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Effect of antioxidants on the oxidation of low density lipoprotein at lysosomal pH

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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.

Hadeel Khalaf Alboaklah

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Posters and Publications

Published abstracts

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- Hadeel. K.M. Alboaklah, & Leake, D. S. 2018 Vitamin E can increase, rather than decrease, low density lipoprotein oxidation at lysosomal pH (in Canada)
- Low density lipoprotein oxidation by iron at lysosomal pH inhibits relaxation of rat aorta (in Amsterdam)

Posters

- Hadeel. K.M. Alboaklah, & Leake, D. S. 2018 Understanding Pathogenesis of Risk Factors of Atherosclerosis Brought Hope Potential Diagnosis and Treatment (London).

- Hadeel. K.M. Alboaklah, & Leake, D. S. 2018 Effect of Antioxidants on the Oxidation of Low Density Lipoprotein at Lysosomal pH (oxford).

Abstract

Oxidised forms of low-density lipoprotein (LDL) are widely belived to be involved in the pathogenesis of atherogenesis, but large clinical trials have not shown protection of cardiovascular disease by antioxidants. Recently, it has been shown that LDL can be oxidised by iron in the lysosomes of macrophages. We hypothesised that antioxidants would protect LDL against oxidation less well at lysosomal pH than at pH 7.4.

LDL was enriched with α-tocopherol by incubating plasma with α-tocopherol and isolating the LDL. This enrichment inhibited LDL oxidation by copper ions (Cu^{2+}) at pH 7.4 ,but not at pH 4.5, as shown by spectrophotometry at 234 nm to measure conjugated dienes and by HPLC to measure individual oxidised lipids. α-Tocopherol enrichment did not inhibit LDL oxidation by Fe^{3+} (2, 5 or 20 μM) at pH 4.5 , but inhibite it by 5 or 20 μM Fe^{2+}, but not 2 μM Fe^{2+}. This might help to explain why α-tocopherol did not inhibit cardiovascular diseases in the large clinical trials.

The antioxidant tempol and probucol inhibited the late phase of LDL oxidation by Fe^{2+} and Cu^{2+} at pH 4.5 more than the early phase, possibly because they were located mainly in the phospholipid monolayer of LDL, rather than in the cholesteryl ester of the LDL particle. There is a suggestion that lysosomal dysfunction plays an important role in atherosclerosis. The lysosomal oxidation of LDL aggregated by sphingomelinase resulted in the production of the advanced lipid peroxidation product (ceroid).

α-Tocopherol enrichment of macrophages did not protect them against apoptosis induced by H_2O_2. The work presented here also demonstrated LDL oxidised Fe^{2+} at pH 4.5, decreased endothelium-dependent vasodilatation of rat aortic rings. This suggests that

lysosomally oxidised LDL released from dead cells in atherosclerotic lesions might damage the endothelium. Taken togather, these results suggest that inhibiting the oxidation of LDLin lysosomes might be a therapy of atherosclerosis.

Contents

Figures and Tables

List of Abbreviations

ACAT	AcylCoA:cholesterol acyltransferase
ABCA1	ATP binding cassette transporter-1
ANOVA	One-way analysis of variance
ApoB-100	Apolipoprotein B-100
ApoE	Apolipoprotein E
BHT	Butylated hydroxytoluene
BP	Bathophenanthrolinedisulfonic acid
BSA	Bovine serum albumin
CE	Cholesteryl esters
CHD	Coronary heart disease
CLOOH	Cholesteryl linoleate hydroperoxide
CVD	Cardiovascular disease
DMSO	Dimethyl sulphoxide
DTPA	Diethylenetriaminepentacetate
DHE	Dihydroxyethidium
EDTA	Ethylenediaminetetraacetic acid
EC	Endothelial cells
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FH	Familial hypercholesterolaemia

FITC	Fluorescein isothiocyanate
FGF	Fibroblast growth factor
PDGF	Platelet derived growth factor
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
IDL	Intermediate density lipoprotein
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
MI	Myocardial infarction
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor-kappa of activated B cells
oxLDL	Oxidized LDL
PBS	Phosphate buffered saline
PLA2	Phospholipase A_2
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

Chapter 1

Introduction

1. Atherosclerosis

1.1 The scale of the problem

Cardiovascular disorder (CVD) is the effective reason of mortality within the world (Garcia et al., 2016, Barnhart et al., 1989). Atherosclerosis is the main cause of CVD and it is the second leading source of death in England (Yang et al., 2015). The World Health Organization (WHO) report that in 2012 globally 17.5 million people died because of cardiovascular diseases and that is responsible for one-third of all deaths worldwide (6.2 million died due to interrupted blood flow in a carotid artery or other artery supplying the brain (stroke) (Natori et al., 2016). In 2011, the British Heart Foundation estimated more than 159,000 people died due to CVDs in the UK with 74,000 of them related to coronary heart disease while strokes caused approximately 42,000 deaths in the UK (Wang and Bennett, 2012). In 2010 the global cost of cardiovascular disease has been evaluated at nearly 863 billion USD. This cost is not just related to the health care system but also national economic growth (Herrington et al., 2016). According to the American Heart Association, the total cost of cardiovascular diseases in the U.S. 2010 was nearly 503.2 billion USD yearly. The typical cost in the year of this disease in Europe was approximately 153 billion USD. In China, the cost of CVD has been determined to be greater than 40 billion USD (Ohsfeldt et al., 2010).

Atherosclerosis is an inflammatory disease of the arteries associated with lipids.

The term atherosclerosis refers to the progression of the atheromatous lesion, which forms a plaque in the arterial wall (Gaudio et al., 2006). This plaque can lead to clinical signs, depending on its size and location, by restricting the blood flow in arteries causing ischaemia or by inducing thrombi that may interfere with blood flow. An embolus can settle in a distal artery (Stary et al., 1995). In the United States, 2004, the early clinical manifestation of atherosclerosis is a heart attack for 66% and 74% men and women, respectively. Sudden cardiac death generally occurs when an artery narrows due to a plaque by about 75% or more (Pipitone et al., 2007). Most clinical manifestations appear after excessive narrowing of arteries or completely closing blood flow to each organ. Peripheral arterial lesion symptoms are numbness and pain within legs from severe narrowing from atherosclerotic plaque in leg arteries (Korneva et al., 2014). Lack of blood amount reached to the brain due to bulky plaque in the carotid and other arteries can produce strokes and symptoms such as difficulty in speaking, weakness, blurred vision, headache, numbness in the face and loss of consciousness (Xu et al., 2016) Another common location of plaque formation is in the coronary arteries, which can result in several symptoms such as chest pain (angina due to myocardial ischaemia), shortness of breath, sweating, dizziness, nausea, arrhythmia and ischaemia (Aboyans et al., 2007).

Several significant factors raise the risk of atherosclerosis, such as high blood pressure, hyperlipidemia, diabetes Mellitus, overweight, smoking, lack of physical activity, fatty diets, and genetic history of early heart disease (Rocha and Libby, 2009).

1.2 Pathogenesis of Atherosclerosis

1.2.1 Histology of normal arteries

The healthy artery wall comprises of three layers. The deepest layer facing the bloodstream named tunica intima, which has a monolayer of endothelial cells that stand on a subendothelial layer of extracellular matrix and proteoglycans. The internal elastic lamina provides a barrier between the endothelial layer and the middle layer of the artery wall, which is called the tunica media. It consists of many layers of smooth muscle cells enclosed with a matrix comprising mostly of different type of fibres like elastic collagen. The exterior elastic member also provides a barrier splits the muscular layers of tunica media and the outer layer, named tunica adventitia. It composed of a network community of fibroblasts cells, collagen fibres and elastin (Spicer et al., 2015, Libby et al., 2010). The structure of the normal and abnormal artery (atherogenic) is displayed in Figure 1.

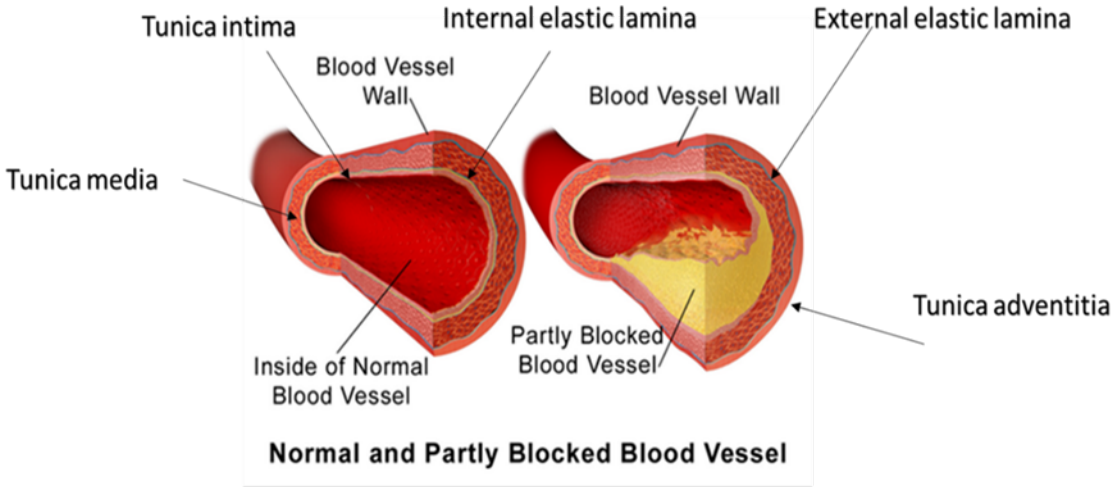


Figure 1.1 Illustration associating a normal blood vessel and incompletely blocked vessel due to the development of an atherosclerotic plaque (Glagov et al., 1987).

1.2.1.2 Endothelial cells structure

Both blood vessels and lymphatics are fully lined by endothelial cells. These cells were thought to be simple coating cells with few functions than to preserve the blood from leaking out of the vessels (Rajendran et al., 2013). However, recent research on endothelial cells has shown that they have an incredible range of functions (McCarron, 2019). Furthermore, they are the vital factor to determines the health of blood vessels and have remarkable effects in pathogenesis cardiovascular disease. These cells are flat, with a central nucleus, with a thickness of around 1-2 µm with a diameter roughly 10-20 µm. The intersections between cells, overlapping areas recognised which lid the vessel. The

intercellular junctions are critical for the health of the vessel. Toxic elements open these intersections and let large particles pass out of the wall (Goncharova , 2017). Consequently, such toxins can possibly damage the blood vessels and cause serious disease (recognised as vascular disease). The cytoplasm is quite simple with few organelles, typically concentrated around the nuclear area. The most apparent feature is the presence of small vesicles (pinocytotic vesicles) bordering the endothelial cell membranes.

This is a mechanism for transporting substances, particularly fluid, transversely from the bloodstream to the underlying tissues. Gases diffuse through swiftly, and this is illustrated in the lung capillaries where there is a very effective transfer of gases, such as oxygen, and anaesthetics. Endothelial cells appear the dynamic impact on the health stability in the body tissue like cartilage, all cell is about 25 µm of a capillary. It is merely 10-15 µm diameter and comprises simply of endothelial cells and attached material named the basal lamina. Endothelial cells also contain Weibel- Palade bodies (WPBs), which have von Willebrand factor (VWF), which is released under many stimulators. VWF has a remarkable role in clinical mechanism such as vascular damage repair and inflammation (Valentijn, 2011).

1.2.2 Lesion-prone area

Atherosclerotic lesion builds up inside the internal curve of the arteries and at the division zone (Chistiakov et al., 2017). The progression of lesions throughout these positions is believed of a reduction in or disrupted shear stress, whereas high shear stress is protective (Davies, 1997). Shear stress is a blood flow forces in endothelial cells that regulate the principle of vascular endothelium function by targeting receptors, that recognise flow and stimulate mechanical signals via mechanosensitive signalling pathways resulting in morphologic and role modifications.It modifies endothelial cell regeneration and their role by the mechanical transduction mechanism by low shear stress initial the stimulation of transcription factors like nuclear factor–kappa B (NF-kB) (Tzima et al., 2005). NF- KB stimulation promote transcription of genes encoding proteins, which implicated in atherosclerosis, like vascular cell adhesion molecule-1 (VCAM-1) that bind with monocytes, assisting their penetration throughout the endothelial junction (Monaco and Paleolog, 2004). Endothelial cells in regions appear higher shear stress, acclimatise to the stream with downregulate response, while low shear stress, NF-kB are triggered in a continual way (Mohan et al., 1999). The expression of these genes is believed early events that happen at the lesion before further indicators of atherosclerosis are obvious (Monaco and Paleolog, 2004).

1. 3 Atherosclerosis process

Atherosclerosis is a pathological disorder that is life-threatening and may initiate growth during childhood life (McGill et al., 2000). This condition is identified by the gathering of lipids in sub inner layer of the artery wall, forming a plaque that reduces blood flow to the target organ (Vitruk, 2013). Atherosclerosis proceeds through steps (Figure 1.2), including:

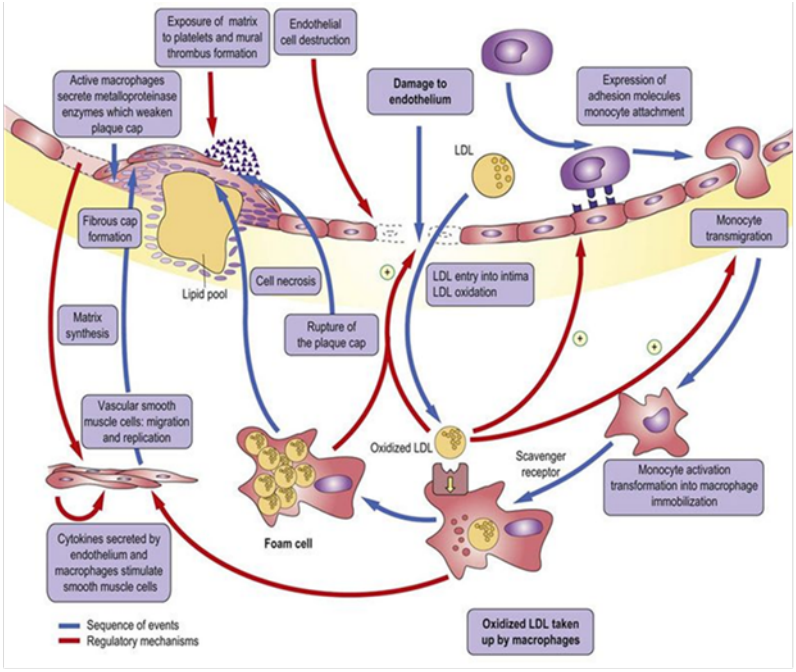


Figure 1.2 Atherogenesis mechanism includes endothelial dysfunction, deposition of lipids in the arterial intima, inflammatory response, migration and proliferation of the vascular smooth muscle cells and thrombosis. There might be a key role of oxidized lipids in the formation of lipid-laden cells and then the lipid pool that becomes the centre of the atherosclerotic plaque (Stocker and Keaney, 2004).

a) Many scientists believe endothelial cell injury is an initial factor of plaque formation starting with damage or dysfunction to the endothelium layer of the artery intima (Mudau et al., 2012, Gimbrone and Garcia-Cardena, 2016). This damage leads to increased permeability and decreases the release of anticoagulant agents, such as a nitric oxide (NO), a fundamental enzyme released from endothelial cells that capable to prevent platelet activity. The continual weakening of NO synthesis and release in porcine aortic endothelial cells, increasing endothelial oxidative stress, consequence reduced the heparan sulfate on endothelial (Zhang et al., 2008, Irokawa et al., 1997). Different materials travelling in the artery, such as cholesterol, are toxic and cellular waste products deposit inside the abnormal area of the intima and continued exposure and accumulation of this material play an important role in information of a large fatty plaque (DiCorleto and Chisolm, 1986).

(b) Lipoprotein deposition occurs when the endothelial layer is damaged. Lipoprotein is deposited in the particular area and chemical reactions occur during this deposition, causing cholesterol molecules to alter by oxidation by free radicals or oxidising enzymes. Low-density lipoprotein (LDL) builds up in the artery wall and initiates an inflammatory response in the injured site (Maxwell, 2000).

(c) There is an inflammatory reaction, where inflammatory cells, including neutrophil, lymphocytes and monocytes from the bloodstream arrive at the injured site. The monocytes become macrophages and phagocytose and digest the modified LDL particles resulting in the macrophages being converted into foam cells, which have a key role in destabilising the plaque leading to rupture.

Foam cells accumulate with the growing arterial plaque and die by apoptosis and necrosis. Metalloproteinases from macrophages cause plaques to rupture and promote blood thrombosis (Stoll and Bendszus, 2006).

(d) Fibrous cap formation. At this time of lipoprotein localization and inflammatory response, smooth muscle cells within the second layer of the artery wall (the media) start to proliferate and migrate to the surface of the fatty plaque. These migrant cells contribute to the formation of the fibrous cup, covering the plaque area (Yano et al., 2000). Atheroma lesions develop when a necrotic core of dead macrophages and extracellular lipid accumulates. The engagement and consequent migration of travelling monocytes into the subintima zone (subendothelial) is assisted by hypercholesterolaemia, which promotes the binding molecules and the release of chemoattractant substances by the endothelial cells. Macrophages formation from monocytes modification, increasing uptake the droplets of lipoprotein, transforming into pro-inflammatory cells leading to more induction of other monocytes and this lead to the advance of atherosclerosis by initiated the inflammation (Libby, 2007). Documents have shown that monocytes are heterogeneous and consist of a minimum of two separate subdivision according to activation of CD14 and CD16 (Ziegler-Heitbrock, 1996). The majority subpopulation display low level of CD16 and high level of CD14 and, whereas the minority subpopulation shows an opposite expression of majority subpopulation on the monocytes surface (Frankenberger et al., 1996).

A subdivision of CD16-positive monocytes can increase inflammatory trigger, leading up-regulates many receptors, counting CCR2, CX_3CR1 and CCR5. They act in a non-supernumerary and self-regulating function in the atherosclerosis mechanism (Ancuta et al., 2003, Tacke et al., 2007). Trigger scavenger receptor in macrophages by oxidised LDL,followed by activation CD36 via stimulation of the transcription factor PPAR-γ, thus producing a 'vicious' feed-forward sequence of cumulative ox-LDL in macrophages, finally this altering of macrophages to the pathological foam cells, according to the review of Gurnell (Gurnell, 2003).

Furthermore, ox-LDL taken up by CD36 stimulates the NF-κB this in turn increases the transcription of pro-inflammatory elements and induces a proinflammatory type (Tak and Firestein, 2001, Schwarz et al., 2017).

1. 3.1 Modification of LDL within Atherosclerotic lesion

Modified LDL might cause endothelial dysfunction, which represents an early sign of atherosclerosis. Considerable modification of LDL stimulates oxidative stress and accelerates senescence in the endothelium (Siennicka and Zapolska-Downar, 2003). A marker of endothelial dysfunction is decreased endothelium-dependent vasodilatation (Szucs et al., 2007).

Many chemical modifications of LDL have been documented since the 1970s, including oxidised LDL, acetylated LDL, glycated LDL, and methylated LDL. Changing LDL components through modification, specifically, apolipoprotein B-100 changes the affinity of LDL for binding to related receptors (Hoff et al., 1993). This event might stimulate atherogenesis via scavenger receptors (SRs) on macrophages and endothelial cells (Iuliano et al., 2001). LDL oxidation is induced via many metal ions and free radicals (Iuliano et al., 2001) and oxidising enzymes such as myeloperoxidase and lipoxygenase (Guo et al., 2001).

The structural changes during LDL oxidation, involve increased density, increased electrophoretic mobility(due in part to the neutralisation of the positive charge of lysyl of residues), changes in phosphatidylcholine(due to hydrolysis by lipoprotein-associated phospholipase A_2), fragmentation of apoB-100, (due to free radical-mediated peptide bond scission) modification of lysine amino groups (by reaction with aldehydes) and creation of fluorescent adducts because of covalent binding of lipid products to apoB-100 (Chehin et al., 2001). Aldehydes produced during the peroxidation of LDL exhibit reactivity with proteins, making a diversity of intermolecular covalent adducts on the apolipoprotein B-100 in LDL particles.

The main consequences as regards atherosclerosis of oxidised LDL are as follows:

(I) Oxidised LDL affects blood cells, stimulating monocyte binding to endothelial cells, by activating of ICAM-1 and VCAM-1 (Quinn et al., 1988) and speeding up the conversion of monocytes to macrophages (Quinn et al., 1987).

(2) Ox LDL plays a crucial role in amplified activation of growth factors, like platelet-derived growth factor (PDGF), which is responsible for inducing smooth muscle cell (SMC) migration (Stiko-Rahm et al., 1992), and basic fibroblast growth factor (FGF), which stimulates smooth muscle cells proliferation during the atherosclerosis process (Lindner et al., 1991).

(3) Ox-LDL contributes to plaque formation and development either by stimulating collagen production from smooth muscle cells, which is responsible for the formation of the fibrous cap of plaques and increasing their size by promoting the relocation of smooth muscle cells from tunica media throughout the elastic membrane to the intima, causing arterial expansion and early angiogenesis in the area (Jimi et al., 1995). On the other hand oxidised LDL may contribute in stimulating fibrous cap thinning through the secretion of matrix metalloproteinases and promoting smooth muscle apoptosis (Loidl et al., 2004), and promoting necrotic core production in plaques (Schwartz et al., 1991). The aggregation and oxidation of LDL in the subendothelial area may show a critical event in atherogenesis. LDL might be oxidised by several mechanisms such as iron, copper, caeruloplasmin, lipoxygenase, superoxide, peroxynitrite, and myeloperoxidase (Schwartz et al., 1991).

This oxidation of LDL happens in both humans and animal models of atherosclerosis, such as mice, rabbits and zebrafish (Witztum and Steinberg, 2001). Ox-LDL may cause apoptosis of foam cells and produce a necrotic lipid centre in the lesion, a feature of an atherosclerotic lesion in both human and murine species.

Cysteine proteases display vital influence in the growth of atherosclerotic plaque. Ox-LDL can release lysosomal cathepsins B and L and may activate cysteine-aspartic proteases (caspases) and stimulate apoptosis (Guicciardi et al., 2000).

Thereby, both cytotoxic ox-LDL and related oxysterols may lead to weakening or destabilization of lysosomes and release the cathepsins, promoting the programmed death of phagocytic cells and progression of the atherosclerotic lesion and necrotic core formation (Emanuel et al., 2014a). This pro-apoptotic process may also apply to smooth muscle cells and endothelial cells. Cathepsin S regulates thrombotic effects of arterial injury (Sukhova et al., 2003) by elastolytic activity, which can degrade collagen. Macrophages trigger elastolytic cysteine proteases during particular situations might stimulate elastin and collagen deprivation at places of inflammation and developing plaque rupture and thrombus formation (Hansson, 2015).

1. 3.2 Endocytosis of oxidised LDL by macrophages

LDL is the major cause of cholesterol accumulation in macrophages. During early plaque, foam cells originated from transforming macrophages, whereas progressive lesions, smooth muscles experienced foam cell conversion.

This event clarified, by the discovery the hypoxia (because of the enlarged arterial wall and diminished oxygen circulation (Crawford and Blankenhorn, 1991)), rises LDL concentration and lipid gathering in SMC (Wada et al., 2002).

Since the ingestion of LDL through the LDL receptor (LDLR) is linked to predatory feedback, the build-up of extreme quantities of LDL in macrophages associated to alteration of LDL, leading swift unrestricted uptake (Brown and Goldstein, 1983). Numerous kinds of LDL alteration described permitting cholesterol internalization *in vitro*, counting aggregation acetylation and oxidation, (Khoo et al., 1988). In vitro acetylation of LDL may encourage cholesterol assembly in macrophages. Nevertheless, not enough indication that acetylated LDL happens *in vivo*. Oxidative of LDL, however, stimulated via all the main cell categories found in the plaque, endothelial cells, SMC and macrophages and lymphocytes, might induce foam cell development, causing atherogenesis. Furthermore, macrophages may encourage oxidation of LDL by exocytosis of iron, resulting from earlier phagocytosis of aged or broken erythrocytes (Yuan et al., 1995).

Iron is obtained from absorbed diet by enterocytes of the duodenal lining. Enterocytes allowed moving iron into the body with help marked molecules. Dietetic iron that absorbed as the ferrous formula (Fe^{2+}) or as heme (Ems, 2020). Under the effect of ferric reductase enzyme ferric Fe^{3+} convert to Fe^{2+}.

Divalent metal transporter 1 (DMT1) carryings several divalent metals throughout the plasma membrane. When the iron is connected with the heme, will be relocated throughout the apical membrane by a particular protein called heme carrier protein (HCP1).

Some oxidised LDL might aggregate in the arterial wall (Aviram et al., 1995) where it is linked with proteoglycan secretion via macrophages (Maor et al., 2000).

Moreover, can be aggregated by different factors, such as sphingomyelinase (SMase). SMase produces the aggregation of LDL through stimulating cleavage of the phosphocholine group of sphingomyelin (SM) developing in creating the hydrophobic moiety, ceramide. The initiation of ceramide produces LDL aggregation by displaying hydrophobic pieces on the LDL monolayer. This hydrolysis will increases the molar ratio of cholesterol to phospholipid in the monolayer part of LDL particle, developing cholesterol crystal nucleation after aggregated LDL.

Enzymes including, SMase, phospholipase A_2 (PLA_2), phospholipase C, might lead to loss of LDL integrity converting to aggregated LDL. Although macrophages show numerous receptors related to lipoproteins modified via chemical alteration (Matsuura et al., 2005), macrophages also have merely limited receptors for native lipoproteins. Therefore, non-oxidised LDL might be internalised by macrophages mainly via fluid-phase endocytosis (Kruth, 2011).

Moreover, aggregated LDL is ingested by phagocytosis receptor which takes place by a clathrin-independent mechanism. Scavenger receptor ingested oxidised LD L and acetylated LDL. Nevertheless, significant indication related to LDL uptake methods based on the shape and function to diverse endocytic locations. This probably linked to the participation of various receptors. 80% of acylated LDL uptake is facilitated by scavenger receptor, whereas 70% of the oxidised LDL ingested is facilitated by CD36 (Schonbeck et al., 2002), and (lectin-like oxidized low density lipoprotein receptor-1) (LOX-1) (Kume et al., 1998). Minor oxidation of LDL produced what has been termed minimally modified LDL (mmLDL), which is known by just LDL receptor rather than the scavenger receptors (Miller, 2003).

Oxidised LDL bind with CD14 and then stimulation CD36, leading accessive uptake of other particles from oxidised LDL (Miller et al., 2003).

In fully oxidised LDL particles, required alteration in both parts of LDL particle, including lipid and protein, and uptake by a different receptor called scavenger receptor, but if there is an alteration in lipid area and producing lipid oxidation product then called minimum modified LDL (MM LDL). MMLDL has a dynamic role in cumulative inflammatory cytokine secretion (Chavez-Sanchez, 2010).

In SMC, LDL receptor-related protein 1 (LRP1) is active compared to scavenger receptor (Llorente-Cortes and Badimon, 2005). LRP1 is usually upregulated by high levels of cholesterol in plasma and facilitates ingested of aggregated LDL in SMC.

Aggregated LDL stimulates plasma membrane engulfment that transports it into a surface-connected section in macrophages, a procedure named patocytosis (Kruth, 2002). The process called actin-dependent, which independently occurred of the LDL receptor.

LDL moved to lysosomes for degradation (Brown and Goldstein, 1983). However, the protein part of oxidised LDL may unaffected to lysosomal breakdown then it collects with a product of degradation (Jessup and Kritharides, 2000).

This lipid assembly process regulates foam cells development. This development in atherosclerosis lesion is started during the cholesterol clearance process inhibited, because of the massive amount of lipid accumulated in macrophages leading to disabled of lysosomes.

Consequently, either decrease the level of LDL or augmented HDL level in plasma, accumulation of excess amounts of LDL in subendothelial might be decreased, although the role of HDL in protecting against atherosclerosis is currently controversial. HDL concentrations are ineffectually associated to cardiovascular disease in epidemiological investigations (Miller, G.J.&Miller, erect 1975) but medications that elevated HDL concentration (cholesteryl ester transfer protein inhibitors) unsuccessfully diminish cardiovascular disease (Barter et al., 2007).

1.3.3 LDL oxidation by transition metals and heme

In the pathogenesis of atherosclerosis, LDL oxidation is an important risk factor (Steinberg, 2009). Oxidative modification of LDL occurs after incubating LDL with copper sulphate overnight (Steinbrecher et al., 1984). Copper sulphate induced oxidative modification of the polyunsaturated fatty acids (PUFA) in the sn2 position of phospholipids can lead to the breakdown of approximately 40% of the phosphatidylcholine, 50-75% of the PUFA (Esterbauer et al., 1987). Non-enzymatic oxidation stimulated by copper sulphate *in vitro* is assumed to based on the existence of LOOH in LDL, may be found in unnoticeable concentration *in vivo* or produced through LDL extraction (Esterbauer et al., 1992).

Copper degrades these hydroperoxides into peroxyl radicals and alkoxyl radicals (Jerome, 2010). The fatty acid moiety of cholesteryl esters is vulnerable to oxidation and the cholesterol moiety, which has an unsaturated bond in its polycyclic sterol ring, is also target to oxidative attack. Low levels of oxidation are adequate to oxidise it to a level where it evolutions different biological characteristics (Berliner et al., 1990). This sort of LDL, named minimally-modified LDL (mmLDL), binds to the LDL receptor, but not to the scavenger receptors (Berliner et al., 1990). Many experiments have shown that mmLDL is a pro-atherogenic factor (Choi et al., 2009). Divalent iron cations (Fe^{2+}) have the ability to stimulate LDL oxidation, but usually to a lesser degree compared with Cu^{2+}. Nevertheless, the complex of iron and protoporphyrin IX is an active LDL-oxidising factor and, in particular, this activation of LDL occurs with a low concentration of peroxides (Miller et al., 1995). In normal conditions, haemoglobin breaks down by catabolism and produces low amounts of free hemin (containing Fe^{3+}) that associate with the red blood cell membrane. In abnormal or pathogenic conditions, such as inflammation and hyperlipidaemia, LDL can be oxidised by binding with hemin in whole blood (Miller and Shaklai, 1999). Moderate amounts of haemoglobin are continuously released from destroyed erythrocytes, especially in arterial bifurcation and aortic bends.

In haemodialysis people with elevated levels of haemolysis, and haemoglobin stimulate LDL to be oxidised has been reported to raise the amount of ox-LDL in patients plasma who suffer from haemolysis (Sevanian and Asatryan, 2002).

LDL oxidation by cells in atherosclerotic plaques possibly needs metal sources of iron or copper as reagents for the oxidation. Plasma iron is carried by the protein transferrin. Acidic pH releases iron from transferrin that catalyses LDL oxidation (Lamb & Leake, 1994). During LDL oxidation process by iron, ion required superoxide anion,which works as reducing factor in present H_2O_2, resulting hydroxyl radical ($OH^{\bullet}$) by the Fenton reaction (Georgi, 2016). Many research has been documented that LDL oxidation mediated by heme takes place *in vivo* (Lynch, 2000*).* The relation of iron form and cardiovascular disease in vivo, however is unpredictable (Sempos, 2002).

Caeruloplasmin is a protein responsible for carrying copper in plasma and then becomes more active in oxidising LDL at acidic pH (Lamb & Leake 1994). Caeruloplasmin is separated from plasma in 1948 for the first time (Holmberg and Laurell, 1948). Although, it is recognised from a long period as acute form react and protein associated with copper transport, there is important role associated with chronic heart failure

(Cabassi et al., 2014).

"moonlighting protein" is another name used to be called Caeruloplasmin because of its diverse activities. Caeruloplasmin contains ferroxidase I, which is the crucial factor of converting Fe^{2+} into Fe^{3+} (form bound in transferrin), consequently restricted Fe^{2+} from contributing in the production of hydroxyl radicals by this action Caeruloplasmin act as an antioxidant. Furthermore, the prooxidant role based on include amine oxidase (Dubick et al., 2015).

1.3.4 LDL oxidation by enzymes

LDL is oxidised by vascular cells in culture. These cells are present in atherosclerotic lesions, including monocyte/macrophages, lymphocytes SMC and endothelial cells (Liao et al., 1994). Several enzyme systems, including lipoxygenase, NADPH oxidase and myeloperoxidase, documented of their ability to oxidised LDL. Vascular cells express one or many of these enzymes. Myeloperoxidase (MPO) enzyme released from blood cells, such as neutrophils, monocytes/macrophages, which produces several oxidants, involving hypochlorous acid and reactive nitrogen specious (RNS), generating chlorinated, nitrated LDL and HDL. Modification of apoA1 occurred when MPO binds to HDL (Undurti et al., 2009). RNS transform LDL to ligand with high ability to bind with CD36, promoting internalises them by macrophages (Podrez et al., 2000).

Studies reported that Myeloperoxidase induced carbamoylation LDL particle transforms it to SR-A ligand (Wang et al., 2007). Both free fatty acids and lysophospholipids are released from glycerophospholipids at the position sn-2under the effect of enzymes called Phospholipase A_2 (PLA_2). Recently, about 30 of these enzymes are sorted based on many factors such as mass, size, role, and calcium necessity. Furthermore, these enzymes have been split into six lines according to specific factors such as size, composition, catalytic component and connection. These lines contain (secretory PLA_2 ($sPLA_2$), cytosolic ($cPLA_2$), Ca^{2+}-independent ($iPLA_2$), platelet-activating factor acetyl hydrolases (PAF-AH), which is a component of LDL, lysosomal PLA_2 and adipose-specific PLA_2).

It is acceptable that PLA_2 can act as a biological focus for the treatment target of numerous illnesses, relating cardiovascular diseases and atherosclerosis. Extraordinary consideration employed on a block of ($sPLA_2$ isoforms) as a therapeutic element, depending on the motivation mechanism during inflammatory infrequent diseases (Quach et al., 2014).

An important role might be played by pro-inflammatory secretory phospholipase A_2 ($sPLA_2$)-IIA, which can act as the mediator of local inflammation in atherosclerosis lesion progression. The sPLA2 –IIA may show a pro-atherogenic outcome in the arterial wall in different steps (Hurt-Camejo et al., 2001). In arterial plaque, this enzyme is present within the lipid centre in both the extracellular matrix and in areas rich in macrophages (Romano et al., 1998). $sPLA_2$-IIA-altered LDL bound to proteoglycan (a protein family that classically experience general posttranslational alteration with sulphated sugar chains) (Iozzo and Schaefer, 2015) undergoes aggregation and fusion and consequently continues the accumulation of lipids in the extracellular matrix and arterial wall (Hakala et al., 2001).

In addition to promoting the retention of LDL particles in the arterial wall, there is proof that $sPLA_2$ alteration may increase macrophage uptake of LDL, resulting in foam cell formation (Aviram and Maor, 1992). $sPLA_2$-IIA releases free fatty acids and lysophosphatidylcholine from LDL. This activity may affect the biological function of vascular cells, and macrophages at the location of accumulation of LDL (Leitinger et al., 1999). NADPH oxidase is a source of superoxide anion in the vascular cells (Lassegue and Clempus, 2003, Seshiah et al., 2002, Zafari et al., 1998), which might be involved in LDL oxidation.

This superoxide leads to an increase in intracellular H_2O_2, proposing a particular role for these oxygen species in vascular growth.

Studies have revealed that 12/15-lipoxygenase is extremely active in macrophages and can oxidise LDL (Takahashi et al., 2005). Thus, purified lipoxygenase can mediate LDL oxidation and conversion of LDL to a cytotoxic (Cathcart et al., 1991).

1.3.5 Lipid peroxidation

In later a long time it has gotten to be caught that lipid peroxidation, may be the vital stage in the mechanical process of numerous disease conditions in mature and newborn patients. This peroxidation is a reaction normally occurred in minor quantities in the body, generally by the influence of numerous ROS e.g. ($H^{\bullet}$, $HOO^{\bullet}$). Lipid peroxidation also produced under influence of some phagocytes. It has been linked with the disease such as atherosclerosis (Mylonas and Kouretas, 1999). Free radicals stimulate LDL oxidation, causing structural alterations of LDL particles.

An initial step of this oxidation is peroxidation of polyunsaturated fatty acids (PUFA) in cholesteryl esters, phospholipids or triglycerides in LDL by removal of a hydrogen atom by a free radical. The reorganisation of the PUFA structure occurs,producing the development of conjugated dienes that absorb UV at wavelength 234 nm. For this reason, we can use a spectrophotometer at wavelength 234 nm to measure the oxidation of LDL (Esterbauer et al., 1989). Oxygen binds to form lipid peroxyl radicals ($LOO^{\bullet}$), which remove a hydrogen atom from another PUFA, which is the beginning of a chain of reactions, leading to lipid hydroperoxides. β-scission forms aldehydes that bind to amino acids on LDL, e.g. lysine

(Esterbauer et al., 1989). Cholesterol (esterified and non-esterified) is oxidised and produces oxysterols such as 7-ketocholesterol. (Figure 1.3A, B)

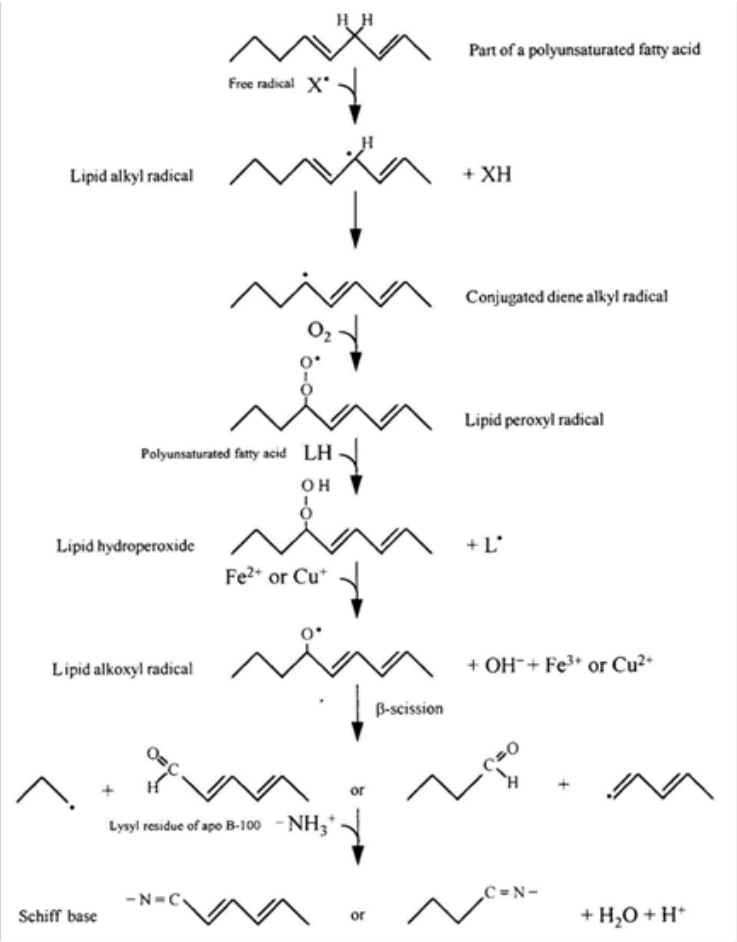


Figure 1.3 A. Diagram shows the free radical chain reaction mechanism of lipid peroxidation of a polyunsaturated fatty acid (PUFA).

(1)The hydrogen atom is abstracted from a PUFA to give a lipid alkyl radical.

(2) Rearrangement and to give a conjugated diene alkyl radical, which can absorb UV at 234 nm.

(3)Reaction with oxygen to give a lipid peroxyl radical.

(4) Hydrogen atom abstracted from another PUFA adds to step 3 give a lipid hydroperoxide and lipid alkyl radical.

(5) The reaction of lipid hydroperoxides with transition metals to give lipid alkoxyl radicals.

(6)Alkoxyl radicals decompose by β-scission to form lipid alkyl radicals and aldehydes.

(7)Aldehydes react with amino acids on apoB -100, especially lysyl residues.

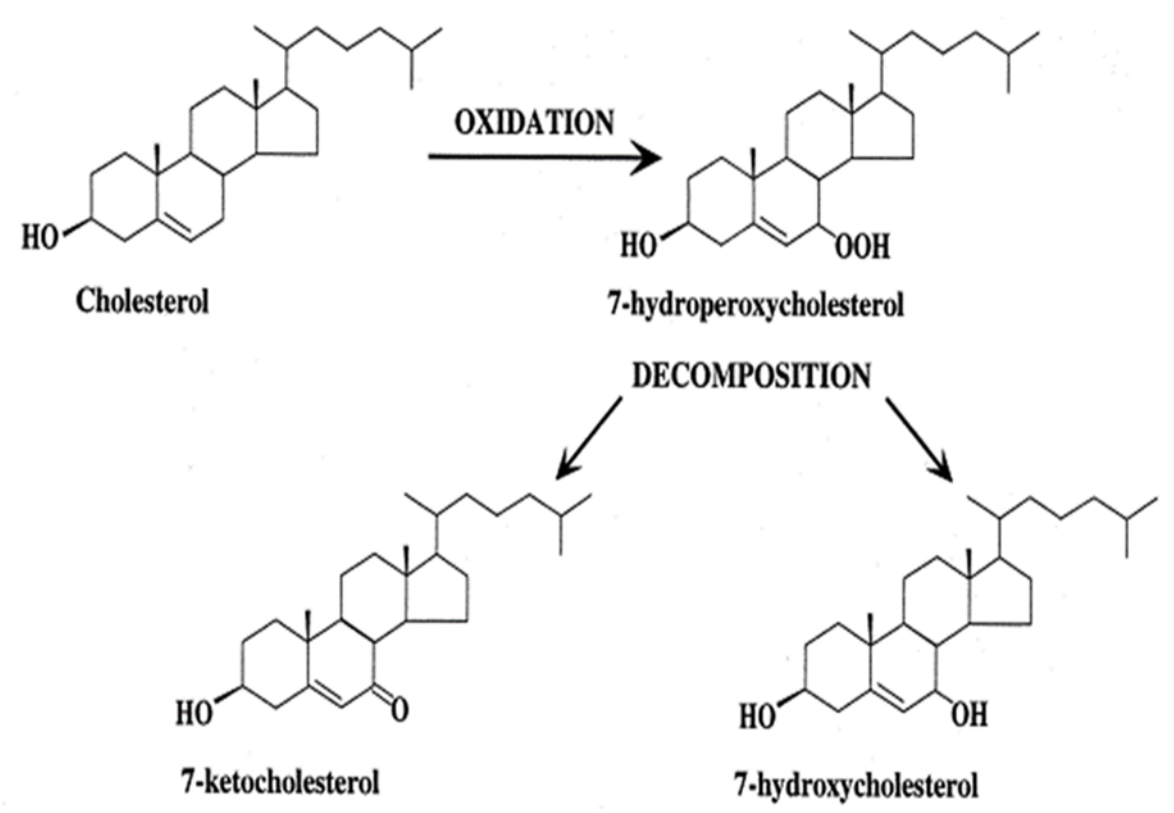


Figure 1.3B. The diagram shows major products of free radical attack of cholesterol are the epimeric 7-hydroperoxy cholesterols. These molecules are labile and are rapidly converted to the epimeric 7-hydroxycholesterols(7α-hydroxycholesterols or 7β-hydroxycholesterols)and 7-ketocholesterol.

