase is found in atherosclerotic lesions and has been proposed to be one of the key enzymes causing aggregation of LDL in interstitial fluid of the lesions (Holopainen et al., 2000, Marathe et al., 1999, Portman and Alexander, 1970). Sphingomyelin constitutes a considerable lipid component of LDL particles and together with phosphatidylcholine forms the polar surface of the lipoproteins (Merrill et al., 1997, Oorni et al., 1998, Oorni et al., 2005). Sphingomyelinase is a

sphingomyelin-specific form of phospholipase C which hydrolyses the phosphodiester bond of sphingomyelin to generate ceramide and phosphocholine. Ceramide is hydrophobic and causes aggregation of LDL particles (Walters and Wrenn, 2008). More recently this laboratory has shown that, human LDL when aggregated with sphingomyelinase (SMase) is rapidly taken up by human macrophages and oxidised inside the lysosomes by iron (Wen et al., 2015). It was further suggested that antioxidants that accumulate in lysosomes have a potential for decreasing atherosclerosis by inhibiting the lysosomal oxidation of LDL.

The macrophages that are treated with modified LDL (e.g. SMase-LDL) would therefore, at any time, contain more of SMase-LDL in their lysosomes than native LDL. Lipid peroxidation is one of the major pathological mechanisms involved in atherosclerosis and lysosomal oxidation of aggregated LDL, including SMase-LDL, may explain why oxidised LDL is present within lesions despite the strong antioxidant protection within the arterial intima (Dabbagh and Frei, 1995). Oxidised LDL formed within the lysosomes might affect cell function or be released into the interstitial fluid; both with potentially atherogenic consequences. Thus, it is important to study the oxidation pattern of modified LDL in detail under lysosomal conditions. Furthermore, it might be necessary for an antioxidant like cysteamine to prevent oxidation of SMase-LDL as a part of its effective treatment against atherosclerosis. Therefore, the aims of this chapter are to study in detail the oxidation pattern of SMase-LDL by iron under lysosomal conditions and also the possible protection by cysteamine in preventing oxidation.

5.2 Methods

SMase-LDL was prepared by incubating freshly isolated native LDL with sphingomyelinase as described in detail in Chapter 2. Methods used to monitor the oxidation of LDL in this section have also been described in detail in sections 2.2 and 2.3.

5.3 Results

5.3.1 Total lipid hydroperoxides in SMase-LDL

In chapter 3, it was established that the oxidation of native LDL takes place even when no pre-existing lipid hydroperoxides are present. In order to see if the same holds true for SMase-LDL, total lipid hydroperoxide levels were measured in SMase-LDL and compared to native LDL. Freshly isolated native LDL was used to prepare SMase-LDL and then analysed for the presence of pre-existing lipid hydroperoxides by the tri-iodide method, as described in detail in the Chapter 2.

In both, native LDL as well as SMase-LDL, no lipid hydroperoxides were detected, in fact they were slightly below the blanks in the assay and there was no apparent difference in the values (Figure 5.1). The levels in native LDL were -3 ± 2 nmol/mg LDL protein and that in SMase-LDL were -2 ± 2 nmol/mg LDL protein ($p>0.05$, $n = 6$). For comparison the levels of lipid hydroperoxides in fully oxidised LDL are about 800 nmol/mg protein (Patterson et al., 2003a).

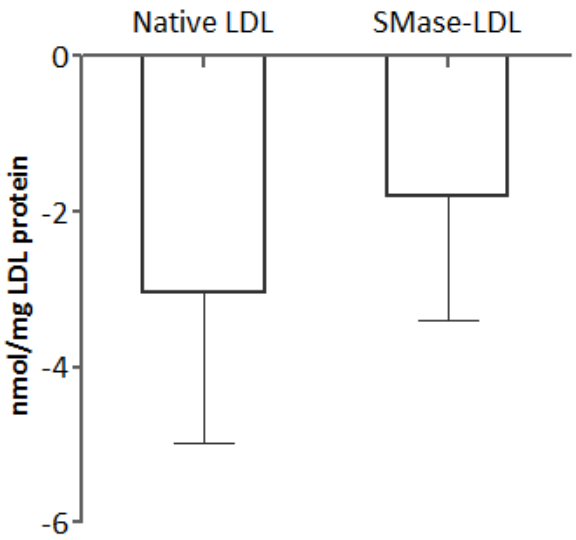


Figure 5.1 Pre-existing lipid hydroperoxides in SMase-LDL and native LDL

Native LDL and SMase-LDL samples were assessed for the presence of pre-existing lipid hydroperoxides by tri-iodide assay. The measured absorbances were slightly below the blank values Graph represent the mean (± SEM) of at least 6 independent experiments.

5.3.2 Measure of oxidation of SMase-LDL by iron at lysosomal pH

Oxidation of native LDL has been extensively studied, whereas little is known about the oxidation characteristics of aggregated LDL, which is presented to the lysosomes for oxidation. It is therefore of interest to investigate the oxidation pattern of SMase-LDL by iron under lysosomal conditions.

Freshly prepared SMase-LDL (50 µg LDL protein/ml) and native LDL were oxidised in NaCl/sodium acetate buffer (pH 4.5) at 37°C with $FeSO_4$ (5 µM). The course of the oxidation was followed by measuring the formation of conjugated dienes at 234 nm and loss of LDL-tryptophan fluorescence (Ex/Em = 282/331 nm).

Incubating, native LDL and SMase-LDL with iron lead to the continuous oxidation of LDL lipids (Figure 5.2). SMase-LDL was oxidised significantly more slowly than native LDL. The time taken to reach an attenuance of 0.1 was 61 ± 4 min in the case of native LDL, compared to 77 ± 3 min for SMase-LDL (Figure 5.2) (p<0.05); mean ± SEM of three independent experiments.

Oxidation of LDL protein was monitored by measuring the loss of fluorescence of LDL-tryptophan during LDL oxidation. The loss of LDL-tryptophan fluorescence was faster in native LDL than SMase-LDL (Figure 5.3). After 100 min incubation, there was 30 ± 1 % loss of LDL-tryptophan fluorescence in the case of native LDL, whereas in the case of SMase-LDL the LDL-tryptophan fluorescence decreased by 21 ± 1% (Figure 5.3) (p<0.05); mean ± SEM of three independent experiments.

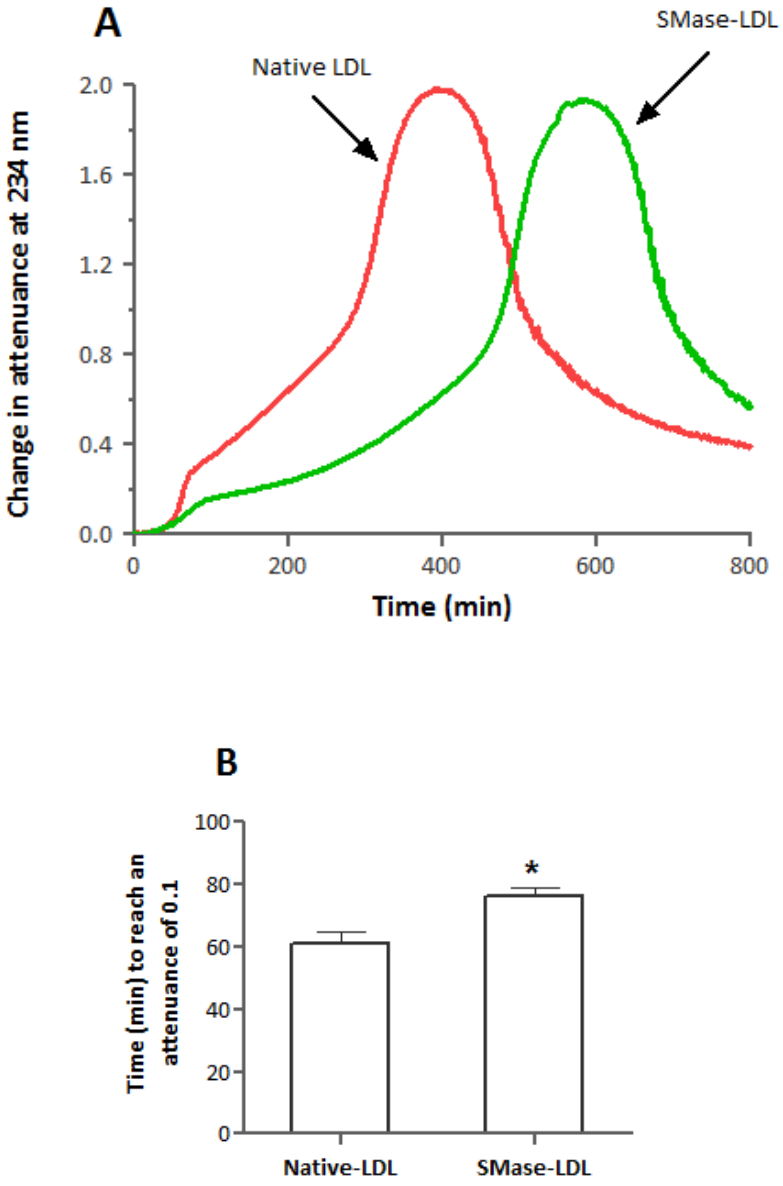


Figure 5.2 Comparison of oxidation of SMase-LDL and native LDL by iron at pH 4.5

(A) SMase-LDL (50 µg protein/mL) and native LDL (50 µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) were incubated with 5 µM $FeSO_4$ at 37°C in capped quartz cuvettes. Oxidation was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of three independent experiments. (B) Time taken to reach an attenuance of 0.1 during the oxidation with iron is shown here. (* indicates $p<0.05$, t-test, n = 3 independent experiments.

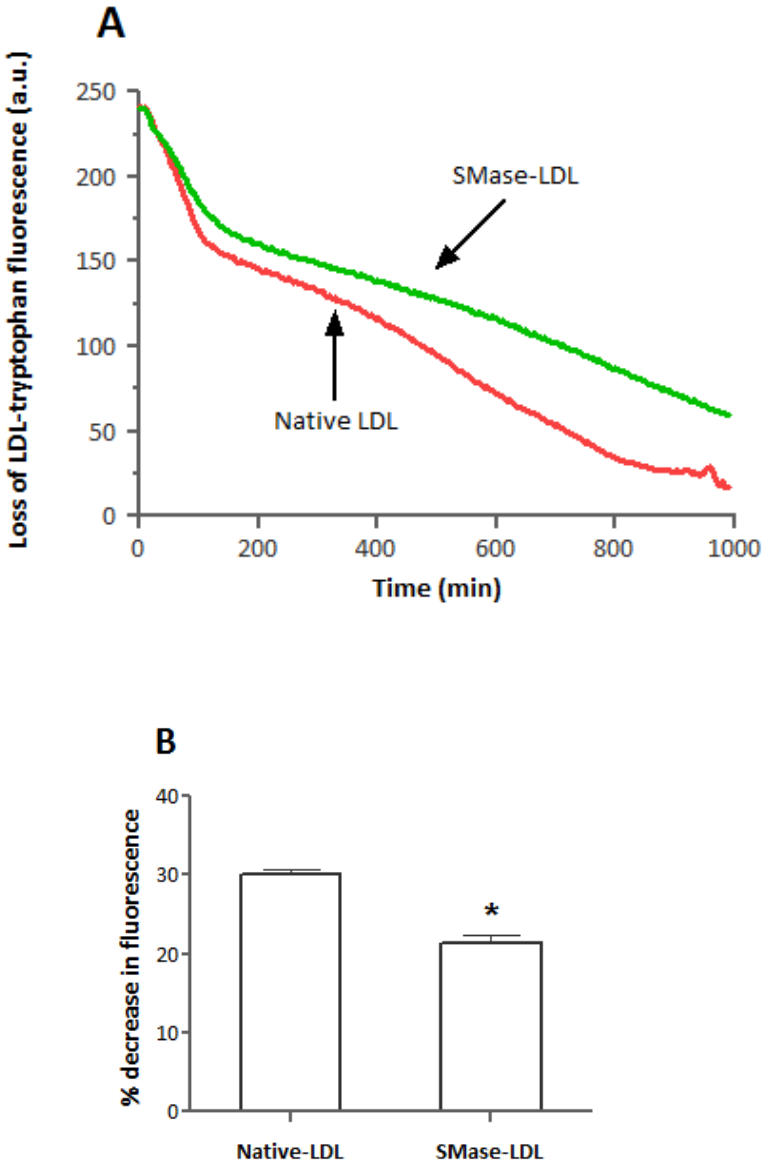


Figure 5.3 Loss of LDL-tryptophan fluorescence during oxidation of SMase-LDL and native LDL by iron at pH 4.5

(A) SMase-LDL (50 µg protein/ml) and native LDL (50 µg protein/ml) were oxidised by 5 µM $FeSO_4$ in NaCl/sodium acetate buffer (pH 4.5) at 37°C in quartz cuvettes and tryptophan fluorescence was measured every 10 min at excitation wavelength of 282 nm and emission wavelength 331nm in a spectrofluorometer. This is a representative example of at least three independent experiments. (B) Shows decrease in LDL-tryptophan fluorescence after 100 min of oxidation (* indicates $p<0.05$, t-test, n = 3 independent experiments).

5.3.3 Chemical characterisation of SMase-LDL oxidation by iron at lysosomal pH

Establishing the lipid composition of SMase-LDL oxidised by iron at lysosomal pH enables information to be gained about the atherogenicity of the oxidised LDL formed in this manner. SMase-LDL (50 µg LDL protein/ml) and native LDL (50 µg LDL protein/ml) were oxidised separately in NaCl/sodium acetate buffer (pH 4.5) at 37°C with $FeSO_4$ (5 µM) in plastic tubes. Samples were taken at different times and were processed for HPLC analysis.

The levels of nonesterified cholesterol declined during the oxidation of SMase-LDL from 978 ± 34 nmol/mg LDL protein at the beginning of the oxidation to 646 ± 68 nmol/mg LDL protein after 24 h, suggesting the cholesterol of LDL was being modified during the reaction (Figure 5.4). This decrease in free cholesterol level in SMase-LDL was not significantly different from the oxidation of native LDL, where the levels decreased from 998 ± 89 nmol/mg LDL protein to 624 ± 52 nmol/mg LDL protein after 24 h.

The major polyunsaturated cholesteryl esters in LDL are cholesteryl linoleate (CL), which has two double bonds, cholesteryl arachidonate (CA) which has four double bonds (CA) and cholesteryl oleate (CO), which has one double bond (Esterbauer et al., 1990). As expected, cholesteryl arachidonate levels decreased faster than those of cholesteryl linoleate, which fell faster than those for cholesteryl oleate. Levels of CL in both SMase-LDL and native LDL decreased continuously during the oxidation process but the decrease was slower in SMase-LDL. The CL levels in SMase-LDL decreased from 1763 ± 79 nmol/mg LDL protein to 1093 ± 203 nmol/mg LDL protein in the first 12 h (Figure 5.4B) and after that there was a

significantly sharp drop in the CL levels to 100 ± 55 nmol/mg LDL protein ($p<0.001$). In the case of native LDL, the levels of CL declined sharply after 6 h from 1285 ± 140 nmol/mg LDL protein to 471 ± 99 nmol/mg LDL protein after 12 h ($p<0.01$). However, after 24 h, levels of CL in native LDL were 87 ± 7 nmol/mg LDL protein, which were not significantly different from those in SMase-LDL.

CA levels also decreased in both SMase-LDL and native LDL during the course of oxidation process (Figure 5.5). Although, the oxidation process of SMase-LDL proceeded at a slower rate than native LDL, the levels in both dropped to the same very low level after 24 h. In native LDL, CA levels dropped from 460 ± 32 nmol/mg LDL protein to 10 ± 5 nmol/mg LDL protein after 24 h of oxidation process. The levels in SMase-LDL dropped from 427 ± 36 nmol/mg LDL protein at time zero to 203 ± 33 nmol/mg LDL protein after 12 h, but the levels dropped to 7 ± 3 nmol/mg LDL protein after 24 h.

There was no apparent difference in the decrease of CO levels between SMase-LDL and native LDL during the course of oxidation (Figure 5.5). In the case of SMase-LDL, the CO levels dropped from 196 ± 10 nmol/mg LDL protein at time zero to 76 ± 11 nmol/mg LDL protein after 24 h, while in case of native, the levels dropped from 208 ± 16 nmol/mg LDL protein to 80 ± 13 nmol/mg LDL protein after 24 h.