1.3.6 LDL uptake within acidification environment

Foam cell accumulation in the intima of the arterial wall is a significant manifestation of atherosclerosis (Gieseg et al., 2009). Foam cells are produced when macrophages start to take up LDL by different processes, involving scavenger receptor activity and phagocytosis (Witztum, 2005). Besides, smooth muscle cells secrete substances to the extracellular material, which engage with aggregated LDL to increase LDL uptake by macrophages (Tabas et al., 1993). Acidification of extracellular fluid increases macrophage uptake of both native and modified types of LDL, via an increase in the levels of proteoglycans on cell surfaces and complexes of LDL-proteoglycans (Plihtari et al., 2011, Lahdesmaki et al., 2012). Extracellular acidosis may increase foam cells derived from monocytes by promoting lipoprotein modification and consequently, stimulating uptake by macrophages (Leake, 1997). Furthermore, Kruth (Kruth et al., 2002) has documented a type of foam cell development not involving either LDL modification or macrophage receptors, when macrophages are incubated with a high concentration of LDL because of fluid-phase pinocytosis of native LDL particles (Zhao et al., 2006). Pinocytosis has been shown to be augmented with an acidic environment (Vermeulen et al., 2004). Macrophages after capturing a large amount of LDL generate incomplete closed chambers on the surface of aggregated LDL (known as lysosomal synapses), causing a decline in pH, and release lysosomal acid lipase. The main function of this enzyme is the hydrolysis of the cholesteryl esters of LDL to unesterified cholesterol and fatty acids. Unesterified lipid is then able to enter inside the cells and the fatty acids cause acidic local environments (Haka et al., 2013). Interestingly, an acidic microenvironment related to macrophages activity, in which they

release cathepsins and H^+ ions, might provide suitable conditions for increased activity of proteases (Haka et al., 2009).

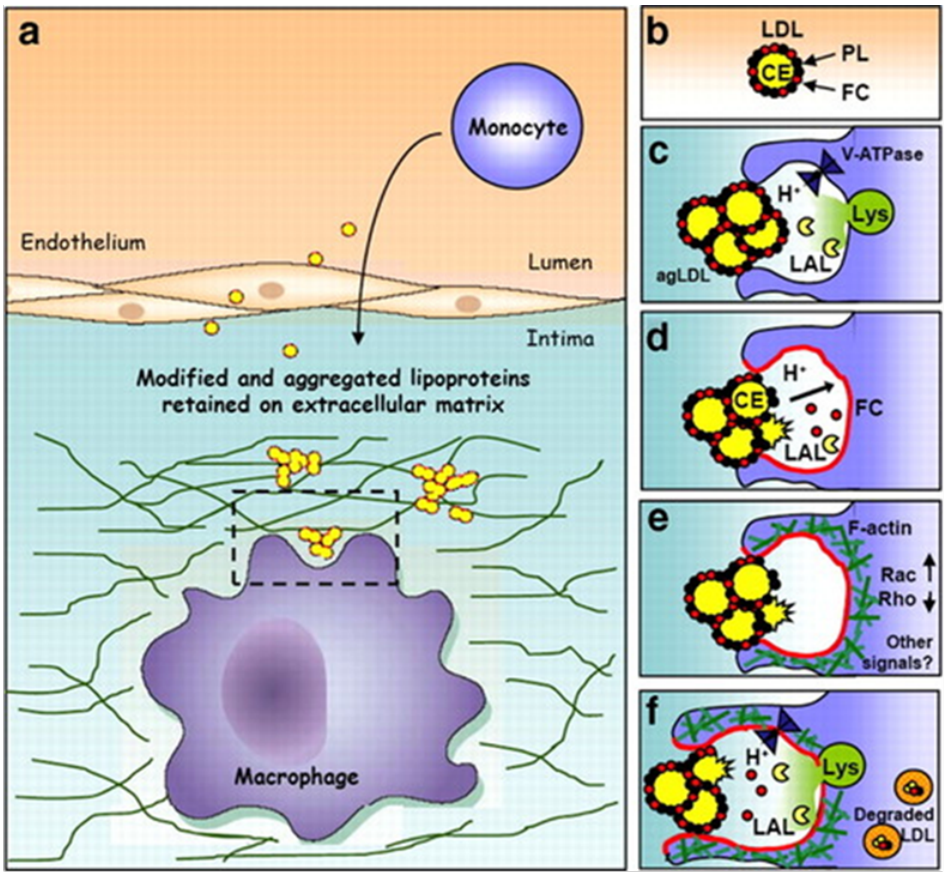


Figure 1.4 Diagram of agLDL macrophage interfaces. (a and b) LDL in the blood stream is accumulated in the subintimal area, where it converts aggregated modified. (c) When macrophages interact with agLDL, a compartment is produced,which is characterised by an extracellular position, acidic and hydrolytic environement,. The acidic pH of the compartment is sustained by V-ATPases in the macrophage plasma membrane. (d) FreeCholesterol may be relocated to the macrophage plasma membrane after hydrolysis of LDL cholesterol ester by lysosm acid lipase that has been provided to the surface-connected compartment from lysosomes. (e) FC in the macrophages may start signaling actions, such as alteration of Rho GTPase activities, which forward to polymerization of actin. (f) Actin polymerization motivations membrane amplification and contact with agLDL, which stimulates more FC release to the membrane, and the maintenance of the sequence. Segments of agLDL are assumed into endosomes, where additional degradation happens (Haka, 2009).

1.3.7 LDL oxidation inside lysosomes

LDL modified by non-oxidative processes, including modification by sphingomyelinase (Xu and Tabas, 1991), phospholipase A_2 (Hakala et al., 1999), and lysosomal cathepsins (Leake et al., 1990) was taken up faster by macrophages. The non-oxidised modified LDL might be taken up by macrophages but oxidised within lysosomes of macrophages (Wen and Leake, 2007).

Ox-LDL in extracellular fluid might only be partially oxidised because interstitial fluid contains effective antioxidant factors (Dabbagh and Frei, 1995) or might be secreted as ox-LDL from foam cells, which lyse due to ox-LDL stimulated apoptosis and necrosis (Zhang et al., 2015). Lysosomes of macrophages contain redox-active iron (Kurz et al., 2004), which might catalyse LDL oxidation in these organelles (Wen and Leake, 2007). Lysosomes contain iron as a result of autophagy of iron-containing organelles or phagocytosis of red blood cells inside arterial plaques with haemorrhage from neovessels (Karlsson et al., 2013). LDL oxidation by iron is much faster at lysosomal pH about (pH 4.5) relative to interstitial fluid pH about (pH7.4) (Wen and Leake, 2007)(Satchell and Leake, 2012). The iron chelator desferrioxamine decreased lysosomal LDL oxidation (Wen and Leake, 2007). Oxidation of LDL in lysosomes might have many consequences, including, macrophages stimulating pro-inflammatory cytokine secretion from macrophages and formation of ceroid (Ahmad and Leake, 2018), which might act as a coating layer to protect lipid droplets blocking lipase activity (Lee et al., 1998) and increasing the pH of lysosomes (Ahmad and Leake, 2019)(Figure 1.5).

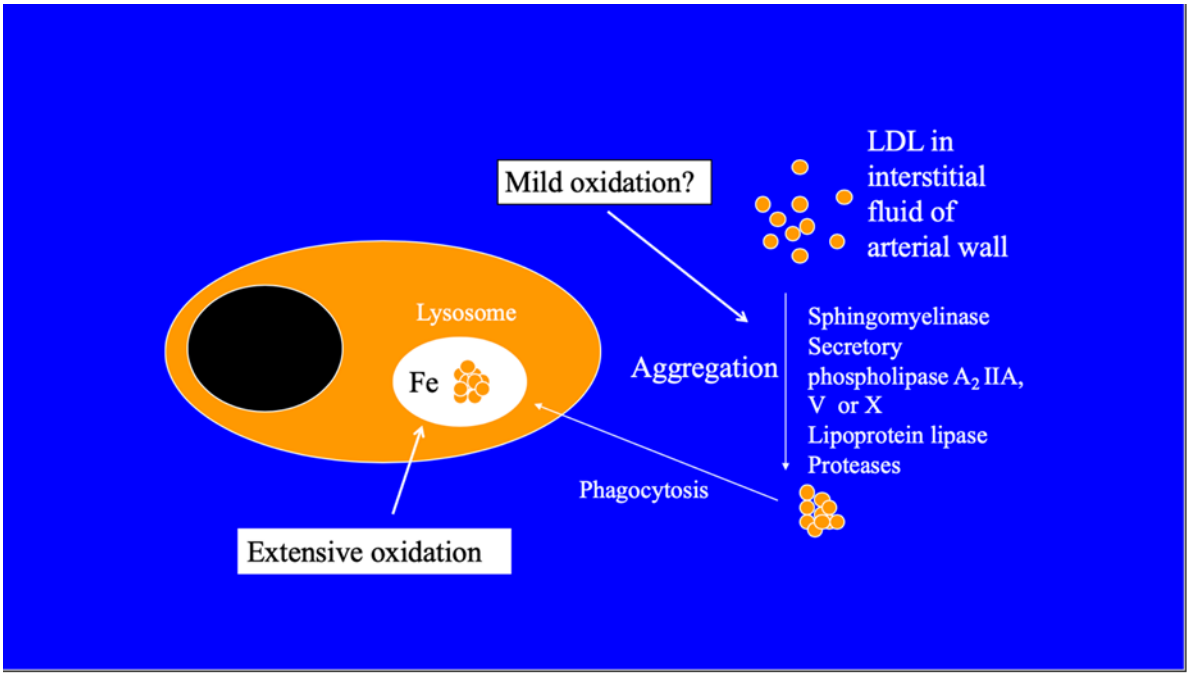


Figure 1.5 An illustration shows LDL oxidation in lysosomes

1.4 Lysosomes

The key role of these microscopic organelles is to work as digestion bodies for cellular elements that have surpassed their lifetime or are then no longer beneficial. In this respect, the lysosomes reutilize the cell's organic features in a process known as autophagy. It is a common term for cytoplasmic elements hydrolysis by lysosomes. Lysosomes hydrolyse cellular constituents, like carbohydrates and proteins, to basic elements, after hydrolysis relocated, the elements return back as new materials to the cytoplasm. To complete the responsibilities related to digestion, the lysosomes exploit about 50 diverse kinds of hydrolytic enzymes, all of which are synthesised in the endoplasmic reticulum and packaged in the Golgi apparatus (Kurz et al., 2008a). Lysosomes are regularly formed from the sheath of the Golgi apparatus and fuse with, late endosomes, which are vesicles that hold materials transported into the cell by endocytosis. The pH inside the lysosome is sustained at about 4.5 by an ATP-dependent proton pump lysosomal membrane (Ohkuma et al., 1982).

1.4.1 Lysosomal iron

Numerous data have confirmed that redox-active iron exists within lysosomes (Yu et al., 2003, Zdolsek et al., 1993, Yuan et al., 1996, Petrat et al., 2001). Lysosomal iron may derive from the breakdown of autophagocytosed ferritin (Yu et al., 2003, Sibille et al., 1989a, Radisky and Kaplan, 1998, Kidane et al., 2006) and iron-rich mitochondrial proteins (Yu et al., 2003, Persson et al., 2001) and the phagocytosis of red blood cells (Yuan et al., 1996). Desferrioxamine (iron chelator) is internalised by fluid-phase endocytosis and delivered to lysosomes (Lloyd et al., 1991, Cable and Lloyd, 1999). Studies have shown that desferrioxamine can deplete the cell cytoplasm of all chelatable iron, suggesting that the cytoplasmic non-ferritin bound pool of iron (identified as the labile iron pool) is derived from lysosomes (Tenopoulou et al., 2005). There is a difference in the levels of iron in different lysosomes, which is possible because of differences in the amount of time passed since the lysosomes have been involved in the breakdown of iron-rich compounds (Kurz et al., 2008a). It is controversial if lysosomes contain ferrous and ferric (Collins et al., 1991), but both might be present (Meguro et al., 2005). The ferrous form might be favoured, however, because of the reducing environment of lysosomes (Terman and Kurz, 2013)

1.4.2 Lysosomal involvement in apoptosis

Subjecting cells to moderate oxidative stress has been reported to increase lysosomal membrane permeability, followed by increase mitochondrial membrane permeability and apoptosis (Brunk et al., 2001). Hydrogen peroxide, which is constantly formed within cells by the damage caused by electrons from the mitochondrial electron transportation chain, can simply diffuse into lysosomes and it remains there as lysosomes do not have the hydrogen peroxide degrading enzymes catalase and glutathione peroxidase (Kurz et al., 2008a). Lysosomal hydrogen peroxide can then react with the lysosomal iron to make the highly reactive hydroxyl radical by the Fenton reaction. Hydrogen peroxide can oxidise various kind of substrates and caused biological damage with the presence of iron (Neyens and Baeyens, 2003, Winterbourn, 1995).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^\bullet$$

The acidic pH of the lysosome and occurrence of reducing equivalents such as cysteine are favourable for the Fenton reaction (Baird et al., 2006).

In oxidative stress, increased quantities of hydrogen peroxide diffuse into lysosomes, causing the formation of hydroxyl radicals, which can harm lysosome membranes, leading to release of lysosomal enzymes, including cathepsin B, and consequent apoptosis.

Desferrioxamine inhibits lysosomal rupture and apoptosis after oxidative stress (Yu et al., 2003). Phospholipase activation might also trigger mitochondrial membrane damage, leading to the release of pro-apoptotic molecules (Zhao et al., 2001). Lysosomal cathepsins

B stimulates the pro-apoptotic protein Bid (Cirman et al., 2004) and result in the reduces the anti-apoptotic proteins like Bcl-2 , Bcl-X_L (Droga-Mazovec et al., 2008).

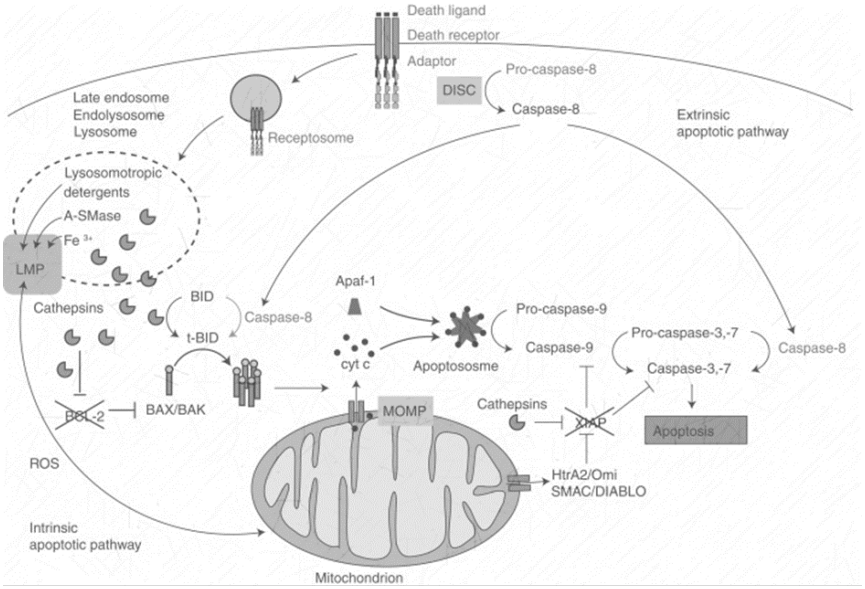


Figure 1.6 Diagram of role lysosomes in apoptosis (Repnik, 2013)

1.4.3 Atherosclerosis as a lysosomal storage disorder

Investigations of lysosomal storage defects, which are measured by the accumulation of ingestible material due to defective lysosomal enzymes, have described that accretion of undigested material within lysosomes can weaken their role and have a devastating effect on the body (Hers, 1972).

Normally, LDL is internalised by macrophages and is carried to lysosomes for the breakdown. Lysosomal acid lipase breaks down the cholesteryl esters in the particle and the products from hydrolysis moves into the cytoplasm (Brown et al., 1983). Excessive

cholesterol converted to fatty acid under influences of enzyme acyl-CoA: cholesterol acyl-transferase (ACAT) and is kept in the cytoplasm as lipid droplets (Rudel et al., 2001). In atherosclerotic lesions, foam cells store lipids within lipid-filled lysosomes and lipid droplets (Haley et al., 1977). Once macrophages bind LDL altered by oxidation or aggregation processes, LDL is swiftly taken up, and cholesterol accumulates. There is diminished lysosomal apolipoprotein breakdown, less cholesteryl ester hydrolysis and eventually diminished cholesterol efflux from lysosomes (Mahlberg et al., 1990, Griffin et al., 2005, Jerome et al., 1998, Yancey and Jerome, 2001).

Oxidised LDL might prevent both lipase and protease activity and is capable of preventing free cholesterol elimination from the lysosomes (Kritharides et al., 1998, Dhaliwal and Steinbrecher, 2000, Hoppe et al., 1994). Cells incubated with oxidised LDL, agLDL or cholesteryl ester with rich lipid distributions had a reduced capability of the lysosomes to maintain an acidic pH, which agreed with the inhibition of cholesteryl ester lipolysis (Cox et al., 2007). It remained proposed that as lysosomal enzymes necessitate an acidic pH to performance correctly, neutralisation of pH of the lysosomes decreases cholesteryl ester hydrolysis. Potential treatments to avoid lysosomal lipid build-up in atherosclerosis include treatments to avoid lysosomal membrane cholesterol accumulation, which inhibits the lysosomal ATP-dependent proton pump and increases the pH of the lysosome (Jerome, 2006).

1.4.4 The role of lysosomes in atherosclerosis

Lysosome organelles are an intracellular structure have an acidic interior of pH about 4.5 (Mindell, 2012), which comprises acidic hydrolases, certain membrane proteins, including mannose-6-phosphate receptor (M6PR). M6PR is marking to classify late endosomes from

lysosomes. The pumps that move H^+ ions opposite their concentration slopes named ATP-dependent proton pumps, which composed from trans member proteins. They hold few binding positions for ATP situated on the cytosolic part of the membrane.

They usually do not convert ATP into ADP and Pi without H^+ ions are alongside transported. According to this constricted connection between ATP breakdown and transport, the energy deposited in the phosphoanhydride bond is not degenerate but served to transfer ions and other molecules reverse an electrochemical slope. The transmembrane pH slope in lysosomal organelles is made and maintained by an ATP-dependent proton pump residing in the organelle membrane (Schneider, 1983).

Lysosomes have similar properties to cell type-specific organelles called lysosome-associated organelles, such as melanosomes, lytic granules, delta granules, lamellar bodies, and other acidic granules (Huizing et al., 2008). Significant evidence indicates that lysosomes play an important role in atherosclerosis.

For example, in fat deposition subendothelial space of lesion expansion, cholesteryl esters (CEs) collect mostly in cytoplasmic droplets, demonstrating that lysosomal role is functioning and propose a significant part for acyl-CoA acyltransferase (ACAT)-1 in producing lipid droplet at this phase (Jerome and Lewis, 1985).

As lesions develop into fibrous plaques, a considerable build-up of CE and free cholesterol happens in lysosomes from an atherosclerotic lesion that collects from mammalian and birds (Miller and Kothari, 1969), demonstrating the defective lysosomal breakdown the cholesterol ester and recycle of cholesterol and signifying that lysosomal disabled is a contributor to the last phase of the disease.

Another lysosomal enzymes, such as cathepsin D and acid sphingomyelinase, are changed during the atherosclerosis process, signifying an association between lysosomes function

and atherosclerosis. Lysosomal storage disorders can refer to the gathering of substances in lysosomes because of inherent disabled in lysosomal hydrolysis. The relation between a defect in a lysosomal enzyme and dysfunction is primary and is associated with the malfunctioning lysosomal enzymes (Platt, 2014) But, in others view, accumulation seems comes from secondary events, which generate inconsistencies in metabolism (Rappaport et al., 2016). This accumulation is the main component of macrophage foam cells clinically significant atherosclerotic (Jerome, 2010).

1.5 Transition metals in atherosclerosis

1.5.1 Iron Homeostasis and Regulation

The only origin of iron balance is absorption from the gastrointestinal tract. Adults slough off 14 μg/kg/day of iron via epithelial shedding (Green et al., 1968). Also, before the menopause women lose the daily equal of 0.9 mg of iron over menstruation (Hallberg et al., 1966). Levels of ferritin in women increase considerably in the age between 50-60 years old, naturally during menopause, while men have a rise in ferritin levels through the late adolescent years (Zacharski et al., 2000). The extra iron level may take place by increased nutritional or complementary consumption (Zotter et al., 2004). Genetic syndromes, such as haemochromatosis, cause excess gut iron absorption (Lynch et al., 1989). Iron insufficiency most usually happens via blooding or insufficient dietary consumption and nutritional blocks of iron absorption (Fleming et al., 1998), or disorders like a coeliac disease characterised by an unusual mucosa that lining gastrointestinal tube causing block

iron absorption (Bardella et al., 2005). Free iron is toxic because of its redox activity (Takeda and Iwai, 2016). Hence, most iron ions in the body exist in a bound structure.

Iron erythrocyte haemoglobin is around 2,500 mg, ferritin in liver and spleen is around 1,000 mg, while myoglobin is 170 mg, besides 3 mg flows in the TPT (Schenck, 1992).

Liver synthesised Hepcidin, which is an iron-regulating hormone (Hentze et al., 2004, Pigeon et al., 2001). The important function of Hepcidin is binding and downregulating ferroportin (Nemeth et al., 2004). Ferroprotein, situated on the external membrane of macrophages, permits the release of iron from these cells and ferroportin to facilitate on enterocytes in the small intestines facilitate absorption of dietary iron (Abboud and Haile, 2000). Consequently, hepcidin stimulates (i) iron confiscation in hepatocytes and macrophages (ii) diminishes duodenal iron uptake via enterocytes. People with haemochromatosis, particularly due to alterations in transferrin receptor 2 (TFR2), have a very low hepcidin concentration causing slight to no iron sequestrations in macrophages (Ganz and Nemeth, 2011). Lower macrophage iron levels in haemochromatosis could clarify their qualified prevention atherosclerosis regardless of their iron excess status. Hepcidin is suppressed by several conditions such as anaemia, erythropoiesis and hypoxia, (Nicolas et al., 2002). Hepcidin activated by an iron response mechanism during inflammation and iron accretion (Milward et al., 2005). A similar case is assumed in anaemia of chronic inflammatory disease when patients have a comparative lack of iron due to it being sequestration in macrophages.

Ratios of cytokines, as well as interleukin-6 (IL-6), which encourages hepcidin activation, are noticeably raised in continuing inflammatory conditions (Andrews, 2004, Weiss and Goodnough, 2005). Raised hepcidin has also been connected with the existence of several

metabolic disorders (Martinelli et al., 2012) and arterial inelasticity assessed by ankle-brachial pulsation wave speed (Kuragano et al., 2011).

1.5.2 Iron and atherosclerosis

Electron transmission to and from iron ions is necessary for transport, usage, and storing of iron in tissue. Redox-active iron stimulates the creation of free radicals able to oxidise LDL (Nagy et al., 2010). The core mechanisms include iron-induced oxidative stress is producing free radicals. The redox-active ferrous type of iron (Fe^{2+}) is mainly responsible for making extremely reactive hydroxyl radicals.

Iron compounds, may stimulate cultured endothelial cell apoptosis, prevent proliferation and stimulate monocyte adhesion (Carlini et al., 2006). ($Apoe^{-/-}$) mice show poor remnant lipoprotein removal with consequent increase of cholesteryl ester-enriched in blood, which stimulate the progress of atherosclerotic plaques).

Atherosclerotic lesions in apo E knockout mice contain significant quantities of iron (Rosenfeld et al., 2000). (A low iron diet diminishes the iron content and the area of the plaques and increases plaque stability. In humans, there is a strong positive relationship between iron type and protein oxidation in the atherosclerotic lesion (Carlini et al., 2006). There are several potential mechanisms by which iron can promote atherosclerosis. Iron can initiate lipid peroxidation by interacting with hydrogen peroxide to create highly reactive $OH^{\bullet}$ according to the Fenton reaction. The ferric iron produced can be converted back to ferrous iron by reaction with superoxide.

In addition to this, iron (de Whalley et al., 1990), ferryl haemoglobin (Paganga et al., 1992) and ferryl myoglobin (Dee et al., 1991) have been documented to catalyse LDL oxidation in vitro. Iron is essential to manufactures enzymes, such as 5-lipoxygenase, that might have a role in atherosclerosis. Iron may also initiate endothelial dysfunction (Sullivan, 2006). Another mechanism by which iron participates in the atherosclerosis process is by catalysing the intralysosomal oxidation of LDL (Wen and Leake, 2007).

1.5.3 Studies of stored iron level and CVD effects

Epidemiologic studies in humans have explored the link between iron status and cardiovascular disease (CVD) (Institute of Medicine Committee on the Prevention et al., 1993, Tuomainen et al., 1998).

Serum ferritin concentrations > 200 μg/l were linked with a 2.2-fold rise in the occurrence of acute myocardial infarction with concentrations < 200 μg/l throughout three years (MacDonald, 1993). Conversely, other studies did not support these findings (Sempos et al., 2000, Ekblom et al., 2011). One reasonable explanation is that raised serum ferritin concentrations do not reflect augmented iron body stores, but are the consequence of infections and other inflammatory developments related to coronary heart disease (Alexander, 1994, Lipschitz et al., 1992). However, more recently, Tuomainen et al (Tuomainen et al., 1998) used the ratio of serum transferrin receptors (as a marker of iron depletion) to serum ferritin and observed a 2.9 fold increase in the incidence of acute myocardial infarction among Finnish men in the lowest tertiles compared to the highest, even after adjusting for inflammation and alcohol intake. Another possibility is that iron stores may relate to established CVD risk factors, such as LDL cholesterol, in increasing the

risk of CVD (de Valk and Marx, 1999). On the other hand, if increased iron stores are causally related to increased CVD risk, a reduction in iron consumption would possibly be a protection factor for CVD. Iron stocks are related to CVD risk factors in women during reproductive age (Ramakrishnan et al., 2002).Carotid atherosclerosis was positively related to serum ferritin in healthy men at 45-65 years and women who were without subclinical inflammation, independently of traditional CVD risk factors (Zacharski et al., 2000).More investigational trials that consider subclinical inflammation, evaluated using numerous markers, such as α_1-acid glycoprotein, are required to discover the pathway of the observed association and to better recognise the fundamental mechanisms (Ahluwalia et al., 2010). The novel proposal of the iron theory did not postulate a mechanism. Numerous mechanisms by which iron reduction might protect from atherosclerosis. Many researchers did not record the important variance in plasma iron levels in patients suffering from diverse kinds of (CHD) equated to healthy controls (Paramonov et al., 2005). There were significantly higher levels of serum iron in patients suffering haemorrhagic plaques in their carotid arteries compared to people in the control group (Tasic et al., 2015). The data of numerous human research strongly recommend a link between serum iron levels and atherosclerosis progress. Based on the epidemiological data, iron levels, observed by measuring serum ferritin levels and transferrin saturation, positively connected with an augmented risk factor of myocardial infarction (Holay et al., 2012, Rajapurkar et al., 2012).

Since atherosclerotic lesion contain redox-active iron, which might stimulate free radical development and then lipid peroxidation proposed important of iron in atherosclerosis (Smith et al., 1992). Recently statistics demonstration that iron may have a participate in

plaque weakness and participate in plaque haemorrhage (Mascitelli and Goldstein, 2014, Michel et al., 2012).

Peroxidation can be augmented by the existence of oxidant factors, such as iron or copper ions (Stadler et al., 2004). Since phagocytosed erythrocytes in macrophages have been recognised in plaque near microvessels, explaining the previous haemorrhage inside the plaque as well as oxidised LDL inside the macrophages (Kopriva et al., 2015).

1.5.4 Mechanisms of iron toxicity in atherosclerosis

Iron compounds, may stimulate cultured endothelial cell apoptosis, prevent proliferation and stimulate monocyte adhesion (Carlini et al., 2006). Atherosclerotic lesions in apo E knockout mice contain significant quantities of iron (Rosenfeld et al., 2000). A low iron diet diminishes the iron amount and the area of the plaques and increases plaque stability. In humans, there is a significant positive relationship between iron status and protein oxidation in the atherosclerotic lesion (Carlini et al., 2006). These are several potential mechanisms by which iron can promote atherosclerosis. Iron can start lipids peroxidation by interacting with hydrogen peroxide to create highly reactive $OH^{\bullet}$ according to the Fenton reaction. The ferric iron is produced can be converted back to ferrous iron by reaction with superoxide.In addition to this, iron (de Whalley et al., 1990), ferryl haemoglobin (Paganga et al., 1992) and ferryl myoglobin (Dee et al., 1991) have been documented to catalyse LDL oxidation in vitro. Iron is essential to produce enzymes, such as 5-lipoxygenase, that might have a role in atherosclerosis. Iron may also initiate endothelial dysfunction (Sullivan, 2006). Another mechanism by which iron participates in the atherosclerosis process is by catalysing the intralysosomal oxidation of LDL (Wen and

Leake, 2007). Furthermore, free radicals can be made *in vitro* under the oxidative condition with existence of ascorbic acid and iron ions. There is another essential, although not commonly documented, an element known as free iron existent in blood named the labile iron pool (Kruszewski, 2004). It was proposed that iron treatment potently encourages the creation of hydroxyl radicals consequently contributing to atherosclerosis (Sengoelge et al., 2005, Feres and Reis, 2005).

1.5.5 Copper concentration in atherosclerotic plaque

Numerous studies have shown that copper is effective in catalysing LDL oxidation, however, the exact mechanism is not yet understood (Esterbauer et al., 1992) (Burkitt, 2001, Horsley et al., 2007).

The copper-containing protein caeruloplasmin can oxidise LDL, especially at low pH (Lamb and Leake, 1994). Nutritional copper supplementation reduces atherosclerosis, however, in rabbit with highly fat-fed (Lamb et al., 1999). Copper might, however, have both pro- and anti-atherogenic influence (Rajendran et al., 2007).

Some investigational studies indicated a relationship between a lack of copper with high cholesterol concentrations and atherosclerosis in coronary arteries (Hamilton et al., 2000). Copper ions can transform superoxide and hydrogen peroxide into the extremely harmful hydroxyl radical that be able to injure the endothelium (Alissa et al., 2006). Lipid peroxidation may occur by copper ion, consequently, alteration of the apoB-100 protein, producing lipid peroxidation products, such as 4-hydroxynonenal (4-HNE), acrolein, and malondialdehyde (MDA). Existence of these oxidised materials on apoB-100 has been serologically demonstrated in atherosclerotic lesions (Uchida et al., 1995, Rosenfeld et al., 1990).

The low concentration of free metal ions, copper ion, insufficient amounts of reducing factor and proteins found in the plasma may protect LDL from oxidation (Uchida et al., 1995). Nevertheless, the mechanism of copper ion-induced LDL oxidation happens in physiological environments remain uncertain (Itabe, 2003).

The initial stage in the copper-stimulated oxidation of LDL thought to be the binding of Cu (II) to several Cu (II)-binding positions on the surface of the ApoB-100. The particular number of these positions is not agreed, but values extending from 3 -40 per apoB-100 molecule have been measured (Roland et al., 2001).

Once bound, Cu (II) can be reduced to Cu (I) by several possible reductants, including amino acid residues (Giessauf et al., 1995) protein and lipid peroxides (Patel et al., 1997) and α-tocopherol (Lynch and Frei, 1993, Schnitzer et al., 1997). Monitoring metal ion level atherosclerotic plaque occurred in many methods. (*Ex vivo* electron paramagnetic resonance (EPR) spectroscopy, together with inductively coupled plasma mass spectroscopy (ICPMS)), in healthy and atherosclerotic carotid artery tissue (Stadler et al., 2004). Iron and copper levels were increased in diseased arteries.

1.6 Antioxidants

Based on the biological perspective, antioxidants described as materials that the level less than that of the oxidisable substrate are proficient at suspending or stopping oxidative reaction (Halliwell, 2012). The main methods to keep biological molecules from oxidation because they become oxidised themselves. The constancy of oxidised antioxidant

molecules is important, so to avoid oxidised antioxidants reacting with other biomolecules (Bors et al., 1990).

1.6.1 Mechanisms of antioxidant protection in the body

All cells in the body are vulnerable to attack by ROS and oxidative if the pro-oxidant-antioxidant equilibrium is troubled because of extreme oxidative factor or insufficient antioxidant protection. The equilibrium between oxidative factor and antioxidant protection is a dynamic circumstance for healthy tissues. Antioxidant mechanisms act as scavenging oxidizing factors, using enzymes or biochemical reactions, diminishing the creation of a single atom of oxygen, chelating necessary transition metal ions required to change weakly reactive species like H_2O_2 or $O_2^{\cdot-}$ into highly reactive ones, and replacing damaged target molecules (Zhang et al., 2014).

Antioxidants can either be small molecules, like proteins and enzymes. The functional antioxidant programs have numerous positions of defence. This antioxidant system starting with, antioxidants inhibit the creation of ROS/RNS, by isolating active redox metal ions and reducing ROOH and H_2O_2 to OH^- and H_2O, correspondingly. In the next line of protection, antioxidants scavenge ROS/RNS before they damage biological particles. In the other protection position, an antioxidant may treat the damage and rebuild injured tissues. Moreover, an insufficient amount of oxidative stress may persuade an effect, which intensifications the creation of antioxidant, consequently transfers them to the correct location at the correct phase and in the accurate amounts (Higdon et al., 2012).

Therefore, antioxidants role supportively and synergistically inside the body to manage oxidative stress and antioxidant balance. Oxidative risk is produced by free and non-free

radical pathway. Both types of oxidants affect and change biological substances, interference of thiol redox roles caused by ROS/RNS (Jones, 2008).

Numerous types of free radicals are implicated *in vivo*, for example, (superoxide ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$), alkoxyl ($RO^{\bullet}$), peroxyl ($RO_2^{\bullet}$), aryloxy ($ArO^{\bullet}$), nitric oxide ($^{\bullet}NO$), nitrogen dioxide ($NO_2^{\bullet}$), thiyl ($RS^{\bullet}$), thiyl peroxyl ($RSOO^{\bullet}$), sulfonyl ($RSO_2OO^{\bullet}$), and carbon-centred radicals ($R^{\bullet}$)). Few radicals are moderately unstable, but some are not. Antioxidants (IH) react with free radicals $X^{\bullet}$. Theoretically, the reactions are explained by different equations based on radicals, antioxidants, and microenvironment.

Equation (a)

Hydrogen abstraction, $X^{\bullet}+IH \rightarrow XH+I^{\bullet}$

Equation (b)

Addition, $X^{\bullet}+C{=}C \rightarrow X{-}C{-}C^{\bullet}$

Equation (c)

Electron transfer, $X^{\bullet}+IH \rightarrow X^{-}+IH^{\bullet +} \rightarrow X^{-}+I^{\bullet}+H^{+}$.

For example (Vitamin E, vitamin C,). Vitamin E may have another role in a signalling mediator, which is independent of the antioxidant role, which is a subject of discussion (Traber and Atkinson, 2007, Azzi, 2007). α-tocopherol has been labelled as a radical-chain breaker which, because of its hydrophobic nature, functions in a lipid environment in membranes and lipoproteins (Azzi, 2007).

1.6.2 EDTA

EDTA (ethylenediaminetetraacetic acid) is a member of an amino polycarboxylic family of ligands. It acts as a hex dentate ("6 toothed") ligand (Figure 1.7) and binds metal ions such as Ca^{2+} and Fe^{3+}.

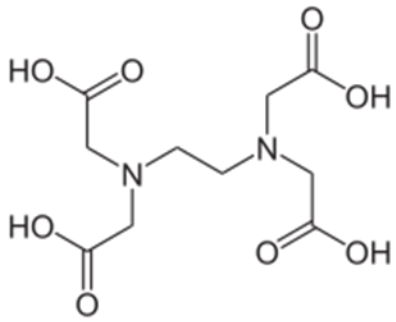
EDTA has a high affinity for transition metals (Xing et al., 1998). During the treatment of (CHD), EDTA provided together with the vitamins intravenously (Ernst, 2000). Nevertheless, global studies have documented that this therapy is unsuccessful as medicine for coronary heart disease, and in many conditions, it has shown unfavourable effects (Ibad et al., 2016).

The main role of EDTA in this situation is to try to decrease the blockages in blood vessels by binding with the metals from the blood or decalcify artery plaques (Bolick and Blankenhorn, 1961). EDTA cannot easily enter mammalian cells (Krishnamurti et al., 1980). EDTA may decrease extracellular metals activity.

EDTA will diminish metal uptake by cells and therefore will inhibit the level of metal intracellularly Metals such as cadmium and lead, which are chelated by EDTA are linked with an augmented risk of (CVD) (Ouyang et al., 2015). The combination EDTA with existing propofol demonstrations no remarkable outcomes on calcium and magnesium contours, haemodynamic indicator in interoperative anaesthesia (Wahr et al., 2000). Researchers demonstrated in the tissue culture media, LDL oxidation by copper or cells is augmented if a low concentration of EDTA is carried over during LDL isolation (Lamb and

Leake, 1992). Low concentration 1-3 µM EDTA can raise LDL oxidation rate by Cu^{2+}, in Ham's F-10 medium, while high concentration 10 µM show inhibition of LDL oxidation by macrophages.

A



B

Figure 1.7 A&B. The diagram shows the EDTA structure, which contains 2 nitrogen atoms with unpaired electrons, and the hydrogen atoms may be removed to give 4 more groups of lone electrons. Figure 7B.The diagram shows that metal ions compete with protons for binding to EDTA. Because metal ions are widely enclosed by EDTA, their oxidised characteristic is often inhibited. Finally, since complexes of $EDTA^{4-}$ are ionic, they tend to be highly soluble in water (Solans et al., 1984).

1.6.3 Vitamin E (α -tocopherol)

Micronutrients can serve as antioxidants, such as vitamin C and, α- tocopherol (Jialal and Fuller, 1993). α-Tocopherol characterises the most biologically powerful member of the vitamin E family in mammals (Machlin and Bendich, 1987). α-Tocopherols are composed

of two rings with a hydrocarbon chain (Figure 8). Natural vitamin E has different types such as α, β, γ, and δ depending on the methyl groups, which are bound to the benzene ring (Brigelius-Flohe and Traber, 1999). Synthetic α -tocopherol consists of eight isomers. RRR-α-tocopherol is believed to be the most important form of vitamin E since it is digested and then absorbed better than other types from the diet by the intestine. The (α-tocopherol transfer protein) is capable for the intracellular transportation of vitamin E. Furthermore, detection of α-tocopherol specific membrane receptors and cytosolic transfer proteins bolster the concept that vitamin E has a role beyond a basic antioxidant function (Engin, 2009) (Sies and Murphy, 1991). Vitamin E is found mainly in vegetable oil and has a role to protect tissues from damage caused by oxidation. It is absorbed by the small intestine and α-tocopherol is retained and dispersed between lipids in cell membranes and plasma lipoproteins. α-Tocopherol captures free radicals during chain reactions. The hydrogen atom from the phenolic hydroxyl group of α-tocopherol is given to the free radical, leading to a more stable free radical in the form of the from vitamin E radical (Sies and Murphy, 1991). Vitamin E is a strong radical-scavenging antioxidant, but there is a limitation in his effectiveness with non-radical oxidants group, including cyclooxygenase, lipoxygenase, and hypochlorite (Niki, 2014). Researchers have shown that α-tocopherol diminished the activity of CD36, decreasing macrophages uptake of oxidised LDL and consequently reduces foam cell formation (Mathur et al., 2015). Vitamin E has been assessed as an antiatherogenic factor by reducing LDL oxidation, preventing the formation of macrophage foam cells and atheroma and inhibiting the immune and inflammatory response (Wallert et al., 2014).

α - Tocopherol appears antioxidant properties under high oxidative circumstances but can show pro-oxidant influences under minor oxidative circumstances (Bowry et al., 1992). The major mechanism by which it acts as an antioxidant is by the scavenger of one radical ($R^{\bullet}$) by a molecule of α-tocopherol (TocOH) (reaction 1), and then inhibition of a next radical by α- tocopheroxyl radical, (reaction 2), causing inhibition of 2 radicals by 1 molecule of α -tocopherol.

TocOH + R• = TocO• + RH (reaction1)

TocO• + R• = non-radical product (reaction 2)

The prooxidant mechansim is as follows

α-toc OH + Mn^{2+} (or (Mn^{3+}) ⟶ α-toc $O^{\bullet}$ + H^{+} + (Mn^{+} or Mn^{2+})

α-toc $O^{\bullet}$ + LH ⟶ α-toc OH + $L^{\bullet}$

$L^{\bullet}$ + O_2 ⟶ $LOO^{\bullet}$

where M^{2+} or (M^{3+}) is a metal ion and LH is a lipid.

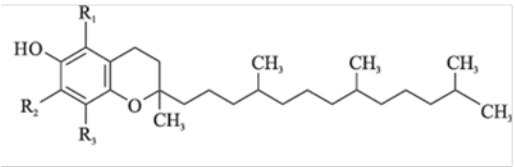
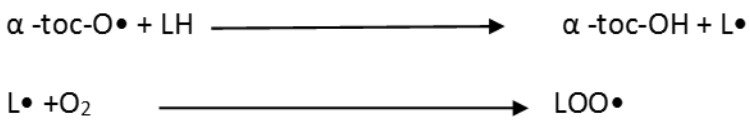


Figure 1.8. An illustration shows the chemical structure of vitamin E family. The difference between tocopherols and tocotrienols, the two subcategories of the vitamin E family of compounds, is that tocopherols have a saturated, while tocotrienols have an unsaturated, phytyl tail connected at the 1-position of a chromane ring structure. Different isoforms within each subgroup differ from each other based on the chromane ring methyl groups (Kamal-Eldin and Appelqvist, 1996, Sylvester and Shah, 2005).

α-Tocopherol can demonstrate as a pro-oxidant agent under low oxidative stress conditions since the α-tocopherol radicals capable remove a hydrogen atom from a PUFA moiety (Bowry, 1992).



vitamin E is present in the intestine as a component of micelles composed of dietary lipids. Micelles are hypothetically important for absorption by the brush border of the enterocyte. Certainly, varied micelles are solubilizing hydrophobic particles and spreading into the (glycocalix) to reach enterocyte (Reboul, 2017). α-Tocopherol is then absorbed into chylomicrons (Sokol et al., 1983). Once it spreads into the bloodstream, absorbed vitamin E is transforming to new lipoproteins under the effect of phospholipid transfer protein (PLTP) and to cells by the action of PLTP and lipoprotein lipase (LPL) (Kostner et al., 1995). The liver also uptake and re-spread vitamin E when chylomicron remnants are taken up and released back to the circulation as a form of low density lipoproteins (VLDL) (Havel, 1994). In liver α-tocopherol bind with a specific protein, the α-tocopherol-transfer protein (α-TTP) (Hosomi et al., 1997), and convert α-tocopherol to several lipoproteins (Brigelius-Flohe and Traber, 1999).

The cytochrome P450 enzymes regulate vitamin E level. γ-Tocopherol absorbed and secret into the bile, while α-Tocopherol release in the bile via the multidrug resistance (MDR) family of carriers. The MDR family contains several members, the most vital being those of the ATP-binding cassette, (Mustacich et al., 2007, Tang et al., 2013).

1.6.3.1 Biological Functions of Vitamin E

statistics provided the fundamental role of vitamin E in health and contributed a good knowledge about vitamin E from different aspects such as biological roles and metabolic outcomes. The finding of α-TTP, which selectively recognise and join to RRR-α-tocopherol, this indicates the importance of this vitamin also emphasises its dominance biologically compared to another isomer. Because of α-tocopherol renewal system as the antioxidant system is mostly responsible for the deficiency in humans and the infrequent diminishing its level in the adult human. Majority of extra tocopherols digestive is expelled in the faeces unaffected, while it's expelled to urine with the side chain condensed with the chromane ring unbroken.

Vitamin E can protection against mitochondrial superoxide generation and may act as a biological modifier independently of its antioxidant properties. By reducing the production of ROS/NOS, vitamin E protects against oxidative damage and also regulates important cellular events (Chow, 2004). Supplementation Vitamin E may increase the threat of haemorrhage in humans (Pastori et al., 2013).

1.6.3.2 Regulation of Cell Survival, Proliferation and Apoptosis by Vitamin E

The first predictable non-antioxidant activities of vitamin E was an inhibitory influence on protein kinase C (PKC) (Mahoney and Azzi, 1988). Vitamin E effects to trigger phosphoserine/threonine phosphatase 2A (PP2A), regulating the dephosphorylation of PKC, on the plasma membrane (Boudreau et al., 2002). The greatest noticeable outcome of vitamin E caused by block PKC pathway, decreasing of cell multiplication. This has been presented to take place in several cells (Zingg, 2007). The disruption in the proliferation of vascular smooth muscle cells (VSMC), leading disruption atherosclerosis mechanism (Boscoboinik et al., 1991, Tasinato et al., 1995). The mitogen-activated protein kinase (MAPK) pathway also blocked by the effect of vitamin E.