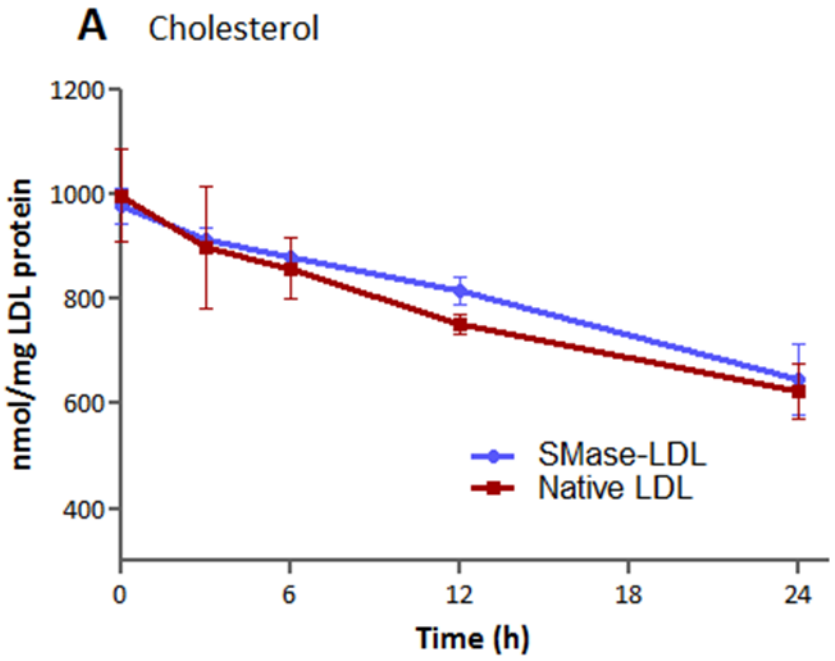
The cholesterol oxidation product, 7-ketocholesterol, in SMase-LDL increased from 0.7 ± 0.6 nmol/mg LDL protein to only 5 ± 3 nmol/mg LDL protein after 12 h of oxidation (Figure 5.6). However, there was a significantly sharp increase to 26 ± 2 nmol/mg LDL protein after 24 h of oxidation ($p<0.001$). In the case of native LDL,

the 7-ketocholesterol levels reached 16 ± 0.3 nmol/mg LDL protein after 6 h and 19 ± 2 nmol/mg LDL levels after 24 h.

Cholesteryl linoleate hydroperoxide (CLOOH) levels showed an increased level initially in both SMase-LDL and native LDL (Figure 5.6), while the levels peaked to 513 ± 32 nmol/mg LDL protein in native LDL after 6 h, the SMase-LDL levels reached a peak value of 388 ± 23 after 12 h. After 24 h the levels in both the LDL forms dropped significantly to 44 ± 6 nmol/mg LDL protein in SMase-LDL and 43 ± 8 nmol/mg LDL protein in case of native LDL.

Figure 5.4 Comparison between lipid profile of SMase-LDL and native LDL during oxidation of by $FeSO_4$ at pH 4.5

SMase-LDL (50 µg LDL protein/ml) and native LDL (50 µg LDL protein/ml) were oxidised with freshly dissolved $FeSO_4$ (5 µM) at 37°C in a sodium chloride/sodium acetate buffer, pH 4.5. At various time points up to 24 h, oxidation was stopped by addition of EDTA (final concentration 2mM) and BHT (2mM). The samples were then assayed for free cholesterol (A) and cholesteryl linoleate (B) by reverse phase HPLC. Graph represent mean (± SEM) of at least 3 independent experiments (** indicates $p<0.01$, *** indicates $p<0.001$ compared with previous time point; $^{++}$ indicates $p<0.01$ between SMase-LDL and native LDL for the same time point, Two-way ANOVA followed by Bonferroni post-test).


A
Cholesterol
1200
1000
800
600
400
nmol/mg LDL protein
0
6
12
18
24
Time (h)
SMase-LDL
Native LDL

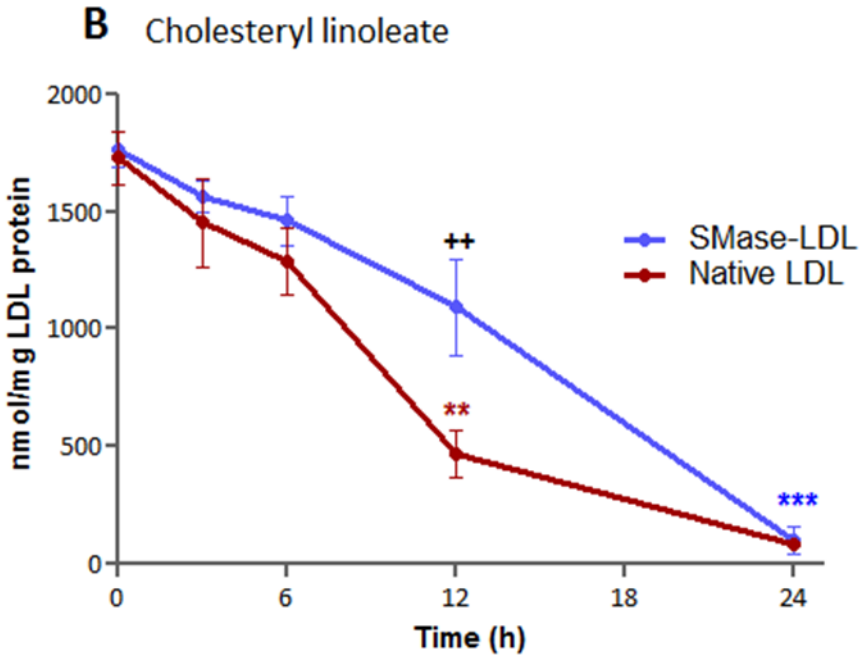
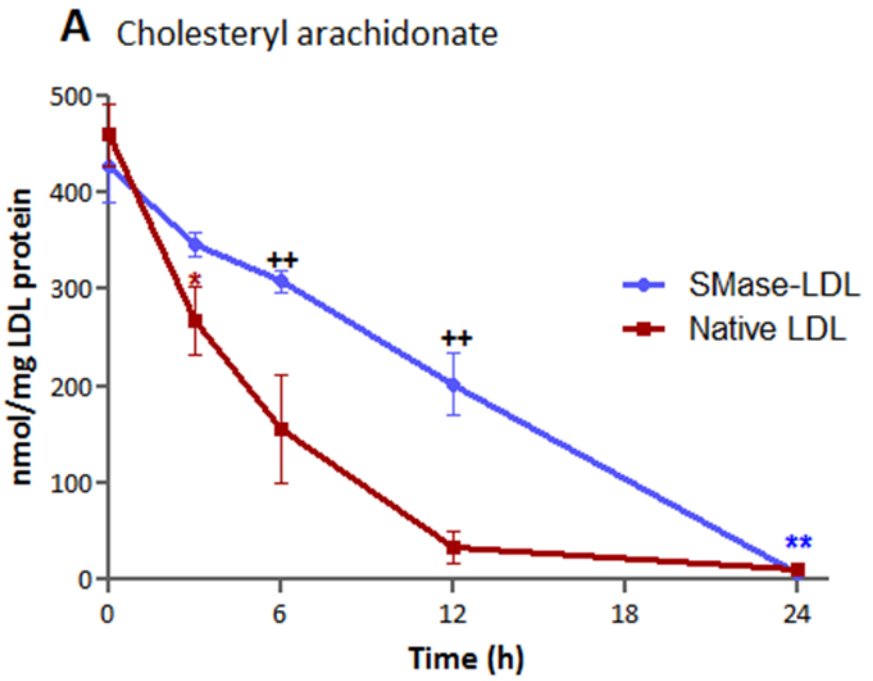

B
Cholesteryl linoleate
2000
1500
1000
500
0
nmol/mg LDL protein
0
6
12
18
24
Time (h)
++
**
***
SMase-LDL
Native LDL

Figure 5.5 Comparison between lipid profile of SMase-LDL and native LDL during oxidation of by $FeSO_4$ at pH 4.5

SMase-LDL (50 µg LDL protein/ml) and native LDL (50 µg LDL protein/ml) were oxidised with freshly dissolved $FeSO_4$ (5 µM) at 37°C in a sodium chloride/sodium acetate buffer. At various time points up to 24 h, oxidation was stopped by addition of EDTA (final concentration 2mM) and BHT (2mM). The samples were then assayed for cholesteryl arachidonate (A) and cholesteryl oleate (B) by reverse phase HPLC. Graph represent mean (± SEM) of at least 3 independent experiments (* indicates $p<0.05$, ** indicates $p<0.01$ compared with previous time point; $^{++}$ indicates $p<0.01$ between SMase-LDL and native LDL for the same time point, Two-way ANOVA followed by Bonferroni post-test).


A
Cholesteryl arachidonate
nmol/mg LDL protein
500
400
300
200
100
0
0
6
12
18
24
Time (h)
++
*
++
**
SMase-LDL
Native LDL

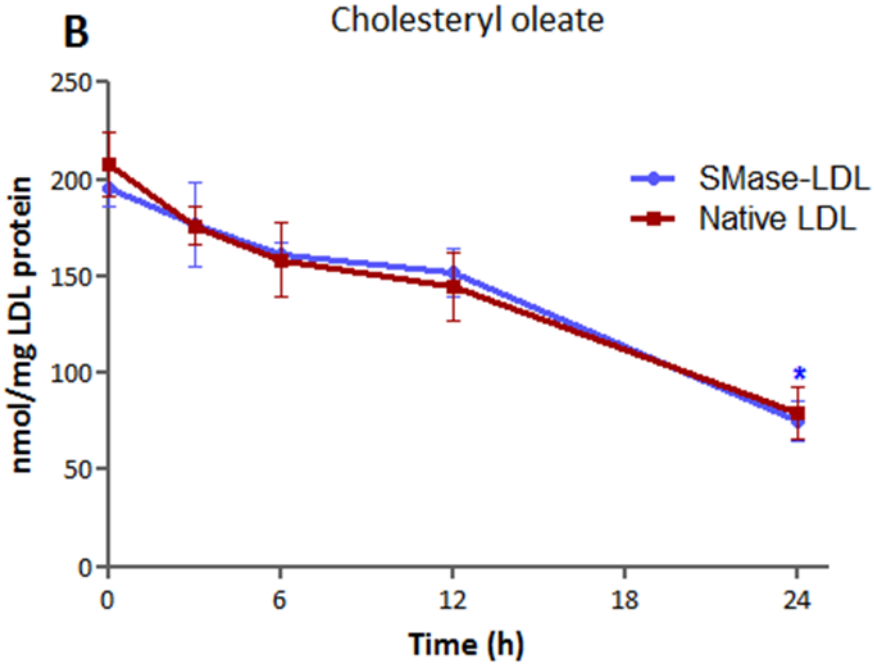
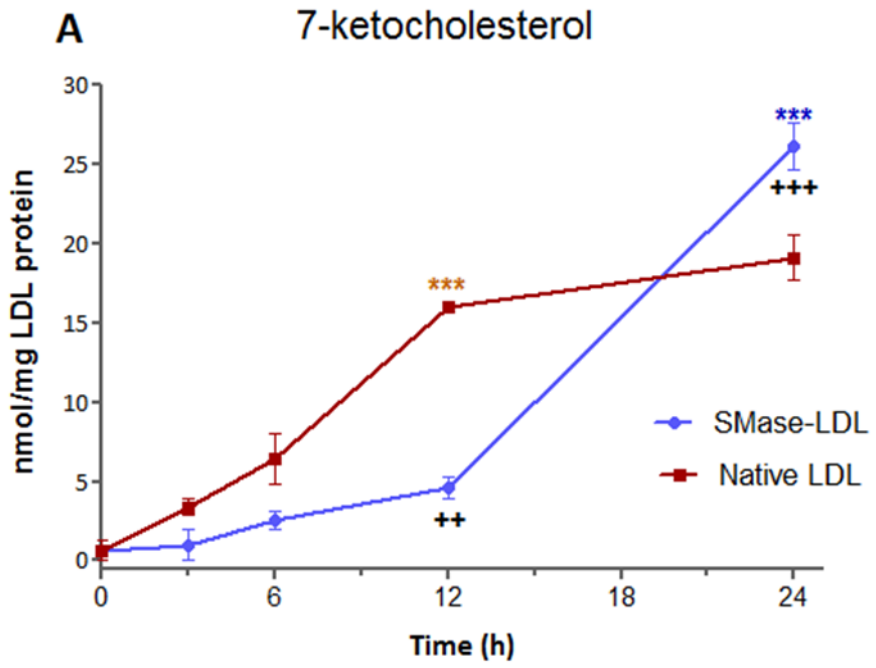
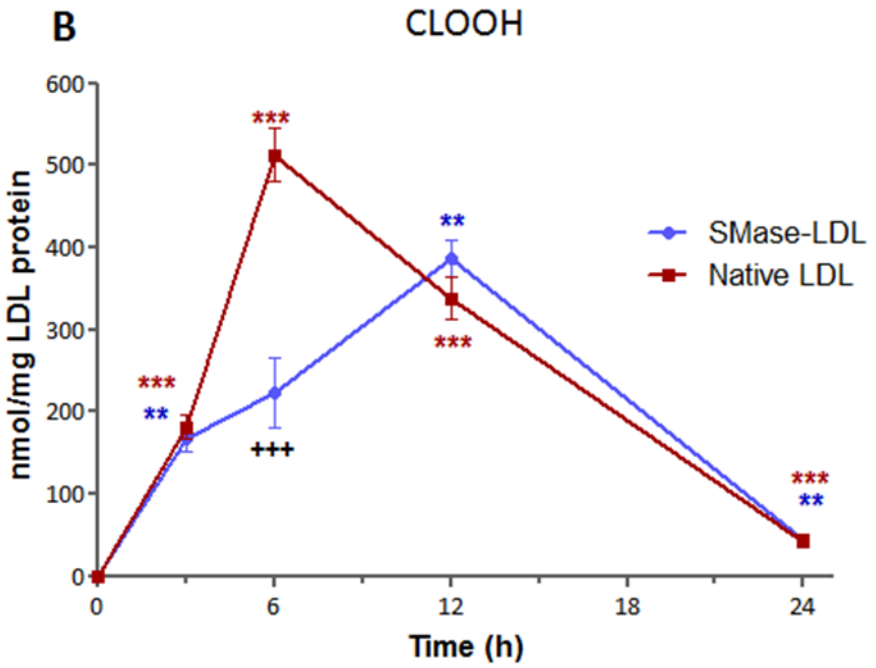

B
Cholesteryl oleate
nmol/mg LDL protein
250
200
150
100
50
0
0
6
12
18
24
Time (h)
*
SMase-LDL
Native LDL

Figure 5.6 Oxidation product levels durng oxidation of SMase-LDL and native LDL by $FeSO_4$ at pH 4.5

SMase-LDL (50 µg LDL protein/ml) and native LDL (50 µg LDL protein/ml) were oxidised with freshly dissolved $FeSO_4$ (5 µM) at 37°C in a sodium chloride/sodium acetate buffer. At various time points up to 24 h, oxidation was stopped by addition of EDTA (final concentration 2mM) and BHT (2mM). The samples were then assayed for 7-ketocholesterol (A) and cholesteryl linoleate hydroperoxide (CLOOH) (B) by reverse phase HPLC. Graph represent mean (± SEM) of at least 3 independent experiments (** indicates $p<0.01$, *** indicates $p<0.001$ compared with previous time point; $^{++}$ indicates $p<0.01$, $^{+++}$ indicates $p<0.001$ between SMase-LDL and native LDL for the same time point, Two-way ANOVA followed by Bonferroni post-test).


A
7-ketocholesterol
nmol/mg LDL protein
Time (h)
SMase-LDL
Native LDL
***
***
+++
++
0
5
10
15
20
25
30
0
6
12
18
24


B
CLOOH
nmol/mg LDL protein
Time (h)
SMase-LDL
Native LDL
***
**
***
**
+++
***
***
**
0
100
200
300
400
500
600
0
6
12
18
24

5.3.4 The effect of cysteamine on oxidation of SMase-LDL by iron at lysosomal pH

Cysteamine was shown to prevent the initial oxidation of native LDL by iron possibly *via* scavenging hydroperoxyl radicals. Cysteamine is a lysosomotropic agent (Pisoni et al., 1995) and therefore should inhibit the oxidation of SMase-LDL under lysosomal conditions. In order to study the antioxidant activity of cysteamine on SMase-LDL oxidation at lysosomal pH, freshly prepared SMase-LDL (50 µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was oxidised with 5 µM $FeSO_4$ in the presence or absence of cysteamine (25 µM) at 37°C in quartz cuvettes. The course of the oxidation was followed by measuring the formation of conjugated dienes at 234 nm and loss of LDL-tryptophan fluorescence (Ex/Em = 282/331 nm).

As in the case of native LDL, cysteamine (25 µM) completely inhibited the initial oxidation of SMase-LDL cholesterol and caused a significant increase in the lag phase at pH 4.5 (Figure 5.7). The time taken for SMase-LDL to reach an attenuance of 0.1 during oxidation by iron was 76 ± 3 min when no cysteamine was added, compared with 352 ± 5 min in the presence of cysteamine ($p<0.001$, $n=3$), which is a 5 ± 0.2 fold inhibition of LDL oxidation.

Incubation of SMase-LDL with ferrous sulphate leads to continuous loss of tryptophan fluorescence, with a sharp loss initially (Figure 5.8). Cysteamine (25 µM) when added at time zero significantly prevented the loss of LDL-tryptophan fluorescence for 500 ± 50 min. The LDL fluorescence decreased by 34 ± 3% after 150 min of LDL oxidation with ferrous iron, whereas in the presence of cysteamine the fluorescence decreased by only 2 ± 0.9% ($p<0.001$, $n =5$).

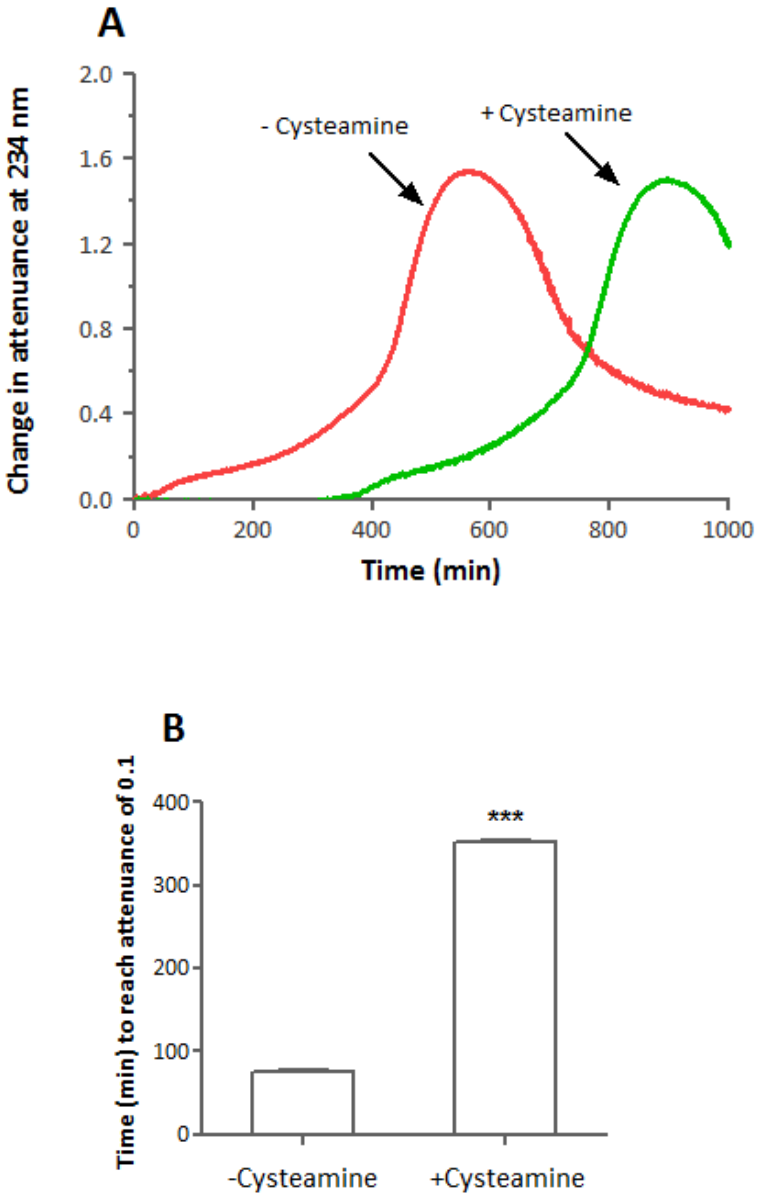


Figure 5.7 The effect of cysteamine on the oxidation of SMase-LDL by iron

(A) SMase-LDL (50 µg protein/mL) in NaCl/sodium acetate buffer (pH 4.5) was incubated with 5 µM $FeSO_4$ in the presence or absence of cysteamine (25 µM) at 37°C in capped quartz cuvettes. Oxidation was monitored by measuring the change in attenuance at 234 nm against appropriate reference cuvettes. This is a representative example of three independent experiments. (B) Time taken to reach an attenuance of 0.1 during the oxidation with iron (*** indicates $p<0.001$, t-test, n = 3 independent experiments.

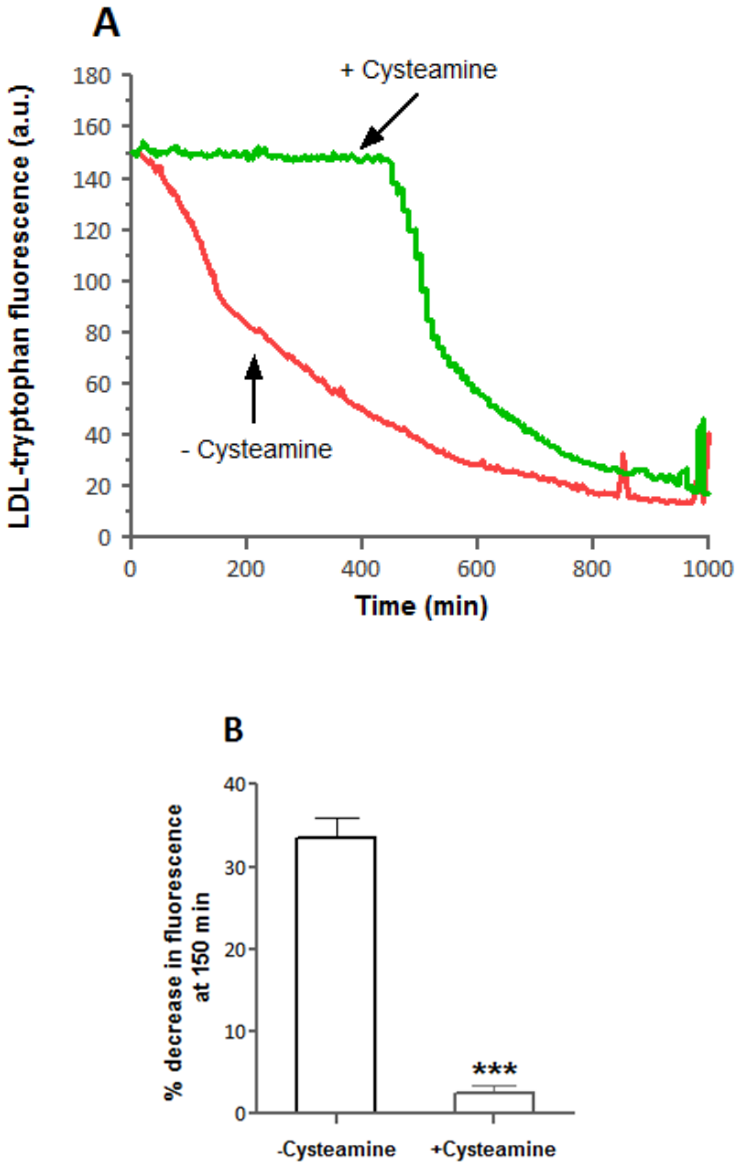


Figure 5.8 The loss of LDL-tryptophan fluorescence during SMase-LDL oxidation by iron

(A) SMase-LDL (50 µg protein/ml) was oxidised by 5 µM $FeSO_4$ in NaCl/sodium acetate buffer (pH 4.5) at 37°C in quartz cuvettes with or without 25 µM cysteamine and tryptophan fluorescence was measured every 10 min at excitation wavelength of 282 nm and emission wavelength 331nm in a spectrofluorometer. This is a representative example of at least three independent experiments. (B) Shows decrease in LDL-tryptophan fluorescence after 150 min of oxidation (*** indicates $p<0.001$, t-test, n = 3 independent experiments).

5.3.5 Measurement of lysosomal lipid peroxidation in human macrophages

Lipid peroxidation might be one of the major pathological mechanisms involved in atherosclerosis. In the lysosomal LDL oxidation theory, it has been shown that aggregated LDL is taken rapidly by the macrophages and oxidised in their lysosomes by iron. Having confirmed that the *in vitro* oxidation of SMase-LDL could be prevented by cysteamine, it was of importance to examine whether these results could apply to the lysosomes of human cultured macrophages.

Human THP1 monocytes were differentiated into macrophages with PMA as mentioned in detail in chapter 2. These macrophages were incubated in RPMI (containing 10% (v/v) fetal calf serum) and treated with no LDL, native LDL (200 µg protein/ml) or SMase-LDL (200 µg protein/ml) in the presence or absence of cysteamine (10 µM or 25 µM) for 24 h to induce lysosomal LDL oxidation. After 24 h the cells were treated with the lysosome-targetable probe, Foam-LPO (2 µM) for 15 min and then analysed by two-colour flow cytometry in the green (FL1) and red (FL2) channels. Macrophages which were treated with SMase-LDL showed decreased fluorescence intensity in the red (FL2) channel compared to control macrophages (Figure 5.9), confirming foam cell formation (Zhang et al., 2015). In the case of macrophages which were treated with SMase-LDL in the presence of cysteamine (10 µM and 25 µM), there was a gain in the fluorescence intensity in the FL2 channel.

The process of lipid peroxidation was quantified by ratiometric analysis of the fluorescence intensities of green channel and red channel (FL1/FL2) (Figure 5.10) (Zhang et al., 2015). The macrophages which were treated with native LDL showed a significant increase in the lipid peroxidation levels compared to control

macrophages ($p<0.001$). The SMase-LDL treated macrophages also showed increased lipid peroxidation activity in their lysosomes which was highly significant compared to control macrophages ($P<0.001$) as well as that in native LDL treated macrophages ($p<0.01$). Addition of the lysosomotropic antioxidant, cysteamine (10 µM and 25 µM) reduced the lipid peroxidation process in the SMase-LDL treated THP1 macrophages ($p<0.01$). Although, the reduction in lipid peroxidation by cysteamine was not concentration dependent, both 10 µM and 25 µM cysteamine brought down the lipid peroxidation level nearer to native LDL treated macrophages.

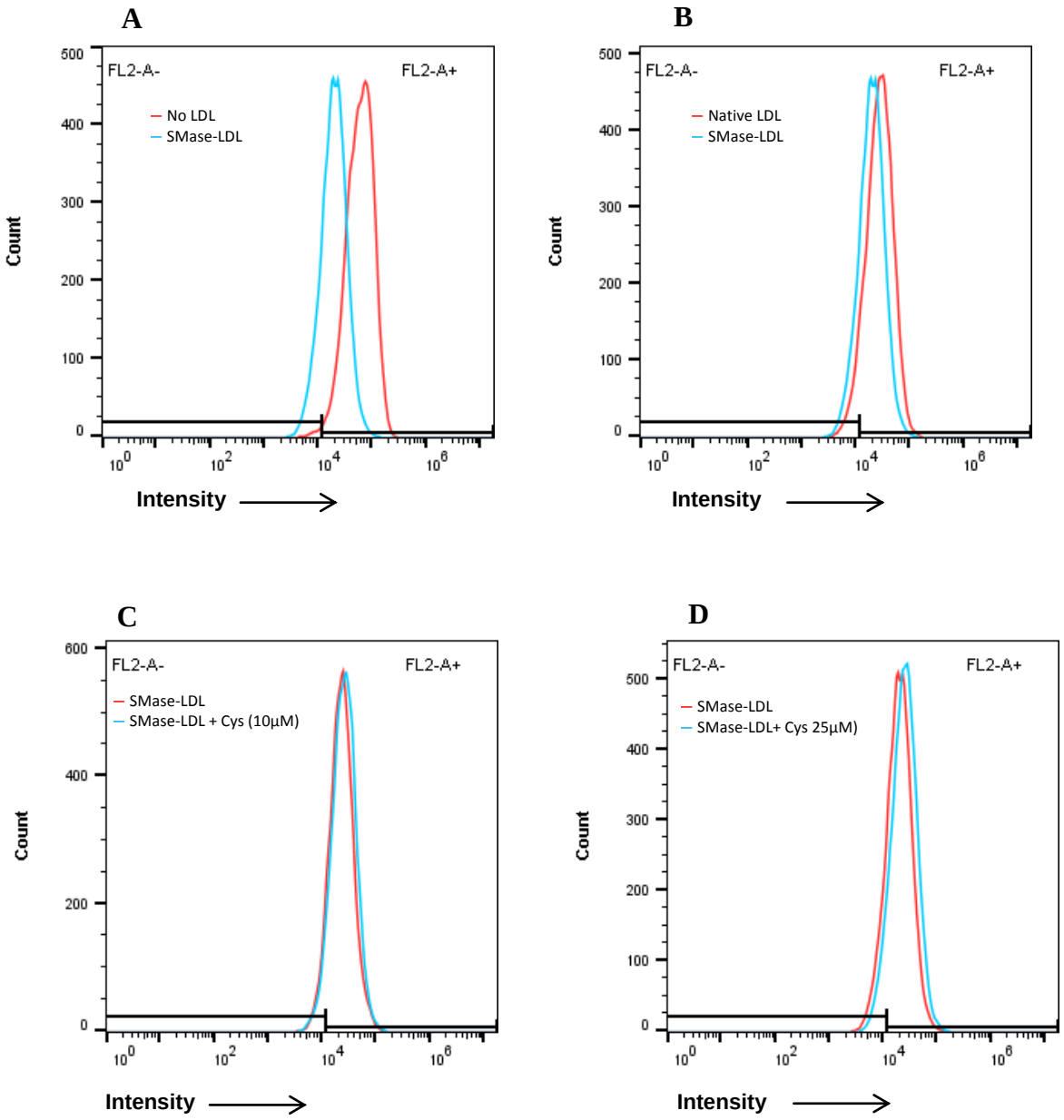


Figure 5.9 Flow cytometry analysis of Foam-LPO in THP1 macrophages

THP1 macrophages were treated with no LDL, native LDL (200 µg protein/ml LDL protein) or SMase-LDL (200 µg protein/ml LDL protein in the presence or absence of 10 µM or 25 µM cysteamine for 24 h. The cells were then incubated with 2 µM Foam-LPO for 15 min, harvested and assayed by flow cytometry. (A) Mean fluorescence intensity (MFI) in red channel (FL2) of healthy macrophages and SMase-LDL treated macrophage, (B) MFI in FL2 of native LDL treated macrophages and SMase-LDL treated macrophages, (C) & (D) FL2 MFI of macrophages treated with SMase-LDL and cysteamine 10 µM and 25 µM respectively. The data shown are a representative of four independent experiments.

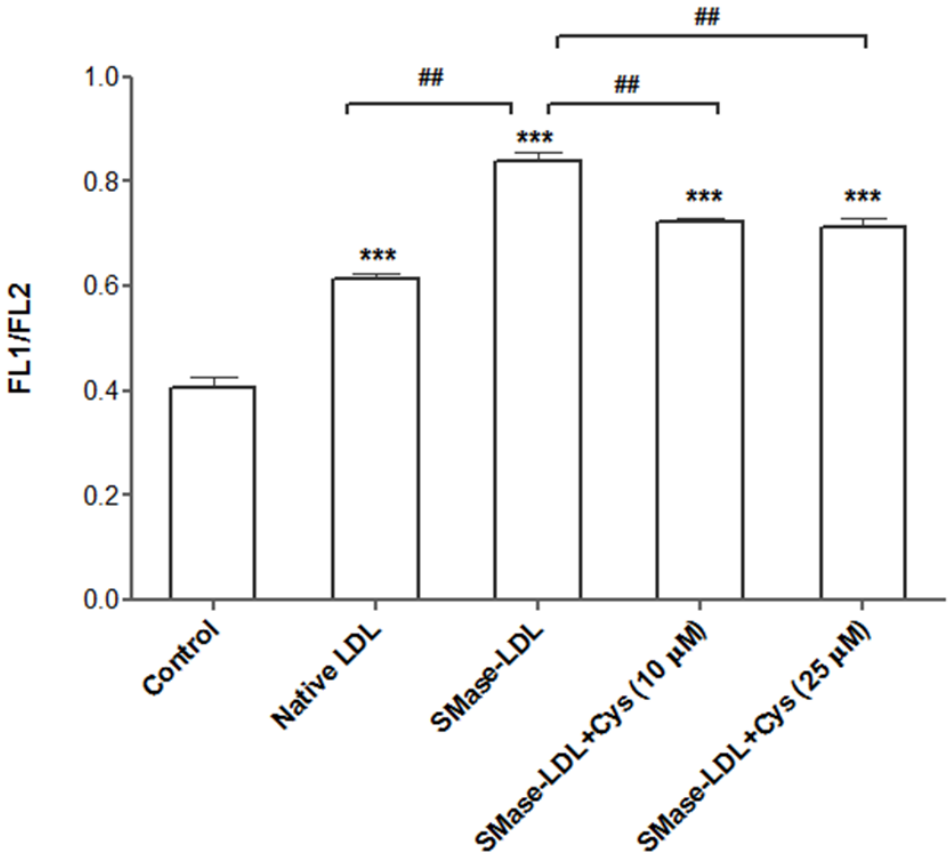


Figure 5.10 Measure of lipid peroxidation by Foam-LPO in lysosomes of macrophages

THP1 macrophages were treated with either no LDL, native LDL (200 µg protein/ml) or SMase-LDL (200 µg protein/ml LDL in the presence or absence of 10 µM or 25 µM cysteamine) for 24 h. The cells were then incubated with 2 µM Foam-LPO for 15 min, harvested and assayed by flow cytometry. Lipid peroxidation was calculated from the ratio between the mean fluorescence intensity of the green channel (FL1) and red (FL2) channel (*** indicated $p<0.001$ compared to untreated control cells, $^{\#\#}$ indicates $p<0.01$ compared to SMase-LDL only treated cells, ANOVA followed by tukey's test, n=3 independent experiments).

5.3.6 Oxidation of SMase-LDL in the lysosomes of macrophages

It has been established that both mechanically modified (vortexed) LDL as well as sphingomyelinase aggregated LDL is oxidised in the lysosomes of macrophages and generates ceroid (Wen and Leake, 2007, Wen et al., 2015). Ceroid (lipofuscin) is a final product of lipid oxidation that consists of insoluble polymerized lipids and is found within foam cells in atherosclerotic lesions (Mitchinson, 1982). Having confirmed that the treatment with SMase-LDL for 24 h increases while treatment with cysteamine decreases the level of lipid peroxidation in the lysosomes of human THP1 macrophages, it was of importance to examine a longer time effect of cysteamine on the lysosomal oxidation of SMase-LDL by ceroid formation. Also, the antioxidant probucol was unable to prevent the oxidation of LDL by iron at lysosomal pH (as discussed in Chapter 3 of this thesis) therefore, to confirm this inefficacy in the lysosomes of macrophages, effect of probucol on intra-lysosomal ceroid formation was also studied.