In VSMC activated when exposed to certain concentration from oxidized LDL, MAPK function and increased cell survival can be reduced under the effect of vitamin E (de Nigris et al., 2000). Protein kinase B pathway also inhibited by vitamin E and stimulated protein tyrosine phosphatase and, increased cell propagation and survival (Zingg, 2007)

1.6.3.3 Improvement of Endothelial Function by VitaminE

Vitamin E has a role to improve numerous characteristics of the endothelium, like antithrombotic role vasodilatation. In macrophages, vitamin E has a blocking impact on arachidonic acid (AA), while causing an intensification in arachidonic acid by endothelial cells. This influence accompanied by inhibition cyclooxygenase (COX) 1 and 2 pathway, another influence is an intensification in the formation of vasodilating prostanoids (PGE2

and PGI2) (Wu et al., 2004). Vasodilatation by phosphorylation endothelial nitric oxide synthase (eNOS) on serine 1177 (Heller et al., 2004a, Heller et al., 2004b),

These outcomes translate to augmented amounts of NO metabolites after vitamin E treatment (Desideri et al., 2002b). Controversially, the influence of vitamin E on vasodilation remains uncertain, as many researchers have documented an improvement in vasodilation after exposure to vitamin E, by a mechanism increasing the phosphorylation of eNOS and inhibition production of O_2^- (Green et al., 1998, Heitzer et al., 1999) but other studies have not shown this (McSorley et al., 2005, McDowell et al., 1994).

1.6.3.4 Regulation of Inflammtory Process by Vitamin E

Vitamin E role documented to prevent numerous inflammatory events that occur during the process of atherosclerosis. The main role Vitamin E inhibition inflammatory chemokines formation by endothelial cells and leukocytes *in vitro* (Wu et al., 1999) (Yoshikawa et al., 1998) and in vivo (Koga et al., 2004). Supplementation of vitamin E with a high dosage, reporting a reduction in adhesion molecules (Desideri et al., 2002b, Desideri et al., 2002a, van Dam et al., 2003).

Moreover, vitamin E prevented the secretion of tumour necrosis factor-α (TNF-α) (Devaraj and Jialal, 2005) and interleukin-1β (IL-1β) (Devaraj and Jialal, 1999, Devaraj et al., 1996). CD36, which recognised oxidized LDL and internalization by macrophages, is inhibited by vitamin E (Munteanu et al., 2004, Munteanu et al., 2006). Lastly, vitamin E suppresses the

function of inducible NOS and NADPH oxidase, thus preventing the macrophage rupture (Khanduja et al., 2005, Cachia et al., 1998).

1.6.3.5 Antioxidant actions by Vitamin E

α -Tocopherol is categorised to be an antioxidant because of its capability to trapped lipid radicals and break the oxidation chain. It can end radical chain reactions mediated by the $LOO^{\bullet}$, inhibiting creating another radical, which continuing the sequences reaction by oxidizing other lipid particles. This process depends on the degree of the reaction between L and $LOO^{\bullet}$, around 1,000-fold less than the degree of the reaction between α-tocopherol and $LOO^{\bullet}$ (10^2 $M^{-1}S^{-1}$) compared to (10^5-10^6 $M^{-1}S^{-1}$). It is questionable that vitamin E may affect the radical sequences response in aqueous phases.

The oxidation chain reaction is generally started with water-soluble particles, someplace vitamin E unavailable because of its lipophilic behaviour. Its reaction with $L^{\bullet}$ is improbable since the degree of the response between $L^{\bullet}$ and O_2 is 100-1,000-fold more equated to that of $L^{\bullet}$and vitamin E. After oxidation of vitamin E, the regeneration process may occur in the presence of vitamin C, resulting in inhibition of vitamin E radicals and prevention lipid peroxidation (Brigelius-Flohe, 2009).

1.6.4 Tempol

Tempol is a low-molecular-weight molecule (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) (Figure 1.9), also identified as 4-hydroxy-TEMPO. Tempol is a radical scavenger which has SOD and catalase actions. Furthermore, tempol inhibition endothelial dysfunction in

diabetic rats and diminishing expansion regional myocardial ischaemia/reperfusion (McDonald et al., 1999).

Tempol is a synthetic component with SOD-like activities (Mehta et al., 2004). It increases the conversion of the superoxide radical into oxygen and hydrogen peroxide.

The tempol free radical is utilized as an activator and a chemical oxidant because it is a stable radical (Rak et al., 2000). It detoxifies ROS and stimulates the disproportionation of superoxide and helps hydrogen peroxide metabolism. Further, it is useful as a free radical scavenger. Oxygen molecules with single electrons are reactive and can cause damage by chemically reacting with cell constituent and leading to destabilization. Clinical data shows that oxidative factors might influence the progress of hypertension. Hypertension is further involved in the progress of cardiovascular diseases, obesity and stroke. Tempol inhibits the production of free radicals by oxidation of iron, thus ending oxidative stress (Januschowski et al., 2014). Being highly cell-permeable (Mitchell et al., 1990b), it saves cells from injury prompted by hypoxanthine/xanthine oxidase and H_2O_2, while it shows no catalase activity.

Tempol also disruption Fenton reaction and formation $OH^{\bullet}$. Whether by SOD-like activity or capture of an electron from metal ions, protecting from oxidative stress (Mitchell et al., 1990b). Experimental evidence shows *in vivo* neuroprotective properties of tempol (Lipman et al., 2006). Tempol decreases the pro-oxidant impact due to fructose overload, reduces lipid peroxidation and decreases NADPH oxidase system by blocking the of p47phox activity in the vasculature (Cannizzo et al., 2014).

Tempol suppressed the appearance of VCAM-1 of in the aorta and diminished the progress of atherosclerosis (Joo et al., 2015). Studies show that tempol decreases oxidative effect by inhibiting the precise assemblage of the Nox2 oxidase in the artery and is capable

of diminishing atherosclerosis. Therefore, tempol is a possible therapeutic drug for treating risk factors related to metabolic disorder (Cannizzo et al., 2014).

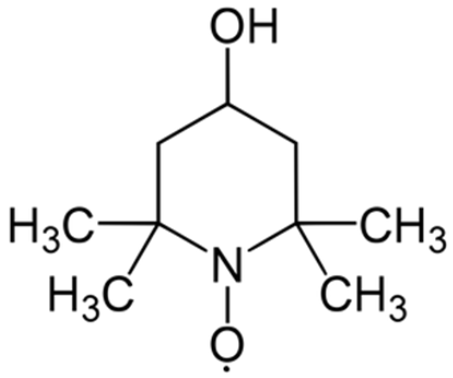


Figure1.9 (4-Hydroxy-Tempol) is a 4-substituted 2,2,6,6-tetramethylpiperidine-1-oxy (TEMPO) derivative. It is a low-molecular-weight compound and has been recommended as a superoxide dismutase mimic (Sledzinski et al., 1995).

1.6.5 Probucol

Probucol is a clinically used cholesterol-lowering and antioxidant drug (Heel et al., 1978). It has been used as a pharmaceutical agent to treat a diversity of infections, traumas, and pathologic disorders, including lowering plasma cholesterol levels in hypercholesterolaemia patients (Zimetbaum 1990). Probucol is lipophilic antioxidant, bis-tertiary butyl-phenol capable of basically providing one of the hydrogens from its phenolic groups and (Figure 1.10). Throughout lipid oxidation process, ($L^{\bullet}$), ($LO^{\bullet}$) or ($LOO^{\bullet}$) simply taken an H atom from probucol, preventing continuous sequences of lipid peroxidation (Jackson et al., 1991). Then, LDL oxidation by these radicals probucol competent to fully inhibit this oxidation (Maiolino et al., 2013). Both *in vitro* and *in vivo*, probucol showed an inhibitory impact on LDL oxidation. Probucol showed diminishing restenosis in patients, nevertheless, probucol impact on atherosclerosis have been inconstant. It did not decrease femoral atherosclerosis in patients (Wallduis et al., 1994).

Probucol oxidation produces toxic material, including spiroquinone and a *bis*-quinone , which involved in initiating QTC prolongation and fatal arrhythmia (Steinberg, 2007). Probucol was permitted in the united state in the 1980s, but in 1995 probucol withdrawn from the US market (Bourne et al., 2007). Several hypotheses have been offered to explain the variability of antioxidant results.

It was proposed that the antioxidant effect was associated with long-term accumulation of this compound that could be essential for its relationship to other antioxidants and leads the maximum benefit. We suggest that the peroxidation process is transmitted within lipoprotein

particles by the reaction of the alpha-tocopheroxyl radical with PUFA moieties in the lipid after α-tocopherol enrichment. This lipid peroxidation pathway can be explained by the recognised chemistry of the α -tocopheroxyl radical and might explain how reagents, such as, probucol which inhibit the α -tocopheroxyl radical, ending lipid peroxidation in LDL enriched with α -tocopherol.

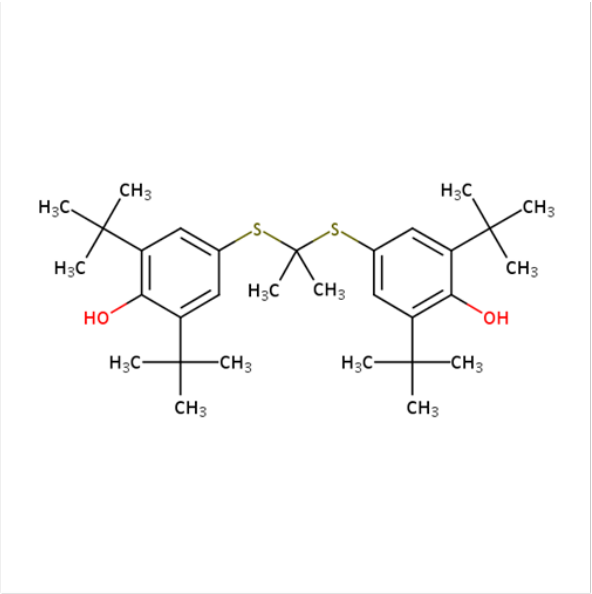


Figure 1.10 Probucol is a bis-phenol antioxidant with antilipidemic activity. Probucol inhibits oxidation of LDL and lowers the level of cholesterol in the bloodstream by increasing the rate of LDL catabolism (Yamashita and Matsuzawa, 2009).

1.7 Thesis aims and hypothesis

Atherosclerosis, a prolonged inflammatory disease of the arterial wall, is the main reason for morbidity and mortality from cardiovascular disease (CVD). There is onsiderable evidence that oxidation of LDL is involved in the pathogenesis of atherogenesis. The large clinical trials of antioxidants (mainly α-tocopherol) have, however, shown no protection against cardiovascular diseaeses. Almost all the studies of LDL oxidation have assumed that LDL oxidation occurs in the extracellular space of atherosclerotic lesions. It has been shown, however, that LDL can be oxidised in the lysosomes of macrophages and that this depends on iron and the acidic pH of lysosomes (Wen and Leake , 2007). The main hypothesis tested in this thesis is that antioxidants do not function as effectively in protecting LDL from oxidation at lysosomal pH as they do at pH 7.4. This hypothesis was investigated by enrichment of LDL with α-tocopherol and oxidising it by different concentrations of copper and ferrous and ferric ion at pH 4.5 and 7.4. Conjugated dienes were measured by an automatic spectrophotometer and oxysterol by using HPLC.

Another aim was to compare the effects of other antioxidants (probucol and tempol) to these α-tocopherol at lysosomal pH.

Another hypothesis was that α-tocopherol enrichment of macrophages would protect them from apoptosis due to oxidative stress. This was investigated by detecting the percentage of apoptotic cells, necrotic cells and secondary necrotic cells by flow cytometry. The final hypothesis was that macrophages, containing LDL that they have oxidised in their lysosomes, die and lyse in atherosclerotic lesions exposing other cells to toxic oxidised LDL. We approached this hypothesis by testing if LDL oxidised under lysosomal conditions (by iron at pH 4.5) decreased endothelium-dependent vasodilatation in rat aortic rings.

Chapter 2

Materials and methods

2.1 General laboratory reagents , solutions and equipments

2.1.1 Laboratory reagents

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

Chemicals	**Supplier**
Alpha-tocopherol	Sigma-Aldrich Ltd
Acetylcholine	Sigma-Aldrich Ltd
Amphotericin	Sigma-Aldrich Ltd
Bio Red®DC ™ PROTEIN ASSAY KIT	Bio Red
Blocking kit	Vector Avidin/Biotin
Bovine serum albumin (BSA)	Invitrogen Ltd
CD 31 rat antibody	AbD Serotec
Chelex-100®	Sigma-Aldrich Ltd
eNOS antibody	Cell signalling
ECL	Fisher Scientific
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich Ltd
EGM-2	Clonetics/Lonza
Ethanol	Sigma-Aldrich Ltd
Folin and Ciocalteu phenol reagent	Fisher Scientific
Fetal calf serum (FCS)	Invitrogen Ltd
Ferrous sulphate	Sigma-Aldrich Ltd

Halt ^{Tm} protease & phosphatase inhibitor	Fisher Scientific
Harris haematoxylin	Sigma-Aldrich Ltd
HRP-Goat Anti Rabbit-IgG	Invitrogen Ltd
HRP-Goat Anti-Mouse IgG	Invitrogen Ltd
HEPES sodium salt	Sigma-Aldrich Ltd
Sodium nitroprusside (SNP)	Fisher Scientific
Sphingomyelinase	Sigma-Aldrich Ltd
Sodium Dodecyl Sulphate	Sigma-Aldrich Ltd
Sodium acetate	Sigma-Aldrich Ltd
Penicillin/streptomycin	Invitrogen Ltd
Phosphate buffered saline	Invitrogen Ltd
Phenylephrine	Fisher Scientific
Phorbol 12-myristate 13-acetate (PMA)	Invitrogen Ltd
p-eNos (S1177) Rabbit antibody	Cell Signalling
PVDF Transfer membrane	Fisher Scientific
Magnesium chloride	Sigma-Aldrich Ltd
Dihydroethidium (Hydroethidine)	Fisher Scientific
Dako Fluorescence Mounting Medium	Dako
Dimethyl sulphoxide(DMSO)	Fisher Scientific
Oil Red O	Sigma-Aldrich Ltd
MCDB131	Atlantic &Georgia
RIPA buffer	Sigma-Aldrich Ltd
RPMI 1640 medium	Invitrogen Ltd

1.2.2 Laboratory equipments

Name	**Company**
Optima ™ XPN ultracentrifuge	Beckman
Lambda-2-6-cell spectrophotometer	PerkinElmer
Lambda Bio 40 8-cell spectrophotometer	PerkinElmer
Agilent 1100 HPLC System	Agilent
BD Accuri ™ C6 flow cytometer	BD Biosciences
Axioskop epifluorescent microscope	Carl Zeiss
Countess II FL automated cell counter	Invitrogen
DMT Myography system	DANSH Myo Technology
HPLC series 200	PerkinElmer

1.2.3 General solutions

High-density KBr solution (HDS) 1.316g/ml for LDL oxidation.

1 L of HDS (2.97 M KBr, 2.62 M NaCl and 297 µM EDTA) was prepared by the addition of 354 g KBr, 153g NaCl, 110.5 mg EDTA.Na_2H_2O and made up to 1 litre of purified water and pH adjusted to 7.4 with NaOH solution.

Low density KBr solution (LDS; density 1.006 g/ml) for LDL oxidation.

(150 mM NaCl and 297 µM EDTA), pH adjusted to 7.4 with NaOH solution.

Lowry solution A for protein measurement.

0.2 M Na_2CO_3, 0.1 M NaOH, 5.7 mM sodium tartrate and 35 mM sodium dodecyl sulphate (SDS).

Lowry solution B for protein measurement

0.16 M $CuSO_4$.

Sphingomyelinase LDL stabilising buffer

$MgCl_2$ 100 mM,150 mM NaCl, 10 mM $MgCl_2$ and 5mM HEPES, pH7.4, used for diluting LDL before adding sphingomyelinase.

Phosphate buffer saline (PBS)

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 1M HCl and final volume made up to 1 litre with ultrapure water. PBS keeps an osmotic balance between the internal and external environment of cells. It maintains physiological pH.

LDL Dialysis buffer

140 mM NaCl, 8.1 mM Na_2HPO_4,1.9 mM NaH_2PO_4 and 100 µM EDTA. The pH was adjusted to pH 7.4, by using pH Meter. LDL is dialysed against this buffer at the end of its isolation.

Sodium chloride/sodium acetate buffer for the oxidation of LDL at pH 4.5

NaCl (150 mM, 8.8g/l), sodium acetate (10 mM, 0.82g/l) adjusted at pH 4.5 with HCl solution. Chelex-100 (washed in ultra-pure water) was added at 1g/l and mixed overnight with stirring. The Chelex was removed by paper filtration and the pH was checked. This buffer was used to dilute LDL to measure conjugated diene formation by a spectrophotometer

Lipid hydroperoxide assay colour reagen

KH_2PO_4 (163 mM), KI (120 mM), K_2HPO_4 (37 mM), Triton X-100 (2 g/l), NaN_3 (150 µM), pH adjusted to 6.0.

MOPS buffer (Chelex -100 treated)

3-[N-morpholino] propane sulfonic 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.

HPLC oxidation mobile phase

Acetonitrile, isopropanol and water (all HPLC grade):40/54/2% by volume. The mobile phase in reverse-phase chromatography has to have the correct polarity and it has to give a reasonable competition for the absorption sites for the analytic molecules.

Oil Red O Stain

Stock solution: Oil Red O (0.35 g) dissolved in isopropanol (100 ml), filtered and stored at room temperature. The working stain was freshly prepared by mixing Oil Red O Stock with ultrapure water 60/40, followed by filtering. It was used to detect intracellular lipid and ceroid

FACS buffer

Hanks balanced salt solution HBSS with 0.6% BSA, 5 mM EDTA for apoptosis detection by using a flow cytometer.

Cell fixing solution (4% (w/v) paraformaldehyde solutions)

4g paraformaldehyde was placed in a 200 ml flask that already contained 60 ml PBS. The combination was warmed to 60 ^{o}C in a fume hood and dissolved with a magnetic stirrer. NaOH (0.5 M) was added dropwise until a clear solution was made. The solution was set to cool and the pH was adjusted using NaOH to pH 7.4. The volume was made up to 100 ml with PBS.

2.2. LDL

2.2.1 Isolation of LDL

LDL (d=1.019 to 1.063g/ml) was isolated by sequential ultracentrifugation of plasma. Venous blood was collected from healthy adult volunteers after checking their haemoglobin level was normal who had fasted for 12 hours before blood donation (Havel et al., 1955, Wilkins and Leake, 1994b). Ethical permission had been given by the University of Reading Ethics Committee. The blood was collected into 50 ml syringes that contained 1 ml of Na_2EDTA (0.15 M) as an anticoagulant and to inhibit LDL oxidation and then transferred to 50 ml centrifuge tubes. The blood was centrifuged at 1500xg for 30 min at 4 ^{o}C and the plasma collected and pooled. High-density KBr solution (HDS, 1.316 g/ml) was added to the collected plasma to adjust its density to approximately 1.019 g/ml, using equation 1 to calculate the volume of HDS to add.

Equation 1: $V_{HDS} = V_{Plasma} (D_{req-} D_{Curr} / D_{Add-} D_{Req})$

Where V_{HDS} is the volume of HDS that was added to the plasma, D_{Add} is the density of the high-density KBr solution, V_{Plasma} is the volume of added plasma, D_{Curr} is the current density of plasma (1.006 g/ml) and D_{Req} is the final required density. The plasma was added to the solution, it was dialysed against 2 litres of 1.019 g/ml density solution for 2 hours in the cold room (4 ^{o}C) to adjust the density to exactly 1.019 g/ml. The plasma was pooled to 35ml Ultra crimp ultracentrifuge tubes (ThermoFisher Scientific Inc., USA) and was centrifuged at 40,000 rpm for 18 hours at (4 ^{o}C). The ultra-crimp tubes were then sliced through the clear band solution in their centre, using a tube slicer. The top part containing VLDL and IDL was disposed of and the lower part containing LDL, HDL and the 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 adding 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 1.063g/ml solution at 4°C for 2 hours. The plasma was then transferred to 35 ml Ultracrimp tubes and centrifuged at 40,000 rpm (145,250 g_{av})for 18 hours at 4°C. The tubes were then sliced below the upper LDL layer, using a tube slicer. There was another centrifugation at 1.063 g/ml (wash spin). The LDL was then dialysed against NaCl/phosphate/EDTA buffer at (4 °C) with multiple changes overnight with stirring to remove the KBr. Finally, the LDL was filter sterilised with a Minisart filter 0.45 µm and stored at 4°C in the dark (Wilkins and Leake, 1994a)

2.2.2 Measuring LDL protein concentration

LDL protein concentration was assessed using a modified Lowry assay (Schacterle and Pollack, 1973). Standard concentrations of bovine serum albumin (BSA) were prepared from a 400 µg/ml stock with purified water. LDL samples (125 µl plus 375 µl water) were made in triplicate. Lowry A reagent (1.5 ml) was added to each assay tube and incubated for 10 min at room temperature. Freshly made Lowry B reagent (150µl) was added to each assay tube and incubated in a water bath at 55 °C for 5 min. The tubes were allowed to cool at room temperature and the absorbance was measured at 650 nm. The principle of the assay is that peptide bonds in the LDL apolipoprotein B-100 or in the BSA standard from a complex with copper ions in the Lowry (A reagent). The complex then reduces the phosphomolybdic-phosphotungstic Folin reagent (Lowry B), producing a blue chromophore which absorbs light at 650 nm.

2.2.3 Aggregation of LDL with sphingomyelinase

The subendothelial aggregation and retention of LDL are important events in atherogenesis. Incubation of LDL with bacterial sphingomyelinase (SMase) in vitro results in the formation of LDL aggregates similar to those that develop in the extracellular matrix and cause macrophage foam cell formation (Schissel et al., 1998). Native LDL was diluted to 2 mg protein/ml with a buffer called stabilizing buffer, which contains NaCl (150mM), $MgCl_2$ (10mM) and HEPES (5 mM), pH7.4. Sphingomyelinase from *Bacillus cereus* (Sigma-S9396-25N) was added at a final concentration of 10mU/ml and the sample was then incubated in a water bath maintained at 37 ^{o}C to aggregated LDL, as described by Walters and Wrenn (Walters and Wrenn, 2008). Aggregation was measured by light scattering at 680 nm in a spectrophotometer (Khoo et al., 1988). Sphingomyelinase aggregated-LDL (SMase-LDL) was 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 a 0.45 μm. Minisart filter before use.

2.2.4 Enrichment of LDL with α-tocopherol

LDL α-tocopherol content was enriched, using the method previously described by Esterbauer et al. (Esterbauer et al., 1991b). Blood was obtained from a healthy volunteer and drawn into 50 ml sterile syringes (containing 3 mM Na_2EDTA) as an anticoagulant. Immediately the blood

was transferred to 50ml centrifuge tubes. The blood was centrifuged at 1500xg for 30 minutes at (4 ^{o}C) and the plasma was collected and pooled. The plasma was incubated at 37 ^{o}C with 1% (v/v of DMSO containing 100 mM (+)α-tocopherol for 3 hours (final concentration 1mM) or with DMSO (1% of plasma volume) alone as control. After the incubation period finished, the LDL was isolated, as described above. α-Tocopherol in the isolated LDL was measuered by the method described by Ramirez-Tortosa (Ramirez-Tortosa et al., 1999). Lipids were extracted from LDL samples (250 µl) using methanol and hexane. Methanol (1 ml) was added to each LDL sample and they were vortexed for 10 seconds. Hexane (3 ml) was then added and vortexed for 30 seconds in order to transfer α- tocopherol of LDL to the hexane. Samples were centrifuged 1500xg for 15 min at room temperature. The upper hexane layer (2 ml) was collected and transferred to 15 ml polypropylene tubes and dried by using an ISSIIO speed vac system. The samples were re-dissolved in 200 µl of ethanol and stored at -20 ^{o}C described in section 2.2.1 and the level of α-tocopherol in the LDL was measured by HPLC. Each sample (20 µl) inserted into a Capital C18 column (250 x 4.6 mm, 5 µm particle size, 5 µm guard column, Capital HPLC Ltd, West Lothian, Scotland). α-Tocopherol was recognised at 298 nm, using the mobile phase (99% methanol 1% water by volume, flow rate of 1.00 ml/min of run time 20 min) in a PerkinElmer HPLC.

2.2.5 Measurement of α- tocopherol in oxidised LDL

Control LDL or enriched with α -tocopherol (1.6ml at a concentration of 50µg protein/ml) was oxidised with freshly dissolved $FeSO_4$, $FeCl_3$, $CuSO_4$ (5 µM) at 37^{o}C in Chelex-100 treated NaCl/sodium acetate buffer (4.5) and $CuSO_4$ 5 µM in MOPS buffer (pH 7.4) in 15 ml polypropylene tubes (Triple Red Ltd, Bucks, UK) in the existence or absences of antioxidants.

At several time points until 24h, the oxidation in each tube was stopped by addition of BHT (final oxidation 80 µM, from a stock of 2mM in ethanol) and EDTA (final concentration 4 mM). Samples were then stored in the dark at 4°C, they were assayed by HPLC for α -tocopherol.

2.2.6 Cuvette preparation

Fused quartz cuvettes were used in the UV spectrophotometer because they do not absorb much UV. It is important that the quartz cuvettes are completely clean to remove any contaminating residues such as iron. After use, the cuvettes were rinsed multiple times with water and soaked in warm soapy water and cotton buds were used to scrub all walls of the cuvettes. The cuvettes were then rinsed with purified water and ultra-purified water. Absolute ethanol than added for 15 minutes, and rinsed with ultra-purified water many times. Diethylenetriaminepentaacetic acid (DTPA) at 100 µM was used to fill each cuvette for 1 hour at room temperature. It is a chelating factor with a very high affinity for metal cations such as iron and has the ability to chelate all 6 ordination sites of iron, making it a successful agent to remove iron (Tang et al., 1997). The cuvettes were then rinsed with purified water and ultra-purified water and finally rinsed with ethanol and left to dry in an oven.

2.2.7 Removing EDTA from LDL by dialysis

LDL was stored in 100 µM EDTA to decrease autoxidation. EDTA was removed from LDL by dialysis, with the dialysis tubing previously soaked with warm water for 30 minutes, and dialysed overnight at 4 ^{o}C with stirring against phosphate buffer solution (140 mM NaCl, 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4 adjusted to pH7.4). The buffer was changed three times.

2.2.8 Monitoring LDL oxidation by the formation of conjucated dienes

The sodium acetate pH4.5 buffer was prepared with NaCl (150 mM), sodium acetate (10 mM) and Chelex-100 (0.1%w/v) dissolved in ultra-purified water and stored overnight at 4^{o}C with stirring Chelex-100 were removed by using Whatman filter paper and adjusted to pH 4.5 if required. The LDL was diluted to 50 µg of protein /ml in the sodium acetate buffer. LDL/buffer solution (2ml) was placed in test quartz cuvettes and 2 ml buffer added to the reference cuvettes, which contained all the component except LDL. The cuvettes were maintained at 37 ^{o}C by a water bath. In a PerkinElmer Lambda 35 or Lambda Bio 40 automate 8-position double –beam spectrophotometer. The program was set to measure the changes in attenuance as a result of conjugated diene formation and LDL aggregation at 234 nm for 1500 minutes at 1-minute intervals. The attenuance is defined as the log decrease in UV spacepassing through the cuvette due to absorbance by the oxidised LDL plus that lost due to scattering of the UV beam by aggregates of LDL.

The attenuance at time zero was subtracted from all the other time points to allow for the initial absorbance of UV to the silica cuvettes and components of the LDL. Free radical attached on polyunsaturated lipids inside the LDL particle cause 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 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 sodium chloride /sodium acetate buffer pH 4.5 or with $CuSO_4$ in Chelex-100 treated MOPS buffer (pH7.4) in capped quartz cuvettes.

2.2.9 Preparation of Lipoprotein -Deficient fetal calf serum

Lipoprotein-deficient serum (LPDS) was made according to a protocol adapted from Renaud et al. (Renaud et al., 1982). A sample of fetal calf serum (virus and mycoplasma screened; GIBCO), was taken for protein assay. 47.71 g KBr (BDH) was added to 120 ml of fetal calf serum to adjust the density to 1.21 g/ml with stirring by hand. It was added to 35 ml Beckman ultracentrifuge tubes, centrifuged in a Sorvall T1250 rotor at 40,000x g at 10 °C for 48 hours. The tubes were sliced, and the lower layer was taken, which contained the lipoprotein –free serum dialysed against PBS pH 7.4 at 4°C with 5 changes over the course of 24 h. The dialysed LPDS was adjusted to the original volume of serum using the dialysis buffer. It was filtered using a 0.22 µm Millex-GV, assayed for protein and stored at -80 °C.

2.3 Monitoring of ferrous iron levels

The ferrous iron chelator bathophenanthrolinedisulfonic acid (BP) was used to measure ferrous iron (Fe^{2+}) levels. BP is chosen to distinguish iron levels in the blood and other biological fluids as it makes a red coloured complex with Fe^{2+} at pH 2-9, with a molar absorption coefficient of 22,140 M^{-1} cm^{-1} at 535 nm (Pieroni et al., 2001). LDL (50 µg protein/ml) was oxidised with freshly dissolved $FeCl_3$ (5 µM in a sodium chloride /sodium acetate buffer pH 4.5) in 15 ml polypropylene tubes in a water bath at 37°C. Samples of 1ml were taken at several time points to new tubes, BP (3 µl of 100 mM) was an add to them and absorbance was measured immediately at 535 nm with a spectrophotometer.

2.4 Monitoring of cuprous copper levels

Cuprous ion quantification was achieved by the chelator bathocuproinedisulfonic acid (BC). BC is selected to identify copper levels in the blood and other biological fluids as it forms a red coloured complex with Cu^{+} at pH 2-9, with a molar absorption coefficient of 12,000 M^{-1} cm^{-1}, at 480 nm (Perugini et al., 1997). LDL (50 µg protein/ml) was oxidised with freshly made $CuSO_4$ (5 µM in a sodium chloride /sodium acetate buffer pH 4.5) in 15 ml polypropylene tubes in a water bath at 37 °C. Samples of 1 ml were taken at several time points into new tubes,

(3 µl of 100 mM) was added to them and absorbance was measured at 480 nm with a spectrophotometer.

2.5 Measurement of lipid hydroperoxide

Lipid hydroperoxides (LOOH) in LDL were measured using a technique modified from that described by El-Saadani et al (el-Saadani et al., 1989). Standard concentrations of H_2O_2 were prepared using ultrapure water. Both samples (oxidised LDL) and standards (250 µl) were added to assay tubes in triplicate and 1ml of colour reagent (0.2M potassium phosphate, 0.12M potassium iodide 0.15 mM sodium azide,2g/Lpolyethyleneglycolmono[p-(1,1',3,3'-tetramethyl-butyl)-phenyl]ether,0.1g/L alkybenzydimethylammoniumchloride, 20 µM of ammonium molybdate (pH 6.0-6.2) was added to each tube. After incubating the tubes in the dark for 1 h, the absorbance at 365 nm was measured. The basis for the assay is that molecular iodine will be created after the reaction between peroxides in the samples or standards and the iodide ions in the colour reagent. Molecular iodine consequently reacts with excess iodide ions in the colour reagent to make the tri-iodide chromophore (I_3^-) which absorbs ultraviolet radiation at 365 nm. The hydroperoxide content of the samples is then measured by comparison with the H_2O_2 standard

2.5.1 Preparing oxidised LDL samples for HPLC analysis of oxidised LDL

Methanol (2 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 into each sample and then vortexed for 30 seconds to move the hydrophobic lipid fraction (cholesterol, cholesteryl esters and triglycerides) of LDL into the hexane phase. The samples were centrifuged for 15 min at room temperature and 1500xg, and 2 ml of the upper hexane layer was transferred to a 5 ml polypropylene tube and dried in a Speed Vac. The dried samples were re-dissolved in 200 µl of an appropriate mobile phase and stored at -20^{o}C until analysis.

2.5.2 HPLC analysis

High-Performance Liquid Chromatography (HPLC) is a system of column chromatography that pumps a sample mixture in a solvent (identified as the mobile phase) at high pressure through a column with chromatographic packing material (stationary phase). Because of this flexibility, HPLC is useful in a diversity of industrial and research applications, such as pharmacological, ecological, forensics, and chemicals.

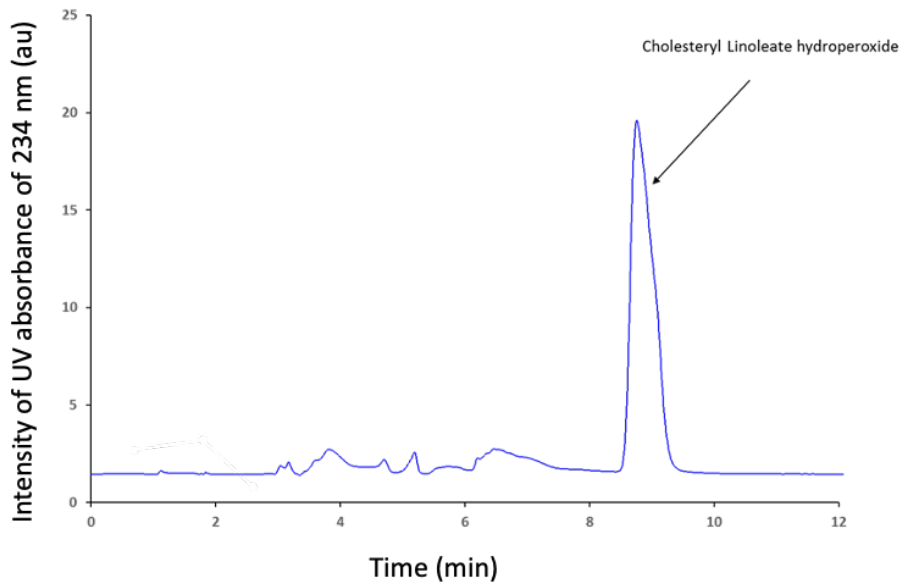
The sample retaining time will differ dependent on the interaction between the stationary phase, the molecules analysed, and mobile phase used. After injection into the column, the analytes interact between the two phases at a different rate, mainly due to diverse polarities in the analytes and are separated (Sharp et al., 2015).

All samples (20 µl) were injected by using an autosampler. The reverse-phase C18 columns have a hydrophobic stationary phase so the more hydrophilic molecules are eluted first (Krokhin and Spicer, 2009). The oxidation products were identified at 234 nm using a suitable mobile phase and conditions as described in Table 2.1. Example chromatograms are presented in Figure 11. The oxidation products were measured by comparison of peak area measurement with those of commercially available standards.

Mobile phase	Wavelength	Flow rate	Time	Lipid detection
44% Acetonitrile 54% Isopropanol 2% water by volume	234 nm	1.2 ml/min	15 min	7-Ketocholesterol, Cholesteryl-linoleate hydroperoxide(CLOOH)

Table2.1 Target lipid species and analysis conditions for HPLC

A



B

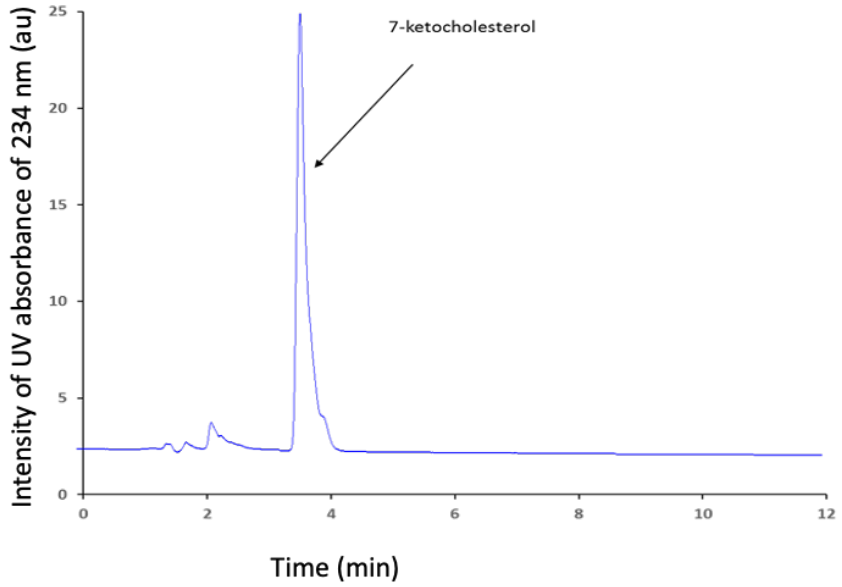


Figure 2.1 Example chromatogram of standards showing (A) Cholesteryl linoleate hydroperoxide and (B) 7-ketocholesterol. A C18 column was used with a mobile phase of Acetonitrile (44%), isopropanol (54%) and water (2%) by volume. Detection was at 234 nm. Minor more polar contaminants were present

2.6 Cell culture

Cell culture is the method in which cells are isolated from an organism and maintained in a fluid medium. In suitable conditions, the cells will live and even proliferate. The growth can be distinguished by cell division and differentiation, throughout which the cells can transform into particular forms that are capable of functions equivalent to those of tissues in the whole organism (Baust et al., 2017).

2.6.1 Human THP-1 cells

THP-1 cells were used to study the interaction of macrophage with oxidised LDL.

Human THP-1 cells, a monocytic cell line derived from an acute monocyte's leukaemia patient, were purchased from the European Collection of Cell Culture (Salisbury, UK). Cells were counted with a Countess II FL automated cell counter (Invitrogen) and seeded at 3x 10^5 cell/ml in PRMI-1640 medium (containing 2 mM L-glutamine, 10 mM HEPES, 1Mm 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 humidified 95% air and 5% CO_2 atmosphere until confluent. Culture medium was changed every 3-4 days. Cells were subculture by transferring the cell suspension under sterile conditions into 50 ml centrifuge tubes and centrifuging at 500 xg for 5 min at room temperature. The culture medium was then removed, and the pellet was re-suspended in fresh pre-warmed culture medium and seeded into new flasks as above.

2.6.2 Cryopreservation of THP-1 cells

Cryopreservation is a technique used at cryogenic temperatures (-191 ^{o}C)to preserve a fine structure of living cells and tissues in a condition of suspended activation at such temperature for any considerable time (Pegg, 2007). THP-1 cells were frozen in cryopreservation media (DMSO). Cells were centrifuged at 500 x g for 5 min at room temperature. The culture media was discarded, and the pellet was enriched with cryopreserve medium (2ml per confluent T150). The cells were relocated to sterile cryopreservation vials and were kept in polystyrene containers (to allow them to freeze gradually) at -80 ^{o}C overnight. Vials were moved to liquid nitrogen for long-term storage. When needed, vials were thawed rapidly in a 37^{o}C water bath and their contents were transferred into 50 ml centrifuge tubes containing RPMI-1640 medium, to dilute the DMSO in the cryopreservation media. They were centrifuged at 500 x g for 5 min at room temperature, new culture medium added and seeded, as described above.

2.6.3 Differentiation of THP-1 cell into macrophages

The THP-1 cells were subcultured in a T75 flask and left to reach a density of 8 x 10^5 cells /ml. To get larger cell numbers, T150 flashed with a maximum of 80 ml of culture medium were used to maintain the activity of the cells, the cell density was not allowed to surpass 1x 10^6 cells/ml. Converting THP-1 monocytes into macrophages was achieved by incubating the cells with phorbol 12-myristate 13-acetate (PMA) dissolved in DMSO (McSwine-Kennick et al., 1991, Park et al., 2007). The transformation was done in 6, 12 or 96 well plates according to the type of experiment by adding PMA (25ng/ml) in the culture medium and incubating with THP-1 monocytes for 72h. After 72h, the non-adherent cells were cleared by washing with sterile PBS and the adherent macrophages were allowed to rest in culture medium without PMA for another 24 h before being used in experiments.

2.6.4 Culturing THP-1 cells to detect ceroid

Glass coverslips 18x18mm were washed with alcohol for a few minutes and added to 6 well tissue culture plates and THP-1 cells at 300,000 cells/ml were add in RPM1640 medium, which contained (penicillin and streptomycin, amphotericin and 10 %(v/v) heat-inactivated foetal calf serum. To each well, tissue culture plate was added 200 µl of RPMI 1640 containing PMA (25 ng/ml) and the cells incubated for 48-72 hours to allow for the conversion of the THP-1 monocytes to macrophages.

Fresh medium (2 ml) for each well was then added for one day. THP-1 cells cultured on glass coverslips were incubated in medium containing either SMase-LDL, native LDL, LDL enriched with alpha-tocopherol or SMase-LDL enriched with alpha-tocopherol at 200 µg protein /ml or without LDL for 24 hours. They were washed 3times with warm PBS (200 µl) and incubated with RPMI medium containing 10 % (v/v) lipoprotein-deficient serum (LPDS) for 7 days with a change of medium every 2-3 days.

PBS was removed and 4% paraformaldehyde in PBS (2ml) was added and incubated for 15min. The cells were washed with PBS, the coverslips treated within 60 %(v/v) ethanol, 90% (v/v) ethanol and ethanol for (3 min) each in sequence. Coverslips were transferred to a glass Petri dish (100x15mm with a cover), which contained 2ml xylene and treated with ethanol, 90% (v/v) ethanol.60 % (v/v) ethanol for (3min) respectively. The coverslips were then soaked momentarily in 60 % (v/v) isopropanol and then incubated with Oil Red O (0.5 %w/v) in 60% (v/v) isopropanol by adding (1.5 ml) to each well for 5-10 min at room temperature. The cells were washed 4 times with distilled water and they were mounted on to slides using Vectashield® mounting medium (Vector Laboratories, UK) and stored at 4 ^{o}C. Ceroid was imaged using light microscopy (Axioskop2, Carl Zeiss Ltd) and images were taken with Axiovision software.

2.6.5 Enriching THP-1 cells with α-tocopherol

α-Tocopherol (2 mM) in ethanol (final ethanol concentration) 0.1% (V/V) was added to heat-inactivated FCS (10 %)(v/v), and incubated with shaking at 37 ^{o}C for 15 min in the dark. RPMI

1640 medium was added and mixed. The solution was then incubated with shaking at 37°C for 15 min. The subsequent medium was sterilized by using a 0.45 µm filter.

One millilitre of the α-tocopherol enriched medium was added to adherent THP-1 cells. After the medium was removed, the cells were washed twice with PBS containing 0.25% (w/v) BSA, followed by two washes with PBS, alone in order to remove α-tocopherol that non-specifically bound to the cell surface (Chan and Tran, 1990), The level of α -tocopherol in the cells was measured by HPLC.

2.7 Apoptosis detection

Apoptosis is a distinct form of cell death. It happens normally during growth, during ageing as a homeostatic procedure to preserve cell populations in tissues and as a defence mechanism such as in immune reactions. It can be rapidly examined by flow cytometry. (Norbury and Hickson, 2001). We have investigated apoptosis of macrophage-like cells induced by oxidative stress.

2.7.1 Measuring apoptosis and necrosis of THP-1 cells

THP-1 macrophage apoptosis and necrosis were detected by flow cytometry, using a commercially available kit (Apo Alert V-FITC apoptosis kit, Clontech Laboratories Inc., Palo Alto, CA, USA). This compound binds to phosphatidylserine, which is transferred to the cell surface during apoptosis and so is suitable to use as a marker of apoptotic cell death. Propidium iodide (PI) can be used to differentiate apoptotic cells from necrotic cells, as it is capable of entering the cell and staining the nuclear DNA during necrosis. Cells with

augmented annexin V binding to phosphatidylserine are undertaking apoptosis and cells with increasing PI staining are undertaking necrosis. Cells with augmented levels of both annexin V and PI labelling are experiencing secondary necrosis, i.e. they were initially apoptotic and then had developed necrosis.