THP1 macrophages grown on cover slips were treated with native LDL or SMase-LDL (200 µg protein/ml) or without LDL for 24 h and were washed and incubated for 7 days in lipoprotein deficient medium with or without the antioxidants, probucol (10µM) and cysteamine (10 µM). The cells were then stained for ceroid which was clearly visible in the form of Oil Red O stained irregularly shaped granules in cells treated with SMase-LDL after soluble lipids had been removed by organic solvents. The ceroid levels in the cells were quantified using ImageJ software as mean integrated density of at least 100 cells and expressed as percent decrease of cells treated with SMase-LDL only.

Many of the THP1 macrophages which were treated with SMase-LDL contained significant amounts of ceroid whereas no significant ceroid was present in cells that had been incubated without LDL or with native LDL (Figure 5.11). Furthermore, treatment with probucol did not prevent the development of ceroid. After 7 days of treatment with probucol (10 μM), there was no apparent decrease in the ceroid levels as they reduced to 86 ± 9% compared to the SMase-LDL treated cells (Figure 5.12). Cells which were treated with cysteamine showed significant reduction in the ceroid formation in their lysosomes. Compared to the SMase-LDL treated cells, cysteamine reduced ceroid development in the macrophages to 44 ± 1% which is similar to that in the cells treated with native LDL (44.3 ± 13%). These results show that lysosomal oxidation of LDL is not prevented by probucol but can be prevented by antioxidants like cysteamine which concentrates in the lysosomes.

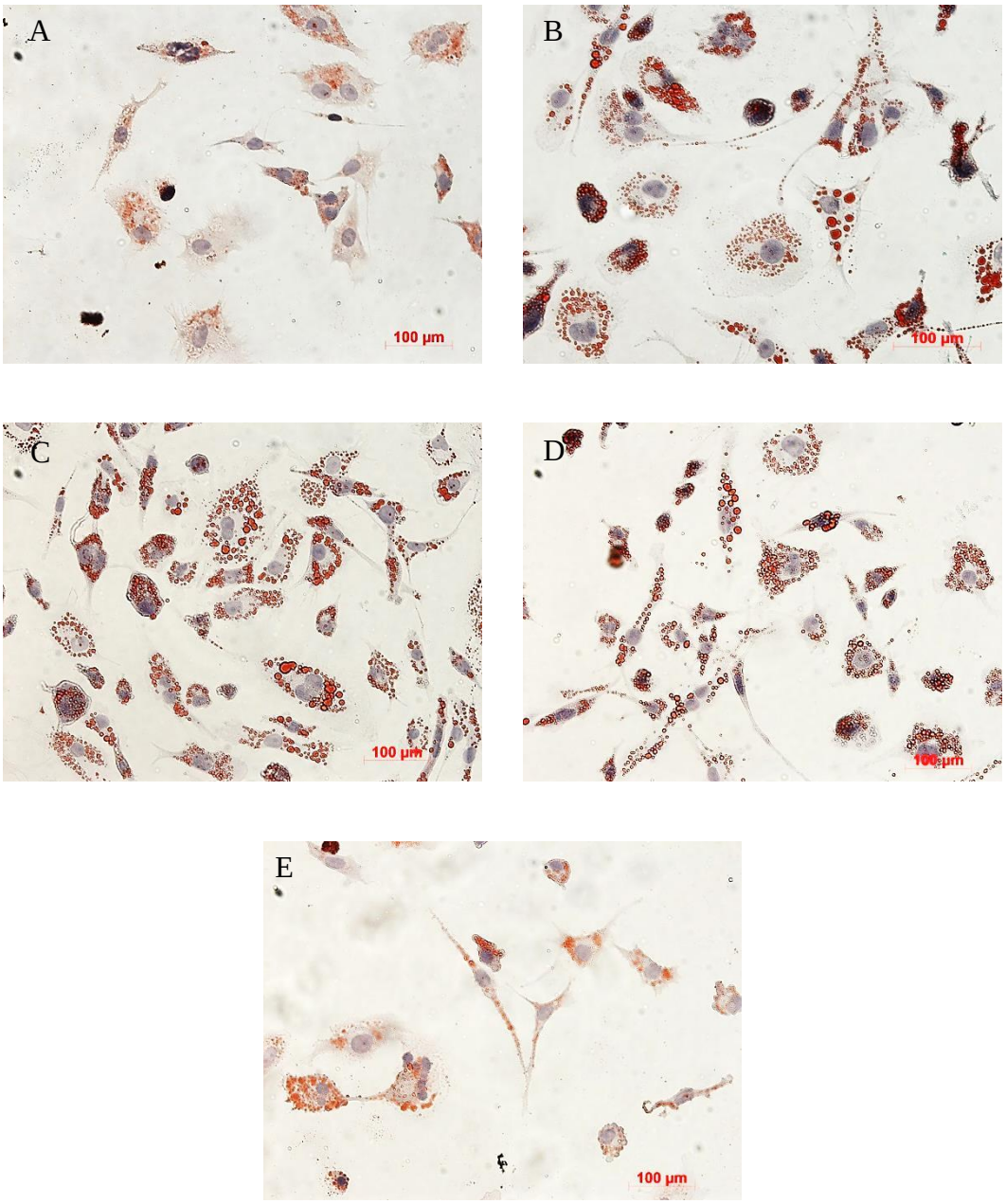


Figure 5.11 Lysosomal ceroid formation

THP1 macrophages were grown on cover slips in 6 well sterile tissue culture plates. Wells were treated with SMase-LDL (200 µg protein/mL) for 24 h and after washing, were further incubated for 7 days in fresh RPMI containing 10% (v/v) lipoprotein deficient serum, either alone (C) or with 10 µM probucol (D) or 10 µM cysteamine (E). Untreated cells were taken as control (A). Native LDL (200 µg protein/mL) treated cells are also shown (B). After 7 days, cells were treated with ethanol and xylene, followed by Oil Red O staining, and examined by light microscopy to demonstrate ceroid. This is a representative of 3 independent experiments.

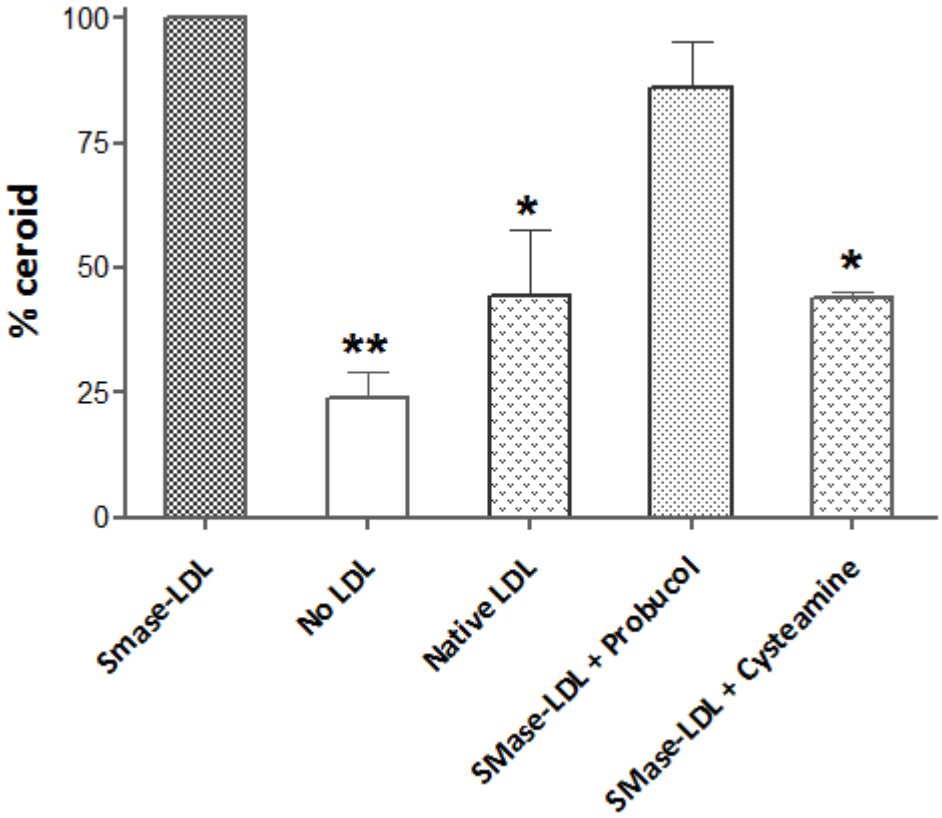


Figure 5.12 Percentage ceroid in THP1 macrophages

THP1 macrophages differentiated on coverslips were treated with SMase-LDL for 24 h. The cells were washed and further incubated for 7 days in RPMI medium with 10% (v/v) lipoprotein deficient serum either alone or with cysteamine (10 µM) or probucol (10 µM). Cells treated with neither LDL or nor antioxidants were used as control and native LDL treated cells were also used for comparison. At the end of the treatment all the wells were processed for ceroid detection by Oil Red O stating procedure. The ceroid levels in the cells were quantified using ImageJ as mean integrated density of at least 100 cells for each treatment and expressed as percentage ceroid compared to SMase-LDL treated macrophages (* indicates $p < 0.05$ compared to SMase-LDL only treated group, ANOVA followed by tukey's test, n = 3 independent experiments).

5.4 Discussion

A prominent feature of atherosclerotic plaque is the cholesteryl ester-loaded macrophage, or foam cell. Because uptake of native LDL is subject to negative feedback regulation (*via* the LDL receptor), accumulation of excessive amounts of LDL-derived cholesterol by macrophages is thought to require modification of LDL that permits rapid unregulated internalization. Several types of LDL modification (e.g. acetylation, oxidation and aggregation) have been shown to permit cholesterol uptake *in vitro*, producing acetylated LDL (acetyl-LDL), oxidized LDL (oxLDL) and aggregated LDL (ag-LDL), respectively (Levitan et al., 2010, Lougheed et al., 1999, Zhang et al., 1997). There is no evidence that acetylation of LDL occurs to any extent *in vivo* and the extracellular oxidation of LDL has been a point of discussion after the failure of major antioxidants trials and is therefore the subject of active debate (Steinberg, 2009). However, there are several lines of evidence implying that LDL aggregation takes place in the arterial wall. Aggregation of LDL has been proposed to represent an essential and central process in atherogenesis (Getz and Reardon, 2015, Pentikainen et al., 1996), and LDL extracted from atherosclerotic lesions has been found to be aggregated or prone to aggregation (Aviram et al., 1995, Bancells et al., 2010b). Furthermore, aggregated LDL has been found to be rapidly endocytosed by macrophages and concentrated in their lysosomes where it is then oxidised (Wen and Leake, 2007). Aggregated LDL found in atherosclerotic lesions has been found to be rich in ceramide (about 10-50 fold higher than un-aggregated LDL) (Schissel et al., 1996). The involvement of ceramide is of particular interest as this lipid has been established as a central messenger in apoptosis (Heinrich et al., 2004, Jin et al., 2008, Kashkar et al., 2005). Taken together, altered sphingolipid metabolism could

represent a risk factor for the development of atherosclerosis and the involvement of SMase and ceramide may play a central role in the development of this disease, providing also a link to apoptosis. Our laboratory has recently shown that LDL aggregated by sphingomyelinase is taken up by human macrophages and concentrated in their lysosomes where it is extensively oxidised by iron (Wen et al., 2015). The oxidation of native LDL by iron at lysosomal pH has been studied in this laboratory previously (Satchell and Leake, 2012), but as mentioned before SMase-LDL is the more pathophysiological form of LDL that undergoes oxidation in the lysosomes of macrophages. Therefore, it is important to know the oxidation pattern of SMase-LDL in depth.

Both native LDL and SMase-LDL were effectively oxidised by iron at lysosomal pH (pH 4.5) and the oxidation process was found to be independent of the presence of pre-existing lipid hydroperoxides. The oxidation of SMase-LDL lipids by iron followed the same pattern as that of native LDL, with an initial lag, followed by rapid oxidation phase, slow oxidation phase and then aggregation and sedimentation phases. However, the oxidation of SMase-LDL was slower than native LDL. The rate of oxidation of the apoB-100 part of SMase-LDL, studied by following the loss of LDL-tryptophan fluorescence, was also found to be slower than that of native LDL. SMase treatment of LDL causes aggregation of LDL, as the increase in ceramide makes it unstable and there are also changes in the secondary structure of apoB-100, such as decrease in α-helix content (Sneck et al., 2012). In the current experimental setup, LDL aggregates of around 200 nm diameter (native LDL is about 22 nm) are formed and therefore, the slower oxidation of SMase-LDL could be due to a reduced accessibility of iron to these

LDL aggregates due to lower surface area to volume ratio (Walters and Wrenn, 2010).

The lipid composition of SMase-LDL oxidised by iron at lysosomal pH, over a 24 h time course, has been characterised and compared to native LDL. Levels of nonesterified cholesterol decreased continuously during the oxidation of SMase-LDL. The decrease was not different from that seen in native LDL, possibly because most of the nonesterified cholesterol is found in the outer monolayer of LDL. The levels of one of the oxidation products of cholesterol, 7-ketocholesterol, increased during the course of oxidation in both SMase-LDL and native LDL. In the case of SMase-LDL, the formation of 7-ketocholesterol was initially slower compared to native LDL but after 24 h, SMase LDL has significantly higher levels than native LDL. The increase in 7-ketocholesterol was much lower than the decrease in free cholesterol, but cholesterol is known to be oxidised to other products (not measured here) such as 7-α-hydroperoxylcholesterol, 7-β-hydroperoxycholesterol, 7-α-hydroxycholesterol, 7-β-hydroxycholesterol, cholesterol α-epoxide and cholesterol β-epoxide (Chang et al., 1997, Min et al., 2015). Oxysterols, such as 7-ketocholesterol, are also present in human atherosclerotic plaques and may exert atherogenic effects (reviewed in Brown and Jessup, 1999). Oxysterols have been detected at increased levels in the plasma of cardiovascular patients (Zhou et al., 2000) and have been found to be relatively higher in atherosclerotic plaques than in normal arteries (Garcia-Cruset et al., 1999, Liu et al., 1997). Oxysterols have been reported to have many pathophysiological effects *in vitro*, such as triggering proinflammatory and cytotoxic reactions towards various cells of the vascular wall (Lizard et al., 1999, Luthra et al., 2008, Pedruzzi et al., 2004, Rosklint et al., 2002). The fact that after

24 h, SMase-LDL oxidised by iron, at lysosomal pH, contains higher amounts of 7-ketocholesterol than native LDL, suggests that it may exert potentially higher atherogenic effects.

The levels of the three major cholesteryl esters measured, cholesteryl linoleate (CL), cholesteryl arachidonate (CA) and cholesteryl oleate (CO), decreased significantly over the course of the 24 h oxidation in SMase-LDL. The rate of decrease of CL and CA levels in SMase-LDL was slower than in native LDL in the first 12 h, but after 24 h both LDL forms had almost negligible amounts of these esters left. However, there was no apparent difference in the decrease of CO levels between SMase-LDL and native LDL, which could be due to overall slower oxidation of CO because of having only one double bond.

The oxidation of cholesteryl esters involves the oxidation of their polyunsaturated fatty acid (PUFA) moieties (described in Chapter 1). PUFAs are oxidised *via* a free radical chain reaction (Esterbauer et al., 1990), which is initiated by the abstraction of a hydrogen atom by a free radical to give a lipid alkyl radical. This alkyl radical then undergoes intramolecular rearrangement to form the more stable conjugated diene alkyl radical which on oxygenation produces a lipid peroxyl radical, which abstracts a hydrogen from another unsaturated lipid, thereby instigating a chain reaction. Here, CL and CA were more rapidly oxidised than CA, probably due to higher number of double bonds present in them. In SMase-LDL, the levels of the cholesteryl linoleate oxidation product, cholesteryl linoleate hydroperoxide (CLOOH), increased significantly up to 12 h of oxidation, and then appeared to decrease again, probably due to its further oxidation in the fatty acyl or cholesterol moiety shifting its time of elution in HPLC. While in case of native LDL, the levels

peaked at 6 h during oxidation and decreased to similar levels as SMase-LDL after 24 h. The slow formation of CLOOH in SMase-LDL could therefore be due to the slower oxidation of CL seen in it when compared to native LDL.

The lysosomotropic thiol antioxidant, cysteamine, completely inhibited the initial oxidation of SMase-LDL cholesterol by iron at lysosomal pH (pH 4.5). No conjugated diene formation was observed in about the first 6 h of oxidation process when cysteamine was added, indicating that no lipid peroxidation took place during that time. The oxidation of apoB-100 of SMase-LDL as measured by tryptophan fluorescence was also prevented effectively by cysteamine during its oxidation by iron at lysosomal pH.