THP-1 cells (5 x10^5 cells/ml) were plated in 6 well tissue culture plates (2 ml/well). RPMI was removed from the cells and was replaced with RPMI containing α-tocopherol (100 µg LDL protein/ml) that had been oxidised by H_2O_2 (100 µM) for 24h. After48 hr, the cells were removed by into plastic sterile 15 ml polypropylene tubes. The mixture was then centrifuged at 250 x g for 10 min at room temperature. The resulting pellets were washed with PBS and the cells were then centrifuged again using the same conditions. Cell pellets were suspended in the kit binding buffer provided (0.5 ml) and centrifuged at 250 x g for 10 min at room temperature. Each pellet was dissolved in 100 µl of a binding buffer containing FITC-annexin V (0.5 µg/ml) and PI (2.5 µg/ml) that previously were incubated for 15 min in the dark. The volume of each sample was made up to 500 µl with binding buffer provided with the kit and the samples were evaluated immediately. Samples were analysed with a Becton Dickinson FACS can flow cytometer (Becton Dickinson, NJ, USA), using Cell Quest software, permitting the percentage of healthy, apoptotic, necrotic cells in the population to be identified.

2.8 Human microvascular endothelial cells

SV40 large T Ag-transformed human dermal microvascular endothelial cells (HMEC-1) were obtained from Center For Disease Control and Prevention (Atlanta, Georgia) and were grownin MCDB131 supplemented with 10% heat-inactivated FBS, L-glutamine, hydrocortisone acetate (1mM) and human epidermal growth factor (10 ng/ml).

2.9 Human aortic endothelial cells

Endothelial cells are found at the edge between the blood and the underlying tissue. Variations in the role of endothelial cells seem to play a key in the pathogenesis of atherosclerosis. Endothelial cells produce and secrete stimulators as well as blockers of both the coagulation system and the fibrinolysis system, in addition to mediators that influence the adhesion and aggregation of blood platelets. Endothelial cells likewise discharge molecules that regulate cell proliferation and modify vessel wall tone. Human aortic endothelial cells (HAEC) secrete antithrombotic and thrombotic elements, such as t-PA and PAI-1 and react to TNF-α by modifying growth characteristics, producing cytokines such as GM-CSF. Human aortic endothelial cells (HAEC) provided by Lonza were isolated from the human aorta. HAEC were cryopreserved at passage by 10 % (v/v) DMSO, 10 % (v/v) FCS and frozen in liquid nitrogen.

They were cultured in Basal Medium-2 (EBM™-2 Medium) and the following growth supplements: human epidermal growth factor (hEGF), 0.5 ml; vascular. endothelial growth factor (VEGF), 0.5 ml; R3 insulin-like growth factor-1 (R3-IGF-1), 0.5 ml. ascorbic acid, 0.5 ml; hydrocortisone, 0.2 ml; human fibroblast growth factor-Beta (hFGF-β), 2.0 ml heparin (0.5 ml); fetal bovine serum(FBS), 10 ml; gentamicin/amphotericin-B (GA), 0.5 ml.

2.10 Effect of LDL oxidised at lysosomal pH on endothelial cells

We investigated the effect of LDL oxidised at pH 4.5 on endothelial function in a variety of ways, as described below.

2.10.1 Rat aorta Vasoreactivity assessment

Endothelial dysfunction is an initial, key distinguishing feature of development of atherosclerotic plaque and consequent complication events. This is described by an impaired bioavailability of endothelium-derived vasodilators, such as a nitric oxide (NO), along with a comparative increase in available vasoconstrictors. Oxidised LDL might damage the endothelium and decrease vasodilatation. We investigated the effect of LDL oxidised by iron ion at lysosomal pH on vasodilatin, as described in chapter 6, as this might be released from lysed macrophages in atherosclerotic lesions.

Wire myography (Mulvany and Halpern 1977) is an *in vitro* research technique for investigating both active and passive characteristics of arteries with the diameters of between 100 and 1000 μm. Blood vessels used in this procedure are arteries such as femoral, mesenteric, carotid, and aorta (Puzserova et al., 2007, Khazaei et al., 2008, Bal et al., 2009, Zaloudikova et al., 2009), from rats and other animals in both physiological and pathological conditions (Spiers and Padmanabhan, 2005). The myography technique involves the following stages: dissecting and mounting of the arteries, normalisation, assessment of tissue viability and construction of a cumulative concentration-response curve (Spiers and Padmanabhan, 2005). The thoracic arteries were obtained from 12-14 week-old male healthy Wistar rats (body weight 261±6 g, n=10) and investigated using wire

myography (Mulvany and Halpern, 1977). The rats were anaesthetized by isoflurane. After cervical displacement, the arteries were carefully dissected, and moved to Kerbs solution and then cleaned to remove the adipose and connective tissues. Arterial segments (approximately 2 mm long) were mounted in a small vessel wire myography (Dual Wire Myography System, DMT) and were stabilized for 30 min. The vessels were contracted with the α_1 –adrenergic agonist phenylephrine (100 nM) and finally relaxed with the M3 agonist acetylcholine at different concentrations (Figures 2.2).

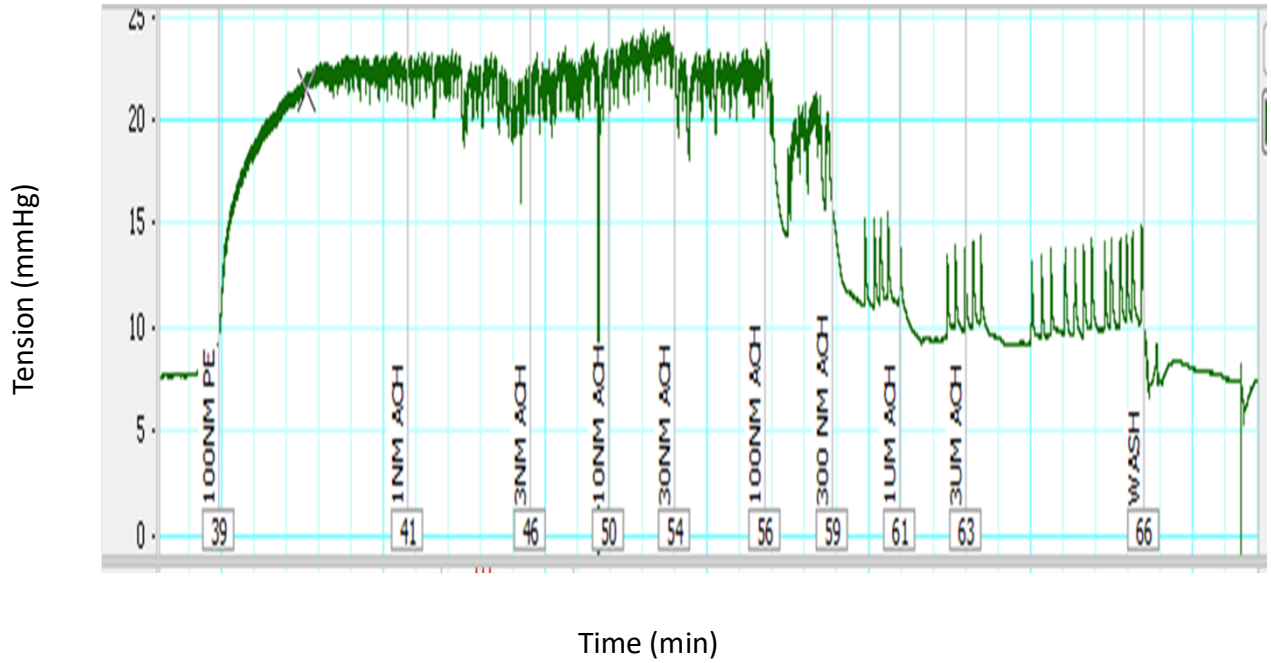


Figure 2.2. Typical tension traces the curve of intraluminal pressure in response to vasoconstriction and vasorelaxation therapy, showing responses of rat arteries rings with 100 nM phenylephrine (to produce contraction), followed by relaxation with different concentration of acetylcholine (1-3,000 nM).

2.10.1 Histological analysis and immunohistochemistry

After the wire myography experiments, the rat aorta was directly frozen in liquid nitrogen-cooled isopentane and mounted in Optimal Cutting Temperature compound (OCT) (TAAB O023) cooled by dry ice/ethanol. Immunohistochemistry was performed on 10 µm cryosections that were air-dried at room temperature for 30 min before the use of block wash buffer (PBS with 5% foetal calf serum (v/v), 0.05% Triton X-100). Antibodies CD-31 primary antibody 1:1000 were diluted in wash buffer 30 min before use (Table 2.1.1) Fluorescence-based secondary antibodies were used to detect all primary antibodies except for CD-31 where the Vectastain ABC-HRP kit was deployed (Vector PK-6100) with an avidin/biotin-based peroxidase system and DAB peroxidase (HRP) substrate (Vector SK-4100). Morphometric analysis of aortic sections was performed by Image-J analysis software.

2.10.3 Measurement of reactive oxygen species in endothelial cells exposed to oxidised LDL

Superoxide production in cultured of human microvascular endothelial cells (HMEC-1) was detected by a dihydroethidium (DHE) assay, according to the manufacturer's instructions. In brief, cells or tissue (arteries) were incubated with oxidised LDL for the specified time. Before treating cells with LDL enriched with α-tocopherol oxidised at pH 4.5 (described in section 2.2.6), free iron, copper, EDTA and BHT were removed from the LDL samples and the pH was adjusted to7.4 (pH 7.4). LDL was dialysed in 500ml of phosphate dialysis buffer containing BHT (80 µM) and EDTA (4mM), with one change of the solution. They were then dialysed

against 500 ml of phosphate dialysis buffer overnight, with repeated changes of the solution, to remove the EDTA and BHT. DHE 10 µM in PBS for 30 min. Fluorescence was measured with a fluorescence microscope with wavelength 488nm and quantified using Image-J analysis software.

2.10.4 Western blot analysis

Proteins from human microvascular cells (HMEC) and human aortic endothelial cells (HAEC)were extracted using RIPA buffer (Sigma-Aldrich) and Halt™ (protease & phosphatase inhibitor). Western blotting was performed (Xie et al., 2012) for total and phosphorylated eNOS.

Proteins in HMEC and HAEC lysates were separated on SDS-PAGE 10-12 % gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked by incubation in Tris-buffered saline (Tris10 mmol/l, pH 7.5, and NaCl 100 mmol/l containing 0.1% (v/v) Tween 20 and 5% (v/v) non-fat dry milk) for 1 h. To decrease non-specific binding, followed by a 24 h incubation at 4°C with rabbit polyclonal eNOS antibody and phosphorylated-eNOS antibody 1:1000 (S1177) (Cell Signalling Technology) and the Developmental Studies Hybridoma Bank (DSHB) mouse monoclonal beta-actin **(**antigen molecular weight 43 kDa). The membranes were washed in Tris-buffered saline containing 0.1% (v/v) Tween 20 before incubation for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (Invitrogen). The membranes were then washed and developed using ECL substrate (Thermo Scientific) to identify proteins, which are bound to the secondary antibody - HRP. The Reagent 1 together with the Reagent 2 provide a reaction catalysed by the enzyme HRP causing the emission of light.

2.11 Statistical analysis

Unless specified otherwise, all results are expressed as means ± standard error of the mean (S.E.M) of joint data from 3 – 5 independent experiments as identified in the figure legends. Testing differences of two means was done using a 2-tailed unpaired Student's t-test. For comparing more than two means, one-way ANOVA (statistical test, which compares the difference in the group means within a sample though considering merely one independent variable) followed by Tukey's post hoc analysis to measure the level of statistical significance between paired differences. Two-way ANOVA was used to (compare the mean variances between groups that have been divided into two independent variables). The main aim of a two-way ANOVA is to recognise if there is an interaction between the two independent variables on the dependent variable. Two-way ANOVA followed by Bonferroni post-tests were employed to compare the results of different HPLC time points of an LDL sample. The level of significance of the difference is shown in the graph as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. ANOVA and post hoc analyses were analysed with Graph Pad Prism 4 software (La Jolla, CA). A *p*-value of <0.05 was taken to be a statistically significant difference.

Chapter 3

Effect of α-tocopherol enrichment on LDL oxidation by transition metals at lysosomal pH

3.1 Introduction

In the last decades, numerous studies have documented the potential role of oxidative stress in atherogenesis (Bonomini et al., 2008). Because of their ability to lose electrons, delocalised transition metals are a thought to be toxic mainly because they can form ROS causing, oxidative damage (Bonomini et al., 2008, Heinecke, 2003).

It is now extensively believed that oxidative alteration of LDL transformed these natural particles into pathogenic (Zuliani et al., 2013, Matsuda et al., 2013), immunogenic (Lopes-Virella et al., 2012, Perrin-Cocon et al., 2013, Matsuda et al., 2013, Gomez et al., 2014) and atherogenic particles (Yang et al., 2012).

Apolipoprotein B-100, has 4536 amino acid residues, with numerous exposed tyrosines and lysines, which can be directly oxidized or altered by lipid oxidation products. *In vivo*, the process of LDL oxidation remains ambiguous and there are divergent mechanisms that may be accountable for the process. They are categorised as enzymatic and non-enzymatic mechanisms. The enzymatic mechanisms contain a number of different enzyme systems, such as lipoxygenases, myeloperoxidase (which induces hypochlorous acid), NADPH oxidases and nitric oxide synthases (Tsimikas and Miller, 2011). The non-enzymatic processes include free transition metal ions such as copper and iron, which catalyse lipid peroxidation. Several studies reported that transition metals may have importance during later stages in the development of atheroma (Yoshida and Kisugi, 2010, Mowri et al., 2000, Ojo and Leake, 2018), but they might possibly be involved in early atherosclerosis as well. Oxidised LDL, which taken directly from atherosclerotic lesions is analogous to lipoproteins oxidized *in vitro*

with copper sulphate (Heinecke et al., 1986). Copper can breakdown performed lipid hydroperoxides (Thomas and Jackson, 1991, Esterbauer et al., 1992). Oxidized LDL (ox-LDL) and metal-catalysed oxidation systems, such as Fe^{3+} with ascorbic acid, increase free radical production (Sener et al., 2009)

The molecular mechanism by which Cu^{2+} induces oxidation in LDL is not clear and it is also unclear if this *in vitro* activity reflects LDL oxidation *in vivo* (Esterbauer and Ramos, 1996). LDL oxidation by copper depends on experimental conditions, such as copper concentrations used or the temperature (Kleinveld et al., 1992). This is also supported by some reports of the presence of low, sub-micromolar concentrations of pro-oxidative copper or iron ions in the arterial wall or in lesions (Dubick et al., 1987, Smith et al., 1992), either free or in complexed forms.

Chelation therapy, a programme of continual intravenous injection of EDTA, regularly in combination with vitamins and minerals, has been reported as a harmless alternative treatment for atherosclerotic vascular disease (Ernst, 2000, Ernst, 1997, Lewin, 1997). One early analysis of uncontrolled trials and unpublished data claimed that EDTA chelation therapy effectively improved the symptoms of cardiovascular disease in over 80% of cases (Seely, 2005). Chelation therapy is thought to not only remove contaminating metals but also to decrease free radical production (Roussel et al., 2009). Clinical trials have shown that chelation therapy is not effective (Villarruz et al., 2002, Seely et al., 2005). Chelation therapy has side effects, including burning at the intravenous site, fever, headache, nausea or vomiting. Rarely, there is hypocalcaemia, sudden drop in blood pressure, drop in bone marrow counts (Babu and Gonzalez-Pena, 1994), heart failure, kidney damage (Weaver et al., 2012) and death (Baxter and Krenzelok, 2008)(Anderson et al., 2003)

Several studies offer evidence for the efficacy of vitamin E as an antioxidant in atherosclerosis (Diaz et al., 1997). However, experimental trials of vitamin E for inhibiting cardiac disorders in patients with recognised coronary artery disease have been generally disappointing (Collins et al., 200) (Vardi et al., 2013, Ginter, 2000,), and this has persuaded many to doubt the validity of the oxidation theory of atherogenesis (Yusuf et al., 2000, de Gaetano, 2001, Heinecke, 2001, Investigators, 2000). *In vitro* studies reported that vitamin E was unsuccessful to inhibit LDL oxidation induced by tyrosyl radical made by myeloperoxidase and the absence of protection of α-tocopherol on LDL apoB-100 and lipid oxidation by lipoxygenase (Savenkova et al., 1994, Ganini and Mason, 2014, Ziouzenkova et al., 1996, Micheletta et al., 2004). Moreover, vitamin E stimulates LDL lipid peroxidation when the flux of radicals is low (Savenkova et al., 1994). These studies highlight the suggestion that in order to understand how to design antioxidant interventions it is necessary to know which oxidative pathways are operative *in vivo*.

Hypothesis and aims

α-Tocopherol is well known to inhibit LDL oxidation at pH 7.4 (Jessup, 1990; Dieber-Rotheneder et al. , 1991),but the large clinical trails of α-tocopherol did not show any protection against cardiovascular disease (Collins et al ., 2002 ; Yusuf et al ., 2000). To try to explain this discrepancy, this project investigated the effect of α-tocopherol supplementation of LDL on its oxidation at lysosomal pH. The hypothesis was that α-tocopherol would not inhibit LDL oxidation as well at pH 4.5 as it does at pH 7.4. If so,this might helps to explain why the clinical trails of α-tocopherol failed.

3.2 Methods

Conjucated dienes in oxidising LDL were measured spectrophotometerly as described in chapter 2.2.8. Cholesterol linoleate hydroperoxide and 7- ketocholesterol were measured by HPLC as described in chapter 2.5.2 and total lipid hydroperoxides were measured by a tri-iodide assay as described in chapter 2.5. Ferrous ion concentrations were measured as described in chapter 2.3,using bathophenanthrolinedisulfonic acid.

Section 3.3.1

The phases of LDL oxidation at pH 4.5 are described.

Section 3.3.2

The effect of EDTA on LDL oxidation at pH 4.5 are described. The rationale for this is that LDL is stored in the presences of EDTA will be carried over into the incubation buffer when LDL is oxidised.

Section 3.3.3

The aim of this section is to determine the effect of α-tocopherol supplementation of LDL on LDL oxidation by Cu^{2+} Fe^{2+} and Fe^{3+} at pH 4.5 (lysosomal pH). Different concentrations of these metals were used because it is known that α-tocopherol can have a pro- oxidant effect on LDL at low oxidative stress (Thomas and Stocker, 2000).

3.3 Results

3.3.1 Monitoring oxidised LDL with iron at pH 4.5 and 7.4

This study investigated the oxidation *in vitro* of LDL with iron at plasma and interstitial fluid pH (7.4) and lysosomal pH (4.5). The oxidation of the LDL was observed by measuring conjugated diene formation by spectrophotometry at 234 nm (Esterbauer et al., 1989). At pH 4.5 there were different phases, including a short lag phase, followed by a rapid oxidation phase and then a slower oxidation phase, an aggregation phase and a sedimentation phase (Figure 3.1). Attenuance (defined as absorbance plus UV scattering) above about 0.7 is due mainly to UV scattering by aggregated LDL, as when all the polyunsaturated fatty acyl group in LDL are oxidised to conjugated dienes (at 50 μg LDL protein/ml) the absorbance is about 0.7. (Esterbauer et al., 1989). The attenuance eventually falls because the LDL aggregates sediment before the beam of UV.

There was very little oxidation noticeable with Fe^{2+} at a pH of 7.4 (Figure 3.1). Using one-way ANOVA to analyse the data, there is a significant difference ($p<0.001$) between the control LDL and LDL oxidised by 5μM $Fe^{2+,}$ while there was no significant difference between the control LDL at pH 4.5 with LDL oxidised by Fe^{2+} at pH 7.4. These phases agree with those shown by Satchell & Leake (Satchell and Leake, 2012). The experiments were run overnight, so that aggregation would be monitored, as LDL aggregates at pH 4.5, but much less at pH7.4 (Satchell and Leake, 2012).

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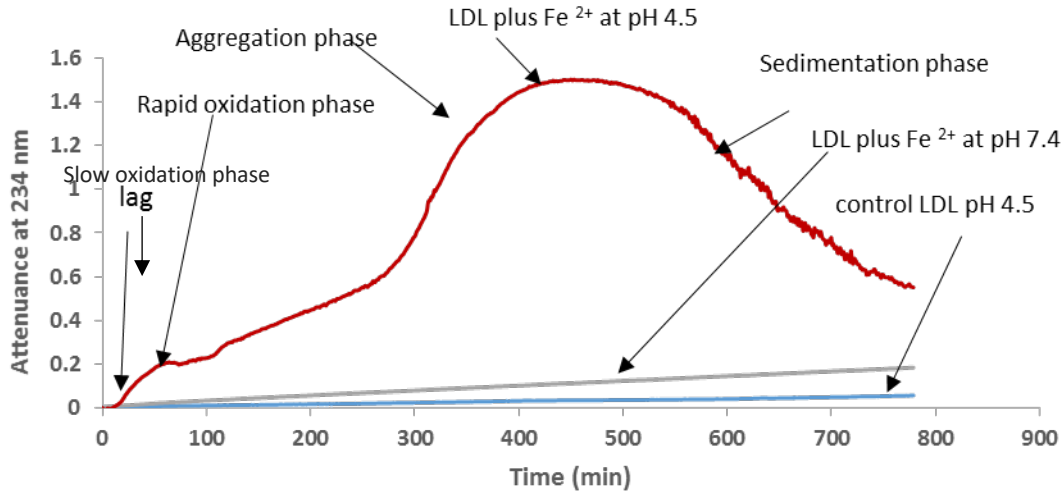

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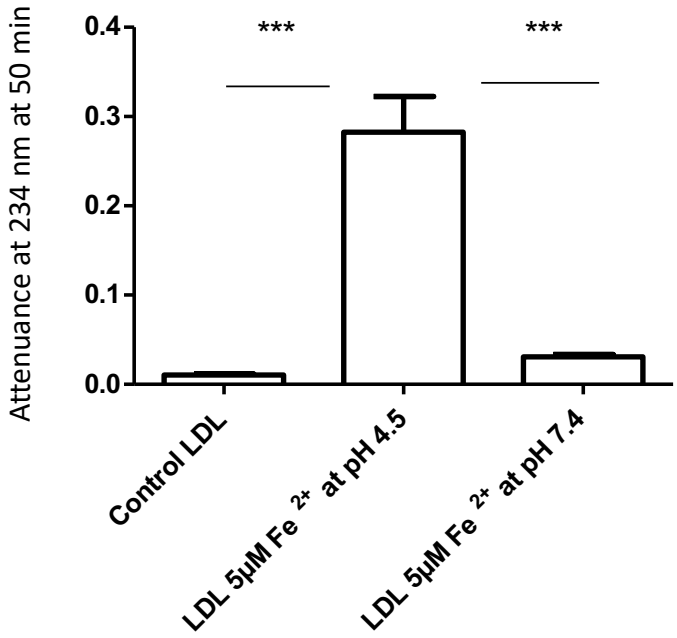

Figure 3. 1 A Graph showing the different phases of LDL oxidation with or without the addition of 5µM $FeSO_4$ at pH 4.5 and 7.4. LDL (50 µg protein/ml) was incubated at 37 °C and pH 4.5 with sodium acetate /NaCl buffer and MOPS buffer of pH 7.4 in quartz cuvettes for roughly 800 minutes. Oxidation was observed by measuring the change in attenuance (absorbance plus UV scattering) due to the formation of conjugated dienes and LDL aggregation in LDL samples at 234 nm. There was a marked difference between control LDL and LDL oxidised by 5 µM Fe^{2+} at pH 4.5, with very little oxidation with 5 µM Fe^{2+} at pH 7.4. A time of 50 minutes was chosen to analyse the results statistically because this corresponds to the mid- to the upper range of the rapid oxidation phase. B The bar chart shows the mean ± SEM of four experements. A one-way ANOVA was significant (*** $p < 0.001$) Tukey's posthoc tests were then carried out.

3.3.2 Effect of EDTA on LDL oxidation by iron at lysosomal pH.

It is standard practice to store LDL in the presence of EDTA to inhibit its oxidation during storage. EDTA can inhibit or increase the oxidation of LDL depending on the conditions(Lamb and Leake, 1992). Therefore, this study investigated the effect of EDTA on LDL oxidation by iron at pH4.5, as EDTA would be carried over from the stored LDL to the incubation solution. The LDL was stored in 100 mM EDTA. If the stored LDL concentration were to be 2 mg protein/ml and LDL is oxidised at 50 μg protein/ml, the carried over EDTA concentration would be 2.5 μM.

LDL was oxidised by 5 μM $FeSO_4$ with addition of 1, 2, 5, 10 μM EDTA. EDTA at 1 or 2 μM partially inhibited LDL oxidation and 5, 10 μM EDTA inhibited it almost totally (Figure 3. 2). Thus equimolar EDTA to Fe^{2+} (5 μM) inhibited the oxidation of LDL almost totally. To confirm that one atom of Fe^{2+} bind to one molecular of EDTA,in this study LDL was oxidised in the presence of 5 or 10 μM Fe^{2+} and 5 or 10 μM EDTA (Figure 3.3). The oxidation given by 5 μM $FeSO_4$ was similar to that given by 10 μM $FeSO_4$ plus 5 μM EDTA, implying that there was 5 μM $FeSO_4$ available in the latter. This means that one molecule of EDTA (which has 6 coordination sites) binds to and inactivates one atom of iron. We can, therefore, calculate the concentration of EDTA carried over from the LDL preparation to the cuvettes and add the same additional molar concentration of $FeSO_4$ to give the same available concentration of iron (usually 5 μM) in all experiments involving different preparations (of different concentrations) of LDL. This should improve the reproducibility of the experiments. This will be important for the experiments involving α-tocopherol supplementation of LDL in which the control and α-tocopherol supplementated LDL will have slightly different concentration of LDL.

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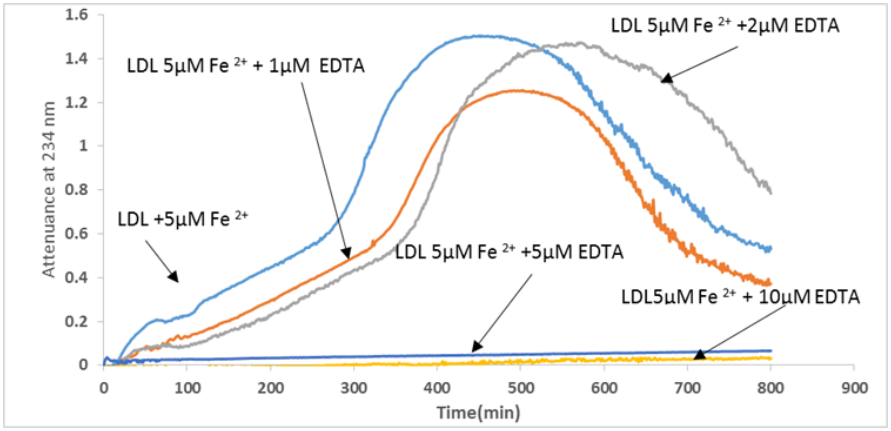

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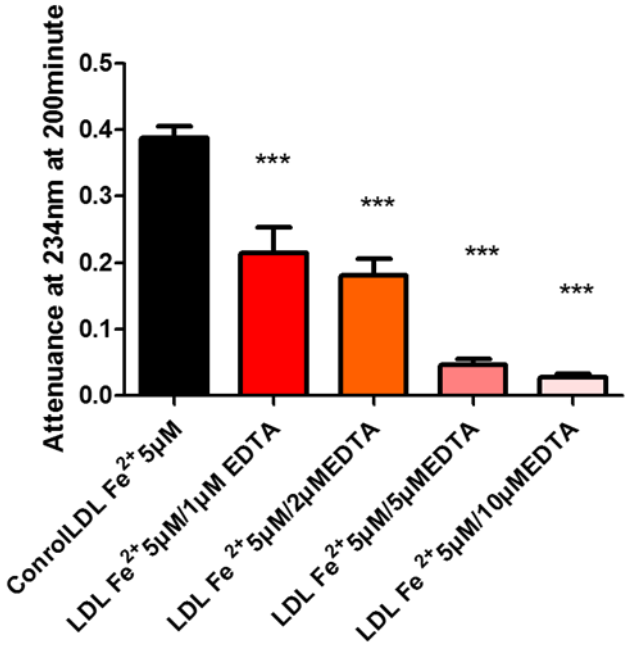

Figure 3.2. Effect of EDTA on LDL oxidation by ferrous ion at pH 4.5. A LDL was dialiysed to remove EDTA and incubated with 5 µM $FeSo_4$ at pH 4.5 in the absence or presence of EDTA (1-10 µM) and the attenuance at 234 nm was monitored.The values in the bar chart were used to perform an ANOVA test, to evaluate the significance of the bindings. The P-value compared to the attenuance with just 5 µM Fe^{2+} as shown. One-way ANOVA followed by Tukey's Multiple Comparison Test showed a significant difference P<0.001 between the control and EDTA groups. Attenuance at 200 minutes was used because it measured oxidation but not aggregation. The bar chart shows the mean ± SEM of the experiments. A one-way ANOVA was significant (*** indicate P < 0.001 compared to the control., n=4) Tukey's posthoc tests were then carried out.

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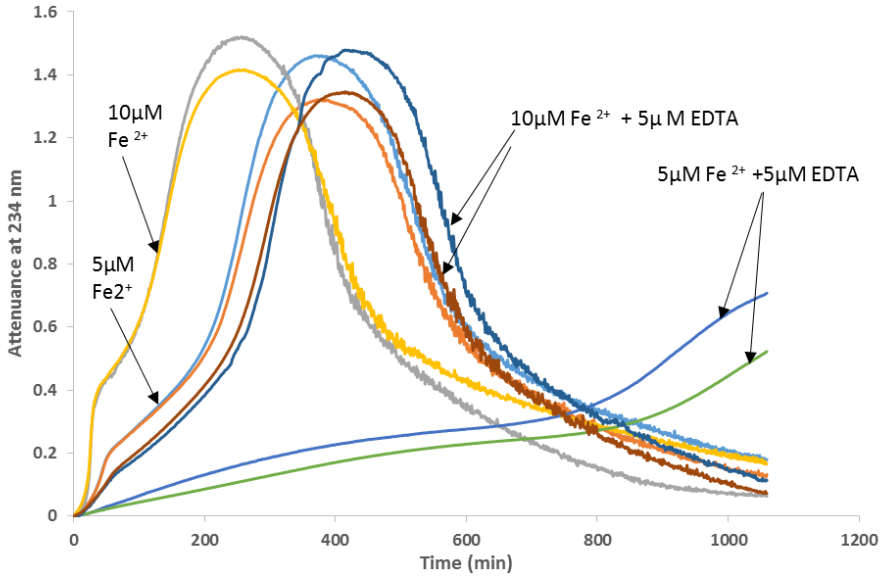

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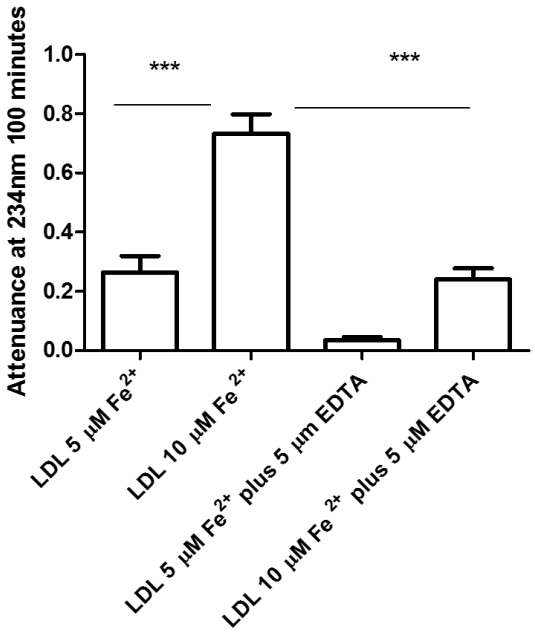

Figure 3.3. Effect of EDTA on LDL oxidation by iron at pH 4.5.
LDL was dialysed against a phosphate buffer overnight before the experiment in order to remove EDTA from the LDL. LDL (50 µg protein/ml) was incubated with pH 4.5 sodium acetate /NaCl buffer in quartz cuvettes at 37° C for roughly 1000 minutes. $FeSO_4$ (final concentration 5 µM or 10 µM with or without EDTA) was added to duplicate cuvettes. The attenuance at 234 nm was monitored. Oxidation was quantified by determining the increase in attenuance at 100 minutes as this corresponds to the time at which about half-maximal oxidation occurred in the absence of EDTA. The bar chart shows the mean ± SEM of four independent experiments. A one-way ANOVA was significant (*** indicates $P < 0.001$, n=4) Tukey's posthoc tests were then carried out.

3.3.3 Effect of α-tocopherol on LDL oxidation at lysosomal pH.

3.3.3.1 Effect of α-tocopherol on LDL oxidation by ferrous ion at lysosomal pH.

It is well known that α-tocopherol can inhibit LDL oxidation at pH 7.4, but the large clinical trials of α-tocopherol did not inhibit cardiovascular disease (Kontush et al., 1996, Huang et al., 2002, Upston et al., 2001, Weinberg et al., 2001). This study, therefore investigated the effect of α-tocopherol on LDL oxidation at lysosomal pH. LDL was enriched with α-tocopherol (final concentration 1%) by adding α-tocopherol in dimethyl sulphoxide (DMSO) to plasma and isolating LDL by ultracentrifugation. α-Tocopherol is soluble in DMSO, DMSO is miscible with and would later be dialysed away. Plasma was incubated with DMSO (1/% v/v) alone as a control. (Adding α-tocopherol to LDL in a simple buffer does not lead to its incorporation into LDL).

LDL was oxidised in NaCl/sodium acetate buffer (pH 4.5) at 37 ^{o}C, in the presence of ferrous iron ($FeSO_4$ 2, 5 or 20 μM). Different concentrations of iron were used because α-tocopherol has a greater pro-oxidant effect at low metal concentrations (Bowry et al., 1995). The oxidation was tracked by measuring the attenuance at 234 nm (Figure 3. 4, 3. 5 and 3. 6). As expected (Satchell and Leake, 2012), LDL oxidation level was faster as the concentration of ferrous ion increased. α-Tocopherol enrichment of LDL did not inhibit (or increase) the oxidisability of LDL by 2 μM ferrous ion ($FeSO_4$) (Figure3.4). It did, however,inhibit the oxidation of LDL by 5 μM (Figure 3.5) or 20 μM (Figure 3.6) ferrous ion.

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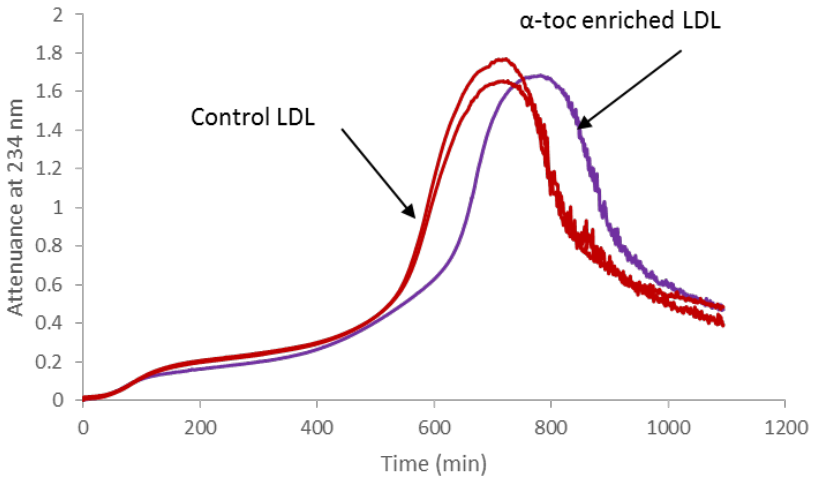

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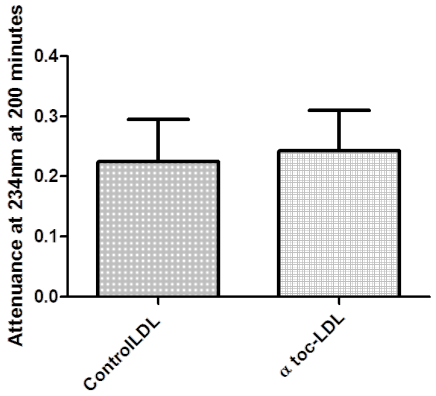

Figure 3. 4. (A) Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol was incubatedin a sodium chloride /sodium acetate buffer (pH 4.5) containing with 2 µM Fe^{2+} at 37 ^{0}C in capped quartz cuvettes. The oxidation was quantified by measuring the change in the attenuance at 234 nm at 200 minutes. (B)bar chart shows the mean ± SEM of four experiments at a time of 200 minutes a time at which substantial oxidation was obtained with the different concentrations of iron used in these experiments. No significant difference was observed in LDL oxidationof the oxidation at 200 min, paired t-test, (n=4 independent experiments).

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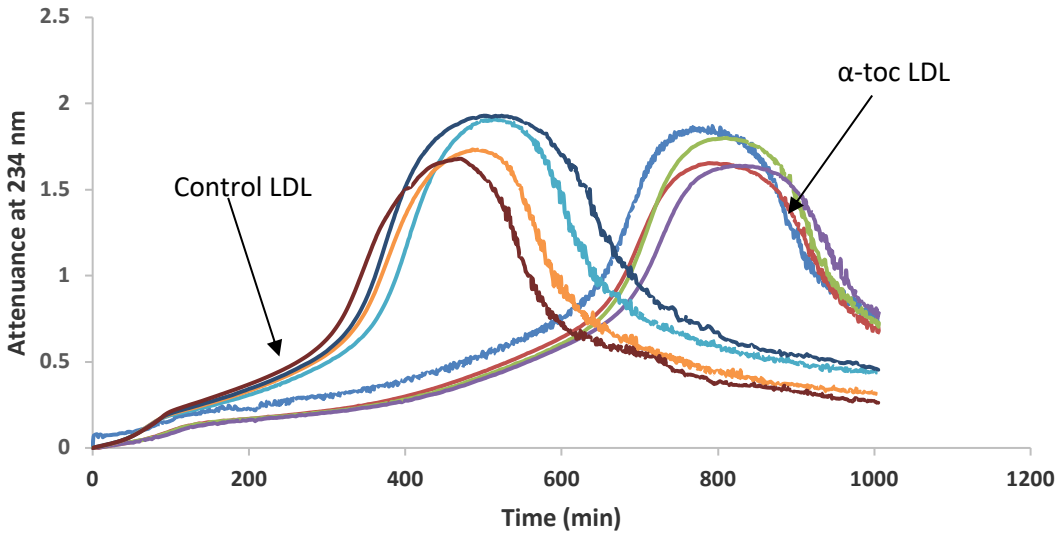

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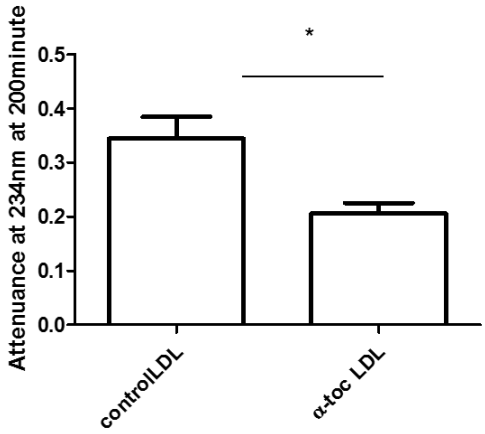

Figure 3. 5 (A)control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in sodium chloride /sodium acetate buffer (pH4.5) incubated with 5 µM Fe^{2+} at 37 ^{0}C in capped quartz cuvettes in quadruplicate. The oxidation was monitored by measuring the change in the attenuance at 234 nm against the appropriate reference cuvettes quadruplicate are shown. (B)Bar chart shows attenuance at 200 min of oxidation (* indicate $p<0.05$, paired t-test, n=4 independents experiments).

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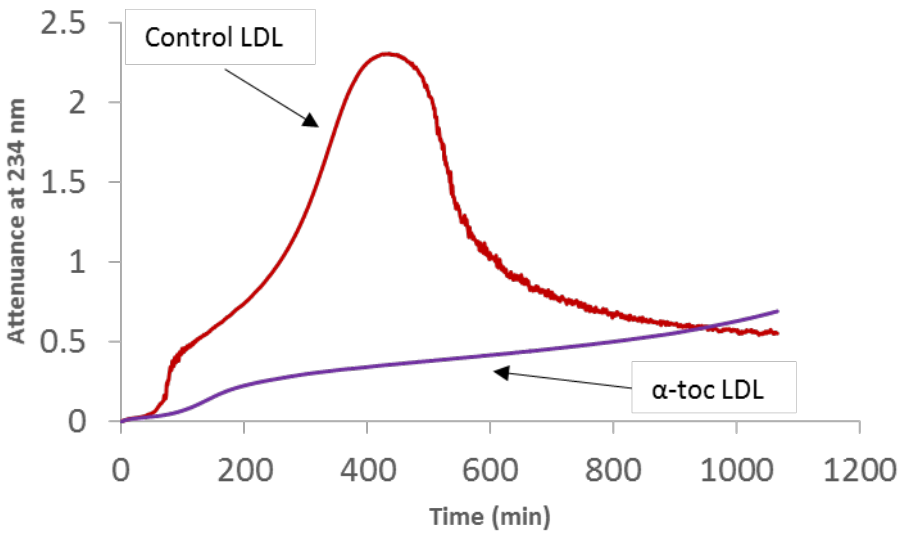

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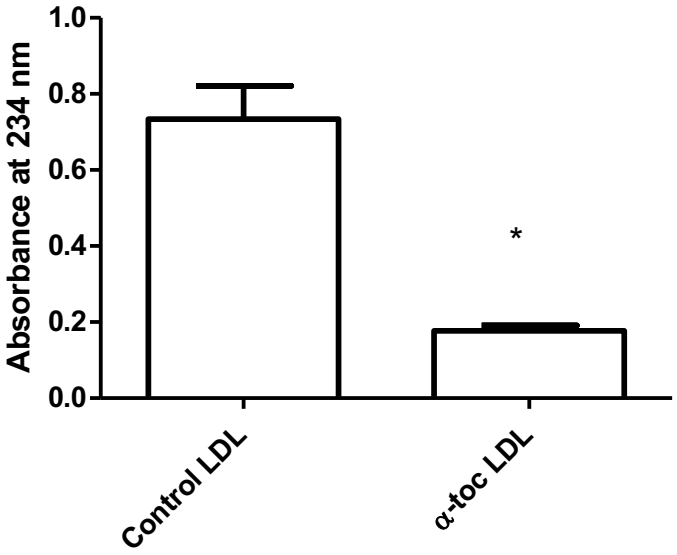

Figure 3. 6. (A) Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in a sodium chloride /sodium acetate buffer (pH4.5) incubated with 20 µM Fe^{2+} at 37 ^{0}C in capped quartz cuvettes. Oxidation was monitored by measuring the change in attenuance at 234 nm against the appropriate reference cuvettes. (B)Bar chart shows decreased LDL enriched with α -tocopherol oxidation at 200 min of oxidation (* indicate $p<0.05$, paired t-test, n=4 independents experiments).

3.3.3.2 Effect of α -tocopherol on LDL oxidation by ferric ion at lysosomal pH.

Lysosomes might contain both ferric (Fe^{3+}) and ferrous (Fe^{2+}) ion (Meguro et al., 2005). As described in previous section. It showed that α -tocopherol did not affect LDL oxidation by a low concentration of ferrous ion (2 µM), but inhibited it by higher concentrations (5 or 20 µM) of ferrous ion. We then investigated the effect of α-tocopherol on LDL oxidation by ferric (Fe^{3+}) ion by measuring the attenuance for control LDL and compare it with LDL enriched with α-tocopherol oxidation by different concentrations of ferric chloride (2 , 5 or 20 µM $FeCl_3$). α-Tocopherol had no effect on LDL oxidation by ferric ion (2 or 5 µM) but increased it at 20 µM (Figures 3. 7, 3. 9). Therefore, α-tocopherol-enrichment of LDL inhibited considerably the oxidation of LDL by 20 µM ferrous ion,but modestly (although statistically) increased by 20 µM ferric ion.

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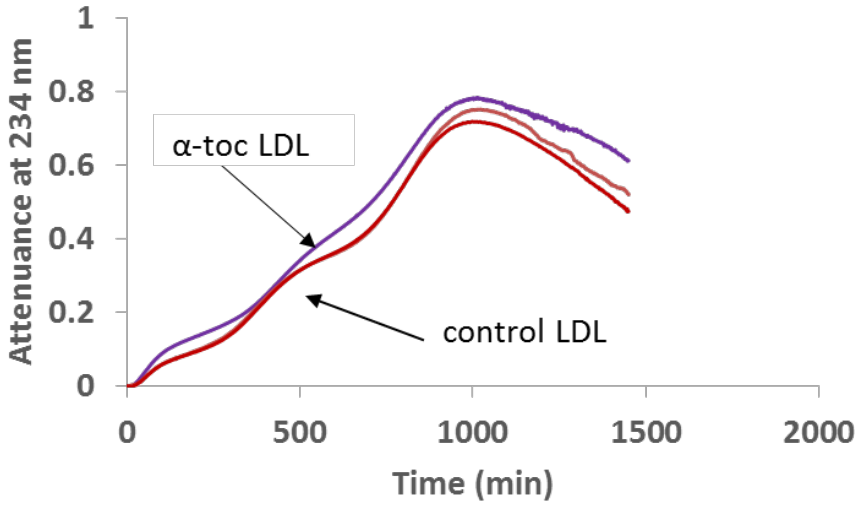

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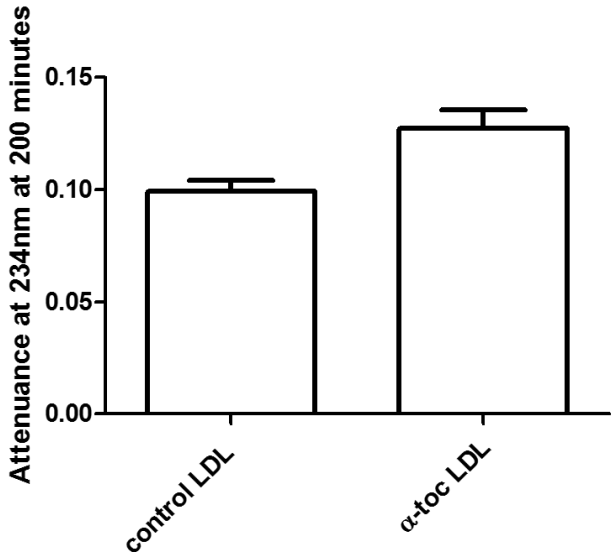

Figure 3. 7 (A)Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in sodium chloride /sodium acetate buffer (pH 4.5) incubated with 2 µM Fe^{3+} at 37 ^{0}C in capped quartz cuvettes. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B)Bar chart shows control LDL and enriched with alpha-tocopherol at 200 min of oxidation (no significant differences, paired t-test, n=4 independents experiments).

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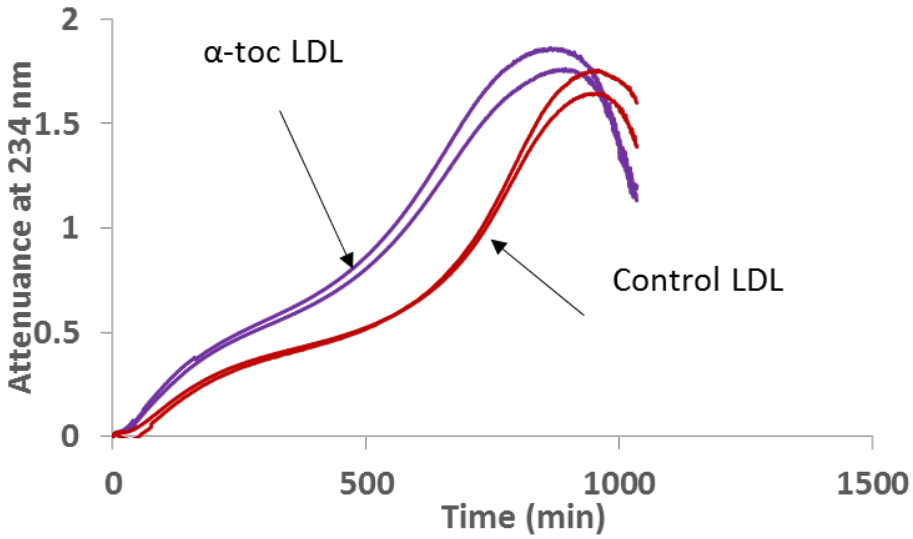

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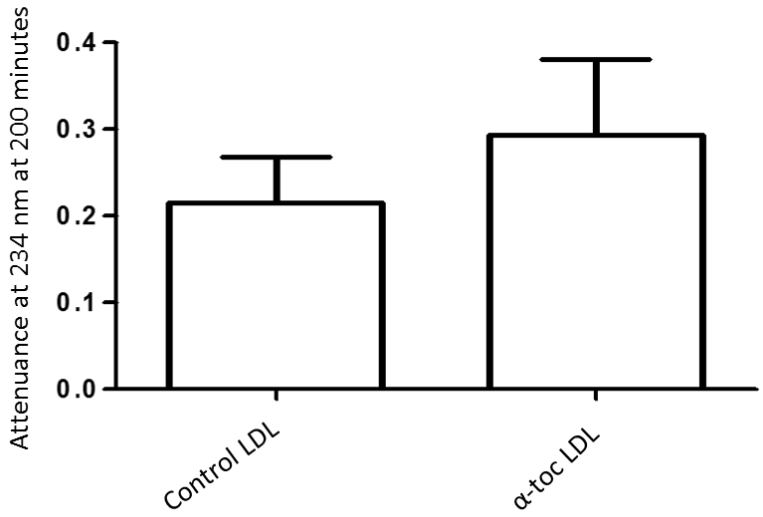

Figure 3. 8 (A) Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in a sodium chloride /sodium acetate buffer (pH4.5) incubated with 5µM Fe^{3+} at 37 ^{0}C in capped quartz cuvettes. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B)Bar chart shows control LDL and enriched with α-tocopherol at 200 min of oxidation (no significant differences, paired t-test, n=4 independents experiments).

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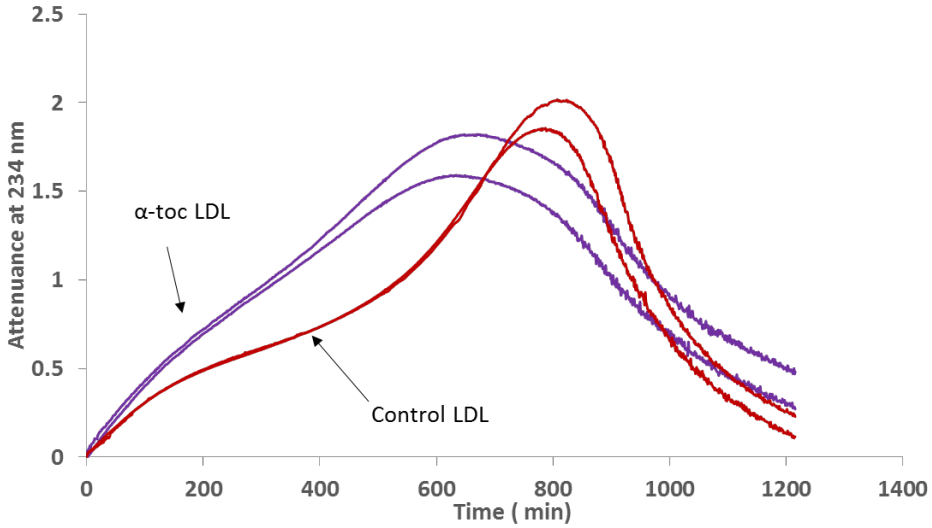

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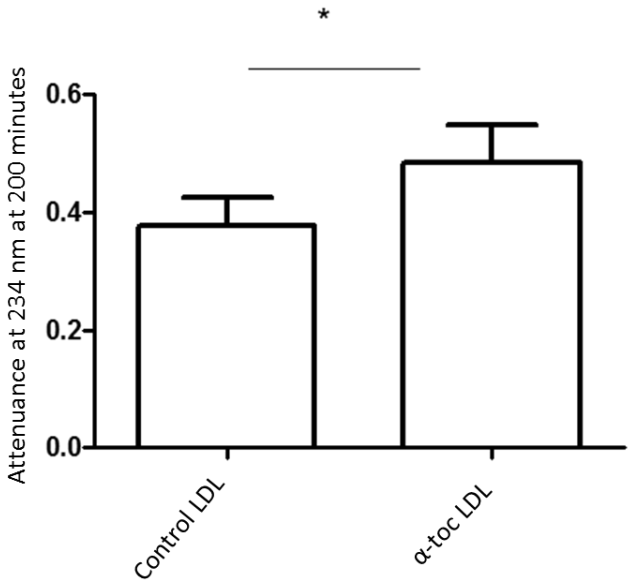

Figure 3. 9 (A)Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in a sodium chloride /sodium acetate buffer (pH4.5) incubated with 20 µM Fe^{3+} at37 ^{0}C in capped quartz cuvettes. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B) Bar chart shows increase LDL enriched with alpha-tocopherol oxidation at 200 min of oxidation (* indicates $p<0.05$, pairedt-test, n=4 independents experiments).

3.3.3.3 Effect of α-tocopherol does not inhibit LDL oxidation by copper at lysosomal pH.

Copper is often used to oxidise LDL *in vitro* studies and it is possible it might have a role in oxidising LDL in lysosomes *in vivo* if copper-containing protein are endocytosed or taken by autophagy. Therefore, in this study we investigated the effect of α-tocopherol on LDL oxidation at pH 4.5 by copper ion . When we used 2, 5 or 20 μM copper, the α -tocopherol had no effect as an antioxidant in diminishing the LDL oxidation (Figure 3. 10, 3. 11 and 3. 12). Similarly to Fe^{2+} or Fe^{3+} at pH4.5, LDL oxidation by Cu^{2+} at pH4.5 showed a short lag phase, a rapid oxidation phase, a slow oxidation phase, an aggregation phase and a sedimentation phase. There was a hint of a pro-oxidant effect ,but it was not statistically significant.

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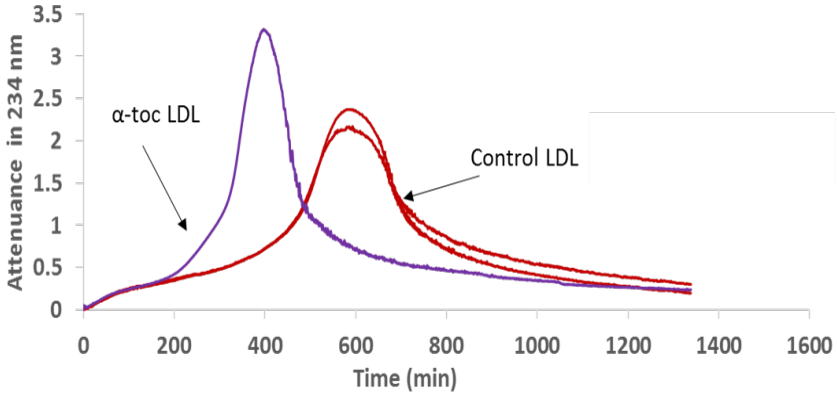

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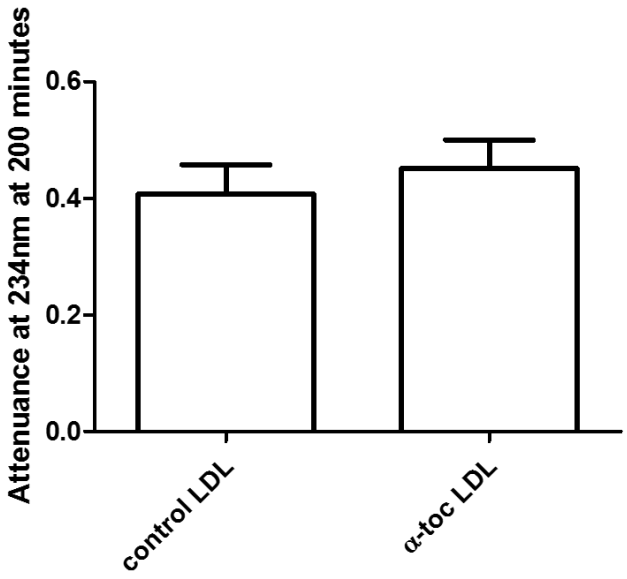
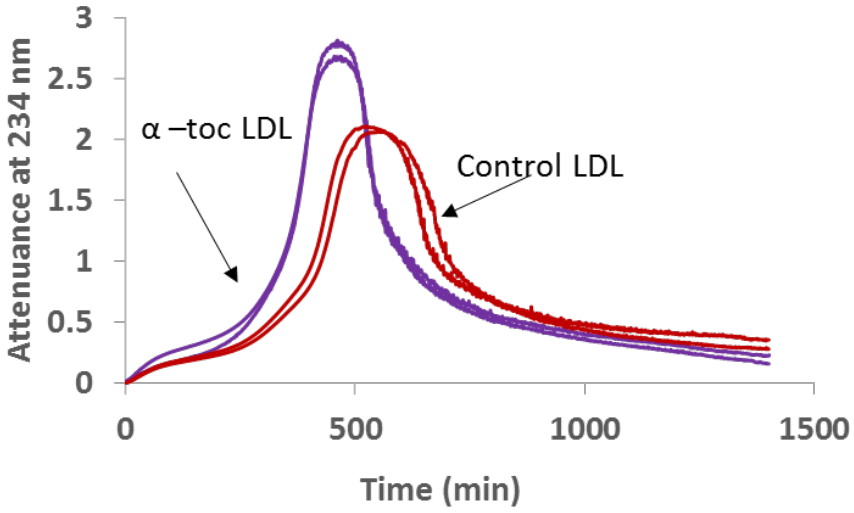


Figure 3. 10. (A) Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in a sodium chloride /sodium acetate buffer pH 4.5 incubated with 2 µM Cu^{2+} at 37 ^{0}C in capped quartz cuvettes. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B) Bar chart shows control LDL and enriched with α-tocopherol at 200 min of oxidation (no significant difference, paired t-test, n=4 independents experiments).

A



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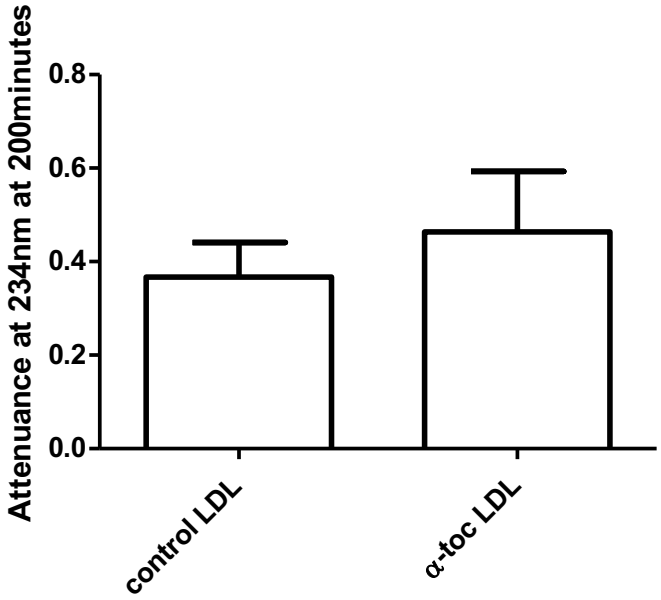
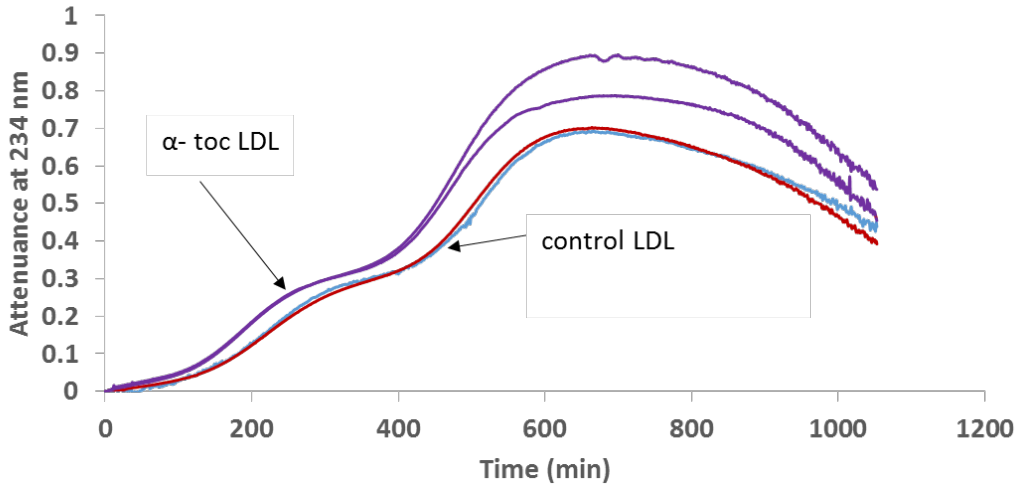


Figure 3. 11. (A)Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in a sodium chloride /sodium acetate buffer (pH 4.5) incubated with 5 µM $CuSO_4$ at 37 ^{0}C in capped quartz cuvettes. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B) Bar chart shows control LDL and enriched with α- tocopherol oxidation at 200 min of oxidation (no significant difference, paired t-test, n=4 independent experiments).

A



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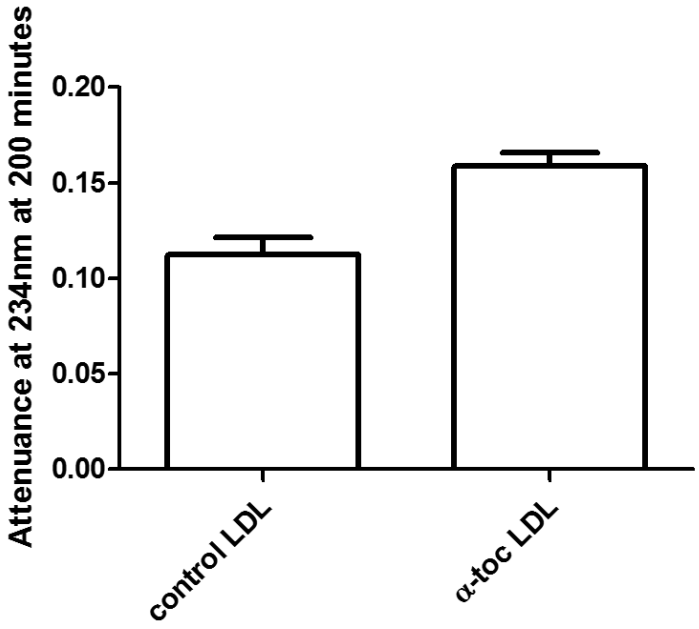
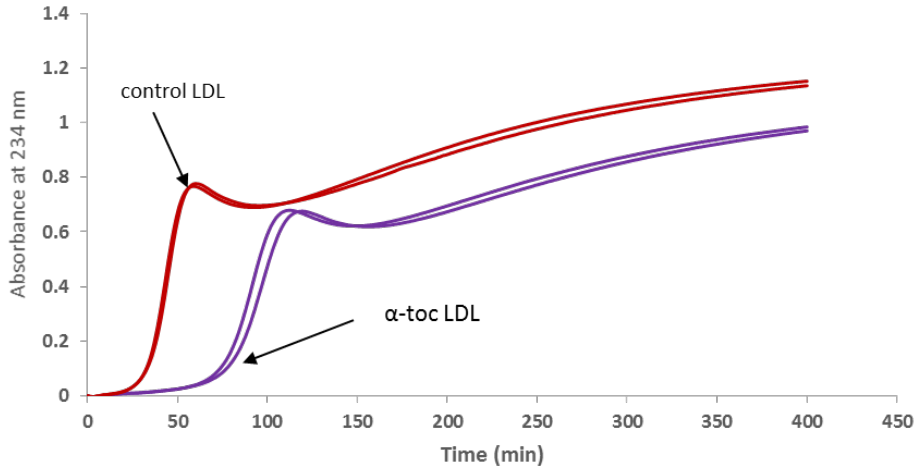


Figure 3. 12. (A) Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in a sodium chloride /sodium acetate buffer (pH 4.5) incubated with 20 µM $CuSO_4$ at 37 ^{0}C in capped quartz cuvettes. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B) Bar chart shows control LDL enriched with α-tocopherol at 200 min of oxidation (no significant difference, paired t-test, n=4 independent experiments).

3.3.3.4 α- Tocopherol inhibit LDL oxidation by copper at pH 7.4

The purpose of the current study is to determine if treatment with α-tocopherol protects LDL from the oxidative effects induced by the transition metal copper at pH 7.4, to compare the action of α- tocopherol at plasma or interstitial pH and lysosomal pH. The results from this compersion might helpe to explain why the large antioxidant trails of α-tocopherol failed to show protection from cardiovascular disease.LDL oxidation at pH 7.4 does not causes aggregate in. The characteristic time sequence of copper-mediated LDL oxidation displays a lag phase, in which conjugated diene absorption shows merely a slight increase, followed by a propagation phase in which 234-nm absorption swiftly increases. In sequence, the 234-nm absorption decreases and is the decomposition phase of lipid hydroperoxides. This is followed by an increase in absorbance due to the formation of lipid decomposion products, which absorbe at 234 nm (Esterbauer et al., 1991). Therefore, the oxidation profile is different from that at pH 4.5. The term absorbances rather than attenuance was used in the figures because there was no aggregation of the LDL and UV scattering at pH 7.4, In marked contrast to Fe^{2+} or Fe^{3+}. LDL oxidation by Cu^{2+} is much faster at pH 7.4 than at pH 4.5. At all copper concentrations (2, 5 and 20 µM), α- tocopherol had a significant influence on reducing the level of LDL oxidation (Figures 3. 13, 3. 14 and 3. 15). α- Tocopherol therefore, as expected,inhibits LDL oxidation at pH 7.4, but does not inhibits LDL oxidation at at pH 4.5.

A



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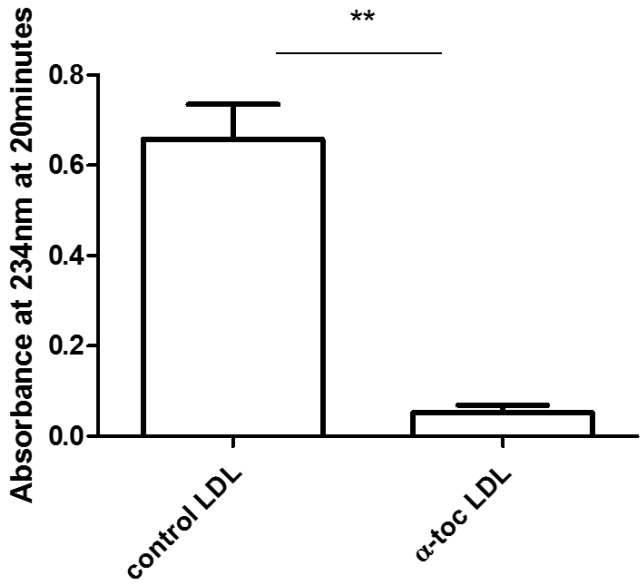
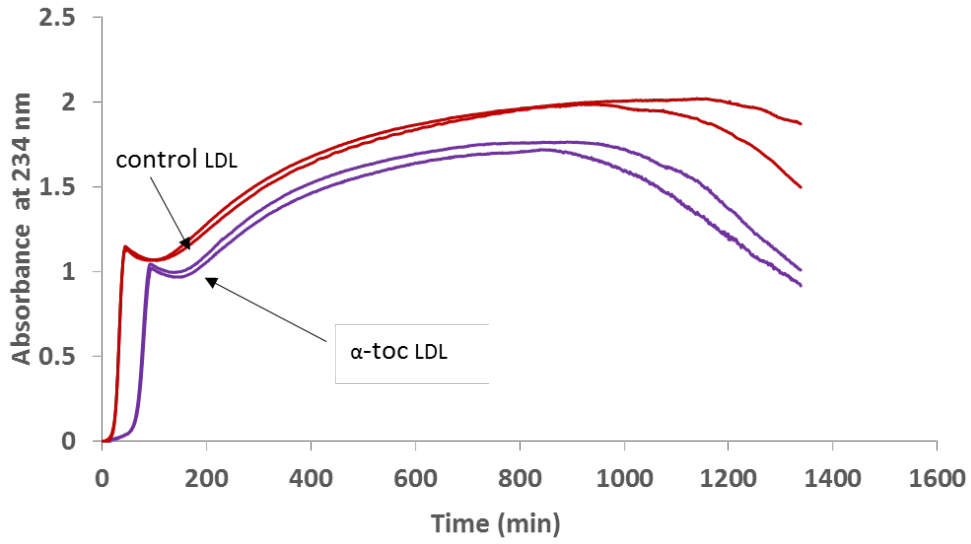


Figure 3.13. (A)Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in a buffer (pH 7.4) incubated with 2 µM $CuSO_4$ at 37 ^{0}C in capped quartz cuvettes. Oxidation was monitored by measuring the change in absorbance at 234 nm. The absorbance at 20 minutes was measured because it corresponds to LDL oxidation before the decomposition phase. (B) Bar chart shows decreased LDL enriched with alpha-tocopherol oxidation at 20 min of oxidation (** indicate $p<0.005$, paired t-test, n=4 independent experiments).

A



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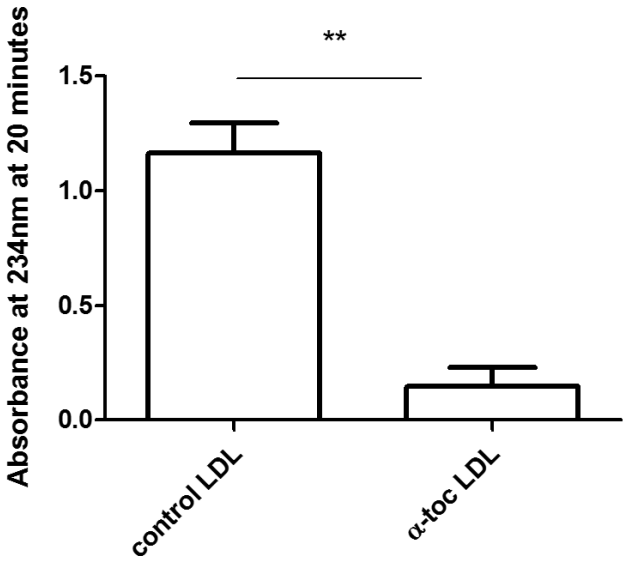

Figure 3. 14. (A) Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in a buffer (pH 7.4) incubated with 5µM $CuSO_4$ at 37 ^{0}C in capped quartz cuvettes. Oxidation was monitored by measuring the change in absorbance at 234 nm. (B) Bar chart shows decreased LDL enriched with alpha-tocopherol oxidation at 20 min of oxidation (** indicate $p<0.005$, paired t-test, n=4 independent experiments).

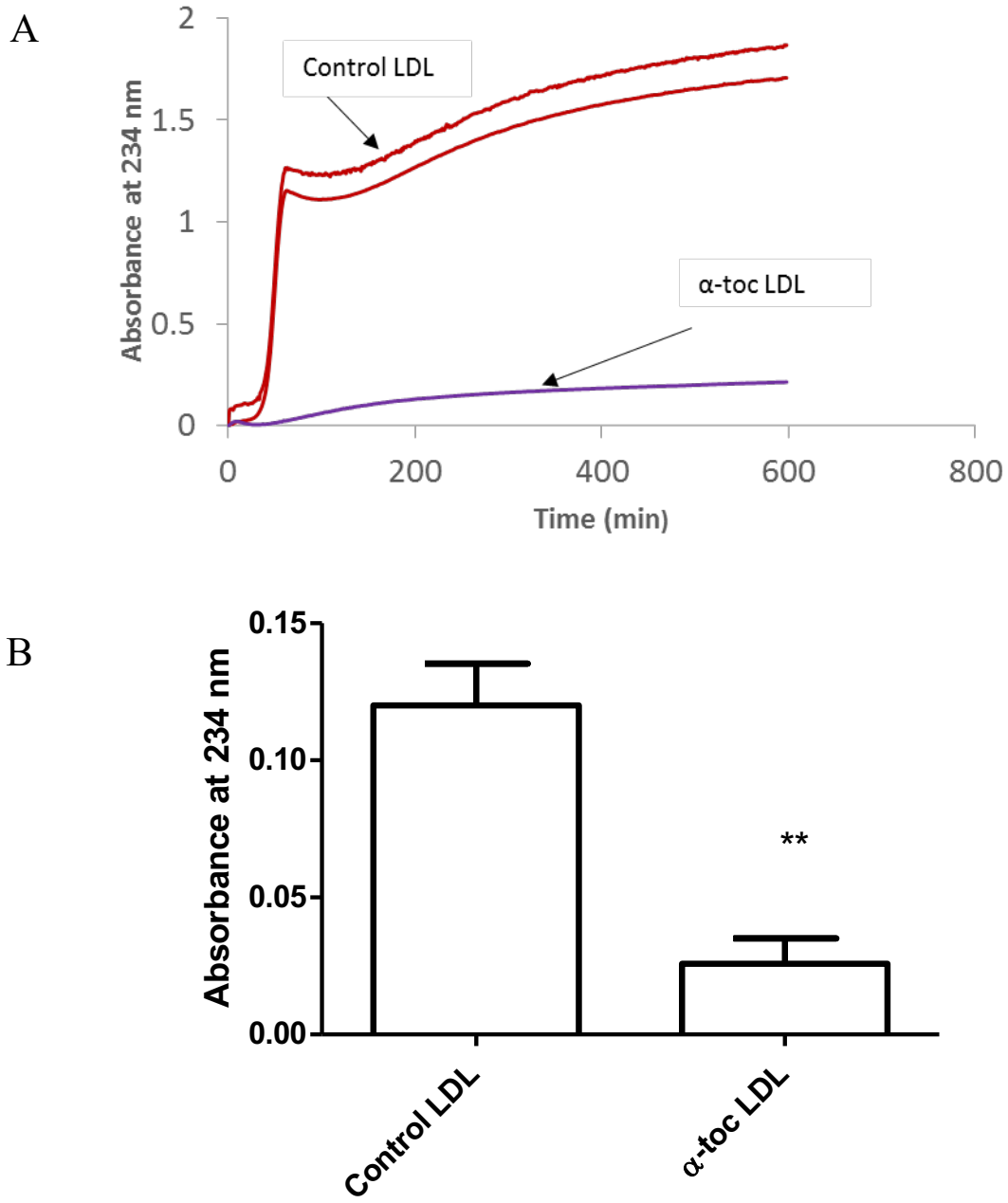

Figure 3. 15.

(A)Control LDL (50 µg LDL protein/ml) and LDL enriched with α-tocopherol in a buffer (pH 7.4) incubated with 20 µM $CuSO_4$ at 37 ^{0}C in capped quartz cuvettes. Oxidation was monitored by measuring the change in absorbance at 234 nm. (B)Bar chart shows decreased LDL enriched with alpha-tocopherol oxidation at 20 min of oxidation (** indicate $p<0.005$, paired t-test, n=4 independent experiments).

3.4 Measurement of ferrous ion levels during LDL oxidation by ferric chloride.

To investigate why α-tocopherol did not inhibit LDL oxidation by ferric ion, in this study we measured the effect of α-tocopherol enrichment of LDL on the rate of conversion of ferric ion to ferrous ion.

The levels of ferrous iron were detected during LDL oxidation by ferric ion using the ferrous iron chelator bathophenanthrolinedisulfonic acid (BP). Control LDL and LDL enriched with α-tocopherol (50 μg LDL protein/ml) was oxidised in NaCl/sodium buffer (pH 4.5) at 37 °C in the presence of ferric ion (FeCl3, 5 μM) in plastic tubes and during the oxidation, samples were collected and measured for the ferrous iron levels using BP (300 μM). During the process of LDL oxidation, all the ferric ions (Fe^{3+}) were converted in the first 30 min (Figure 3.16). The conversion of ferric to ferrous increased faster with LDL enriched with α-tocopherol than control LDL (the concentrations were 5.02± 0.2 μM compared to 2.10± 0.4 μM at 60 min ($P<0.001$). This sharp increase relates to the rapid oxidation phase of LDL. These results show that ferric ion is reduced to ferrous iron during the rapid phase of LDL oxidation and that α-tocopherol is able to reduce ferric ion to ferrous iron and this might help to explain why α-tocopherol did not inhibit LDL oxidation by ferric ions, as discussed in the discussion section.

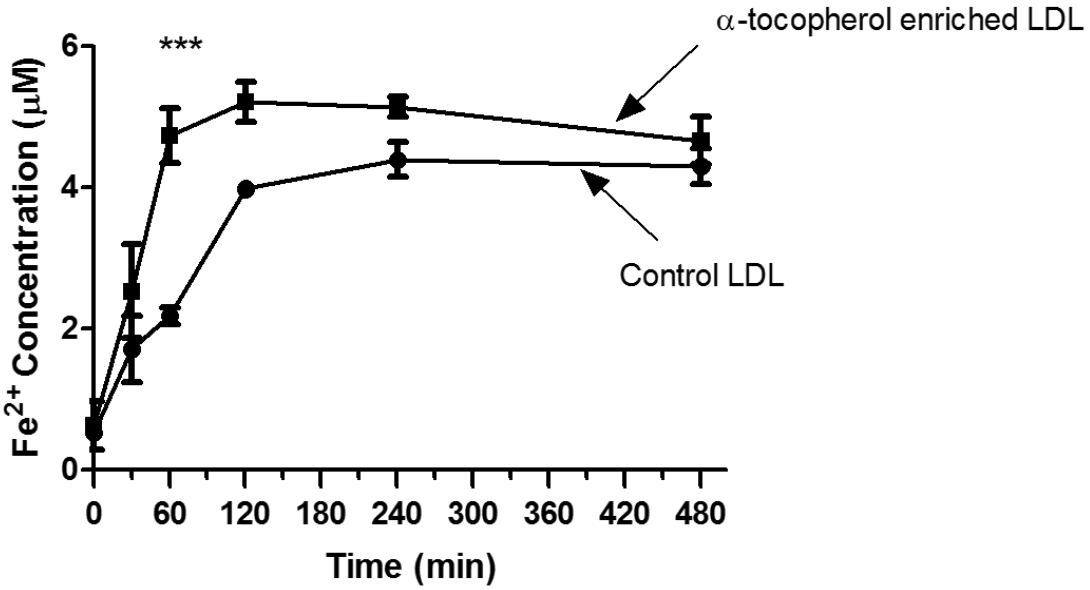

Figure 3.16 Kinetics of ferrous ion formation during LDL oxidation by $FeCl_3$ at pH 4.5

Control LDL and LDL enriched with α-tocopherol (50 μg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) were incubated with 5 μM $FeCl_3$ in plastic tubes at 37 °C. At certain time points, samples were collected, and ferrous ion concentration measured by measuring absorbance at 535 nm after treating with the iron chelator bathophenanthrolinedisulfonic acid (300 μM). (*** indicate $p<0.001$, two-way ANOVA followed by Bonferroni post-test , n=4 independent experiments).

3.5 Measurement of Cu^{+} levels during LDL oxidation by copper at lysosomal pH.

In this study examined if α-tocopherol in LDL could reduce the higher valency state of copper (Cu^{2+}) to the lower valency state of copper (Cu^{+}). The ability to control LDL and LDL supplemented with α- tocopherol to reduce cupric ions (Cu^{2+}) to cuprous ion (Cu^{+}) was measured using chelator bathocuproinedisulfonic acid. Cuprous concentrations increased more rapidly with α- tocopherol-enriched LDL than control LDL (5.0± 0.3Mm compared to 2.30± 0.1 at 120 min (***$p<0.001$) (Figure 3.17). Investigation of the time course of Cu^{2+} reduction in tocopherol enriched and control LDL shows of Cu^{2+} reduction to Cu^{+} at early time points. This suggests a pro-oxidant role for tocopherol under lysosomal conditions of LDL oxidation. As described later, this might help to explain why α- tocopherol did not inhibit LDL oxidation at acidic pH.

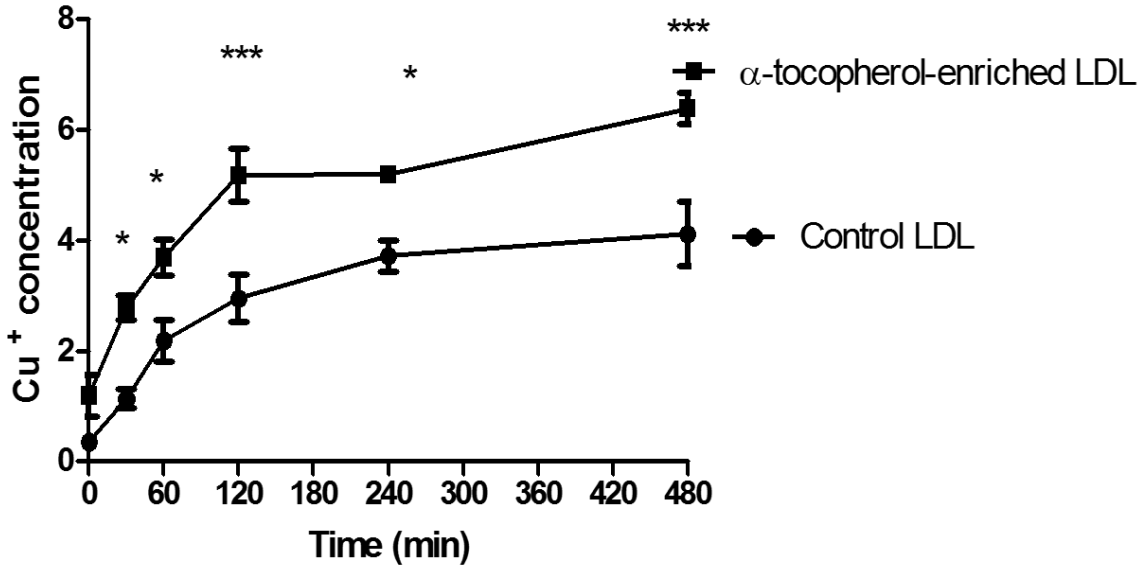

Figure 3.17 Kinetics of cuprous ion levels during LDL oxidation by $CuSO_4$ at pH 4.5

Control and LDL enriched with α- tocopherol (50 µg protein/ml) in NaCl/sodium acetate buffer (pH 4.5) was incubated with 5 µM $CuSO_4$ at plastic tubes maintained at 37 °C. At several time points, samples were taken and Cu^+ ion concentration detected by measuring absorbance at 480 nm after treating with the cuprous copper chelator bathocuproinedisulfonic acid (300 µM). (* indicates $p<0.05$ and *** indicate $p<0.001$, two-way ANOVA followed by Bonferroni post-test. , n=4 independent experiments).

3.6 Monitoring α-tocopherol Content of LDL particles during the oxidation process

α-Tocopherol is known to be consumed when it acts as an antioxidant during LDL oxidation (Proudfoot et al., 1997). Hence, the purpose of the present study was to determine if α-tocopherol is consumed faster when its levels in LDL are increased. Control and LDL supplemented with α-tocopherol were incubated in NaCl/sodium acetate buffer (pH 4.5) with 5 μM $FeSO_4$ and samples were taken at different time points during the oxidation process and were analysed for α-tocopherol by HPLC. There was a significant difference in α-tocopherol levels between control LDL and LDL enriched with α-tocopherol, with an approximately two-fold enrichment (24.3± 0.3 nmol/mg LDL protein (Figure 3.18) compared to 14.4±0.2 nmol/mg LDL protein (***$p<0.001$). This corresponds to about 12 and 7 molecules of α-tocopherol per particle of LDL. The level of α-tocopherol in α-tocopherol- enriched LDL decreased faster during LDL oxidation by 5 μM $FeSO_4$. This might explain why α-tocopherol-enriched LDL converted Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^{+} faster.

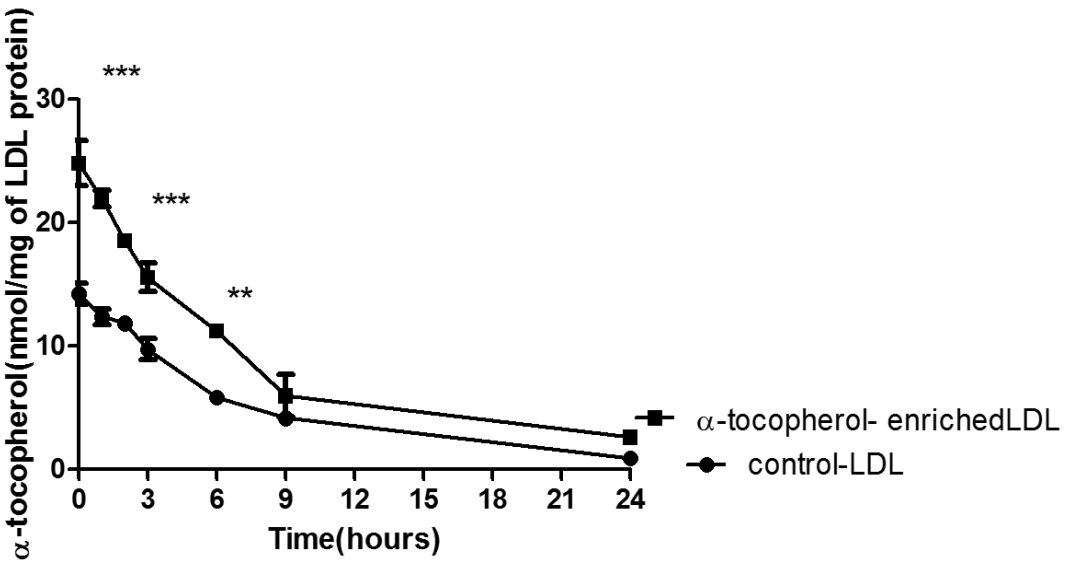

Figure 3.18. The depletion of α-tocopherol in LDL during oxidation.

Control and LDL enriched with α- tocopherol (50 µg protein/ml) was incubated in NaCl/sodium acetate buffer (pH 4.5) with 5 µM $FeSO_4$ in plastic tubes maintained at 37 °C. α- Tocopherol content in LDL was measured periodically. (** indicate $p<0.005$ and *** indicate $p<0.001$, two-way ANOVA followed by Bonferroni post-test , n=5 independentexperiments).

3.7 Total lipid hydroperoxides in oxidised LDL

A spectrophotometric technique was used for the measurement of lipid hydroperoxides, which depends on the capability of lipid hydroperoxides to oxidize iodide (I^-) to iodine (I_2), which reacts with unreacted iodide (I^-) to form the triiodide anion (I_3^-). The aim of this study to investigate the role of α-tocopherol as an antioxidant to reduce the formation of total lipid hydroperoxide.

Total lipid hydroperoxide levels were measured by a tri-iodide assay in control LDL and LDL enriched with α-tocopherol oxidised by 5 μM $FeSO_4$ at pH 4.5. There was a modest, but statistically significant,decrease in hydroperoxide formation by α-tocopherol (Figure 3.19). This result agreed with the conjugated diene results (Figure 3.5).

The levels of hydroperoxides at 24 h were consistent with those of fully oxidised LDL (Satchell and Leake, 2012).

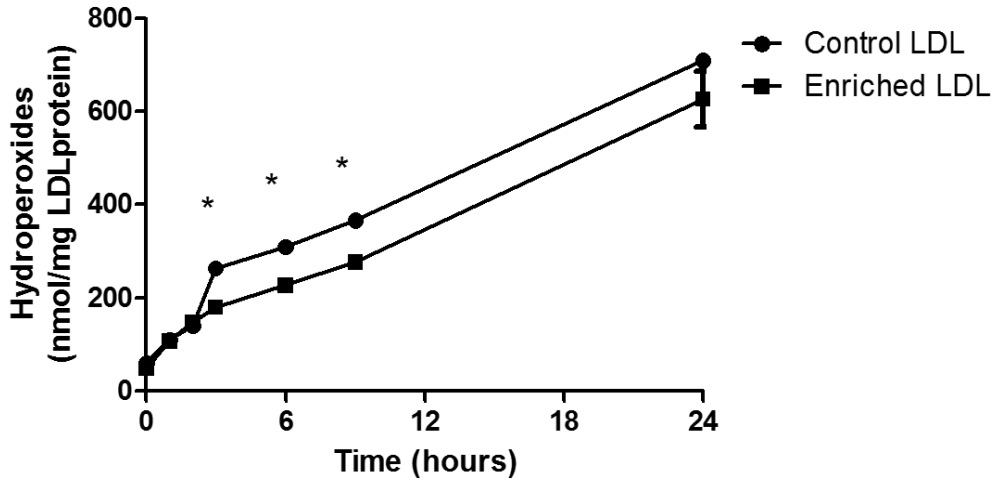

Figure 3.19 Effect of enrichment of LDL with α- tocopherol on total lipid hydroperoxides during LDL oxidatio by 5 µM $FeSO_4$. Control LDL and LDL enriched with α- tocopherol was oxidised by 5 µM $FeSO_4$ at 37 ^{o}C at pH 4.5 and then evaluated for the presence of lipid hydroperoxides by a tri-iodide assay at different time points. (*indicate $p<0.05$, two-way ANOVA followed by Bonferroni post-test,n=5 independent experiments).