Similar results were noticed *in vivo* in the lysosomes of macrophages, where incubation of macrophages with SMase-LDL caused increased lipid peroxidation in their lysosomes, after 24 h, compared to native LDL and treatment with cysteamine significantly prevented this lysosomal peroxidation (Figure 5.10). The high levels of lipid hydroperoxides present in SMase-LDL oxidised by iron in lysosomes may confer atherogenic properties to the LDL by accumulating in the lysosomes of macrophages which might affect the subsequent hydrolysis of cholesteryl esters (Jerome et al., 2008, Kritharides et al., 1998) and can lead to ceroid formation (Carpenter et al., 1990, Shimasaki et al., 1995).

Ceroid, an end product of LDL oxidation consisting of a complex of insoluble oxidised lipids and proteins, is found both intracellularly and extracellularly within atherosclerotic lesions (Mitchinson et al., 1985). Our laboratory has already established that intralysosomal ceroid is produced in human macrophages when they are treated with aggregated LDL (Wen and Leake, 2007, Wen et al., 2015).

Here, human THP1 macrophages treated with SMase-LDL contained high quantities of intralysosomal ceroid compared to the untreated cells and native LDL-treated cells. The amount of ceroid was quantified as total integrated density and expressed as percentage of ceroid in SMase-LDL treated cells. THP1 macrophages which were treated with the lysosomotropic antioxidant, cysteamine for 7 days after SMase-LDL treatment had significantly lower ceroid present in their lysosomes, whereas the macrophages which received probucol treatment did not show any decrease in the ceroid percentage (Figure 5.12).

Cysteamine is a superoxide scavenger (discussed in Chapter 3) and therefore, would be able to prevent the lipid peroxidation of SMase-LDL by scavenging the hydroperoxyl radical ($HO_2^{\bullet}$) formed during the redox cycle of lysosomal iron. Treatment with cysteamine every day for 7 days would thus continuously inhibit the lysosomal oxidation of LDL cholesterol and protein in the macrophages and therefore is able to decrease the formation of intralysosomal ceroid.

Probucol has a long history of clinical application for the treatment of cardiovascular diseases (Yamashita et al., 2008, Yamashita and Matsuzawa, 2009). It was initially introduced as a cholesterol-lowering drug in early 1970's, and later it was also found to attenuate atherosclerotic lesion development in most animal models (Carew et al., 1987, Kita et al., 1987, Sasahara et al., 1994, Yoshikawa et al., 2008). It was assumed that probucol inhibited atherosclerosis by decreasing the oxidation of LDL, but Stocker's group suggested that probucol might inhibit atherosclerosis by upregulating heme oxygenase-1 (Deng et al., 2004). However, the findings of The Probucol Quantitative Regression Swedish Trial (PQRST) in 1995 showed that the treatment of hypercholesterolaemia

patients with probucol did not have any beneficial effects (Walldius et al., 1994). Under the current experimental conditions, probucol was unable to prevent the lysosomal oxidation of LDL in the macrophages which could be due to its inability to protect the oxidation of LDL by iron at lysosomal pH (as discussed in Chapter 3). The results here provide further evidence of the role of hydroperoxyl radicals in initiating the oxidation of LDL by iron under lysosomal conditions.

Chapter 6-

Implications of lysosomal oxidation of LDL

6.1 Introduction

The presence of lipid laden macrophage foam cells is a characteristic feature of atherosclerosis (Yu et al., 2013). The atherosclerotic foam cells derive the majority of their lipid from uptake of modified lipoprotein, such as aggregated LDL or oxLDL, and other extracellular lipid particles (Aviram, 1993, Khoo et al., 1988). Under normal conditions, receptor-mediated uptake of lipoproteins trafficks the particles to lysosomes where at an acidic pH, the lysosomal enzymes break down the protein and lipid components of LDL to products that can readily transverse the lysosomal membrane (Ghosh et al., 2010, Goldstein and Brown, 2009). Modified LDL such as aggregated LDL has been reported to produce foam cells by receptor- independent uptake by macrophages (Steinbrecher and Lougheed, 1992, Zhang et al., 1997). Oxidised LDL is recognised and taken up by scavenger receptors found on macrophages and because of no feedback regulation for such uptake, leads to accumulation of cholesterol in these cells. Aggregated LDL on the other hand can be taken up by macrophages through processes like phagocytosis, pinocytosis or patocytosis (Kruth, 2002, Kruth et al., 1999) and presented to the lysosomes where it can be oxidised (Wen and Leake, 2007). Aggregation of LDL by the enzyme sphingomyelinase has been reported to cause a 10 fold increase in cholesteryl ester accumulation in macrophages compared to native LDL (Xu and Tabas, 1991).

There is good evidence to suggest that lysosomal dysfunction plays an important role in foam cell formation and plaque development (Reviewed by Jerome and Yancey, 2003). Recently it has been reported that oxLDL and cholesterol are able to cause profound lysosomal dysfunction in mouse macrophages through

disruptions in the pH, proteolytic capacity and membrane integrity of their lysosomes (Emanuel et al., 2014). It is also reported that treatment of human macrophages with oxLDL or vortexed LDL can affect the functions of lysosomal acid lipase (LAL) and overall loss of lysosomal function by increasing the lysosomal pH (Cox et al., 2007). As mentioned earlier, mouse macrophages rapidly phagocytose aggregated LDL into their lysosomes and oxidise it with iron (Wen and Leake, 2007). Recently it has been shown that LDL aggregated by sphingomyelinase (SMase-LDL), a more pathophysiological form of LDL, is also rapidly internalised by macrophages and oxidised in their lysosomes (Wen et al., 2015). Oxidation of lipoproteins in lysosomes leads to the formation of 7-ketocholesterol (Wen and Leake, 2007), a major component of oxidised LDL, which is reported to promote accumulation of unesterified cholesterol in the lysosomes of macrophages (Maor et al., 1995) and to induce permeabilization of the lysosomal membrane in human endothelial cells (Li et al., 2011). Accumulation of 7-ketocholesterol and other oxysterols in the lysosomes during oxidation of LDL generates ceroid/lipofuscin which has been reported to diminish the lysosomal proteolytic activity in cultured human fibroblasts (Terman and Brunk, 1998).

In the previous chapter of this thesis, it was shown that inhibiting the lysosomal oxidation of LDL by the antioxidant cysteamine generates less Intralysosomal ceroid in human THP-1 macrophages and also reduced the overall lysosomal lipid peroxidation process in them. Taking into consideration the above mentioned data, targetting lysosomal dysfunction might be an important step in decreasing atherosclerosis. Therefore, it is important to investigate if the lysosomal oxidation of SMase-LDL produces lysosomal dysfunction in human macrophages and also if

inhibiting this oxidation by the lysosomotropic antioxidant, cysteamine, may restore normal lysosomal function in them.

Ceroid or lipofuscin is an auto-fluorescent lipid-protein complex produced after extensive oxidation of lipoproteins in the lysosomes of macrophages and is frequently found in the foam cells (Ball et al., 1987, Wen and Leake, 2007, Yin and Brunk, 1998). Ceroid was first reported to be present in human atherosclerotic lesions in 1946 (Pappenheimer and Victor, 1946) and since then, several reports have described ceroid in the full spectrum of atherosclerotic lesions (Burt, 1952, Haka et al., 2011, Hartroft, 1953, Mitchinson, 1982, Verbunt et al., 1992). Ceroid is believed to have detergent like properties which can disrupt membranes and thus release lysosomal contents into the cytosol leading to cell injury or necrosis (Gupta et al., 2001) and can therefore, lead to plaque progression.

Ceroid is often used as a histological index of cellular senescence and ageing (Garay et al., 1979, Jung et al., 2010). This is because the accumulation of lipofuscin (or ceroid) has been found to have a linear correlation with age in many animals (Hosokawa et al., 1994, Munnell and Getty, 1968, Nakano and Gotoh, 1992, Sheehy et al., 1995). In humans, it has been demonstrated that the accumulation of lipofuscin in myocardium (Strehler et al., 1959), plasma and saliva (Feng et al., 2015) are directly related to the age of a person.

Atherosclerosis is classed as a disease of ageing, as increasing age has been found to be an independent risk factor for the development of this disease. Atherosclerosis is also seen to be associated with biological ageing, as atherosclerotic plaques show evidence of cellular senescence characterized by reduced cell proliferation, apoptosis, elevated DNA damage, epigenetic

modifications, and telomere dysfunction (reviewed in Brunk and Terman, 2002). Not only is cellular senescence associated with atherosclerosis, there is growing evidence that cellular senescence may promote atherosclerosis. For example, cellular senescence has been shown to cause vascular smooth muscle dysfunction (Gorenne et al., 2006, Wang et al., 2015) and endothelial cell dysfunction (Minamino et al., 2002), both of which can exert profound effects on atherogenesis and stability of advanced plaques (Minamino and Komuro, 2007). It has been proposed that oxidative stress-induced damage to cellular components, probably due to the combination of higher reactive oxygen species (ROS) and impaired antioxidant defence, is the main contributor to the ageing process (Moon et al., 2001). Although it is well established that oxidised lipoproteins and their products are able to induce ROS-dependent senescence in cells (Imanishi et al., 2004, Riahi et al., 2015, Watt et al., 2016), it is not known if lysosomal oxidation of LDL can induce senescence in human cells. Therefore, it is of interest to investigate if oxidation of SMase-LDL in the lysosomes of macrophages can induce senescence in them and if these senescent signs can be reversed by cysteamine. The most commonly used biomarker for assessing the process of senescence in mammalian cells is to measure the activity of lysosomal enzyme β-galactosidase, which is highly expressed in pre-senescent and senescent cells (Dimri et al., 1995).

Inflammation participates in atherosclerosis during initiation and throughout all stages of plaque development. Expression and secretion of inflammatory cytokines, like TNF-α, by the cells in the arterial intima is another characteristic feature of atherosclerosis. TNF-α is seen to participate in the atherosclerotic process through various mechanisms, such as stimulation of adhesion molecule

expression (Khan et al., 1995), stimulation of scavenger receptor expression and cholesterol loading (Li et al., 1995), regulation of plaque stability (Libby et al., 1995), and stimulation of growth factors important for proliferation (Filonzi et al., 1993).

Several studies have shown that oxidised LDL and acetylated-LDL can activate macrophages, including inflammatory reactions in the vessel wall. Modified LDL can stimulate monocyte migration (Navab et al., 1991), expression of adhesion molecules (Khan et al., 1995), and production of TNF-α, IL-8, and MCP-1 (Pollaud-Cherion et al., 1998, Wang et al., 1996). Even native LDL has been suggested to possess immune-stimulating properties, like stimulation of adhesion molecule expression on vascular endothelial cells, chemokine receptor expression, monocyte chemotaxis (Han et al., 1998) and even potentiation of lipopolysaccharide (LPS)-induced production of TNF-α in human macrophages (Netea et al., 2002). The role of aggregated LDL in the expression of proinflammatory cytokines is controversial with some studies suggesting that it can decrease the secretion of many proinflammatory cytokines such as TNF-α, IL-6 and IL-8 by human macrophages (Persson et al., 2006, Satchell, 2007), while a recently published report suggesting that loading of human macrophages with aggregated LDL promotes their release of proinflammatory cytokines (Estruch et al., 2013). All these studies used the same form of aggregated LDL produced by mechanical vortexing and there is not much known about the effect of enzymatically aggregated LDL, a more pathophysiological form of aggregated LDL, like SMase-LDL, on the expression of pro-inflammatory cytokines. Data are presented here, which show that LDL modified by sphingomyelinase can potentiate the LPS-induced expression of TNF-α.

6.2 Methods

The methods used have been explained in detail in section 2.5.

6.3 Results

6.3.1 Effect of SMase-LDL and cysteamine on lysosomal function

The lysosomal function of the macrophages was measured by using the lysosomotropic dye called LysoTracker Red. Human THP-1 monocytes were differentiated into macrophages with PMA as described in detail in chapter 2. These macrophages were incubated in RPMI (containing 10% v/v fetal calf serum) and treated with no LDL, native LDL (100 µg protein/ml) or SMase-LDL (100 µg protein/ml) in the presence or absence of cysteamine (10 µM or 25 µM) every 24 h for 72 h. The macrophages were treated with LysoTracker Red (500 nM for 30 min) and then analysed by flow cytometry in the red channel (FL2). The results were expressed as percentage loss of signal compared to control cells which received no LDL. A decrease in LysoTracker intensity relative to baseline suggests disruptions in lysosomal function, integrity, or quantity.

THP-1 macrophages that were treated with native LDL showed no significant shift in LysoTracker Red (LTR) signal compared to control cells, whereas macrophages which were treated with SMase-LDL for 72 h showed a significant loss in the LTR signal compared to the control (Figure 6.1). Native LDL treated cells showed only 6 ± 4 % decrease in the LTR signal after 72 h which was not statistically different, whereas in macrophages which were treated with only SMase-LDL for 72 h showed a significant decrease of 26 ± 2% (p<0.001) in the LTR signal compared to the control (Figure 6.2). The loss in LTR signal due to SMase-LDL was largely

reversed by 25 μM cysteamine, but not by 10 μM cysteamine. The percentage loss of LTR signal when cells received 10 μM cysteamine was 24 ± 1% (p<0.001) compared to control, while in macrophages which received 25 μM cysteamine, the SMase-LDL induced loss of LTR signal was brought down from 26 ± 2% to 9 ± 3% (p<0.01), similar to that in native LDL treated macrophages.

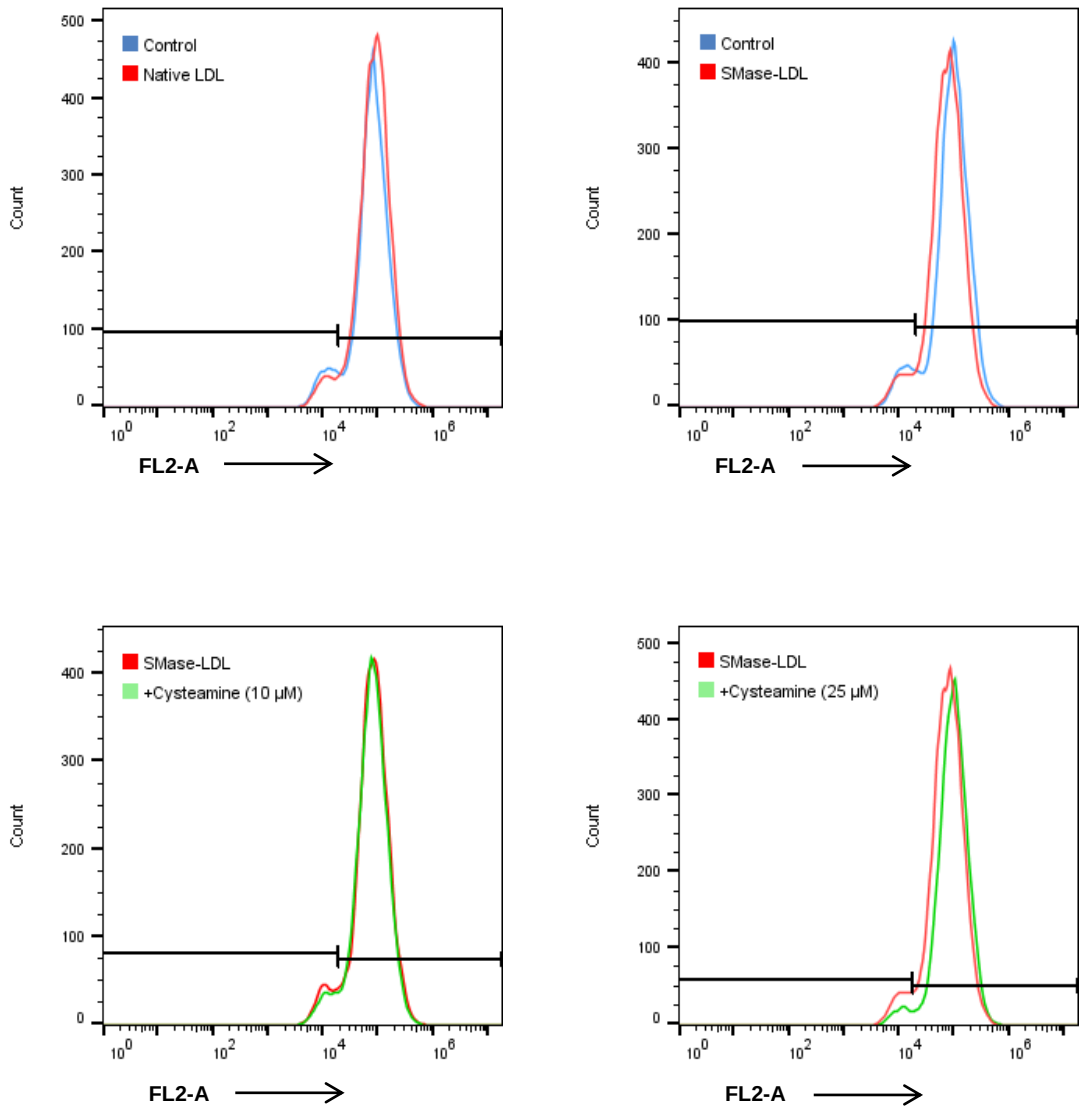


Figure 6.1 Effect of SMase-LDL and cysteamine on lysosomal function of macrophages

THP-1 macrophages (1 x 10^6) were cultured in 12 well tissue culture plates in RPMI medium (containing 10% v/v FCS) alone or containing native LDL or SMase-LDL with or without cysteamine (10 µM or 25 µM) for 72 h. All LDL concentrations were 100 µg protein/ml. After 72 h, cells were treated with 500 nM Lysotracker Red for 30 min and then assayed by flow cytometry. Mean fluorescence intensity peak of Lysotracker Red in the FL2 channel was then measured. The data shown are a representative of four independent experiments.

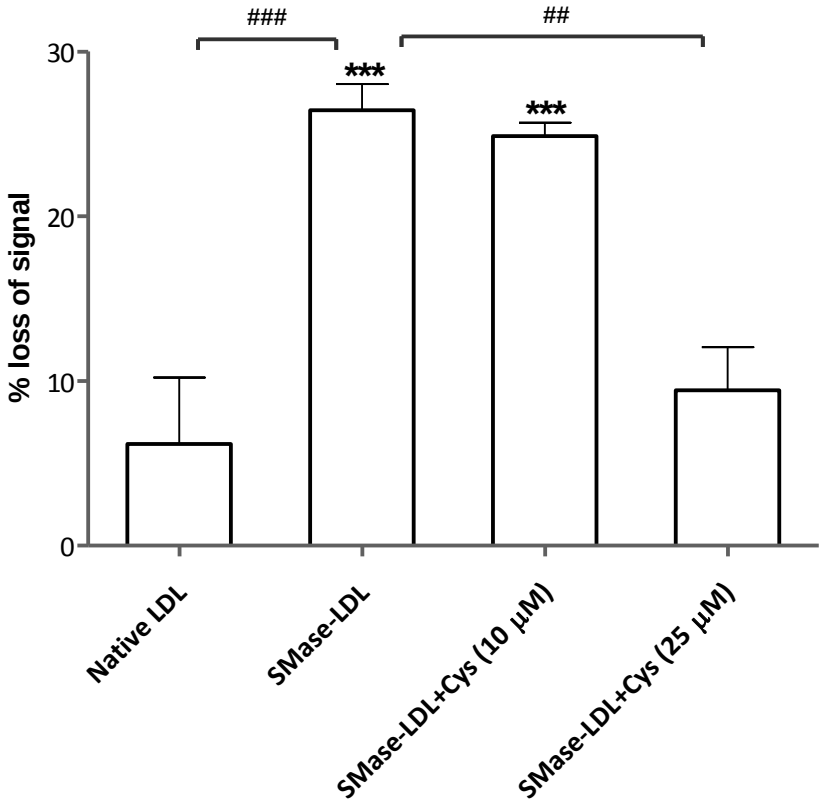


Figure 6.2 Effect of lysosomal oxidation of LDL on lysosomal function of macrophages

THP-1 macrophages (1 x 10^6) were cultured in 12 well tissue culture plates in RPMI medium (containing 10% v/v FCS) alone, or containing native LDL, or SMase-LDL with or without cysteamine (10 µM or 25 µM) for 72 h. All LDL concentrations were 100 µg protein/ml. After 72 h cells were treated with 500 nM Lysotracker Red for 30 min and then assayed by flow cytomtery. The data were expressed as percentage loss of mean fluorescence intensity of Lysotracker Red in the FL2-A channel compared to untreated control macrophages. (*** indicates p<0.001 compared with untreated cells, $^{\#\#}$ indicates p<0.01, $^{\#\#\#}$ indicates p<0.001 compared with SMase-LDL treated cells, ANOVA followed by tukey's test, n = 4 independent experiments).

6.3.2 Effect of SMase-LDL oxidation and cysteamine on lysosomal pH of macrophages

For the optimal function of the hydrolytic enzymes of lysosomes, the acidic pH is an essential requirement (Mindell, 2012) and any increase in this pH has been shown to affect the function of the lysosomal enzyme systems (Ohkuma and Poole, 1978). There are reports that modified LDL leads to accumulation of cholesterol in the lysosomes of macrophages and can affect lysosomal hydrolysis due to changes in lysosomal pH (Cox et al., 2007, Orso et al., 2011). In order to determine if the lysosomal oxidation of SMase-LDL affects lysosomal function due to changes in the lysosomal pH, a ratiometric lysosomal pH indicator dye called LysoSensor Yellow/Blue DND-160 was used to measure the pH of the lysosomes in macrophages.

Briefly, differentiated THP-1 cells at 1 x10^5 cells per well (96 well black microplate) in RPMI medium (containing 10% v/v FCS) were incubated with no LDL, native LDL (100 µg protein/ml) or SMase-LDL (100 µg protein/ml) every 24 h for 72 h in the presence or absence of cysteamine (10 µM or 25 µM). The cells were then washed and treated with 5 µM Lysosensor Yellow/Blue and the pH of the lysosomes was calculated as described in section 2.5.7.

The pH of the lysosomes of untreated THP-1 macrophages was 4.9 ± 0.3 which is within the normal range of 4.5 to 5 (Figure 6.3). Native LDL treatment did not significantly increase the pH of the lysosomes. Treatment with SMase-LDL for 72 h increased the lysosomal pH to 6.2 ± 0.2, which was found to be statistically significant compared to both control macrophages (p<0.001) and native LDL treated macrophages (p<0.01). Cysteamine treatment (10 µM or 25 µM) prevented

the SMase-LDL induced increase in lysosomal pH. In the macrophages, which received 10 µM cysteamine, the pH was significantly lower than SMase-LDL-treated macrophages at 5.0 ± 0.1 (p<0.001), whereas 25 µM reduced the SMase-LDL induced lysosomal pH increase to 4.6 ± 0.2 (p<0.001).

To determine if the action of cysteamine on lysosomal pH was due to inhibition of lysosomal oxidation of LDL or due to direct pH lowering effect of cysteamine, the effect of cysteamine was studied separately on untreated THP-1 macrophages and native LDL-treated macrophages. It was found that treatment with 10 µM or 25 µM cysteamine had no significant effect on the lysosomal pH of THP-1 macrophages in the absence of LDL (Figure 6.4A). The pH of lysosomes in untreated cells was found to be 3.9 ± 0.3, while the lysosomal pH was 4.6 ± 0.2 with 10 µM cysteamine treatment (p>0.05) and 4.5 ± 0.2 with 25 µM cysteamine treatment (p>0.05).

Similarly, cysteamine (10 µM or 25 µM) showed no significant effect on the lysosomal pH of THP-1 macrophages when treated with native LDL (Figure 6.4B). The pH in the cells which were incubated with only native LDL was found to be 5.8 ± 0.3, while in presence of cysteamine, 10 µM and 25 µM, the pH of the THP-1 lysosomes was 5 ± 0.2 (p>0.05) and 5.3 ± 0.1 (p>0.05) respectively.

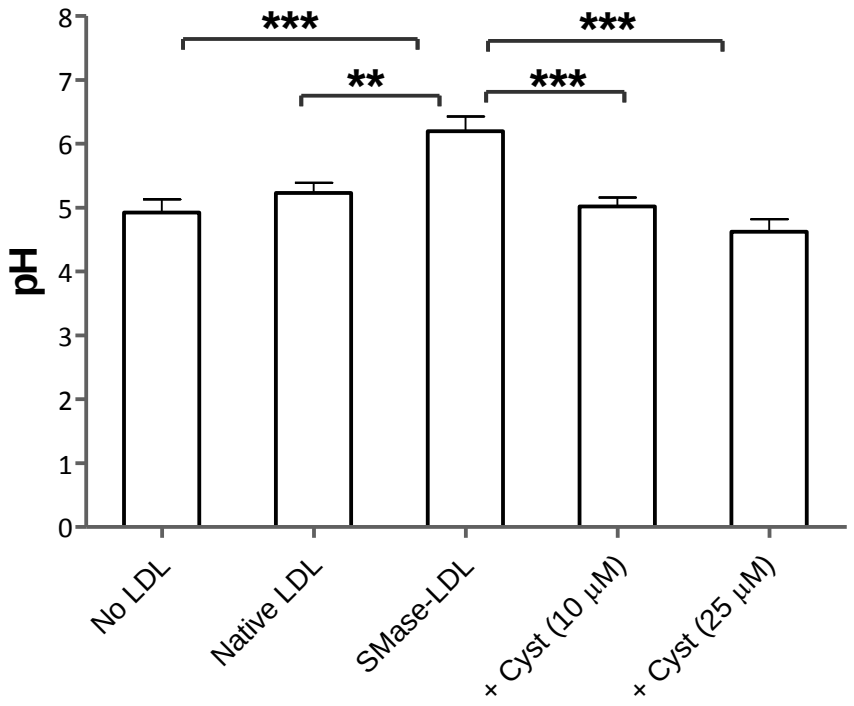


Figure 6.3 Effect of lysosomal oxidation of SMase-LDL on the pH of lysosomes in THP-1 macrophages.

THP-1 macrophages were cultured in black 96-well microplate at 1 x 10^5 per well in RPMI medium (containing 10% v/v FCS) with no LDL, native LDL (100 µg protein/ml) or SMase-LDL (100 µg protein/ml) with or without cysteamine (Cyst) (10 µM or 25 µM) for 72 h. The cells were then treated with 5 µM Lysosensor Yellow/Blue for 30 min at 37°C. The samples were then read in a Fluo Star Optima fluorometer (BMG Labtech), with excitation at 355nm. The ratio of emission at 440nm and 535nm was then calculated for each sample and the pH values determined from the standard plot. (****** indicates $p<0.01$ and *** indicates $p<0.001$ compared to SMase-LDL treated macrophages, ANOVA followed by tukey's test, n=6 independent experiments).

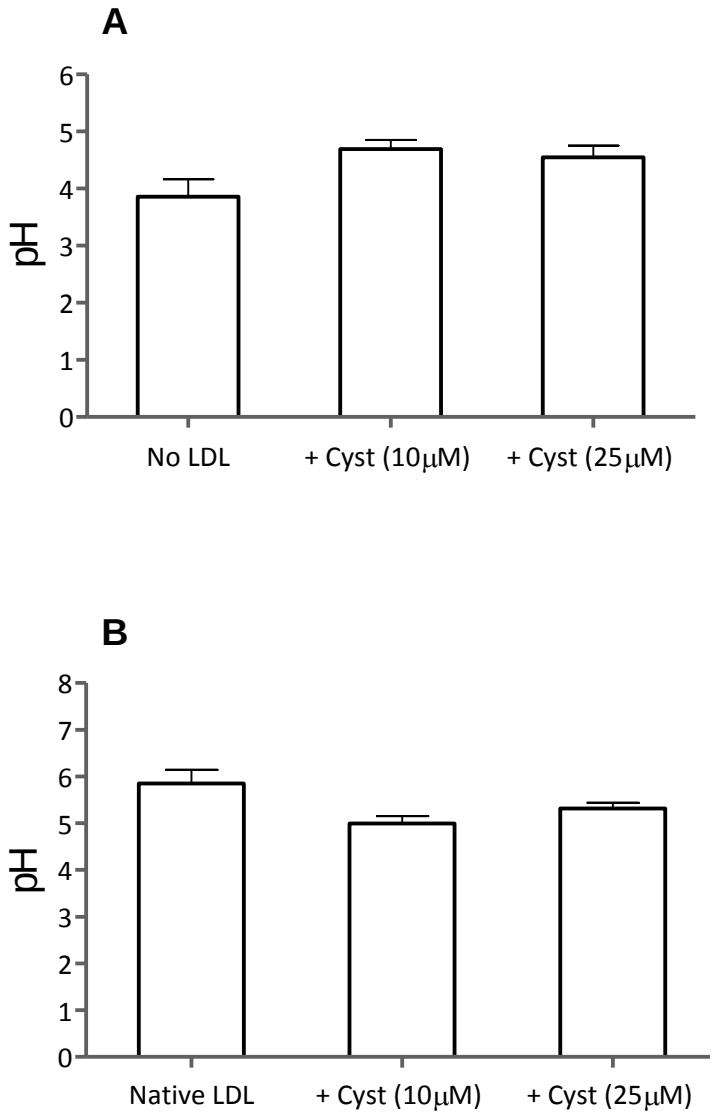


Figure 6.4 Effect of cysteamine on lysosomal pH of macrophages

THP-1 macrophages were cultured in black 96-well microplate at 1 x 10^5 per well in RPMI-1640 medium (containing 10% v/v FCS) with no LDL, or native LDL (100 µg protein/ml) with or without cysteamine (10 µM or 25 µM) for 72 h. The cells were then treated with 5 µM Lysosensor Yellow/Blue for 30 min at 37°C with 5% CO_2. The samples were then read in Fluo Star Optima fluorometer (BMG Labtech), with excitation at 355nm. The ratio of emission at 440nm and 535nm was then calculated for each sample and the pH values determined from the standard plot. (A) shows effect of cysteamine on control cells, (B) shows effect of cysteamine on native LDL treated cells (ANOVA followed by tukey's post-test, n=4 independent experiments).

6.3.3 Effect of lysosomal oxidation of SMase-LDL on cell senescence

Intralysosomal accumulation of ceroid (or lipofuscin) has been associated with cellular ageing (Georgakopoulou et al., 2013, Katz et al., 1984) and there is evidence that cellular senescence is present in atherosclerosis (Alique et al., 2015). To investigate if lysosomal oxidation of LDL induces the expression of senescence markers, human THP-1 macrophages were treated with SMase-LDL (100 µg protein/ml) for 72 h in the presence or absence of cysteamine (10 µM) and then stained for the expression of senescence-associated β-galactosidase (as described in detail in chapter 2), a lysosomal enzyme seen in senescent human cell types (Dimri et al., 1995). Cells were also treated with native LDL (100 µg protein/ml) and untreated THP-1 macrophages were taken as control. The results were expressed as percentage of senescent cells (i.e. blue-green stained) in each group.

Incubation of THP-1 macrophages with native LDL increased the senescence-associated β-galactosidase (SaβG) levels in their lysosomes (Figure 6.5). SMase-LDL treated macrophages showed higher expression of SaβG compared to native LDL-treated cells and cysteamine treatment reduced the SMase-LDL induced SaβG expression. About 23 ± 4% of untreated control cells were found to be senescent (Figure 6.6), while native LDL treatment increased the senescent cell percentage to 46 ± 2% ($p<0.001$). In the case of cells which were treated with SMase-LDL, 69 ± 3% of cells were seen to express the senescent marker SaβG, which was highly significant compared to both control ($p<0.001$) and native LDL treated macrophages ($p<0.001$). However, treatment of macrophages with cysteamine reduced the SMase-LDL induced number of senescent cells to 38 ± 25 % ($p<0.001$).

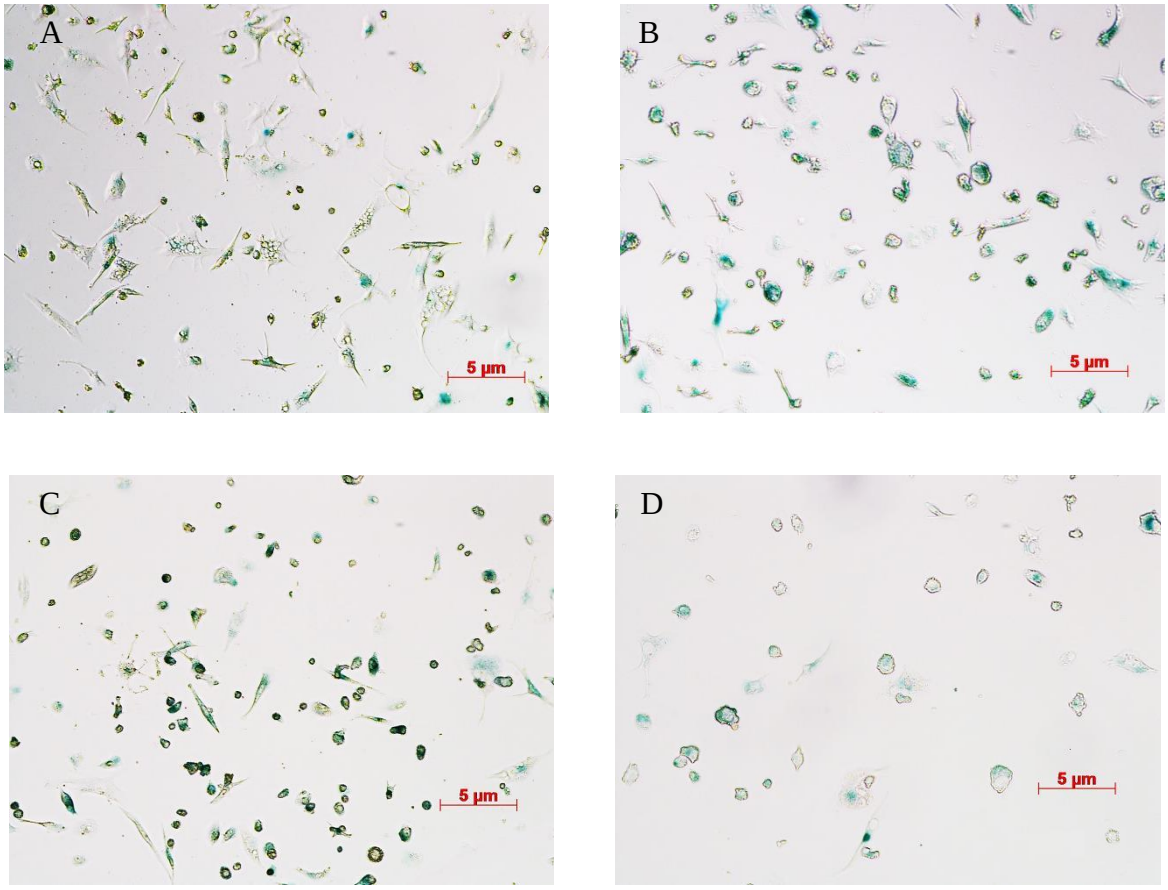


Figure 6.5 Effect of lysosomal oxidation of LDL on senescence of THP-1 macrophages

THP-1 macrophages were cultured in 12 well tissue culture plates at 3000 cells per well in RPMI medium (containing 10% v/v lipoprotein-deficient serum) containing either no LDL (A), native LDL (100 µg protein/ml) (B), SMase-LDL (100 µg protein/ml) alone (C) or SMase-LDL (100 µg protein/ml) with 10 µM cysteamine (D) for 72 h. The cells were then stained to identify any senescent cells by visualisation under bright-field microscopy for blue-green colouration indicating increased levels of lysosomal β-galactosidase activity. The images shown are representative of three independent experiments.