3.8 HPLC analysis of oxidised lipids in LDL

To investigate the effect of α-tocopherol on LDL oxidation at lysosomal pH in more detail, in this study I examined the level of individual of oxidised lipids ,such as 7-ketocholesterol and cholesteryl linoleate hydroperoxide (CLOOH), which absorb UV radiation at 234 nm by using reverse-phase HPLC

Control LDL and LDL enriched with α-tocopherol (50 μg LDL protein/ml) were oxidised in sodium chloride/sodium buffer (pH 4.5) at 37 °C by 5 μM $FeSO_4$. Samples were taken at several times and were treated for HPLC analysis as declared in detail in the Methods section in chapter 2.

Throughout LDL oxidation by iron, there was continuous production of 7-ketocholesterol which had an increased rate of production in α-tocopherol-enriched LDL (Figure 3.20). The levels of CLOOH decreased after 12 h either because the hydroperoxide groups were degraded (for instance into an alkoxyl or peroxy radical) or another part of the CLOOH molecules become oxidised and the molecules elute from the HPLC column at a different time. The 7-ketocholesterol increased at a lower rate than the oxidisation of the cholesteryl linoleate fatty acyl group, having just one double bound instead of two. The levels of CLOOH and 7-ketocholesterol observed were compatible with previous result (Satchell and Leake, 2012)

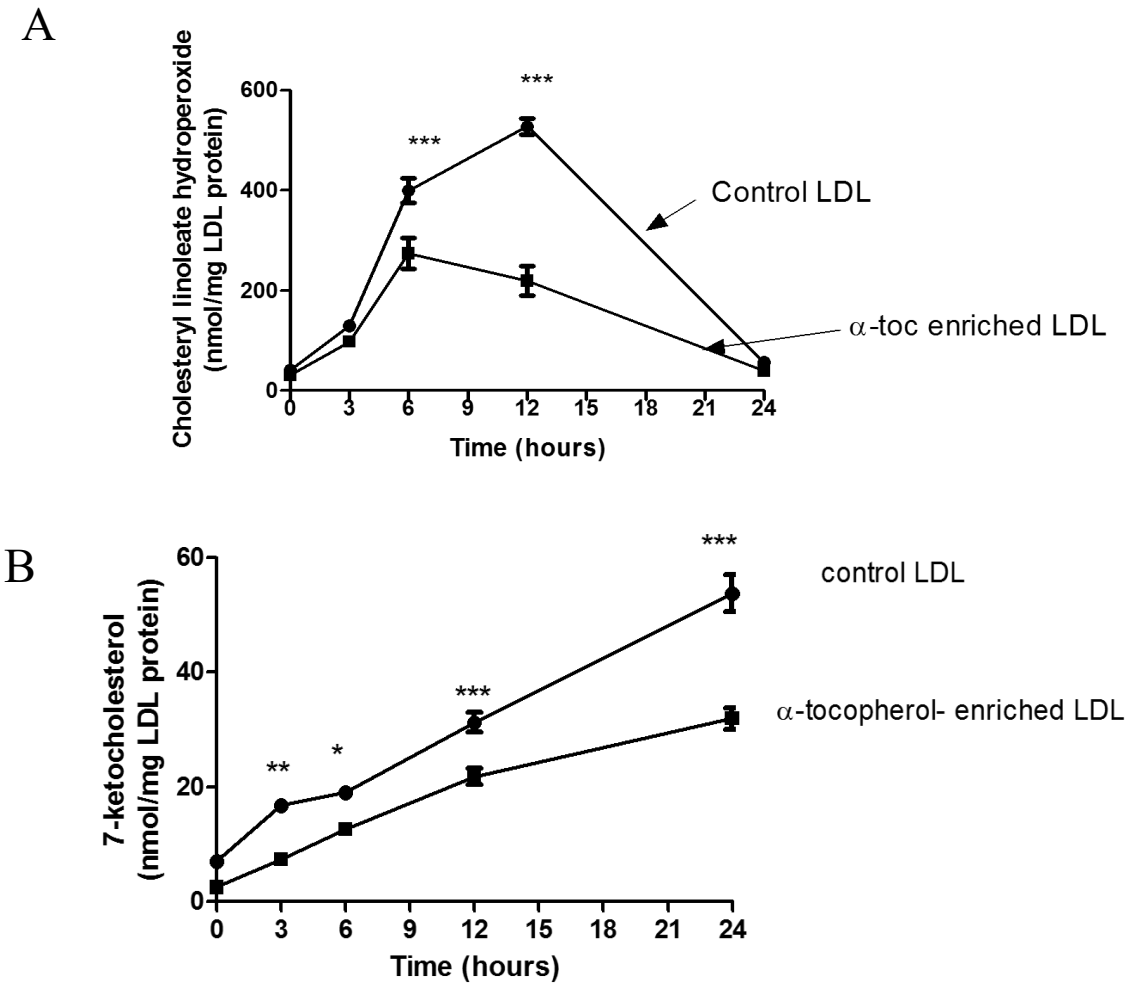

Figure 3.20. Effect of α- tocopherol enrichment on LDL oxidation products during oxidation by $FeSO_4$ (5 μM) at pH 4.5.

Control LDL and LDL enriched with α- tocopherol (50 μg LDL protein/ml) were oxidised with freshly dissolved $FeSO_4$ (5 μM) at 37 °C in a sodium chloride/sodium acetate buffer of pH 4.5. At several times until 24 hours, oxidation was blocked by adding EDTA (final concentration 4mM) and BHT (80 μM, form stock 2mM in ethanol). The samples were then assayed for cholesteryl linoleate hydroperoxides (A) and 7-ketocholesterol (B) by reverse-phase HPLC. (* indicate $p<0.05$, ** indicate $p<0.005$ *** indicates $p<0.001$, n=4 two-way ANOVA followed by Bonferroni post-test) compared with the control LDL.

3.9 Discussion

This chaper shows for the first time that α-tocopherol is less effective at inhibitory LDL oxidation at lysosomal pH then at pH 7.4.

Iron is a candidate for oxidising LDL *in vivo*. Animal investigations have presented a positive correlation between iron localised in plaques and atherosclerosis (Lee et al., 1999). Human atherosclerotic lesions contain iron and are capable of stimulating LDL oxidation by macrophages (Lamb et al., 1995). In addition to this, iron has been noticed *in vivo* at higher levels in atherosclerotic lesions compared with the normal artery (Rajendran et al., 2007). Redox-active iron has been documented to exist in lysosomes (Yu et al., 2003, Petrat et al., 2001) ,possibly because of processes, such as the breakdown of ferritin (Sibille et al., 1989b, Radisky and Kaplan, 1998),the degradation of iron-containing organelles (Persson et al., 2001) and the phagocytosis of erythrocytes (Yuan et al., 1996).

Consistent with experiments carried out earlier in the Leake laboratory (Wen and Leake, 2007), LDL is oxidised successfully by iron at lysosomal pH 4.5 (Figure 3.1) but ineffectively oxidised at the pH of blood plasma or interstitial fluid (pH 7.4). It is likely that acidity may increase the solubility of iron permitting it to oxidise LDL more effectively at low pH (Morgan and Leake, 1995) or convert superoxide radicals into the much more reactive hydroperoxyl radicals (Ahmed & Leake,2018). Extracellular fluids have the high antioxidant ability, with just a low percentage of serum or interstitial fluid greatly inhibiting the ability of metals to oxidise LDL (Leake and Rankin, 1990). LDL oxidation by iron inside lysosomes may reconcile the suggestion for iron as oxidising an agent *in vivo*, with the data for an overwhelming antioxidant defence is the interstitial fluid of the inner layer of arteries.

LDL oxidation with ferrous iron at pH 4.5 progresses with a particular pattern including a lag, swift oxidation, slow oxidation, and aggregation and sedimentation phases. This pattern of oxidation with ferrous iron was quite similar to that with ferric iron but was faster (Figure 3.6). Iron may be capable to start the oxidation of LDL by reacting with existing lipid hydroperoxides in the LDL particle, as follows

$Fe^{2+} + LOOH \longrightarrow Fe^{3+} + OH^- + LO\bullet$ (Fast)

$Fe^{3+} + LOOH \longrightarrow Fe^{2+} + LOO\bullet + H^+$ (Slow)

$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O^-_2\bullet$

$O^-_2\bullet + H \longrightarrow HO_2\bullet$

$HO_2\bullet + LH \longrightarrow H_2O_2 + L\bullet$

$L\bullet + O_2 \longrightarrow LOO\bullet$

$HO_2\bullet$ is much more reactive than $O^-_2\bullet$ (Bedwell et al., 1989) and can dissolve in the lipids of LDL.

The reaction of ferrous iron with lipid hydroperoxides is quicker than the reaction of ferric ions and this may help to explain why the oxidation of LDL is slower with ferric iron. Ferrous ion also gives an electron to oxygen, forming the superoxide anion. The rapid phase might be due to the cholesteryl ester core oxidising and the slow phase might be due to the phospholipid monolayer oxidising (Ahmed & Leake, 2018).

Redox-active iron is present in lysosomes of macrophages and foam cells in atherosclerotic lesions (Stadler et al., 2004, Yuan et al., 1996). In this study, it has been shown that the addition of different concentrations of EDTA (1, 2, 5, and 10 µM) inhibited LDL oxidation by 5 µM iron at pH 4.5 in a concentration-dependent manner (Figure 3.2), LDL oxidation proceeded at a similar rate with 10 µM iron plus 5 µM EDTA as at 5 µM iron ion, indicating that EDTA molecules bind to the iron atom at 1:1 ratio (Figure 3.3). Extra iron can, therefore,

be added equimolar with the EDTA carried over from the LDL preparation to allow for the different EDTA concentrations carried over from individual LDL preparation. This should improve the reproducibility of experiments. It was important to establish this as EDTA can sometimes have pro-oxidant as well as antioxidant properties (Lamb and Leake, 1992).

α-Tocopherol is the most important lipid-soluble antioxidant in LDL, with typically six molecules per LDL particle (Esterbauer et al., 1991a). Previous experiments have shown that enrichment of LDL with α -tocopherol inhibits LDL oxidation by macrophages (Jessup et al., 1990) or copper ion at pH 7.4 (Esterbauer et al., 1991a). It was hypothesised that supplementation with α-tocopherol would diminish the incidence of cardiovascular disease, but, large clinical trials have found no protective effect of α-tocopherol supplementation (Yusuf et al., 2000, de Gaetano, 2001, 2002).

We approximately doubled the α-tocopherol content of LDL (Figure 3.18) by adding α-tocopherol to plasma, incubating and isolating the LDL by ultracentrifugation (Esterbauer et al., 1991b). In the presence of oxidative stress by copper (2,5 or 20 μM) at pH7.4, the rate of the oxidation of α-tocopherol-enriched LDL was lengthier than with non-enriched LDL (Figure 3.15), as expected, while when α-tocopherol -enriched LDL was oxidised by ferrous and ferric ion at pH 4.5, there was no inhibition of LDL oxidation (Figure 3.7- 3.9). α-Tocopherol acts as an antioxidant, particularly by scavenging lipid peroxyl radicals. The α-tocopherol radical is then able to react with a further radical to create a non-radical product. In a mildly oxidising environment, it is less expected that the α-tocopherol radical will encounter another radical within the relatively small LDL particle (about 22 nm in diameter) and so it may react with a polyunsaturated fatty acid to form a lipid alkyl radical instead, a process named tocopherol-mediated peroxidation (Upston et al., 1999).

α-tocopherol-OH + LOO• ⟶ α-tocopherol –O• +LOOH

α-tocopherol –O• + R• ⟶ Non-radical product

α-tocopherol –O• + LH ⟶ α-tocopherol –OH + L•

L• + O_2 ⟶ LOO•

α-Tocopherol had an antioxidant effect with 5 and 20 µM Fe^{2+} (Figure 3.5and 3.6) but not with 2µM Fe^{2+} at pH 4.5 (Figure 3.4).

This finding is in contrast to previous reports (Lynch and Frei, 1995, Schnitzer et al., 1997) suggesting that the α-tocopherol content of LDL may not be rate-limiting in Cu (II) reduction. α-Tocopherol enrichment increased the rate of Cu (II) reduction to Cu (I) by LDL at pH4.5 of (Figure 3.17). Bathocuproine was not present throughout the incubations but was added just before samples were taken for Cu (I) measurement. Bathocuproine was therefore not giving artefactual results by pulling the equilibrium over in fevour of Cu^{+} in the reaction below.

$Cu^{2+\cdot}$ + α -toc-OH ⟶ $Cu^{+\cdot}$ + α -toc-O• + H^{+}

LDL oxidation by high copper concentrations was calculated by kinetic simulation by Abuja et al. (Abuja et al., 1997). According to this simulation, the following reactions determine the kinetics of initiation:

Cu^{2+}(or Fe^{3+} + α-toc-OH ⟶ Cu^{+}(or Fe^{2+}) +α -tocO• + H^{+}

α -toc-O• + LH ⟶ α -toc-OH + L•

L• +O2 ⟶ LOO•

Cu^{2+} (or Fe^{3+}) + LOOH ⟶ Cu + (or Fe^{2+}) LOO• + H^{+}

Cu^{+} (or Fe^{2+}) + LOOH ⟶ Cu^{2+} (or Fe^{3+}) + LO + OH^{-}

I was able to show that increasing the α-tocopherol content of LDL increased the rate of Cu^{2+} reduction to Cu^{+} (Figure 3.17) and Fe^{3+} reduction to Fe^{2+} (Figure 3.16). LDL oxidation by copper at pH 4.5 was much slower than at pH 7.4 (in marked contrast to what was observed with iron). The reason for this is unclear, but there might be a mechanism by which LDL becomes oxidised at pH 7.4 that does not operate at pH 4.5, e.g. Involving the oxidation of tryptophan,upsteire on methionine residues by copper. Giessauf et al, found that 8 or 9 of the 37 trytophan residues in apo B-100 seem to be vulnerable to chelation with Cu^{2+} leading to a rapid decrease in tryptophan fluorescence (Giessauf et al., 1995). The rate of LDL oxidation by Cu^{2+} at pH 4.5 was not increased as the concentration of Cu^{2+} increased (Figure 3.10-3.12). In a previous study it was observed that the rate of LDL oxidation at pH 4.5 by ferric ions (Fe^{3+}) was not increased by increasing the concentration of Fe^{3+} (whereas it was by increasing the concentration of Fe^{2+}) (Satchell and Leake, 2012).

In marked contrast to the antioxidant effect of α-tocopherol on LDL oxidation by Cu^{2+} at pH 7.4, α-tocopherol did not inhibit LDL oxidation by Cu^{2+} (2, 5 or 20 μM) (Figure 3.10-3.12) at pH 4.5. The reason for this is unknown, but it might be speculated that the rapid mechanism for LDL oxidation by Cu^{2+} at pH 7.4 is inhibited by α-tocopherol, but the slower oxidation at pH 4.5 is not inhibited by α-tocopherol. α-Tocopherol has both antioxidant and pro-oxidant effects and the net balance of these effect on LDL oxidation depends on the nature of the metal ions, its concentration and the pH. One possibility is that the hydroperoxyl radical at pH 4.5 might convert α-tocopherol into a pro-oxidant

α -toc-OH + $HO^{\bullet}_2$ ⟶ α -toc-O• + H_2O_2

α -toc-O• + LH ⟶ α -to-OH + L•

α-Tocopherol lost its antioxidant effect on LDL with Cu^{2+} at pH 4.5 and did not inhibit LDL oxidation by Fe^{3+} at pH 4.5 or 2μM Fe^{2+} at pH 4.5, although it did inhibit with 5 or 20μM Fe^{2+}

at pH 4.5. This might help to explain why the large clinical trials of α-tocopherol supplementation did not decrease cardiovascular disease if the oxidation of LDL occurs mainly in the lysosomes, which have an acidic pH. Since LDL preparations frequently have small amount of pre-formed lipid hydroperoxides, the breakdown of these to alkoxyl and peroxyl radicals would appear a feaisble place to start when addressing mechansim of copper-induced LDL oxidation. It is largely accepted that α-tocopherol can quicken this oxidation process *in vitro* by converting Cu (II) to Cu (I), which can then convert the hydroperoxides to alkoxyl radicals. The concentration of Cu (I) started to increase significantly from 30 minuts and plateaued at about 480 minutes for both control and α-tocopherol enriched LDL. α-Tocopherol accelerated the conversion Cu (II) to Cu (I) (Figure 3.17),. These findings suggest that the pro-oxidant effect of α-tocopherol enriched LDL might be due to the conversion of Cu^{2+} to more reactive Cu^{+} . This study provides new information on the weaker antioxidant effects of α-tocopherol at lysosomal pH then at pH7.4. If LDL oxidation in atherosclerotic lesions occurs mainly in lysosomes, rather than in the interstitial fluid,it might helps to explain why the large antioxidant trails of α-tocopherol failed to provide protection against cardiovascular disease.

Chapter 4

Effect of tempol and probucol on low density lipoprotein oxidation at lysosomal pH

4.1Introduction

4-Hydroxy-2, 2, 6, 6-tetramethylpiperidine-1-oxyl (Tempol) is a membrane-permeable aminoxyl-type free radical scavenger, with antioxidant properties. Tempol is a steady piperidine nitroxide, free radical with low molecular weight, which infiltrates biological membranes and scavenges superoxide anions in vitro (Araki et al., 2006). This complex shows a superoxide dismutase mimetic action to diminish superoxide radicals. The beneficial effect of aminoxyl action is partly due to prevent the results of Fenton's reaction ($Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^{\bullet -}$) induced by Fe^{2+} ions (Soule et al., 2007a). Preclinical studies, with a variety of rat models, have shown the possibility for numerous clinical uses, including regulation of hypertension (possibly by increasing the availability of nitric oxide), weight gain, defence from ionising radiation and DNA damage, and inhibition of doxorubicin treatment-related cardiomyopathy (Bartha et al., 2011). To date, most of the considerations have been paid to the capacities of nitroxides to lower superoxide and peroxide, (Krishna et al., 1996a) prevent the effects of Fenton reactions (Mitchell et al., 1990b) and undergo radical–radical reorganisations (Soule et al., 2007a) particularly for carbon-centered radicals. Tempol which has been demonstrated to modify sensitivity to radioactivity, tumourigenesis and diet-stimulated body mass gain (Mitchell et al., 2003). Tempol (at 10 mg/g of food) has inhibitory properties on body weight increases in C3H mice and inhibits obesity-induced alterations in leptin levels. Leptin is a hormone manufactured from white adipocytes, which binds to its on receptors in the hypothalamus to control food consumption and energy expenditure. Alterations in leptin and its receptor lead to genetic obesity in rodents and humans (Mitchell et al., 2003, Phillips et al., 1996). Leptin can encourage the production of the effective vasoconstrictor, endothelin, and intensification of the expression of its receptors in both

endothelial and vascular smooth muscle cells (Chao et al., 2007, Juan et al., 2008). Tempol enhanced NO signalling by increasing cGMP levels in models of oxidative stress (Wilcox and Pearlman, 2008). Similar influence on mass increase has recently been described for mice on a high-fat diet (Li et al., 2013). Tempol has also been documented to decrease production of reactive oxygen species in diabetes (Frances et al., 2011) and hypertension (Wilcox and Pearlman, 2008, Schnackenberg et al., 1998), with the latter due to increased nitric oxide ($NO^{\cdot}$) production by endothelial cells (Wilcox and Pearlman, 2008) and resulting vasodilatation (Patel et al., 2006). Tempol also protects the endothelium in spontaneously hypertensive rats and small arteries from the effect of visceral lipid in obese subjects (Patel et al., 2006). Nitroxides have also been suggested to modify atherosclerotic lesions and vascular inflammation by decreasing vascular adhesion molecule-1 (VCAM-1) and by diminishing the amounts of particular NADPH oxidase-2 (NOX2) subunits (Cannizzo et al., 2014). Tempol has been shown to inhibit obesity-stimulated alterations in adipokines and to increase plaque collagen and macrophage numbers, and to decrease lipid levels (Kim et al., 2015). Tempol was used as a redox probe (Tada et al., 2013). Nitroxyl radicals (nitroxides) such as 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (hydroxy-TEMPO, Tempol) have been widely reported to have protective properties against oxidative stress and degenerative illnesses in cells and in animals (Soule et al., 2007b, Yamada et al., 2006). These properties depend on their reaction with reactive oxygen species (ROS) (Krishna et al., 1996a, Krishna et al., 1996b, Takeshita et al., 2002). The antioxidant reactions of tempol have been investigated in detail as superoxide dismutase (SOD) mimics (Krishna et al., 1996a) and for their catalase-like ability (Krishna et al., 1996b). However, the swift transformation of nitroxyl to hydroxylamine in vivo requires the use of a high amount of nitroxyl to understand its therapeutic properties. The main cause of transformation of nitroxyl to hydroxylamine is the

one-electron reaction by several types of reductants, for instance, ascorbic acid (AsA). Both enzymatic and non-enzymatic antioxidants work in a network way to employ their protective effects, no single antioxidant could provide the whole antioxidant properties in plasma. Hence, plasma antioxidant status is the result of communication and cooperation of various antioxidants. The idea of total antioxidant capacity (TAC) was established considering the synergistic role of those antioxidants rather than the simple amounts of individual antioxidants (Wang et al., 2013, Gaziano, 2004).

This study have investigated the effect of both α-tocopherol and tempol alone and in combination on LDL oxidation. Combination treatment was used because antioxidants can complement each others action.

Probucol is a clinically used cholesterol-lowering drug, Probucol has also been used as a pharmaceutical agent to treat a variety of infections, traumas, and pathologic conditions, including, lowering plasma cholesterol levels in hypercholesterolaemia patients (Zimetbaum 1990). Probucol is a lipid-soluble, bis-tertiary butyl-phenol capable of giving one of the hydrogens from its phenolic-OH groups and consequently performing 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 (Mao et al., 1991, Jackson et al., 1991). Then, 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 oxidative stress (Maiolino et al., 2013). LDL oxidation is inhibited by probucol (Parthasarathy et al., 1986, Jackson et al., 1991, Leake and Rankin, 1990). Probucol inhibits atherosclerosis in most animal models (Carew et al., 1987, Sasahara et al.,

1994).Probucol did not, however, protect against human femoral artery atherosclerosis in the PQRST trail (Walldius et al., 1994).

4.2 Material and Methods

LDL was enriched with α-tocopherol, as described in the methods chapter. Its oxidation by iron or copper at lysosomal pH was then measured by monitoring the formation of conjugated dienes at 234 nm or by measuring the formation of cholesterol linoleate hydroperoxide and 7-ketocholesterol by HPLC in presence or abscence of the antioxidants tempol or probucol. The aim was to ascertain if tempol or probucol are good antioxidants for LDL at lysosomal pH and if a combination of tempol or probucol and α-tocopherol enrichment would be an effective antioxidant combination therapy. It was wondered if these antioxidants might convert the α-tocopheroxyl.

4.3 Results

4.3.1 Effect of α-tocopherol and tempol on LDL oxidation by 5µM ferrous iron at pH 4.5

Two antioxidants, α-tocopherol and tempol, on LDL oxidation by ferrous ion at lysosomal pH (Figure 4.1). The oxidation showed the usual pattern of lag, slow oxidation, rapid oxidation, aggregation and sedimentation phases (Satchell and Leake, 2012). The rate of oxidation of LDL was decreased when it was supplemented with α-tocopherol, but it did not alter the pattern of oxidation and aggregation (Figure 4.1). Tempol (10 µM) decreased the rate of oxidation of both control and α-tocopherol-enriched LDL (Figure 4.1). Unlike α-tocopherol, it altered the pattern of oxidation, in that it inhibited the slow phase of oxidation much more than it inhibited the rapid phase of oxidation. This is similar to the effect of tempol observed with LDL oxidation by the iron-storage protein ferritin (Ojo and Leake, 2018) and the effect of probucol on LDL oxidation by ferrous ion (Ahmad and Leake, 2018) at lysosomal pH. Tempol inhibited CLOOH and 7-ketocholesterol formation in LDL and LDL enriched with α-tocopherol, with the inhibition being more after 3h than before 3h (Figure 4.2). These data suggest, that tempol changs the pattern of LDL oxidation by iron in both control LDL and LDL enriched with α -tocopherol. However, there was no significant difference between control LDL plus tempol, α -tocopherol -enriched LDL plus tempol and enriched LDL with α -tocopherol, but if we compared these groups with control LDL, there were significant differences. This indicates that tempol shows its antioxidant properties at the late slow phase of oxidation, rather than the earlier rapid phase.

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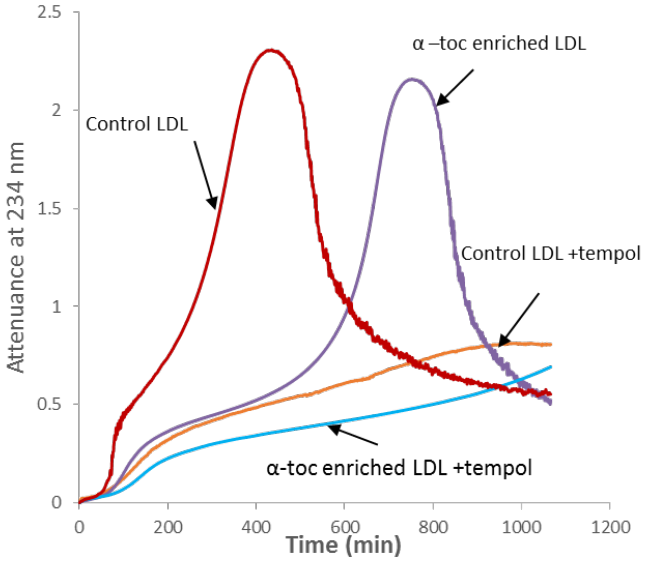

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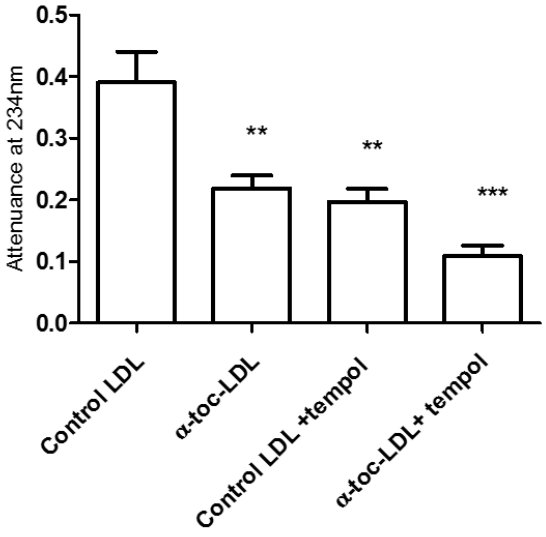
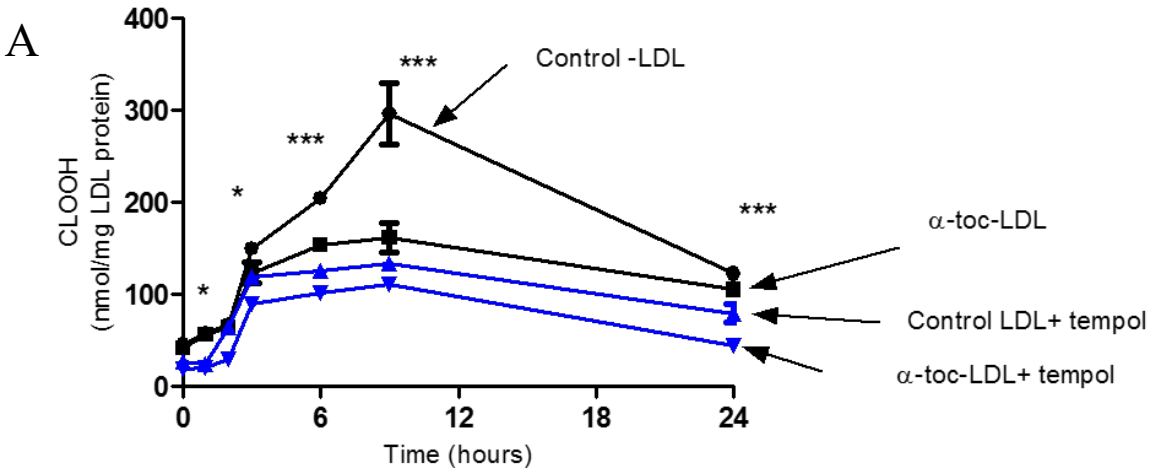


Figure 4.1. Effect of α-tocopherol and tempol on LDL oxidation by ferrous ion. (A) Control LDL and LDL enriched with α-tocopherol (50µg protein/ml) were oxidised by 5µM $FeSO_4$ at pH 4.5 and 37 $^{\circ}$C with or without 10 µM tempol. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B) The increase in attenuance at 100 min, around the transition between the rapid and slow phases of oxidation. The bar chart shows the mean ± SEM of the four experiments during the oxidation period of 100 min. A one-way ANOVA followed by Tukey's posthoc tests were significant (**P<0.01, ***P<0.001, n=4) compared to control.



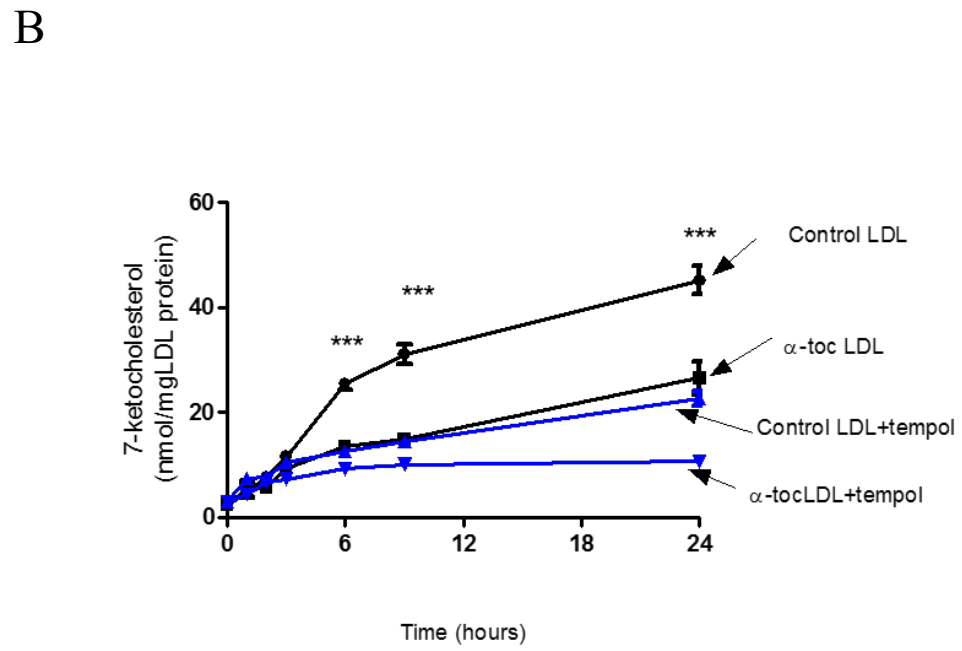


Figure 4. 2. Effect of α-tocopherol and tempol on LDL oxidation by ferrous ion in the presence or absence of 10 µM tempol. Control LDL and LDL enriched with α-tocopherol (50 µg LDL protein/ml) were oxidised by 5 µM $FeSO_4$ at pH 4.5 and 37 ^{o}C. (A) CLOOH and (B) 7-ketocholesterol were measured by HPLC. The mean± SEM of 3 independent experiments is shown. Two-way ANOVA followed by Bonferroni post hoc tests were significant (* P< 0.05 ***P<0.001, n=3) compared control LDL with LDL enriched with α- tocopherol and tempol.

4.3.2 Effect of tempol on LDL oxidation by 5μM Ferric ion at pH 4.5

Regarding chapter 3, α–tocopherol had no effect to inhibit LDL oxidation by different concentrations of ferric ion at lysosomal pH. The aim of this study was to investigate the effect of the antioxidant tempol on LDL oxidation by 5μM ferric as well as its effect in combination with α-tocopherol.

As expected, (Ahmad and Leake, 2018, Satchell and Leake, 2012) LDL oxidation by ferric ion was slower than by ferrous ion at lysosomal pH (Figure 4.3). Tempol (10μM) did not inhibit the oxidation of either LDL or α- tocopherol-enriched LDL by ferric ions, in marked contrast to the inhibition observed with ferrous ions. HPLC showed that tempol did not inhibit LDL oxidation of either control LDL or α- tocopherol-enriched LDL (Figure 4.4). Instead, tempol increased the oxidation of α- tocopherol-enriched LDL. The rate of LDL oxidation was increased, rather than decreased, by α-tocopherol enrichment. α-Tocopherol enrichment of LDL did not inhibit the formation of CLOOH, and 7-ketocholesterol (Figure 4.4). This finding suggests that, tempol did not inhibit α-tocopheroxyl radical.

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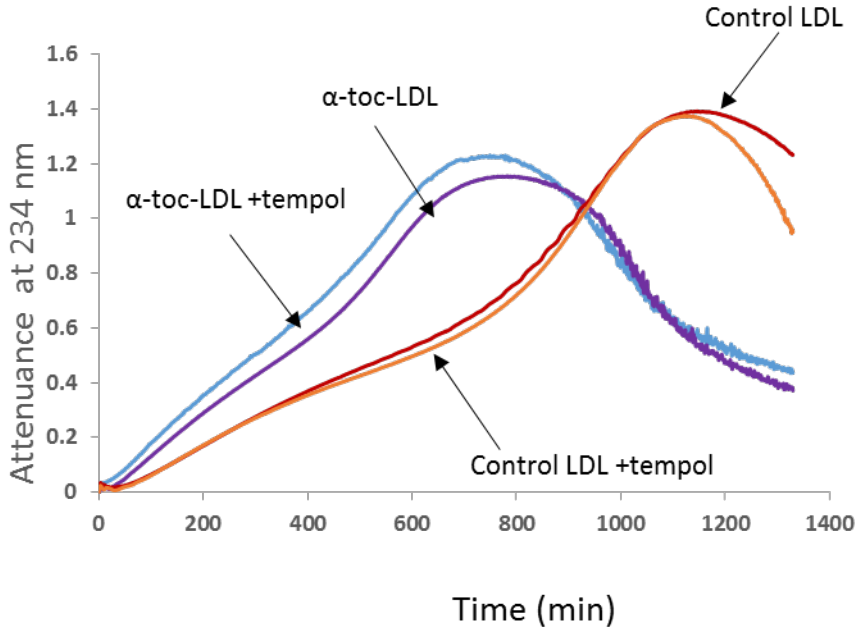

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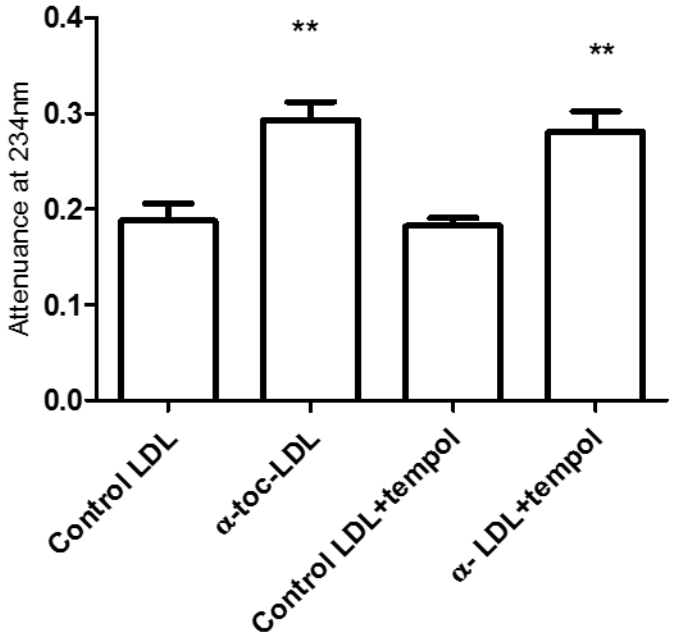

Figure 4. 3. Effect of α-tocopherol and tempol on LDL oxidation by ferric iron.
(A) Control LDL or LDL supplemented with α-tocopherol (50 µg protein/ml) were oxidised by 5 µM $FeCl_3$ at pH 4.5 and 37 ^{o}C in the presence or absence of 10 µM tempol. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B) The increase in attenuance at 200 minutes (the transition between the rapid and slow phases of oxidation) for the experiments was measured. The bar chart shows the mean ± SEM of the three experiments during the oxidation period of 200 min. A one-way ANOVA followed by Tukey's posthoc tests were significant (**P<0.01, n=3) compared to control.

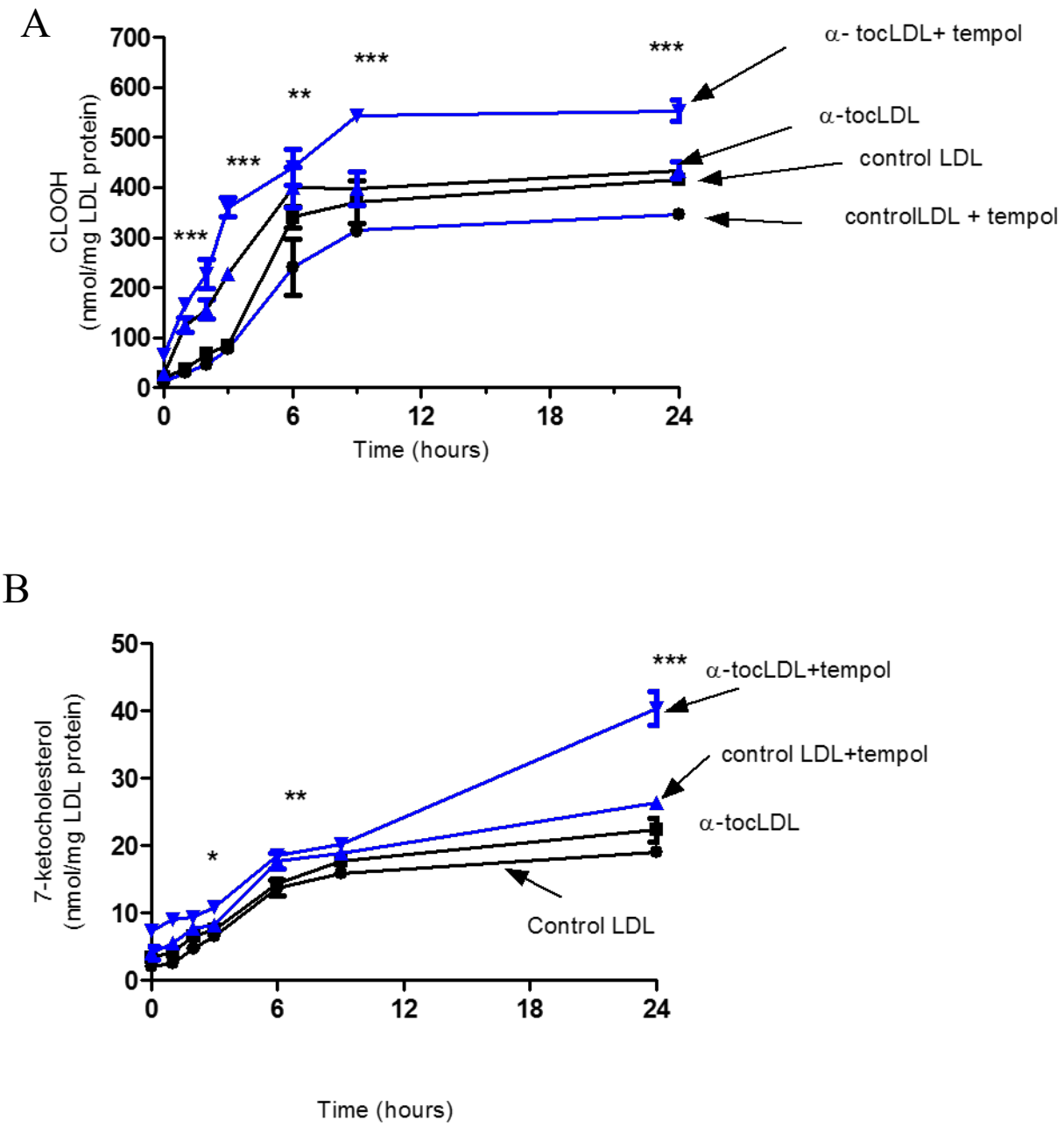

Figure 4.4. Effect of α-tocopherol and tempol on LDL oxidation by ferric iron.

Control LDL or LDL supplement with α-tocopherol (50 µg protein/ml) were oxidised by 5 µM $FeCl_3$ at pH 4.5 and 37 ^{o}C in the presence or absence of 10 µM tempol. (A) CLOOH and (B) 7-ketocholesterol were measured by HPLC. Two -way ANOVA, followed by Bonferroni post hoc tests were significant (*P< 0.05, ** P< 0.01 ***P<0.001, n= 3) compared to control LDL for LDL enriched with α-tocopherol and tempol.

4.3.3 Effect of α-tocopherol and tempol on LDL oxidation by 5μM copper at pH 4.5

LDL oxidation by copper at lysosomal pH showed the oxidation profile as that shown by iron at this pH, i.e., there was a short lag phase, a rapid oxidation phase, a slow oxidation phase, an aggregation phase and sedimentation phase (Figure 4.5). The rate of LDL oxidation during the slow oxidation phase was increased, rather than decreased, by α-tocopherol. When adding 10 μM tempol, there was a decrease in the oxidation of LDL supplemented with α-tocopherol at the later times but not the early times. α-Tocopherol enrichment increased the formation of CLOOH and 7-ketocholesterol. (Figure 4.6). Tempol decreased the rate formation of CLOOH and 7-ketocholesterol in α-tocopherol-enriched LDL. These results show that tempol does not inhibit the early oxidation of LDL by copper at lysosomal pH, but inhibits the later oxidation.