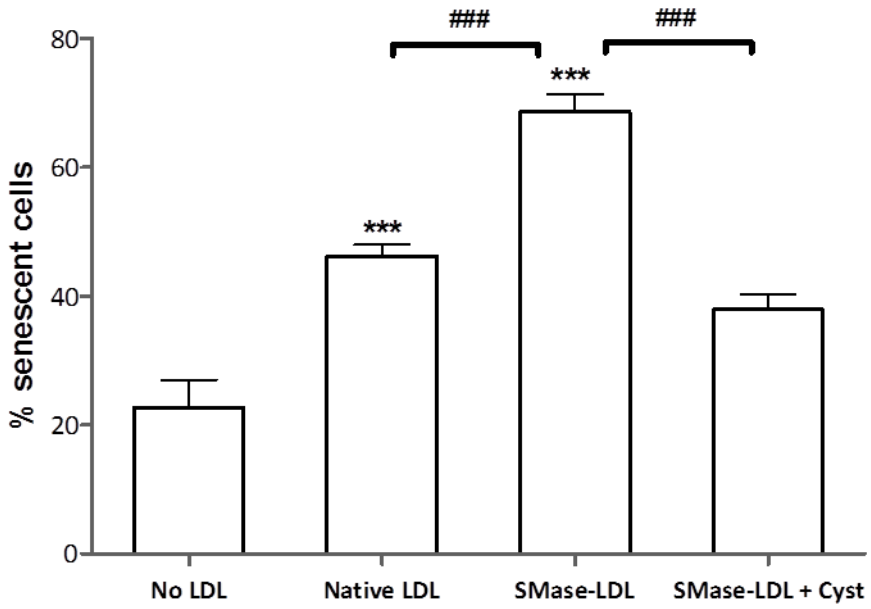


Figure 6.6 Effect of cysteamine on SMase-LDL induced senescence in THP-1 macrophages

THP-1 macrophages cultured in 12 well tissue culture plates at 3000 cells per well in RPMI medium (containing 10% v/v lipoprotein deficient serum) were incubated for 72 h with no LDL, with native LDL (100 µg protein/ml) or with SMase-LDL (100 µg protein/ml), with or without 10 µM cysteamine (Cyst). The macrophages were then assessed for the presence of senescent cells by the senescence associated β-galactosidase assay. The results where expressed as percentage of senescent cells in 100 cells. (*** indicates $p<0.001$ compared to untreated cells, ### indicated $p<0.001$ compared to SMase-LDL treated cells, One-way ANOVA followed by tukey's post-test, n = 4 independent experiments).

6.3.4 The effect of lysosomal oxidation of SMase-LDL on macrophage TNF-α expression

Preliminary experiments were conducted to assess the effect of lysosomal LDL oxidation on macrophages TNF-α expression, using a ELISA kit, as described in Chapter 2. SMase-LDL was used in these experiments, so that the LDL could enter the macrophage lysosomes in a non-oxidised form, in order to exclusively assess the effects of oxidation occurring intracellularly. THP-1 macrophages were cultured in RPMI-1640, supplemented with 10% (v/v) FCS to prevent extracellular LDL oxidation. The macrophages were incubated for 12 or 24 h with native LDL or LDL that had been aggregated with sphingomyelinase to greatly increase its uptake by the macrophages. To investigate the effect of the antioxidant cysteamine on TNF-α secretion, THP-1 macrophages were treated with cysteamine (10 µM or 25 µM) prior to incubation with SMase-LDL, so as to prevent the initial oxidation of LDL in the lysosomes. After LDL treatments, the macrophages were washed and then stimulated with LPS (10 ng/ml) in culture medium for 4 h at 37°C. At the end of the incubation time, the medium was collected and assayed for TNF-α secretion.

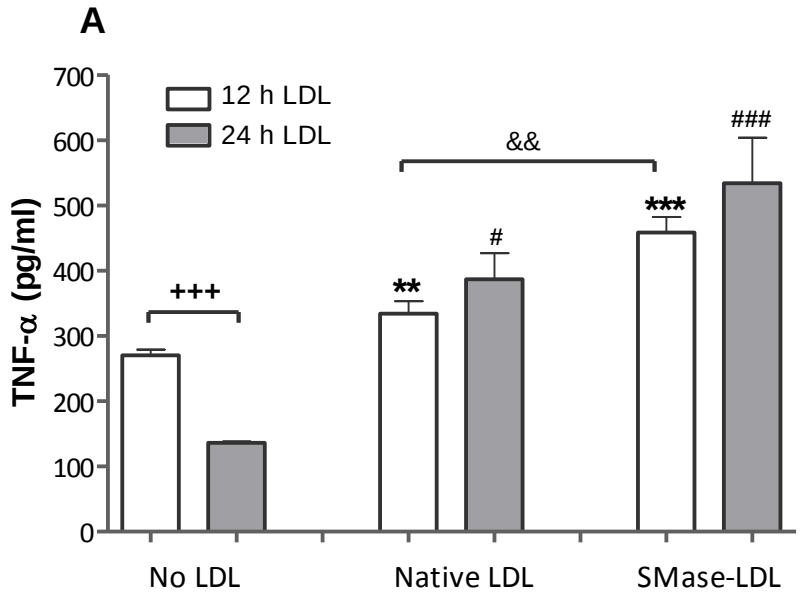
The THP-1 macrophages showed a significant increase in the LPS stimulated TNF-α secretion after both 12 and 24 h incubation with native LDL or SMase-LDL (Figure 6.7). While the secretion of TNF-α, although insignificant, showed an upward trend from 12 h to 24 h LDL treatment, interestingly in the control cells, which received no LDL, the TNF-α levels dropped from 270 ± 9 pg/ml after 12 h incubation to 137 ± 2 pg/ml after 24 h incubation (p<0.001). Treatment of THP-1 macrophages with native LDL for 12 h increased the TNF-α secretion to 334 ± 19 pg/ml (p<0.01), while SMase-LDL increased it to 458 ± 24 pg/ml (p<0.001) after 12

h compared to the control cells. In macrophages which were treated with native LDL for 24 h, the TNF-α concentrations were found to be 386 ± 40 pg/ml (p<0.05 compared to control), while incubation with SMase-LDL increased the concentration to 534 ± 70 pg/ml (p<0.001 compared to control). There was a significant difference in the TNF-α concentrations between native LDL-treated and SMase-LDL treated cells after 12 h, however no significant difference was found in the 24 h treated cells.

Prior treatment of cysteamine for 24 h was seen to reduce the SMase-LDL associated secretion of TNF-α compared to the macrophages incubated with only SMase-LDL (Figure 6.7). In the macrophages which were incubated with SMase-LDL for 12 h, cysteamine produced similar, but significant reduction in the TNF-α concentrations from 458 ± 24 pg/ml to 355 ± 23 pg/ml (p<0.05) and 352 ± 12 pg/ml (p<0.05), respectively, by 10 µM and 25 µM cysteamine. Although, cysteamine reduced the TNF-α secretion in the macrophages treated with SMase-LDL for 24 h from 534 ± 70 pg/ml to 505 ± 18 pg/ml and 418 ± 23 pg/ml with 10 µM and 25 µM concentrations, respectively, but it was not found to be statistically significant.

Figure 6.7 Effect of SMase-LDL on TNF-α expression in THP-1 macrophages

THP-1 monocytes were differentiated into macrophages with 25 ng PMA /ml in 12 well tissue culture plates at a density of 3.5 x 10^5 cells/ml in RPMI-1640 medium (with 10% v/v FBS) for 72 h. The cells were washed and rested for another 24 h, after which they were then incubated in fresh RPMI-1640 medium (with 10% v/v FBS), alone or with native LDL or SMase-LDL (both at 50 µg protein/ml) for either 12 h or 24 h (A). Two sets of wells were pre-incubated with cysteamine (10 µM or 25 µM) for 24 h prior to SMase-LDL treatment (B). After LDL treatment, the cells were washed with PBS and then stimulated with LPS (10 ng/ml) for 4 h at 37 °C and the medium was collected and assayed for TNF-α. In (A) $^{+++}$ indicates $p<0.001$, ** and *** indicates $p<0.01$ and $p<0.001$ respectively compared to 12 h untreated cells, $^{\#}$ and $^{\#\#\#}$ indicates $p<0.05$ and $p<0.001$ compared to 24 h untreated cells, $^{\&\&}$ indicates $p<0.01$. In (B) * indicates $p<0.05$ compared to SMase-LDL treated cells. The data shown are from 4 independent experiments and the statistics shown are One-way ANOVA followed by tukey's post-test.


A
700
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TNF-α (pg/ml)
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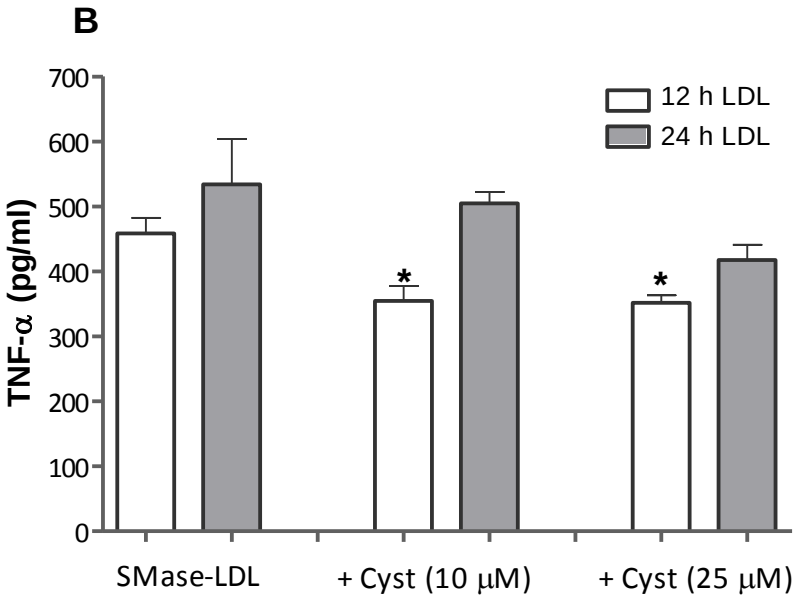
SMase-LDL


B
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TNF-α (pg/ml)
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SMase-LDL
+ Cyst (10 μM)
+ Cyst (25 μM)

6.4 Discussion

Acidic pH is essential for the optimal function of degradative enzymes present in the lysosomes of cells. Almost all of the lysosomal enzymes are acid hydrolases, which are active at acidic pH (between 4.5 and 5) (Coffey and De Duve, 1968) and any change in this pH can cause lysosomal dysfunction (Ohkuma and Poole, 1978). Lysosomes generate and maintain their pH gradients by using the activity of a proton-pumping V-type ATPase, which uses metabolic energy in the form of ATP to pump protons into the lysosome lumen (Ohkuma et al., 1982). The lysosome receives extracellular cargo (via endocytosis) and cytoplasmic material (via autophagy) for degradation (Eskelinen and Saftig, 2009). Failure of the lysosome to process its contents efficiently leads to an accumulation of undigested material inside the lumen and can cause lysosomal dysfunction (Platt et al., 2012).

The accumulation of lysosomal free cholesterol has been shown to directly cause an increase in lysosomal membrane cholesterol content (Cox et al., 2007). The data presented here have shown that treatment of human THP-1 macrophages with SMase-LDL for 3 days decreases lysosomal function in them in terms of Lysotracker Red accumulation (Figures 6.1 and 6.2). The lysosomotropic antioxidant, cysteamine is able to improve lysosomal function, with a 25 µM concentration being able to almost completely reverse the SMase-LDL induced lysosomal dysfunction. It was also noticed that treatment with native LDL did not have any considerable effect on the lysosomal function of the THP-1 macrophages. The data here also suggest that the decrease in lysosomal function, due to atherogenic lipid loading in the THP-1 macrophages appears to be due to the loss of lysosomal acidification (or rise in lysosomal pH). SMase-LDL treatment

for 72 h was seen to increase the lysosomal pH of the THP-1 macrophages considerably from 4.9 to 6.2 (which represents a decrease in hydrogen ion concentration of 20 times), compared to the baseline pH of untreated cells and native LDL treated cells (Figure 6.3). The increase in pH would decrease the degradation of LDL and lead to more lipid accumulation in lysosomes, leading to more lipid-laden foam cells. It was also noticed that cysteamine was able to prevent the SMase-LDL induced increase in the lysosomal pH. Furthermore, it was noted that cysteamine had no great effect on the lysosomal pH of macrophages in absence of LDL or in the presence of native LDL. In chapter 4 it was shown that macrophages treated with SMase-LDL for 24 h showed increased levels of lipid peroxidation in their lysosomes which was greatly inhibited by cysteamine. In the present experimental conditions, the continuous treatment of THP-1 macrophages with SMase-LDL for 72 h would have induced extensive lipid peroxidation in their lysosomes by iron generating lipid peroxidation products like 7-ketocholesterol, 4-hydroxynonenal and malondialdehyde which have previously been shown to inhibit the activity of lysosomal v-ATPase causing lysosomal dysfunction (Cox et al., 2007, Krohne et al., 2010a, Sudo et al., 2015).

Cysteamine, on the other hand had little effect on the lysosomal pH of control or native LDL treated macrophages suggesting that the effect of cysteamine in preventing the SMase-LDL altered lysosomal pH is due to the inhibition of lysosomal peroxidation of aggregated LDL and is independent of any effect on the lysosomal v-ATPase.

There is strong evidence suggesting that decreased lysosomal proteolytic activity and increased lysosomal pH occurs as a consequence of aging in long-lived post

mitotic cells (Martinez-Vicente et al., 2005, Rajawat et al., 2009, Terman and Brunk, 2006). In fact, increasing lysosomal function is being considered as a plausible avenue for anti-ageing interventions so as to increase the longevity of cells (Carmona-Gutierrez et al., 2016).

It has been proposed that oxidative stress-induced damage to cellular components, probably due to the combination of higher reactive oxygen species (ROS) and impaired antioxidant defence, is the main contributor to the ageing process (Moon et al., 2001). The accumulation of oxidation products of cholesterol (oxysterols) has been seen to induce senescence in human cells, through the generation of reactive oxygen species (Carmona-Gutierrez et al., 2016, Zarrouk et al., 2014). Data presented here have shown that both native LDL and SMase-LDL treatment for 3 days induces senescent like properties in human THP-1 macrophages (Figures 6.5 and 6.6). The incidence of senescence was found to be much higher with SMase-LDL compared to native LDL and treatment with cysteamine significantly decreased the SMase-LDL induced senescence. Superoxide radicals are considered by some to be the major cause of ageing in cells (Hamilton et al., 2001, Jacobson et al., 2007, Sawada and Carlson, 1987) and as explained earlier lysosomes are ideal places for the generation of superoxide anions ($O_2^{\bullet -}$) from oxidation of catalytically active ferrous ions, which under lysosomal pH conditions can form more toxic hydroperoxyl radicals ($HO_2^{\bullet}$) (De Grey, 2002, Grady and Chasteen, 1991). $HO_2^{\bullet}$, a stronger oxidant, is capable of initiating lipid peroxidation (Bielski et al., 1983) under lysosomal conditions and therefore can generate intralysosomal ceroid, an ageing pigment (Kieseier et al., 1996, Yin et al., 2011). Cysteamine, being a superoxide scavenger (Sunman et al., 1993), is able to inhibit the lipid peroxidation of LDL in the lysosomes and thus

prevent the formation of senescent-associated intralysosomal ceroid in human macrophages.

Inflammation plays a key role in the initiation, progression and rupture of atherosclerotic lesions (reiewed in Libby, 2012). Both minimally oxidised LDL and oxLDL have been shown to cause the secretion of proinflammatory cytokines by macrophages (Bekkering et al., 2014, Miller et al., 2005) by enhancing the expression of toll-like receptor-4 (TLR-4). However, some studies have shown that oxidised LDL inhibits the production of inflammatory cytokines by macrophages in response to inflammatory stimuli such as lipopolysaccharide (LPS) (Page et al., 1999, Thai et al., 1995). The effect of vortexed and acetylated LDL on the expression of the proinflammatory cytokine, TNF-α, is controversial with some reports showing a decrease in its levels (Ares et al., 1995, Satchell, 2007) and others showing an increase (Estruch et al., 2013). LPS is considered a classical ligand of TLR4 receptors (Lu et al., 2008). Here, we sought to determine whether lysosomal oxidation of SMase-LDL by human THP-1 macrophages, could alter the LPS induced secretion of TNF-α levels.