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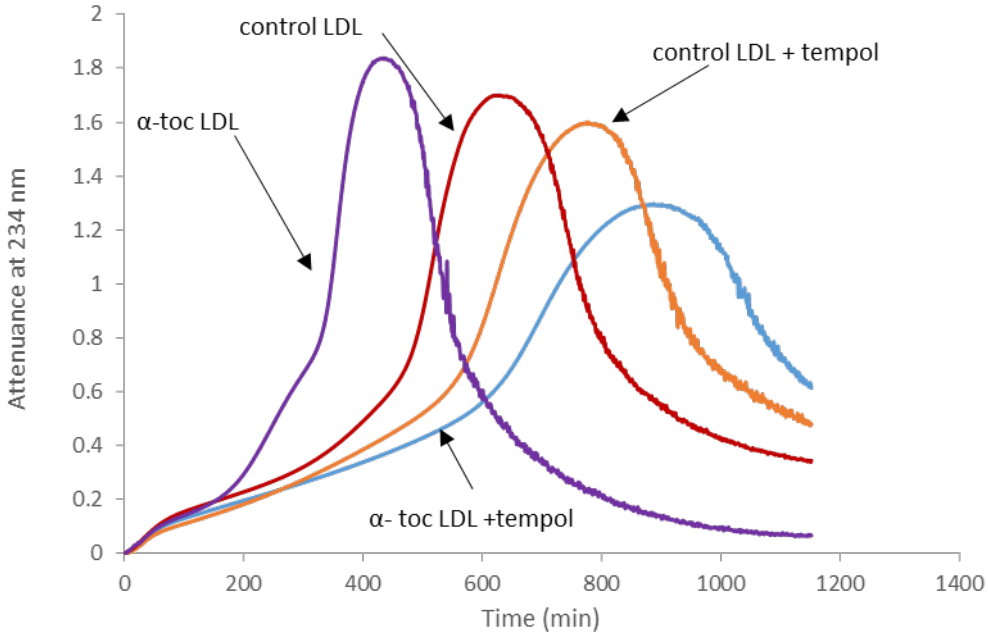

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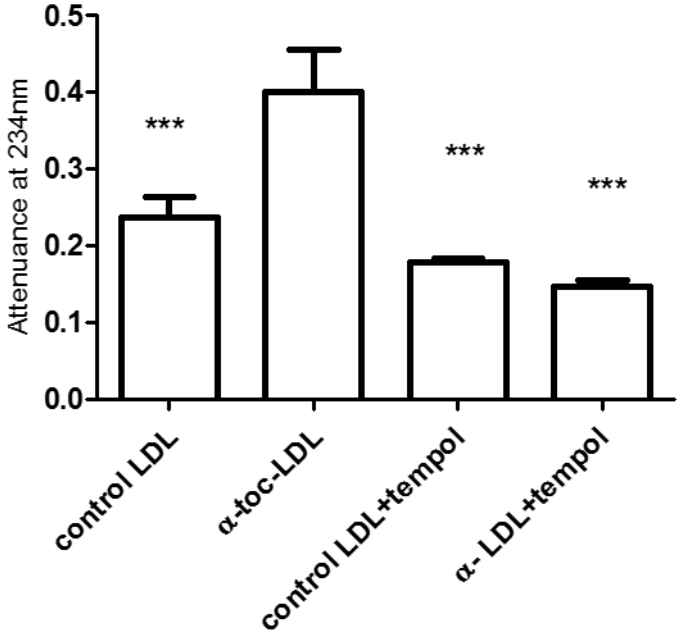

Figure 4.5. Effect of α-tocopherol and tempol on LDL oxidation by copper ion at lysosomal pH.

(A) Control LDL and LDL enriched with α-tocopherol (50 µg protein/ml) were oxidised by 5µM $CuSO_4$ at pH 4.5 and 37 ^{o}C. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B) The bar chart shows the mean ± SEM of the three experiments during the oxidation period of 200 min before the aggregation phase was reached. A one-way ANOVA followed by Tukey's posthoc tests were significant (***P<0.001, n=3) compared to LDL supplemented with α-tocopherol.

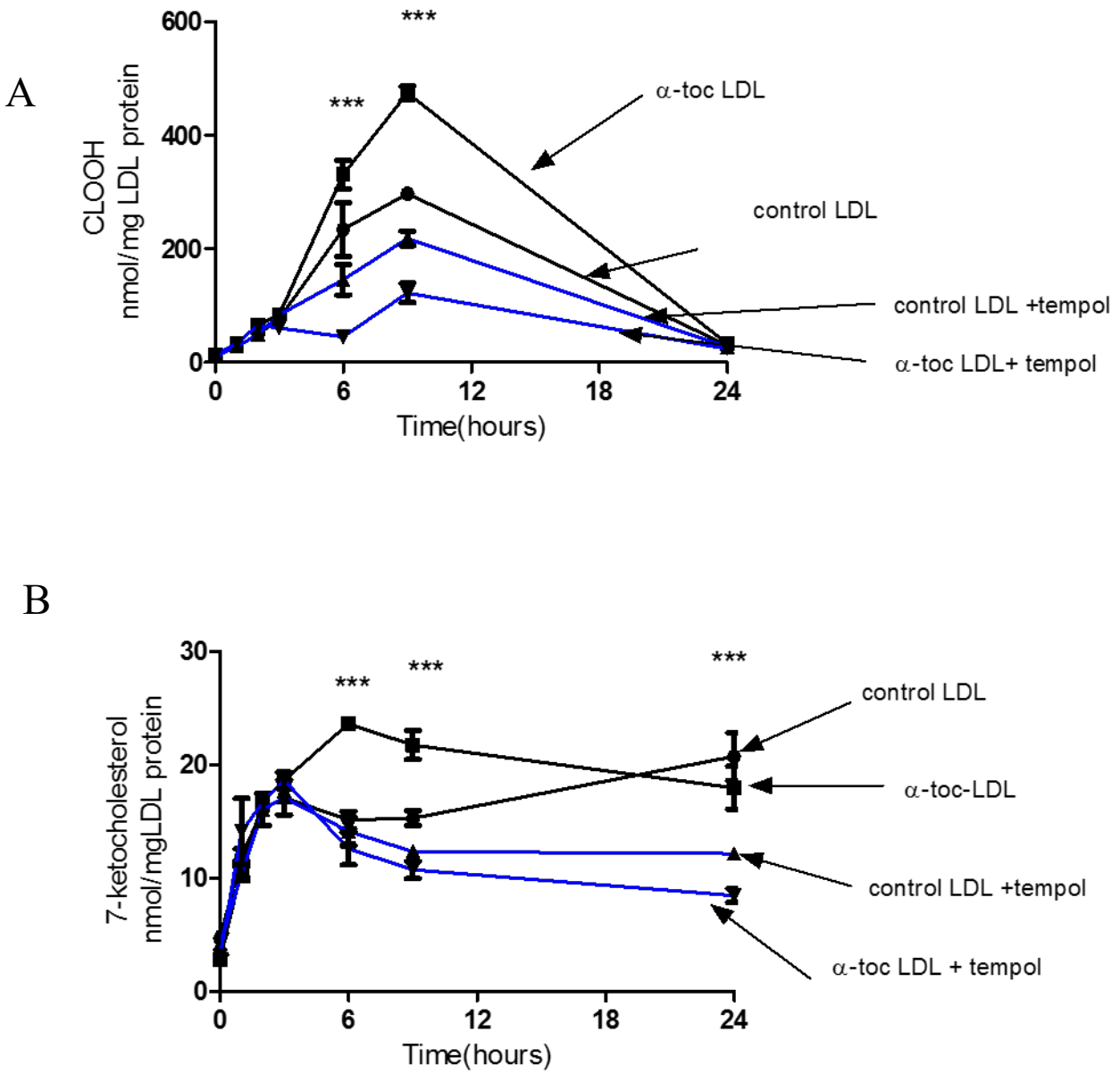

Figure 4.6. Effect of α-tocopherol and tempol on LDL oxidation by copper ion at lysosomal pH.

Control LDL and LDL enriched with α-tocopherol (50 µg protein/ml) were oxidised by 5 µM $CuSO_4$ at pH 4.5 and 37 ^{o}C. (A) CLOOH and (B) 7-ketocholesterol were measured by HPLC. Two -way ANOVA, followed by Bonferroni post hoc tests were significant ***P<0.001, (n=3) compared to control LDL with LDL enriched with α- tocopherol and tempol.

4.3.4 Effect of α-tocopherol and tempol on LDL oxidation by 5 μM copper ion at pH 7.4

LDL oxidation by copper ions at pH 7.4 was much faster than at pH 4.5 (Figure 4.7), in marked contrast to what was observed with ferrous or ferric iron. The pattern of oxidation was as expected (Esterbauer at al., 1989), with no aggregation observed at pH 7.4. As expected, (Jessup et al., 1990; Dieber-Rotheneder et al., 1991), enrichment of LDL with α-tocopherol decreased its rate of oxidation but did not totally inhibit LDL oxidation. Tempol (10 μM) decreased the rate of oxidation of LDL and α-tocopherol-enriched LDL but only at later time points, there is no inhibition initially. α-Tocopherol enrichment of LDL decreased the rate of formation of CLOOH and 7-ketocholesterol (Figure 4.8). Tempol also decreased significantly the rate of formation of these two oxidised lipids (Figure 4.8). Thus,α-tocopherol inhibits LDL oxidation by copper considerably at pH7.4 , with tempol having a more modest inhibition.

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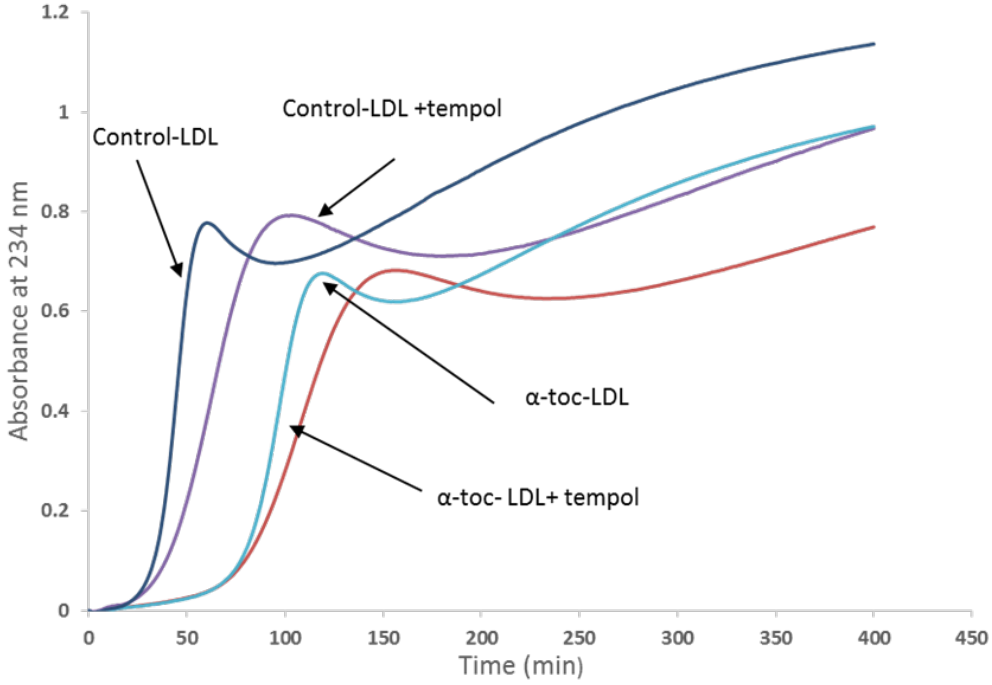

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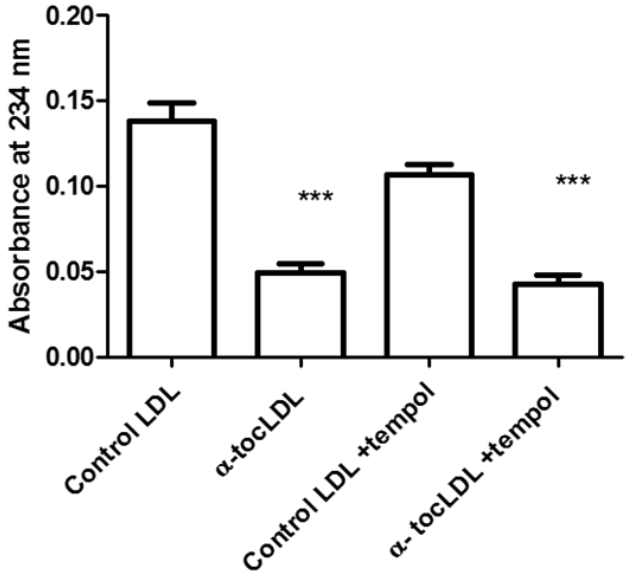

Figure 4.7. Effect of α-tocopherol and tempol on LDL oxidation by copper ion at pH7.4.

(A) Control LDL and LDL enriched with α-tocopherol (50 µg protein/ml) were oxidised by 5µM $CuSO_4$ at pH 7.4 and 37 ^{o}C with or without tempol (10 µM). Oxidation was monitored by measuring the change in attenuance at 234 nm. This is a representative example of three experiments. (B) The bar chart shows the mean ± SEM of the three experiments during the oxidation period of 50 min. A one-way ANOVA followed by Tukey's posthoc tests were significant (***P<0.001, n=3) compared to control LDL.

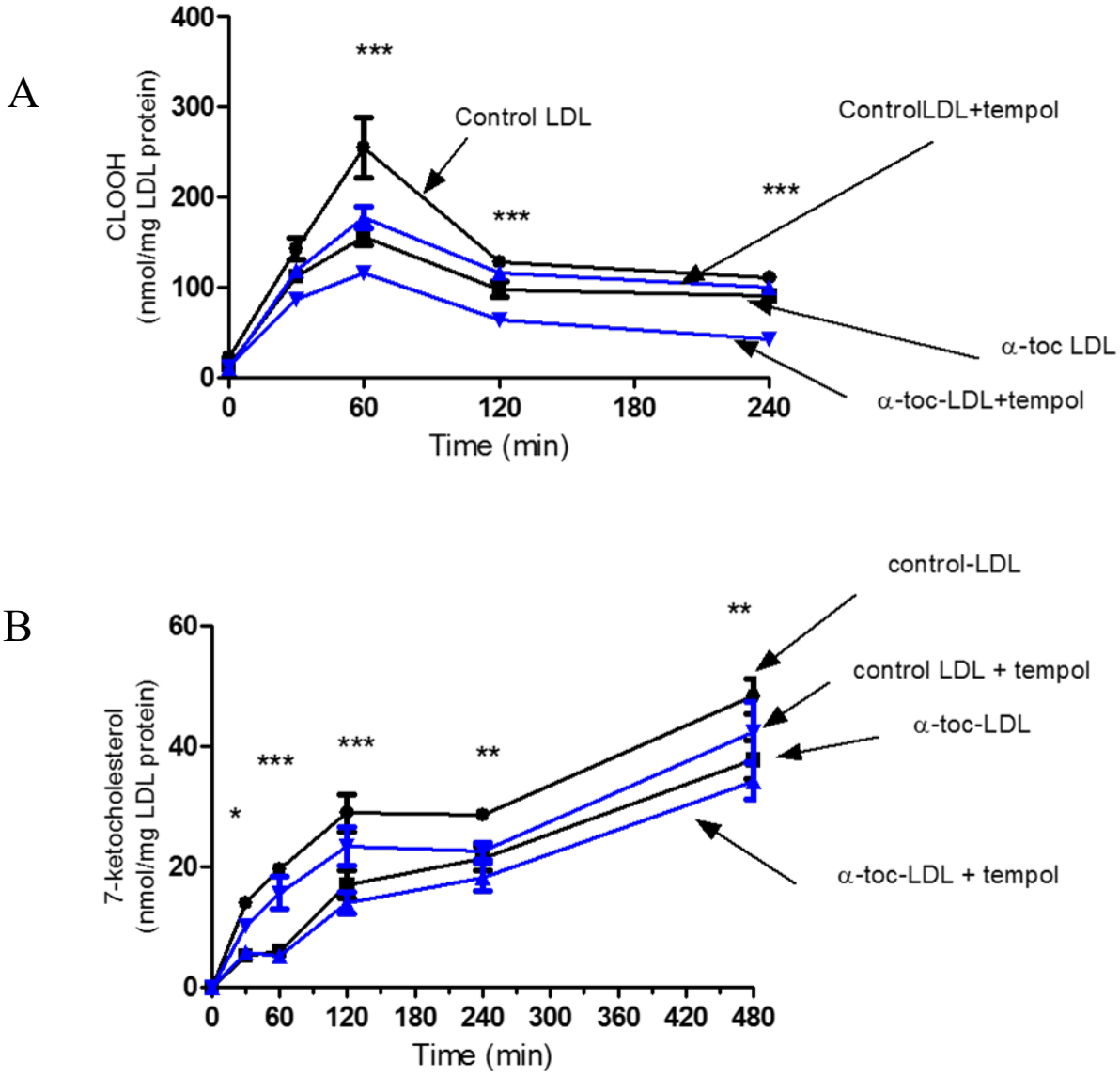

Figure 4.8. Effect of α-tocopherol and tempol on LDL oxidation by copper ion at pH 7.4. Control LDL and LDL enriched with α-tocopherol (50 µg protein/ml) were oxidised by 5 µM $CuSO_4$ at pH 7.4 and 37 ^{o}C with or without tempol 10 µM. (A)CLOOH and (B) 7-ketocholesterol were measured by HPLC. (Two -way ANOVA, followed by post hoc Bonferroni tests were significant*P< 0.05,** P< 0.01 ***P< 0.001, n=3) compared to control LDL with LDL enriched with α- tocopherol plus tempol.

4.3.5 Effect of Probucol on LDL oxidation by 5µM Fe^{2+} at pH 4.5

A diversity of agents have been employed in the treatment of hyperlipidaemia, including probucol (Jialal and Grundy, 1992). Probucol is also a good antioxidant for LDL at pH 7.4. Our hypothesis was that a combination of probucol and α- tocopherol would give a greater antioxidant effect because probucol might possibly scavenger alpha-tocopheroxyl radicals by donation of a hydrogen atom fromits hydroxyl groups. In agreement with the result in chapter 3 (Figure 3.5), α- tocopherol enriched with LDL delayed but not change the pattern of LDL oxidation by 5 µM $FeSO_4$ at pH 4.5. Adding probucol (5 µM) (Figure 4.9), greatly reduced the pattern of oxidation for both control LDL and LDL enriched with α-tocopherol, but was less affective at early times. α-Tocopherol enrichment decreased the formationl of CLOOH and 7-ketocholesterol during LDL oxidation by 5 µM Fe SO_4 at pH 4.5 (Figure 4.10). The addition of probucol (5 µM) greatly decreased the formation of these oxidation products in both control LDL and LDL enriched in α-tocopherol, especially CLOOH. Probucol greatly inhibited LDL oxidation by copper at pH7.4.

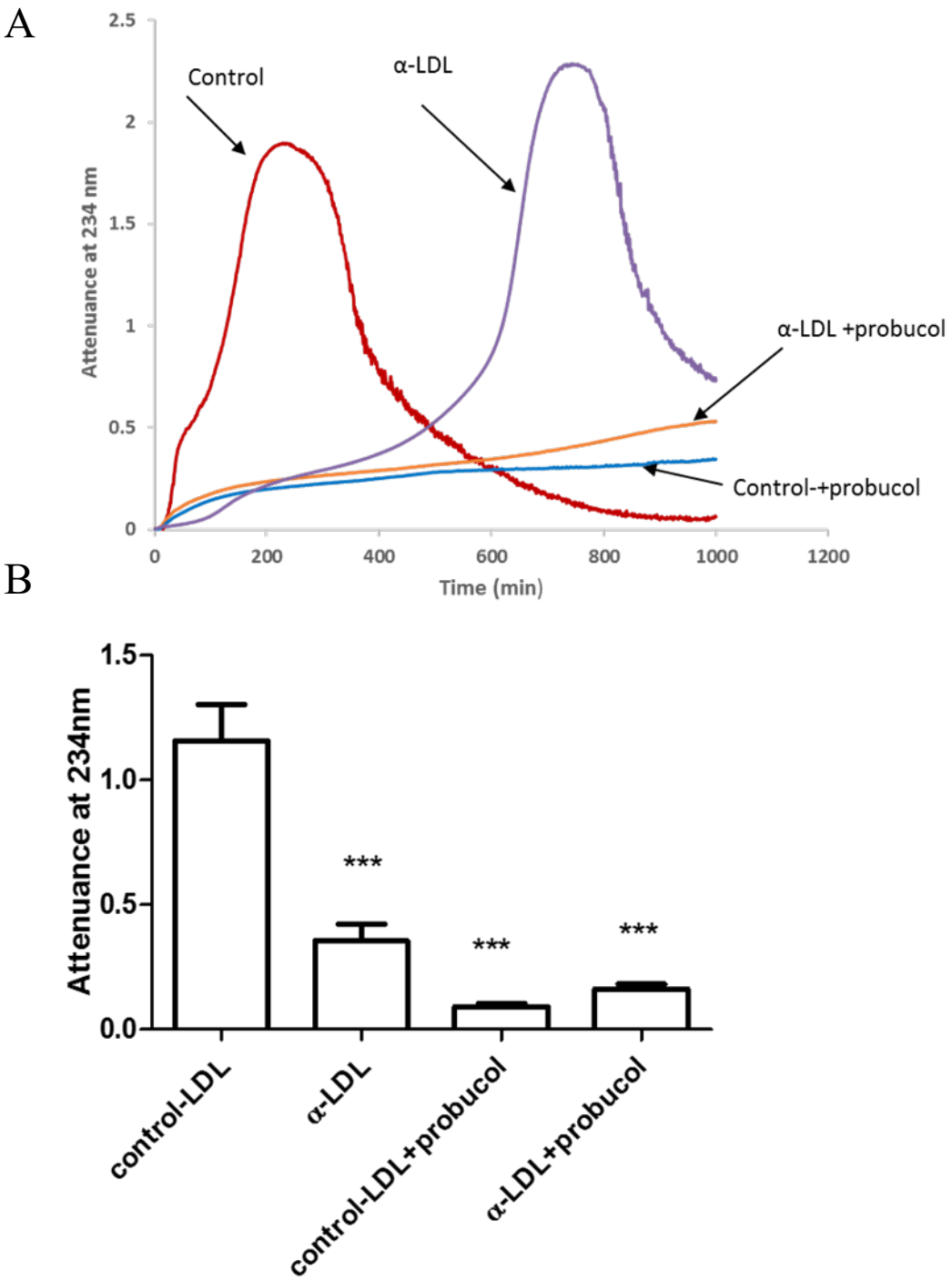

Figure 4.9 Graphs showing the effect of probucol (5 µM) on LDLoxidation by ferrous iron.

(A) Control LDL and LDL enriched with α-tocopherol (50 µg LDL protein/ml) were oxidised by 5 µM $FeSO_4$ at pH 4.5 and 37°C. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B) The bar chart shows the mean ± SEM of the four experiments during the oxidation period of 200 min. A one-way ANOVA followed by Tukey's posthoc tests were significant (***P<0.001, n= 4) compared to control LDL.

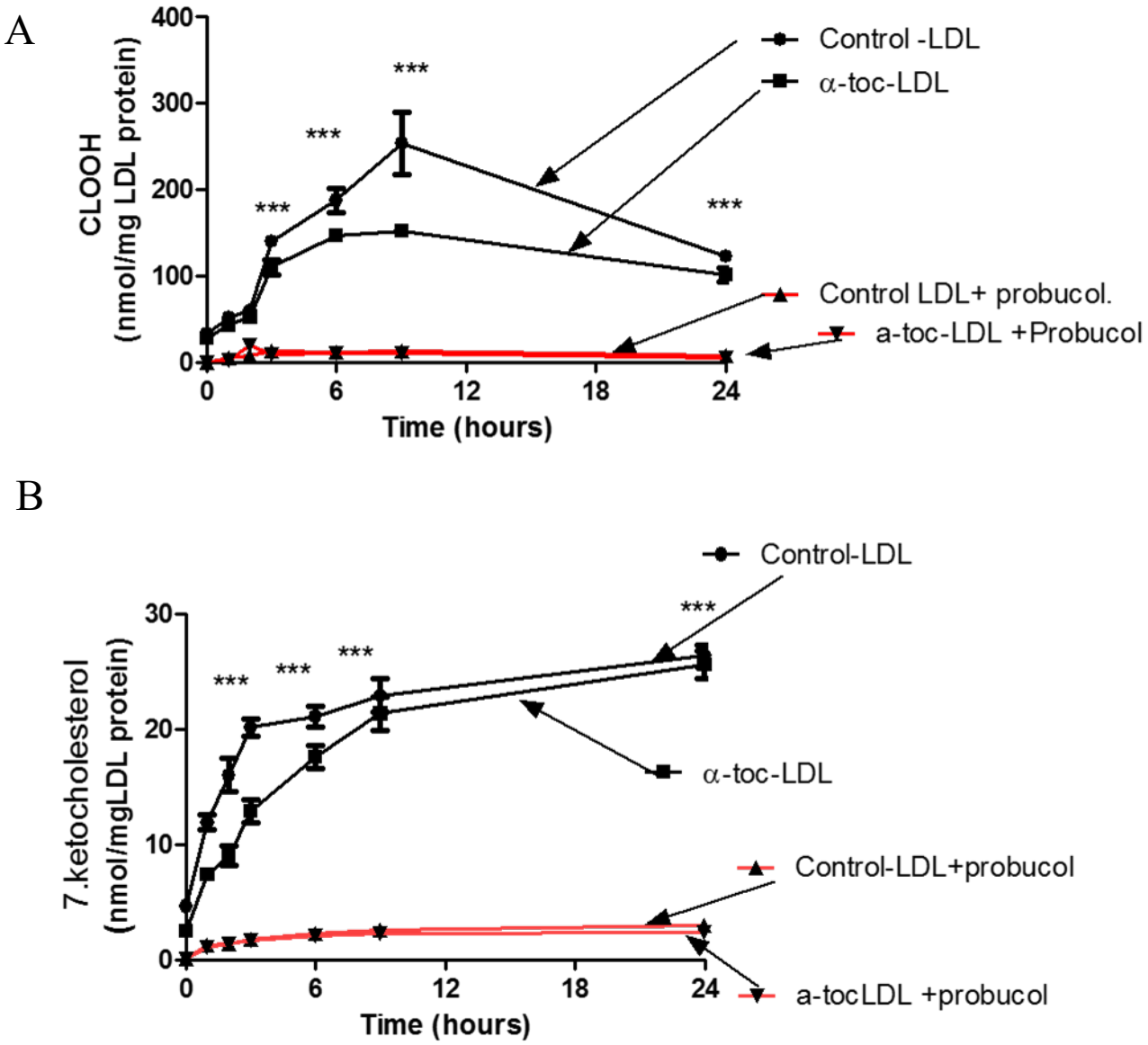

Figure 4.10 Effect of α-tocopherol and probucol on LDL oxidation by ferrous ion.

Control LDL and LDL enriched with alpha-tocopherol (50µg protein/ml) were oxidised by 5µM $FeSO_4$ at pH 4.5 and 37^{o}C in the presence or absence of probucol (5 µM). At different time points, the oxidation was stopped by adding EDTA (final concentration 4µM) and BHT (80µM). The samples were assayed for (A) CLOOH and. (B) 7-ketocholesterol, by C18 reversed-phase HPLC. (Two -way ANOVA, followed by post hoc test Bonferroni tests was significant (***P< 0.001, n=4) compared to control LDL with LDL enriched with α- tocopherol plus probucol.

4.3.6 Effect of probucol on LDL oxidation by 5μM Fe $^{3+}$ at pH 4.5

This study aimed to examine the therapeutic effects of the combined use of probucol and α -tocopherol on LDL oxidation by ferric ion at lysosomal pH. In agreement with the results in chapter 3, α-tocopherol enrichment did not inhibit LDL oxidation by Fe^{3+} at pH 4.5 (Figure 4.11.). in the presence of probucol (5 μM), there was a considerable decrease in the oxidation rate of both control and α-tocopherol enriched LDL (Figure 4.11). Probucol (5 μM) greatly decreased CLOOH and 7-ketocholesterol formation during the course of oxidation of control LDL and α -tocopherol -enriched LDL (Figure 4.12). These results show that probucol with or without α-tocopherol can greatly inhibit the oxidation induced by Fe^{3+} at pH 4.5 of the cholesteryl ester-rich hydrophobic core of LDL as well as the oxidation of non-esterified cholesterol, which is mainly in the surface monolayer of LDL.

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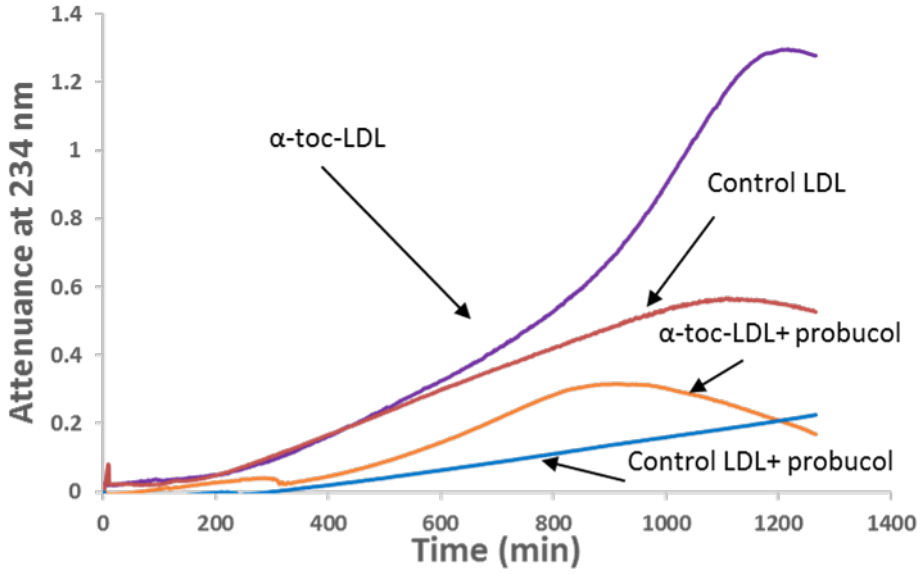

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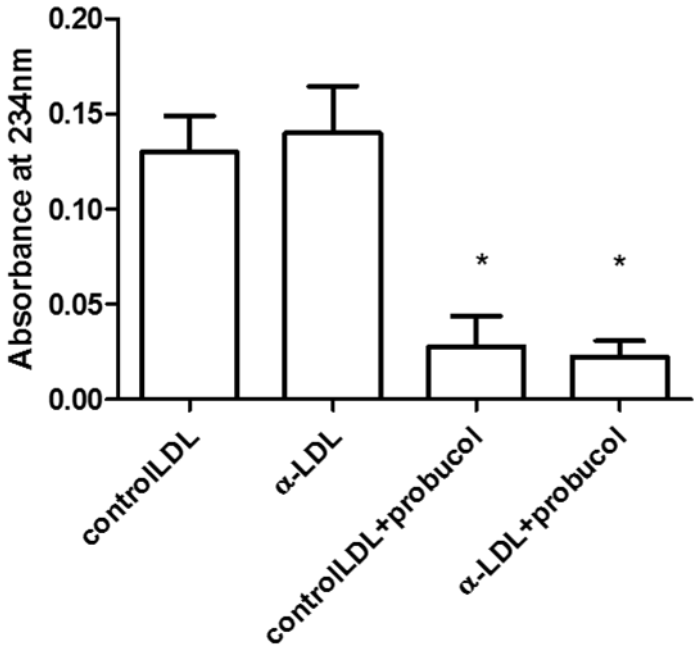

Figure 4.11 Graphs showing the effect of probucol (5 µM) on LDL oxidation by ferric iron.

(A) control LDL and LDL enriched with α-tocopherol (50µg LDL protein/ml) were oxidised by 5µM Fe^{3+} at pH 4.5 and 37°C. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B) The bar chart shows the mean ± SEM of the four experiments during the oxidation period of 300 min. A one-way ANOVA followed by Tukey's posthoc tests were significant (*P<0.05, n= 4) compared to LDL supplemented with α-tocopherol.

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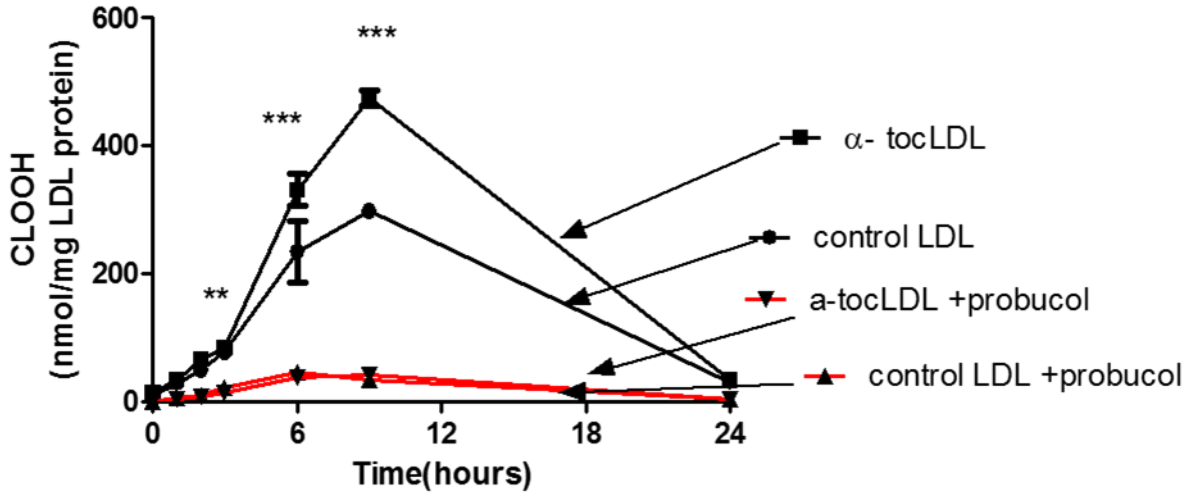

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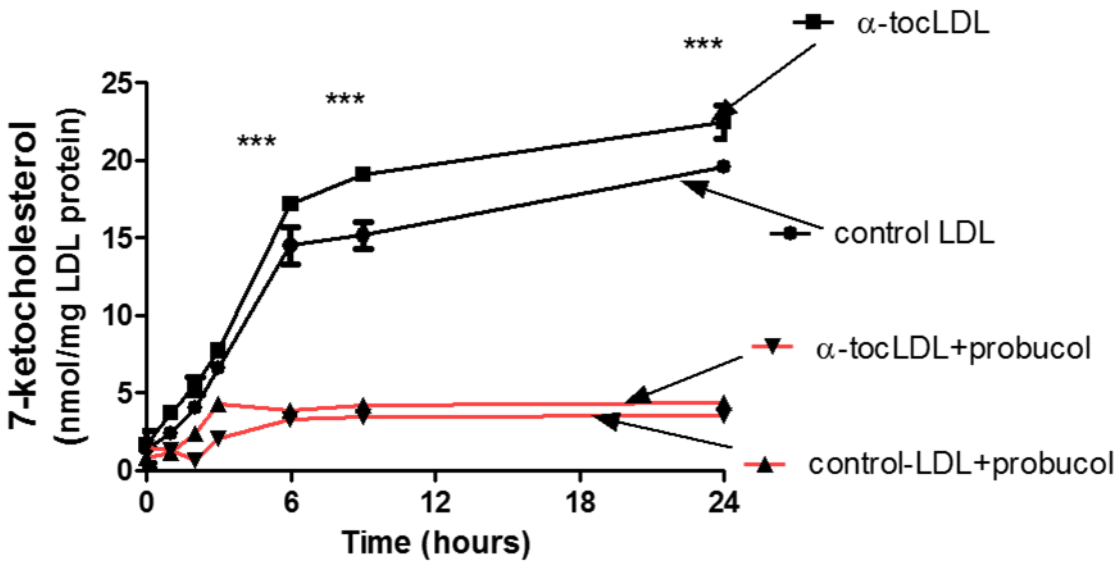


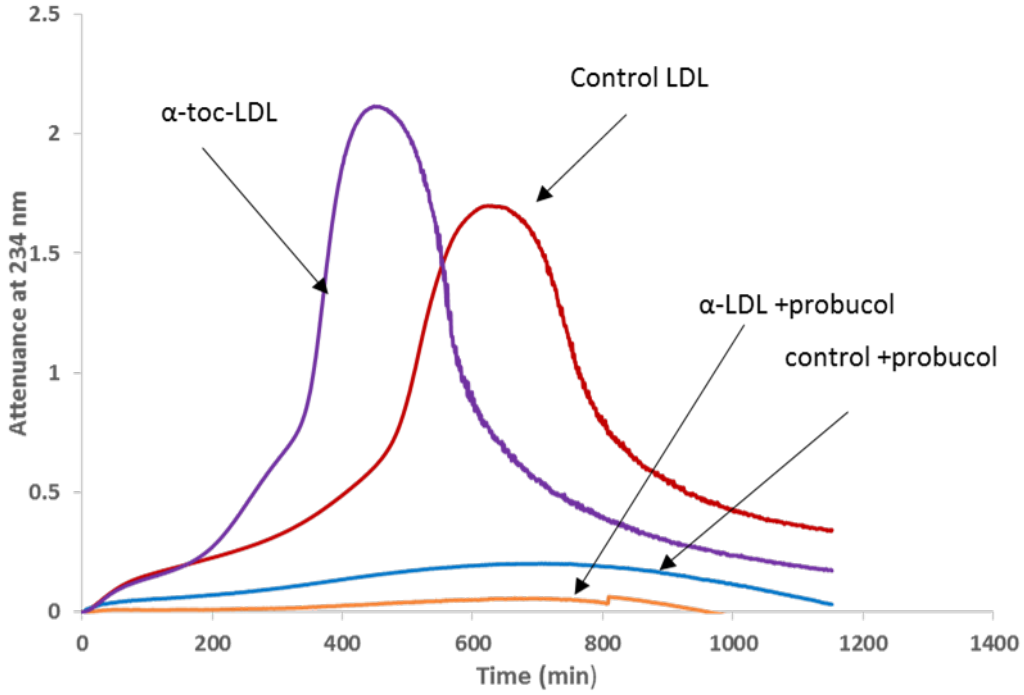
Figure 4.12 Effect of α-tocopherol and probucol on LDL oxidation by ferric iron.

Control LDL and enriched LDLwith α -tocopherol (50 µg LDL protein/ml) were oxidised by 5µM $FeCl_3$ at pH 4.5 and 37^{o}C in the presence or absence of probucol (5 µM). At different time points, the oxidation was stopped by adding EDTA (final concentration 4mM) and BHT (80µM). The samples were assayed for (A) CLOOH and (B) 7-ketocholesterol by C18 -reverse-phase HPLC. (Two -way ANOVA, followed by post hoc test Bonferroni tests were significant ***P< 0.001, n=4) compared to control LDL with LDL enriched with α- tocopherol plus probucol.

4.3.7 Effect of probucol on α-tocopherol-enriched LDL oxidation by 5μM Cu^{2+} at pH 4.5

LDL the oxidation by 5μM Cu^{2+} at pH 4.5 was not inhibited by α-tocopherol supplementation, but in theses experiments was increased by it (Figure 4.13). Addition of probucol (5 μM) greatly inhibited the LDL oxidation induced by copper at lysosomal pH in both control and LDL enriched with α-tocopherol. There was also much less formation of CLOOH and 7-ketocholesterol in the presence of probucol (5μM) (Figure 4.14).

A



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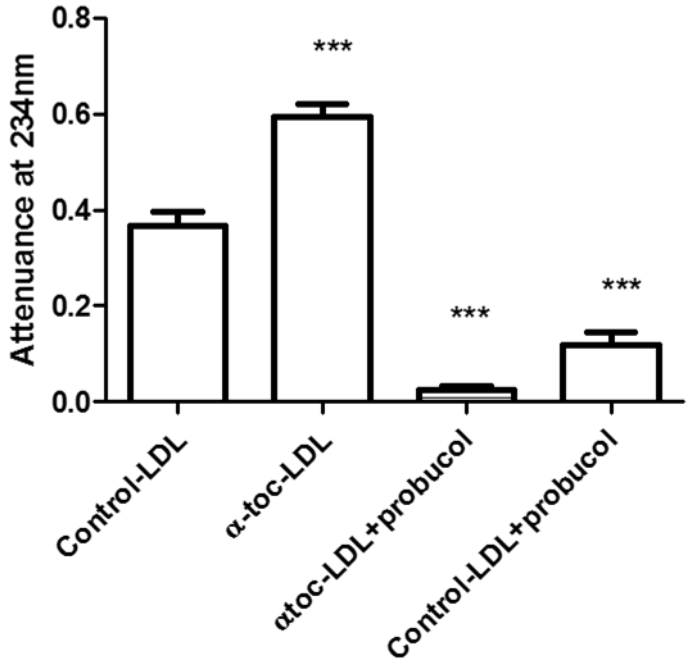


Figure 4.13 Graphs showing the effect of probucol (5 µM) on LDL oxidation by copper ion at lysosomal pH.

(A) control LDL and LDL enriched with α-tocopherol (50µg LDL protein/ml) were oxidised by 5µM Cu^{2+} at pH 4.5 and 37°C. Oxidation was monitored by measuring the change in attenuance at 234 nm. (B) The bar chart shows the mean ± SEM of the four experiments during the oxidation period of 300 min. A one-way ANOVA followed by Tukey's posthoc tests were significant (***P<0.001, n= 4) compared to control LDL.

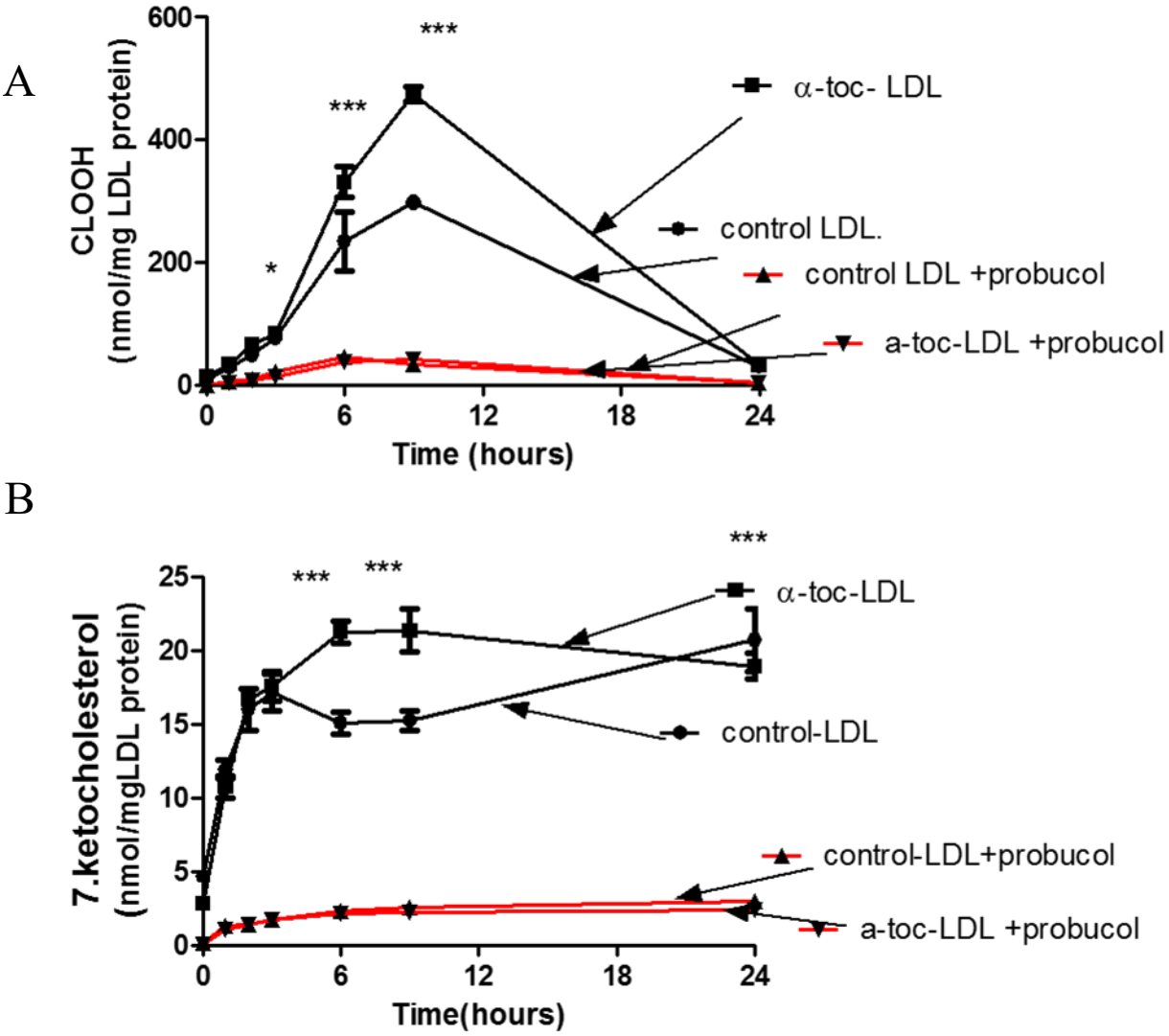


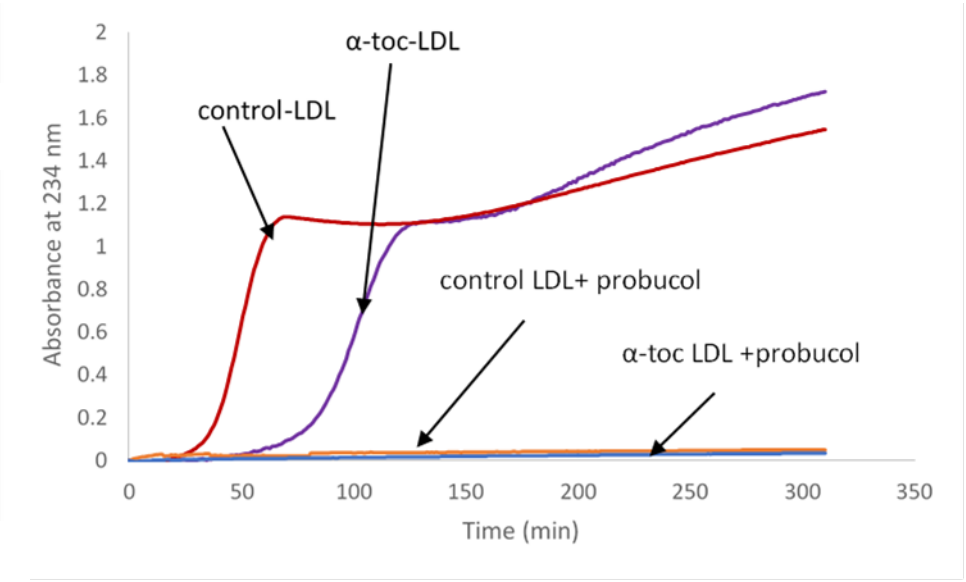
Figure 4.14 Effect of α-tocopherol and probucol on LDL oxidation by copper ion at pH 4.5.