The data presented here suggests that both native LDL and SMase-LDL treatment for 12 h increases the LPS induced expression of TNF-α in THP-1 macrophages (Figure 6.7). The secretion of TNF-α was seen to be considerably higher with SMase-LDL after 12 h compared to both control macrophages and native LDL treated macrophages. The potentiation of LPS-induced TNF-α secretion by native LDL is in agreement with the previous studies by Netea et al. (2002). Although the LPS induced TNF-α secretion was seen to increase after 24 h LDL treatment compared to 12 h, the difference was not statistically different. The TNF-α levels

secreted by the control macrophages that were incubated for 24 h were lower than the ones incubated for 12 h, for unknown reasons. Pre-incubation with cysteamine for 24 h decreased the secretion of TNF-α by macrophages incubated with SMase-LDL for 12 h (but not for 24 h).

The explanation for increased expression of TNF-α by both native LDL and SMase-LDL could be due to increased expression of CD14 receptors on the THP-1 macrophages (Esfahani et al., 1993). CD14 is a component of the innate immune system, expressed on human monocytes and macrophages and is a receptor for LPS (Fenton and Golenbock, 1998). In addition, SMase-LDL treatment might increase the expression of toll-like receptor-4 (TLR4) on the macrophage surface, either due to the ceramide present in SMase-LDL (Tawadros et al., 2015) or due to the oxidative stress caused by the lysosomal oxidation of LDL (Powers et al., 2006). The increased expression of TNF-α by lysosomal LDL oxidation in human macrophages can participate in the atherosclerosis process possibly by stimulating adhesion molecule expression (Bevilacqua, 1993), stimulating growth factors important for proliferation (Filonzi et al., 1993) and regulation of plaque stability (Libby et al., 1995). TNFα once released enhances through NF-κB activation the expression of acyl-CoA-cholesterol transferase 1 to promote cholesterol uptake and cholesteryl ester-laden cell formation from differentiating monocytes (Lei et al., 2009). TNFα has been seen to reduce the life span of endothelial cells (EC) in culture in a concentration- and time-dependent manner through increased rate of apoptosis (Csiszar et al., 2007, Gaur and Aggarwal, 2003). Increased TNFα formation and concentration in the vascular wall of the aorta and of carotid and coronary arteries contribute along with EC apoptosis to impaired endothelial function (Csiszar et al., 2004). TNFα also

stimulates NOX and superoxide dismutase (SOD) in VSMC, consequently the formation of ROS and H_2O_2 is enhanced. ROS and/or H_2O_2 facilitates the activation of other stress related ERK and p38MAPK pathways and therefore the growth and movement of VSMC (Xu et al., 2009). The exact mechanism of increased TNF-α secretion by SMase-LDL needs detailed investigation, as does the effect of lysosomal oxidation of SMase-LDL on the expression of other proinflammatory cytokines.

Chapter 7- General discussion

7.1 General discussion

This thesis has many potential applications in the field of atherosclerosis. The main outcomes of the research presented in this thesis can be summarised as:

- The initial oxidation of LDL by iron at lysosomal pH (pH 4.5) occurs in the hydrophobic cholesteryl ester core first and doesn't depend on the pre-existing lipid hydroperoxides in LDL.

- Under lysosomal conditions protonated superoxide ions known as hydroperoxyl radicals ($HO_2^{\bullet}$) are the main oxidising species capable of causing LDL oxidation and superoxide scavengers like cysteamine are able to inhibit this oxidation, while antioxidant probucol is unable to prevent it.

- Incubation of human macrophages with SMase-LDL leads to intralysosomal ceroid generation in human macrophages which is greatly inhibited by lysosomotropic antioxidant cysteamine.

- Lysosomal oxidation of LDL can lead to lysosomal dysfunction in human THP-1 macrophages by altering the lysosomal pH and inhibiting the oxidation by cysteamine restores the functions of lysosomes.

- Lysosomal oxidation of LDL induces senescence in human THP-1 macrophages which is greatly inhibited by cysteamine.

- Lysosomal oxidation of SMase-LDL potentiates LPS-induced TNF-α secretion in human THP-1 macrophages whereas pre-incubation with cysteamine greatly decreases it.

The existence of modified forms of LDL to convert macrophages into foam cells has been in the limelight since 1970's (Goldstein and Brown, 1973, 1974). The presence of oxidised LDL in atherosclerotic plaques has been demonstrated and a plethora of atherogenic properties of oxidised LDL have been discovered (reviewed in Stocker and Keaney, 2004). Oxidised LDL was widely believed to be the main culprit in the development of atherosclerosis, doubt about its importance has grown due to the lack of effect of the large antioxidant trials using vitamin E. In fact, this laboratory has recently shown that vitamin E enrichment of LDL actually increased the initial oxidation of LDL by iron at lysosomal pH (Satchell and Leake, 2012). The lack of effect of the clinical trials does not therefore necessarily invalidate the oxidised LDL hypothesis of atherosclerosis, but the actual site and the mechanisms by which it is produced *in vivo* are still a matter of debate (reviewed in Steinberg and Witztum, 2010). The use of copper ions at pH 7.4 is a widely employed method for producing oxidised LDL *in vitro*, however, it is questionable as to whether copper oxidises LDL *in vivo*. Recently it was shown that LDL aggregated by vortexing could be oxidised by redox active iron within the lysosomes of macrophages in a cell culture system (Wen and Leake, 2007). LDL oxidation by iron at lysosomal pH has been characterised in this laboratory previously (Satchell and Leake, 2012), however the exact mechanism through which iron is able to oxidise LDL is yet to be established.

Work presented in this thesis has shown that at lysosomal pH, the hydroperoxyl radical ($HO_2^{\bullet}$) is the main oxidising species which can induce extensive oxidation of LDL. Due to the acidic and reducing environment within lysosomes, most of the iron present in them occurs in the ferrous state (Schafer et al., 2000), which on oxidation is converted to the ferric form (Figure 3.4) with the generation of

superoxide anion (O_2^-) (Morgan and Leake, 1993). Superoxide anion is a weak oxidant, but at lysosomal pH most of it will be protonated to form stronger oxidant, hydroperoxyl radical ($HO_2^\bullet$) which has a pK_a of 4.8, close to the lysosomal pH of about 4.5 (Bielski, 1985, Grady and Chasteen, 1991). Our HPLC data (Figure 3.11) revealed that initial oxidation of LDL by iron at lysosomal pH occurs in the cholesteryl ester-rich hydrophobic core of LDL particles, which is not prevented by the chain breaking antioxidant, probucol (Figures 3.8 and 3.11), due to probucol's limited access to the LDL core, but is completely prevented by the more hydrophobic chain breaking antioxidant, DPPD. Although DPPD is able to prevent the oxidation in LDL's core (Figure 3.9) it is unable to prevent the oxidation of the apoB-100 part of LDL (Figure 3.10). Cysteamine, which is a thiol based lysosomotropic antioxidant, offers complete inhibition of LDL oxidation by iron *in vitro* at lysosomal pH, including the inhibition of lipid peroxidation in the hydrophobic core and phospholipid monolayer as well as inhibiting the oxidation of apoB-100 (Figures 3.12, 3.13 and 3.14). The fact that cysteamine completely inhibits the oxidation of all the parts of LDL suggests that oxidation of LDL by iron under lysosomal conditions is mediated by protonated superoxide ions ($HO_2^\bullet$), which because of their neutral charge can freely enter the hydrophobic core. Cysteamine was also found to be better at preventing LDL oxidation by iron at lysosomal pH when compared to another thiol molecule, cysteine (Figure 4.2), as well as other known lysosomotropic drugs such as propranolol (Figure 4.3), amiodarone (Figure 4.4) and 7,8-dihydroneopterin (Figure 4.5).

Native and oxidised-LDL are prone to aggregation and atherosclerotic plaque formation is believed to be initiated by the extracellular aggregation and deposition of lipoproteins in the subendothelial matrix. The results presented here

demonstrate that cysteamine is able to prevent the oxidation-induced aggregation of LDL (Figure 4.6). In addition, the antioxidant activity of cysteamine at pH of 7.4 was also demonstrated, should the oxidation of LDL occur under interstitial pH conditions (Figure 4.7).

Foam cell formation via the incubation of macrophages with oxidised LDL does not accurately reflect the *in vivo* environment, as most LDL in atherosclerotic plaques is found in the aggregated state and bound to subendothelial matrix. It has been shown that enzymes secreted in the arterial intima like lipoprotein lipase (LPL), sphingomyelinase (SMase), phospholipase A_2 (PLA2), cholesteryl ester hydrolase (CEH), matrix metalloproteinases (MMP) and plasmin can cause LDL aggregation to produce atherogenic LDL forms that are rapidly internalized by macrophages. Previous work from this laboratory has shown that sphingomyelinase-aggregated-LDL (SMase-LDL) is rapidly endocytosed by human macrophages in their lysosomes and oxidised by redox active iron present in them (Wen et al., 2015). In this thesis, the oxidation of SMase-LDL by iron at lysosomal conditions has been characterised and results have been compared to the oxidation of native LDL (Figure 5.4, 5.5). Although, SMase-LDL is oxidised more slowly than native LDL, after 24 h both the forms of LDL reach similar levels of oxidation generating products like 7-ketocholesterol and cholesteryl ester hydroperoxides (Figure 5.6). The presence of cysteamine inhibits this oxidation significantly (Figure 5.7).

The data presented here also demonstrate that cysteamine, unlike probucol, is able to prevent the oxidation of SMase-LDL in the lysosomes of human macrophages. Probucol, initially introduced as a cholesterol-lowering drug, was found to attenuate atherosclerotic lesion development in animals (Carew et al.,

1987); however the findings of The Probucol Quantitative Regression Swedish Trial (PQRST) in 1995 showed that the treatment of hypercholesterolaemia patients with probucol did not have any beneficial effects (Walldius et al., 1994). In the current experimental conditions, probucol was unable to prevent the lysosomal oxidation of LDL in the macrophages, which could be due to its inability to protect the oxidation of LDL by iron at lysosomal pH. The lysosomotropic property of cysteamine, allows it to concentrate more in the lysosomes of cells where it is able to greatly inhibit the oxidation of LDL as indicated by the decrease in the overall intralysosomal lipid peroxidation process and reduction in the SMase-LDL induced intralysosomal ceroid formation (Figure 5.11). These results therefore further provide evidence of the role of hydroperoxyl radicals ($HO_2^{\bullet}$) in initiating the oxidation of LDL by iron under lysosomal conditions.

Acidic pH is essential for the optimal function of degradative enzymes present in the lysosomes of cells (Ohkuma et al., 1982). Almost all of the lysosomal enzymes are acid hydrolases, which are active at acidic pH (between 4.5 and 5) and any change in this pH can cause lysosomal dysfunction (Platt et al., 2012). Lysosomes generate and maintain their pH gradients by using the activity of a proton-pumping V-type ATPase, which uses metabolic energy in the form of ATP to pump protons into the lysosome lumen (Ohkuma et al., 1982). The lysosome receives extracellular cargo (via endocytosis) and cytoplasmic material (via autophagy) for degradation. Failure of the lysosome to process its contents efficiently leads to an accumulation of undigested material inside the lumen and can cause lysosomal dysfunction. The data presented here have shown that treatment of human THP-1 macrophages with SMase-LDL decreases their lysosomal function when incubated continuously for 3 days with SMase-LDL. The lysosomotropic antioxidant,

cysteamine is able to improve the lysosomal function and with a 25 µM concentration, [which is attained in cystinosis patients treated with cysteamine (Dohil et al., 2006)] being able to completely reverse the SMase-LDL induced lysosomal dysfunction (Figure 6.1, 6.2). It was also noticed that treatment of native LDL did not have any considerable effect on the lysosomal function of macrophages. The data here also suggest that the decrease in lysosomal function, due to atherogenic lipid loading in the THP-1 macrophages appears to be due to loss of lysosomal acidification (or rise in lysosomal pH). SMase-LDL treatment for 72 h increased the lysosomal pH of the THP-1 macrophages considerably, compared to the baseline pH of untreated cells and native LDL-treated cells (Figure 6.3). It was also shown that cysteamine was able to prevent the SMase-LDL induced increase in the lysosomal pH. Furthermore, it was noted that cysteamine had little effect on the lysosomal pH of macrophages in the absence of LDL or in the presence of native LDL (Figure 6.4).

As mentioned above, macrophages when treated with SMase-LDL show increased levels of lipid peroxidation in their lysosomes, which is greatly inhibited by cysteamine. Lipid peroxidation products like 7-ketocholesterol, 4-hydroxynonenal and malondialdehyde have been shown to induce lysosomal dysfunction by inhibiting the activity of lysosomal v-ATPase (Krohne et al., 2010b, Sudo et al., 2015). In the present experimental conditions, the continuous treatment of THP-1 macrophages with SMase-LDL for 72 h would have induced extensive peroxidation in their lysosomes and thus increasing their pH. Cysteamine, on the other hand, had no effect on the lysosomal pH of control or native LDL treated macrophages suggesting that the effect of cysteamine in maintaining the SMase-LDL altered lysosomal pH is due to the inhibition of

lysosomal lipid peroxidation of aggregated LDL and is independent of any effect on the lysosomal v-ATPase.

Inflammation is considered an important element in vulnerable plaque formation and plaque rupture. It is also a key component in the initiation and progression of the disease (Libby, 2002). An attempt was made in the work presented in this thesis, to determine whether intralysosomal LDL oxidation altered the expression of the inflammatory cytokine, tumour necrosis factor-alpha (TNF-α). While both native LDL and SMase-LDL increased the LPS-induced secretion of TNF-α by human THP-1 macrophages, the effect was more pronounced with SMase-LDL (Figure 6.7). Prior treatment of macrophages with cysteamine produced significant reduction in the SMase-LDL induced TNF-α secretion possibly due to prevention of intralysosomal LDL oxidation. Further investigation is required in this field, looking at the effect of lysosomal oxidation of SMase-LDL on other inflammatory cytokines. The increased secretion of TNF-α by both native LDL and SMase-LDL could be due to increased expression of the LPS receptor, CD14 (Esfahani et al., 1993) or of Toll-like receptor-4 (TLR4) on the surface of THP-1 macrophages, probably due to the ceramide present in SMase-LDL (Tawadros et al., 2015) or due to the oxidative stress (Powers et al., 2006) from the spilling of oxidised lysosomal contents in the cytosol. Cysteamine might possibly block all these effects, thus reducing the secretion of TNF-α. The exact mechanism of increased TNF-α secretion by oxidised LDL needs detailed investigation, as well as the effect of lysosomal oxidation of SMase-LDL on the expression of other proinflammatory cytokines.

Superoxide radicals are considered by some to be a major cause of ageing in cells (Hamilton et al., 2001, Jacobson et al., 2007). $HO_2^{\bullet}$, a stronger oxidant, is capable of initiating lipid peroxidation (Bedwell et al., 1989, Bielski, 1985, Yin et al., 2011) under lysosomal conditions and generating intralysosomal ceroid, an ageing pigment (Kieseier et al., 1996). There is mounting evidence that lysosomal dysfunction is associated with many age-related diseases and accumulation of oxidation products of cholesterol (oxysterols) has been seen to induce senescence in human cells, through the generation of reactive oxygen species (Carmona-Gutierrez et al., 2016, Zarrouk et al., 2014). Data presented here have shown that both native LDL and SMase-LDL treatment for 3 days induces senescence like properties in human THP-1 macrophages in terms of β-galactosidase activity (Figure 6.5, 6.6). The incidence of senescence was found to be much higher with SMase-LDL compared to native LDL and treatment with cysteamine significantly inhibited the SMase-LDL induced senescence. Cysteamine, being a superoxide scavenger (Sunman et al., 1993), is able to inhibit the lipid peroxidation of LDL in the lysosomes and thus might prevent the increase in the senescence marker β-galactosidase.

Therefore, therapeutic strategies to target lysosomes with antioxidants like cysteamine to prevent the intralysosomal oxidation of LDL, seems a plausible avenue to decrease the development of atherosclerosis.

7.2 Limitations of the study

Although the work presented in this thesis has many potential applications in the field of atherosclerosis in terms of targetting the lysosomal oxidation of LDL in

macrophages with lysosomotropic drugs, there are some limitations which need further investigation. Although, it was established through various experiments that hydroperoxyl radical ($HO_2^{\bullet}$) is the main oxidising species during LDL oxidation with iron but the generation of $HO_2^{\bullet}$ ions could not be studied. Furthermore, in the current study only THP-1 monocytes derived macrophages were used to study the lysosomal oxidation of LDL and sue to the time constraints the results could not be further confirmed on peripheral human blood monocyte derived macrophages.

7.3 Future work

Due to the above mentioned limitations there is a scope for further investigation of the current work like:

- Monitoring the generation of $HO_2^{\bullet}$ radicals during iron mediated LDL oxidation under lysosomal conditions by electron spin resonance (ESR).
- Study the effect of lysosomal oxidation of LDL on the function of lysosomal acid lipase.
- If the oxidation of LDL by iron at pH 4.5 and in lysosomes produces bioactive oxidised phospholipids and if it is inhibited by cysteamine.
- If oxidized lipids formed at lysosomal pH increase the secretion of other inflammatory cytokines and metalloproteinases and if it is inhibited by cysteamine.
- If cysteamine can reduce the progression of existing atherosclerosis in mice.

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