Control LDL and LDL enriched with α-tocopherol (50µg LDL protein/ml) were oxidised by 5µM $CuSO_4$ at pH 4.5 and 37^{o}C in the presence or absence of probucol (5 µM). At different time points, the oxidation was stopped by adding EDTA (final concentration 4mM) and BHT (80µM). The samples were assayed for (A) CLOOH and (B) 7-ketocholesterol by C18- reverse-phase HPLC. (Two -way ANOVA, followed by post hoc test Bonferroni tests were significant (*P< 0.05 ***P< 0.001, n=4) compared to control LDL with LDL enriched with α- tocopherol plus probucol.

4.3.8 Effect of probucol on α-tocopherol-enriched LDL oxidation by 5μM Cu^{2+} at pH 7.4

In agreement with the result shown in chapter 3, α-tocopherol enrichment delayed LDL oxidation by Cu^{2+} at pH 7.4 (Figure 4.15). In the presence of 5μM probucol, there was an almost total decrease in oxidation in both control and α-tocopherol-enriched LDL. α-Tocopherol enrichment inhibited the formation of CLOOH and 7-ketocholesterol (Figure 4.16). Probucol greatly inhibited the formation of both CLOOH and 7-ketocholesterol of both control and α-tocopherol enriched LDL (Figure 4.16). It may be concluded that oxidation of LDL can be delayed by α-tocopherol supplementation, but did not totally inhibit, but addition of probucol can almost totally inhibit LDL oxidation at pH 7.4.

A



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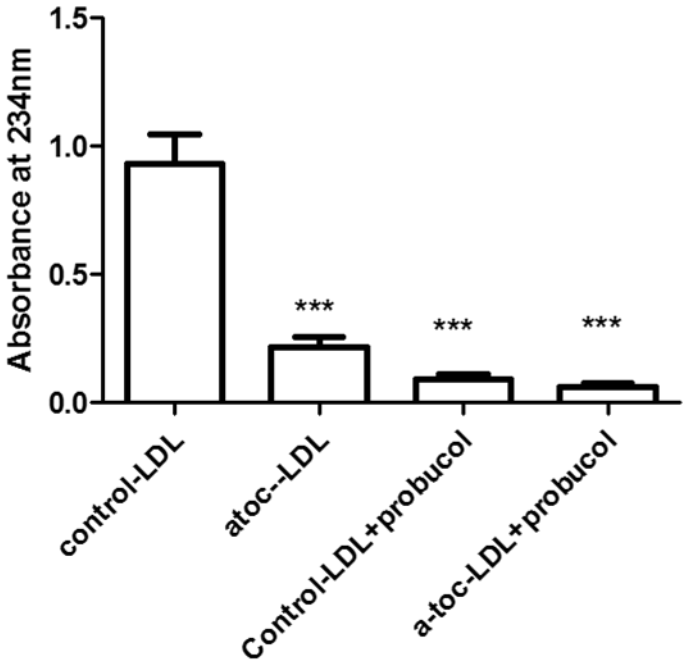


Figure 4.15 Effect of α-tocopherol and probucol (5µM) on LDL oxidation by copper ion at pH 7.4.

(A) control and LDL (50µg LDL protein/ml) was oxidised by 5 µM $CuSO_4$ at pH 7.4 and 37^{o}C. Oxidation was monitored by measuring the change in absorbance at 234 nm. (B) The bar chart shows the decrease in absorbance during the propagation phase of oxidation at 50 min. A one-way ANOVA followed by Tukey's posthoc tests were significant (***P<0.001, n= 4) compared to control LDL.

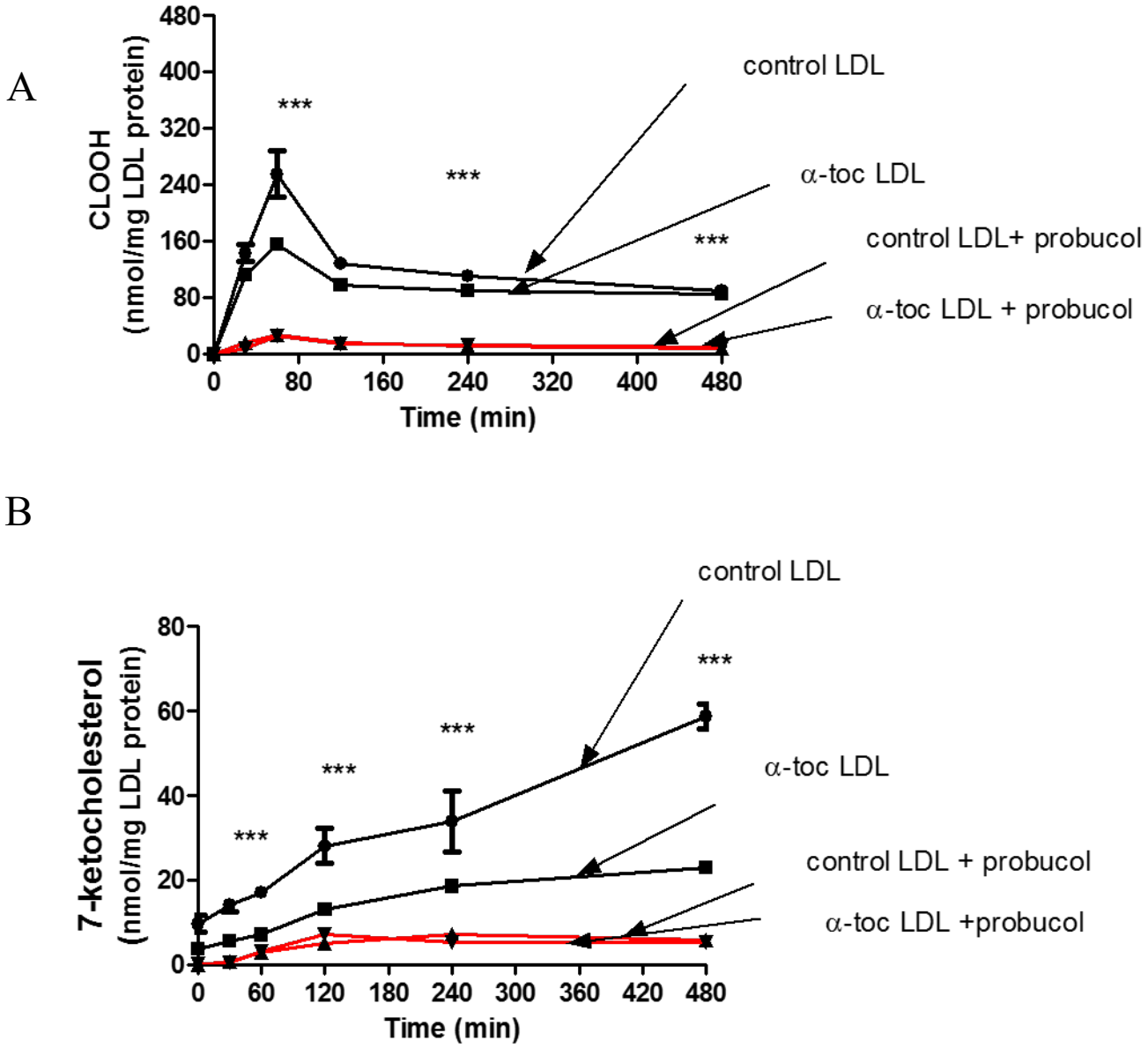


Figure 4.16 Effect of α-tocopherol and probucol on LDL oxidation by copper ion at pH 7.4.

Control LDL and LDL enriched with α -tocopherol (50µg LDL protein/ml) were oxidised by 5 µM $CuSO_4$ at pH 7.4 and 37°C in the presence or absence of probucol (5 µM). At different time points, the oxidation was stopped by adding EDTA (final concentration 4mM) and BHT (80µM). The samples were assayed for (A) CLOOH and (B) 7-ketocholesterol by C18- reverse-phase HPLC. (Two -way ANOVA, followed by post hoc test Bonferroni tests were significant (***P< 0.001, n=4) compared to control LDL with LDL enriched with α- tocopherol plus probucol.

4.4 Discussion

Our group have raised the possibility that LDL might be oxidised in the lysosomes of macrophages in atherosclerotic lesions and be catalysed by redox-active iron (Wen and Leake, 2007). α-Tocopherol usually inhibits LDL oxidation at pH 7.4 as shown in numerous studies (Jessup et al., 1990, Dieber-Rotheneder et al., 1991),but Stocker's group (Bowry et al., 1992, Upston et al., 1999, Ingold et al., 1993) have shown that α-tocopherol can increase the rate of LDL oxidation if the oxidative stress is low. Under low oxidative stress conditions, the α-tocopheroxyl radicals can abstract a hydrogen atom from a polyunsaturated fatty acyl group in LDL particles, unless it becomes reduced by a co-antioxidant, such as a vitamin C or ubiquinol-10. In chapter 3, the results showed that the antioxidant/ pro-oxidant activity of α-tocopherol depends on the pH and kind of oxidant. α-Tocopherol is transformed to α-tocopheroxyl radical during oxidative reactions. To regenerate α -tocopherol, it is required to get a hydrogen atom either from another polyunsaturated fatty acid or co-antioxidant. Therefore, α-tocopherol requires a co-antioxidant to quench the α -tocopheroxyle radical. In this study, tempol and probucol were chosen to perform as possible co-antioxidants.

The stable aminoxyl faces radical tempol inhibited the oxidation of LDL by Fe^{2+} at pH 4.5, especially the later stage of oxidation but was less effect at inhibitoring the initial oxidation. Tempol has a hydroxyl group and four methyl group and is soluble in both water and ethanol. It would, therefore, be expected to reside in the phospholipid monolayer of LDL, rather than the hydrophobic cholesteryl ester core. This might explain why it inhibited the later oxidation of LDL more than the initial oxidation, as we believe the core of LDL oxidises

before the surface monolayer at pH 4.5 (Ahmad and Leake, 2018). Tempol might scavenge hydroperoxyl radicals or phospholipid or non-esterified radicals in the surface monolayer.

tempol$^{\bullet}$ + $HO_2^{\bullet}$ ⟶ tempol OOH

tempol$^{\bullet}$ + PLOO$^{\bullet}$ ⟶ tempol OOPL

tempol$^{\bullet}$ + cholesterol OO$^{\bullet}$ ⟶ tempol OO cholesterol

Tempol might also scavenge the pro-oxidant α-tocopheroxyl radical.

α-toc O• + tempol$^{\bullet}$ ⟶ α-toc O tempol

α-Tocopherol-enrichment of LDL in these experiments reduced the oxidation of LDL by 5 μM ferrous ion, but in contrast the lack of antioxidant effect observed with ferric ion (Figure 4.3 & 4.4). The reason for this difference is unknown, but might be due to a difference in the balance between antioxidant and pro-oxidant effects observed with ferrous and ferric ion. Tempol inhibited the oxidation of LDL (especially LDL enriched with α-tocopherol) by Cu^{2+} at pH 4.5 (Figure 4.5), which might have been due to a direct antioxidant effect of tempol or possibly due to the conversion of the α-tocopheroxyl radical to α-tocopherol . It did not, however, inhibit with Fe^{3+} at pH 4.5 (Figure 4.3). The reason for this is unknown and is unexpected as both metal ions were in their in their higher valency states (Cu^{2+} Fe^{3+} rather than Cu^{+} and Fe^{2+}).

Probucol is a diphenol structure with antioxidant and anti-inflammatory properties (He et al., 2017). It inhibited LDL oxidation by Cu^{2+} at pH 7.4 very effectively (Figure 4.15& 4.16),

in agreement with previous studies (Parthasarathy et al., 1986, Leake and Rankin, 1990), but it does not decrease atherosclerosis in humans (Walldius et al., 1994) or decrease the primary endpoints in cardiovascular clinical trials (Yamashita et al., 2008, Tardif et al., 2008). Probucol did not inhibit so effectively the oxidation of LDL at lysosomal pH (Figure 4.9, 4.11 or 4.13). Probucol would be expected to reside in the surface phospholipid monolayer of LDL, as it contains two hydrophilic hydroxyl groups, and there is evidence for this based on the distribution of ^{14}C probucol in lipoproteins according to their phospholipid content (Bard et al., 1994). If LDL oxidation at lysosomal pH starts mainly in the hydrophobic core, probucol in the surface monolayer might not effectively inhibit it. If LDL is oxidised in lysosomes *in vivo*, this might help to explain why it does not decease atherosclerosis in patients very effectively. In conclusion, tempol inhibited the later oxidation of LDL more effectively than the early oxidation of LDL by Fe^{2+} or Cu^{2+} at pH 4.5. It did not inhibit LDLoxidation by Fe^{3+}at pH 4.5 and modestly inhibited the later oxidation of LDL by Cu^{2+} at pH 7.4. Probucol inhibited the oxidation of LDL by Cu^{2+} at pH 7.4 very effectively,but not quite as effectively at pH 4.5. Probucol inhibited the later oxidation of LDL by Fe^{2+} more effectively than the early oxidation of LDL at pH 4.5, but inhibited the early oxidation of LDL by Fe^{3+} at pH 4.5. The detailed chemistry to explain these complex effect remains to be explained.

Chapter 5

The effect of LDL oxidised at lysosomal pH on cultured macrophages.

5.1 Introduction

Scavenger receptors are known by their ability to endocytose modified (e.g. acetylated or oxidised) types of LDL and were first described by Goldstein and Brown in 1979 (Goldstein and Brown, 1979). LDL contains a cholesteryl ester-rich core wrapped by phospholipids and apoB-100, which targets uptake by the LDL receptor. Both the lipid and protein of LDL are subjected to oxidative damage through the action of free radicals. There is a debate about the exact nature of the chemical alterations to LDL *in vivo.* Oxidised LDL is widely belived to involved in the pathogenesis of atherosclerosis (Chisolm and Steinberg, 2000). The classical LDL receptor is downregulated by increasing cellular cholesterol levels but scavenger receptors are not down-regulated. Modified LDL therefore converts macrophages to foam cells, where cytoplasm and lysosomes are swollen with lipid droplets. SR-A knock out mice show a reduced size of atherosclerotic lesions, suggesting a proatherogenic role for SR-A (Babaev et al., 2000). Numerous lines of evidence indicate that lysosomal dysfunction is a serious step in foam cell establishment and plaque progression (Emanuel et al., 2014b). Ceroid pigments, which are analogous to lipofuscins, are formed under pathological circumstances associated with ageing and are known to accumulate in lysosomes producing fluorescence between 360 and 430 nm. Oxidized LDL accumulation results in intense lysosomal dysfunction in cultured macrophages, resulting in disruptions in lysosomal pH, proteolytic ability, membrane integrity, and morphology of their lysosomes (Emanuel et al., 2014b). Earlier studies have described the variable impact of different sorts of modified LDL on build-up of cholesterol, in the cytosol or in lysosomes, with oxidised LDL and aggLDL leading to accumulation of cholesterol in lysosomes, whereas acetylated LDL and LDL

modified by enzymes lead mostly to cytosol cholesterol accumulation (Yancey and Jerome, 2001, Griffin et al., 2005, Orso et al., 2011).

In our laboratory, mouse macrophages swiftly phagocytosed aggregated LDL into their lysosomes and oxidised it with iron (Wen and Leake, 2007). Macrophages endocytose native LDL and aggregated LDL by the LDL receptor, and modified lipoproteins through SR such as SR-A1 and CD36 (Steinberg et al., 1989, Greaves and Gordon, 2009). Cholesteryl esters resulting from the lipoproteins are hydrolysed in late endosomes/lysosomes to release free cholesterol, which is then trafficked to the plasma membrane and peripheral organelles. Additional free cholesterol is re-esterified then in the macrophages on the endoplasmic reticulum via acyl-CoA: cholesterol acyltransferase1 (ACAT1) and stored in cytoplasmic lipid droplets (Chang et al., 2006). This lipid-scavenging role of macrophages is originally useful, but under specific conditions of unregulated or increased lipid engulfment, it leads to extreme accumulation of cholesteryl ester in macrophages that lead to foam cell formation (Accad et al., 2000). Macrophages might phagocytose LDL in the atherosclerotic lesion and oxidised it with iron in their lysosomes. Macrophages death (by apoptosis or necrosis) is common in atherosclerotic lesions and contributes to the "necrotic" lipid core in atheroma. Other macrophages might be expected to endocytose lysosomally oxidised LDL from dead and lysed macrophages. The uptake of dead cells or fragments of cells containing oxidised lipids might be toxic to macrophages. In this study the aim was to investigate the effect of giving antioxidants to macrophages as possible protection against these toxic effects.

5.2 Material and Methods

Lipid accumulation and lysosomal LDL oxidation was induced macrophages using sphingomylinase -aggregated LDL as described in chapter 2. The effect of tempol and probucol on lipid accumulation and ceroid formation was measured. The effect of α-tocopherol, tempol and probucol on reactive oxygen species-generation by macrophages as a result of oxidised LDL exposure was assessed, as described in chapter 2. Finally, the effect of α-tocopherol treatment of the macrophages on apoptosis due to oxidative stress (H_2O_2) was investigated, as described in chapter 2.

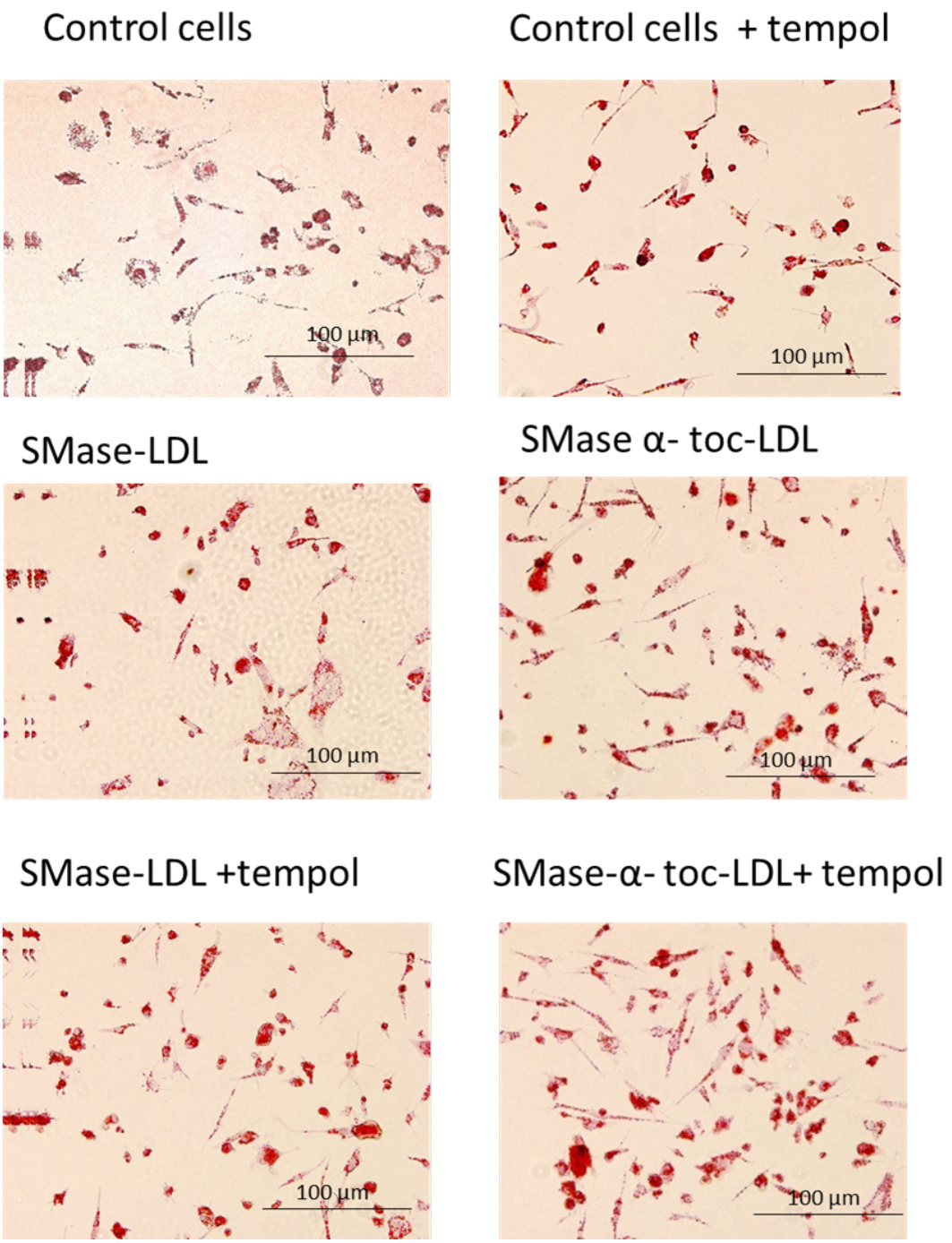
5.3 Results

5.3.1 Measurement of intracellular lipid accumulation in macrophages

A typical feature of initial atherosclerotic lesions in the presence of lipid-laden macrophage foam cells (Groh et al., 2018). It has been recognised that both physically altered (vortexed) LDL, as well as SMase LDL, is oxidised in the lysosomes of macrophages and eventually produces ceroid. Ceroid (lipofuscin) is an ultimate product of lipid oxidation that contains insoluble polymerized lipids and is observed within foam cells in atherosclerotic lesions. Ceroid accumulation is detected via its fluorescence and cytochemical staining characteristics (Haka et al., 2011). The aim of this study was to investigate the effect of antioxidants on intracellular lipid and ceroid accumulation in macrophages.

Incubation of THP-1 human macrophages with LDL aggregated by sphingomyelinase produced extensive total lipid accumulation, (Figure 5.1) . The enrichment of LDL with α-tocopherol, followed by its aggregation by sphingomyelinase SMase-α-LDL, did not affect the lipid content of macrophages compared to (SMase- LDL). Adding the antioxidant tempol (10 μM) did not affect lipid accumulation in macrophages. Tempol slightly increased lipid accumulation in macrophages incubated with aggregated α-tocopherol-enriched LDL.

A


Control cells
Control cells + tempol
100 µm
100 µm
SMase-LDL
SMase α- toc-LDL
100 µm
100 µm
SMase-LDL +tempol
SMase-α- toc-LDL+ tempol
100 µm
100 µm

B

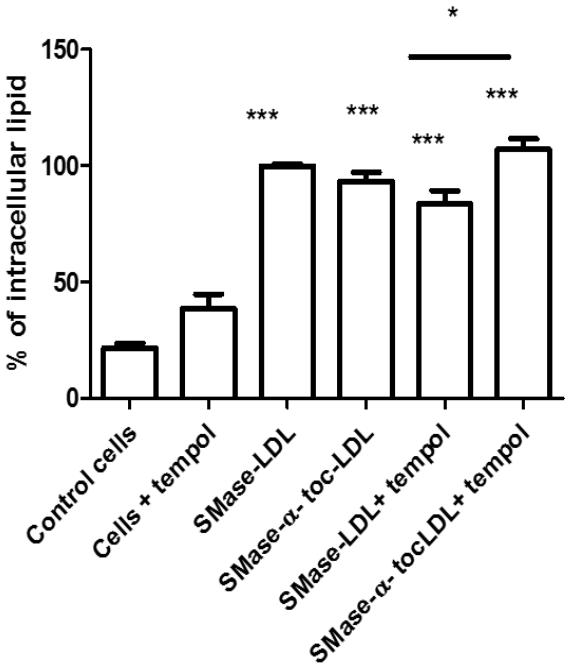


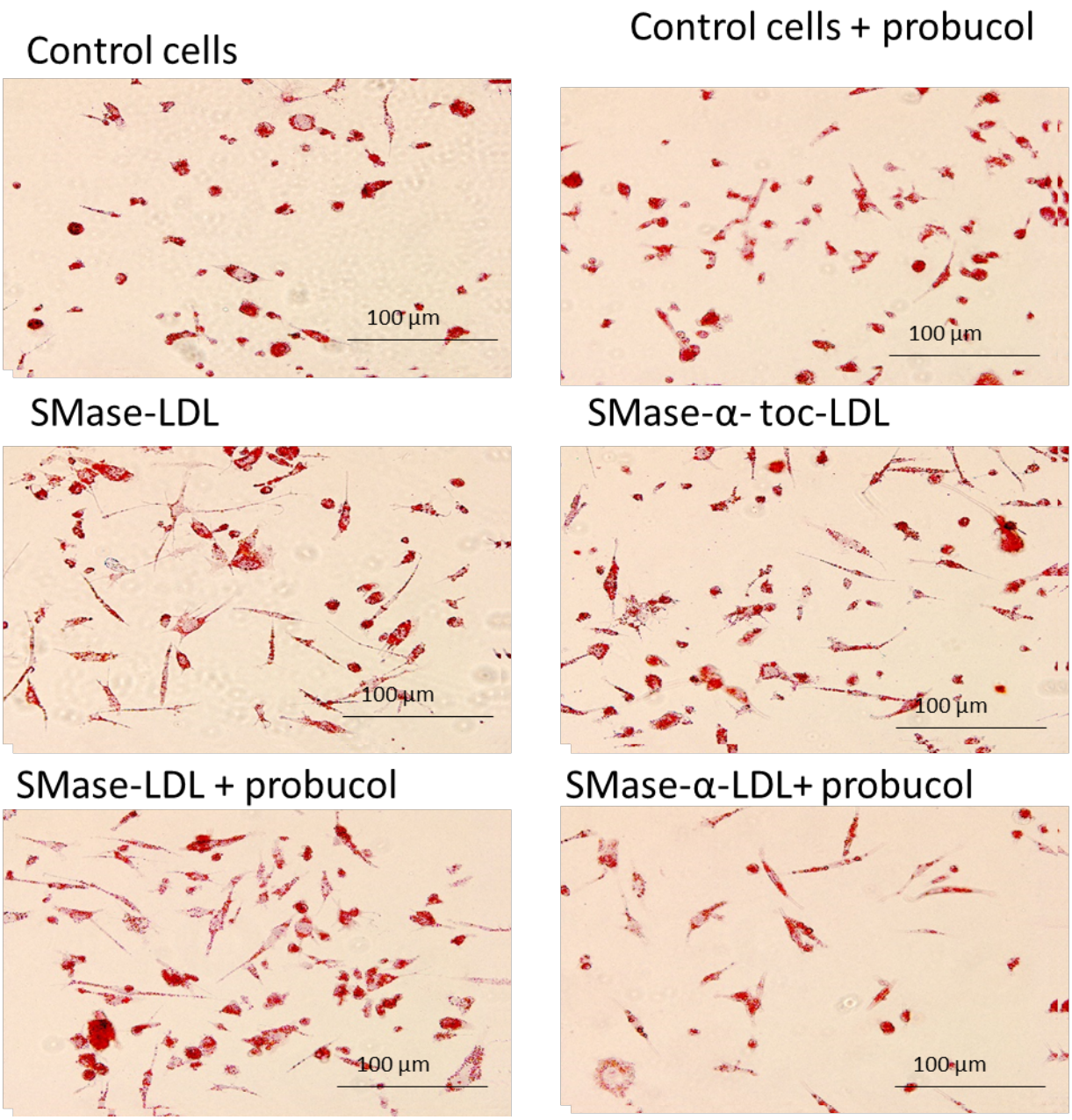
Figure 5.1. Measuring intracellular lipid accumulation in macrophages with or without tempol.

(A)THP-1 macrophages were grown on coverslips in 6 well sterile tissue culture plates. cells were treated with LDL aggregated by sphingomyelinase (Smase-LDL) or α-tocopherol-enriched LDL aggregated by sphingomyelinase (200 µg protein/mL) for 24 h and after washing were further incubated for 7 days with and without tempol 10 µM in fresh RPMI containing 10% (v/v)lipoprotein – deficient serum as a control. After 7 days, cells were washed, fixed and treated with, Oil Red O staining, and examined by light microscopy to demonstrate intracellular lipids. (B) The bar chart shows intracellular lipid accumulation in macrophages as a percentage of that in the cells incubated with Smase-LDL. A one-way ANOVA followed by Tukey's posthoc tests were significant (***P<0.001, n= 4) compared to control LDL.

5.3.2 Intracellular lipid accumulation in presences of probucol in macrophages

Probucol has been experimentally used as an antiatherogenic drug, not only for of its lipid-lowering outcome but because of its antioxidative properties that inhibit oxidative modification of LDL shown *in vitro* (Gotoh et al., 1992) and *in vivo* models (Carew et al., 1987). The aim of this study is to investigate the effects of antioxidants on intracellular lipid accumulation caused by aggregated LDL. The probucol (5 μM) was added to the THP-1 macrophages together with SMase-LDL or SMase-α-tocopherol supplemented LDL. Probucol did not prevent lipid accumulation with SMase-LDL but markedly decreased lipid accumulation with SMase-α-tocopherol supplemented LDL (Figure 5.2). The concentration of probucol added to the medium was nearly equivalent to the plasma concentration of the drug regularly obtained from patients after treatment with probucol.

A


Control cells
Control cells + probucol
SMase-LDL
SMase-α- toc-LDL
SMase-LDL + probucol
SMase-α-LDL+ probucol
100 µm

B

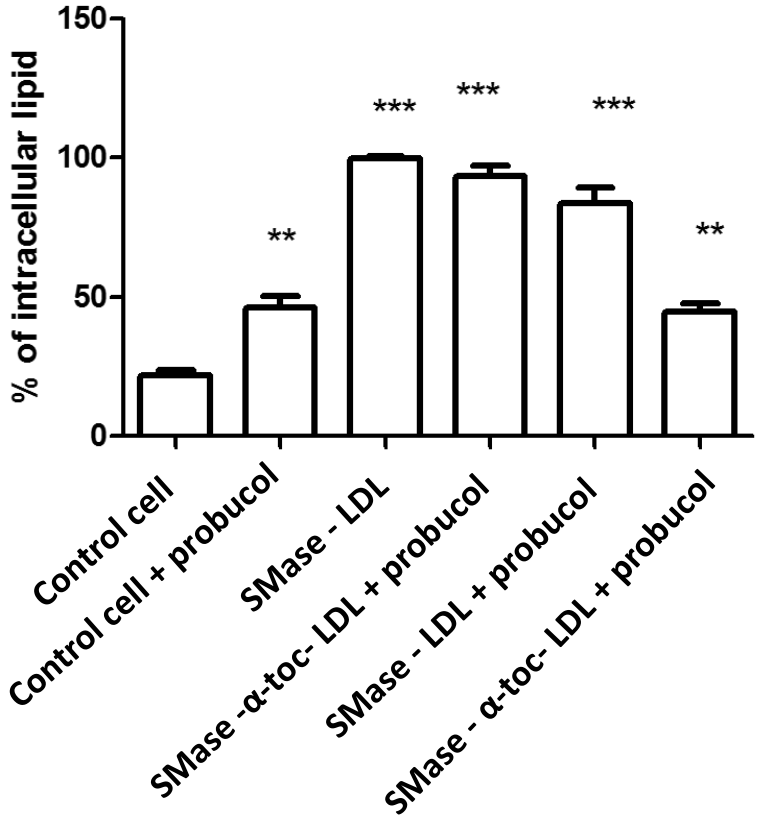


Figure 5.2. Measuring intracellular lipid accumulation in macrophages with or without probucol.

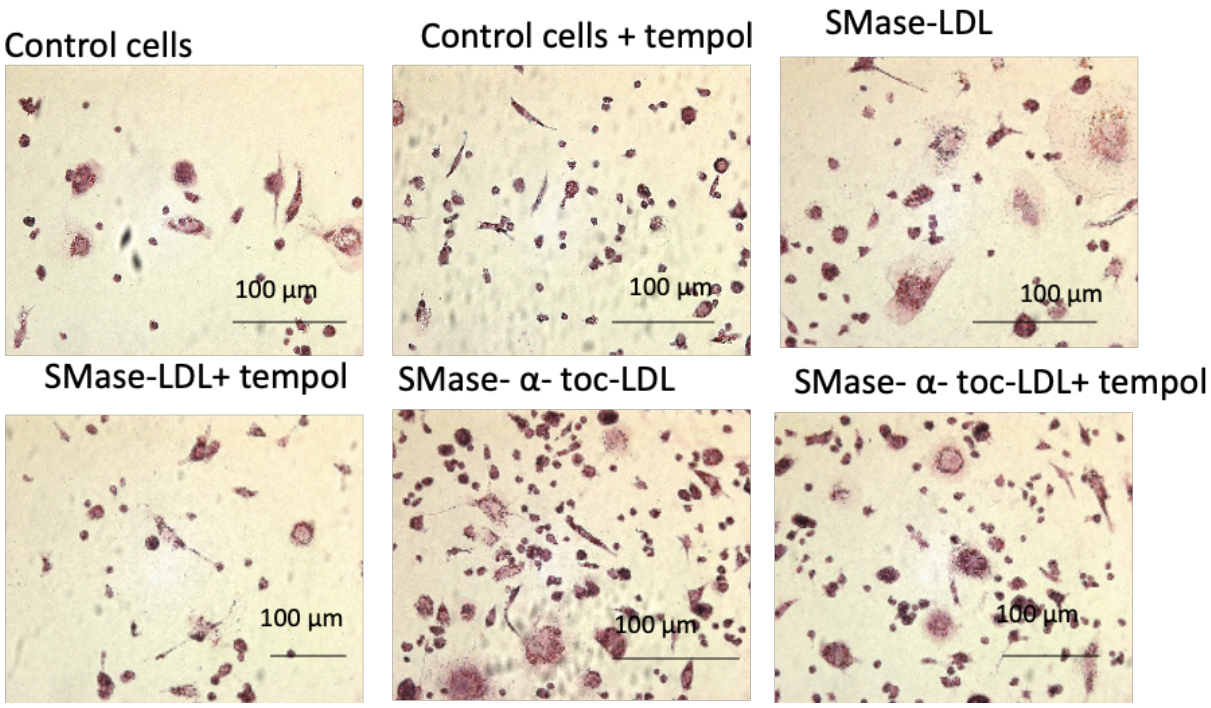
(A)THP-1 macrophages were grown on coverslips in 6 well sterile tissue culture plates. Wells were treated with LDL aggregated by sphingomyelinase (SMase-LDL) or α-tocopherol-enriched LDL aggregated by sphingomyelinase (200 µg protein/ml) for 24 h and after washing were further incubated for 7 days in fresh RPMI containing 10% (v/v) lipoprotein -deficient serum with or without probucol. After 7 days, cells were washed, fixed and stained with Oil Red O, and examined by light microscopy to demonstrate intracellular lipids. (B) The bar chart shows the intracellular lipid accumulation in macrophages as a percentage of that incubated by SMase-LDL. A one-way ANOVA followed by Tukey's posthoc tests were significant (**P< 0.005, ***P<0.001, n= 4) compared to control LDL.

5.3.3 Measurement of ceroid formation in macrophages

5.3.3.1 Measurement of ceroid formation in macrophages in the presence of tempol

Foam cell lysosomes contain the peroxidised lipid-protein complex ceroid (Ball et al., 1987). Ceroid is predicted to have detergent characteristics that liquefy membranes, triggering cell injury and necrosis, and initiating release of the toxic complex into the centre of the plaque. In tissues, it is identified histochemically by insolubility in lipid solvents and stainability with neutral lipid dyes (Di Guardo, 2015, Lang and Insull, 1970). Ceroid levels were increased in macrophages incubated with SMase aggregated-LDL or SMase aggregated α-tocopherol-supplemented LDL (Figure 5.3). This is consistent with the finding is not as effective an antioxidant for LDL at pH 4.5 as it is at pH 7.4,as shown in chapter 3. There was a significant decrease in the formation of ceroid with SMase LDL in the presence of tempol. Tempol addition to SMase -α- tocopherol enriched LDL did not protect cells from advanced LDL oxidation and therefore ceroid formation.

A


Control cells
100 µm
Control cells + tempol
100 µm
SMase-LDL
100 µm
SMase-LDL+ tempol
100 µm
SMase- α- toc-LDL
100 µm
SMase- α- toc-LDL+ tempol
100 µm

B

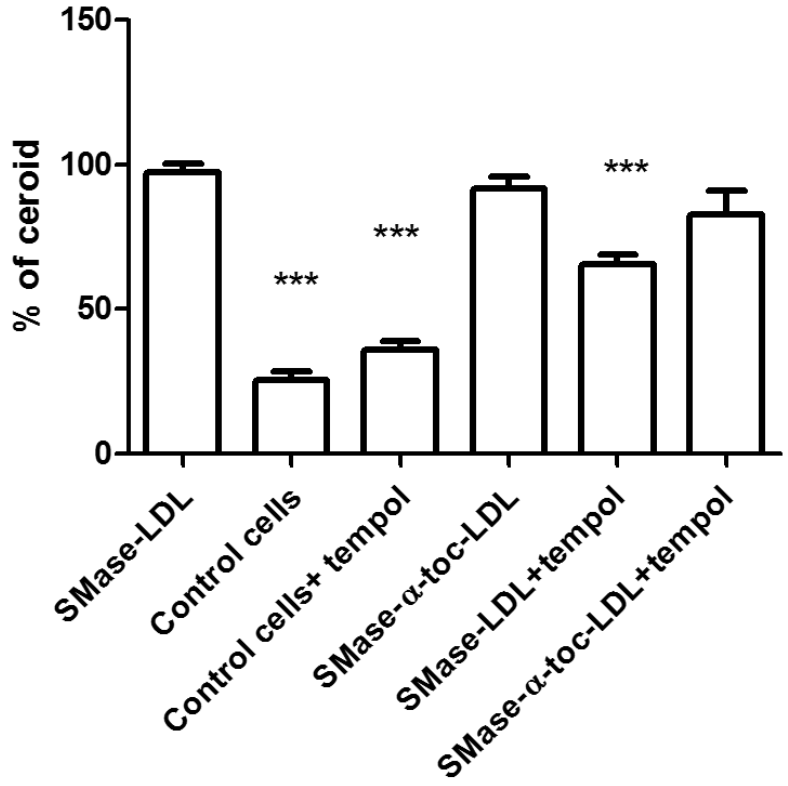


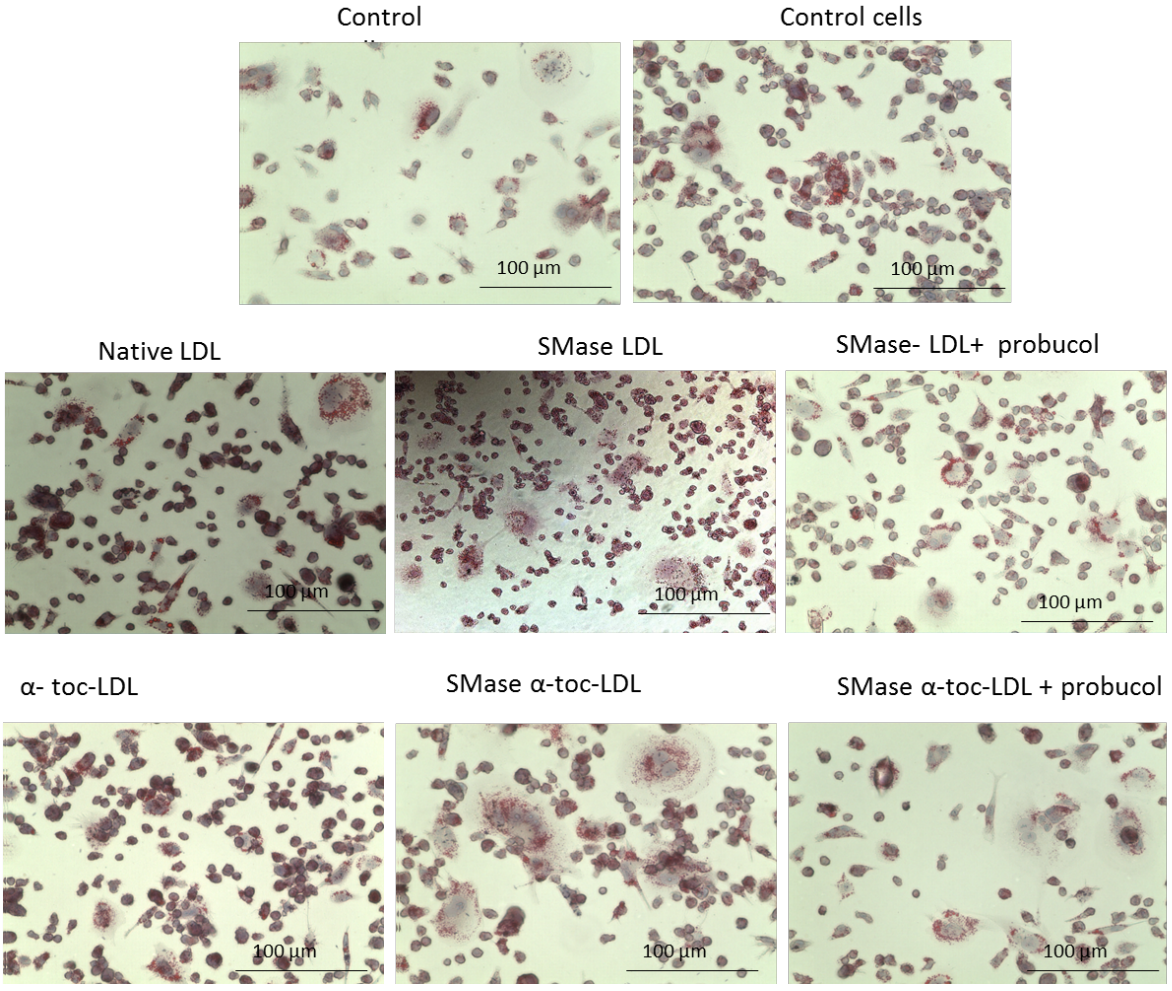
Figure 5.3 Measuring ceroid formation in macrophages with or without tempol in macrophages

(A) THP-1 macrophages were grown on coverslips in 6 well sterile tissue culture plates. Wells were treated with SMase-LDL and α-tocopherol enriched 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 or with 10 µM tempol. After 7 days, cells were treated with ethanol and xylene, followed by Oil Red O, and examined by light microscopy to demonstrate ceroid. (B) The bar chart shows the ceroid levels in macrophages as a percentage of that with SMase-LDL. A one-way ANOVA followed by Tukey's posthoc tests were significant (***P<0.001, n= 4) compared to the SMase-α-toc-LDL.

5.3.3.2 Measurement of intracellular ceroid formation in macrophages with the presence of probucol

In this study I investigated the effect of probucol on ceroid formation in macrophages (Figure 5.4). Enriched LDL with α-tocopherol did not inhibit ceroid formationaccumulation. Surprisingly probucol alone increased the accumulation of ceroid in cells compared to control cells. There was also more ceroid formation with native α-tocopherol-enriched LDL than native control LDL. In contrast, probucol, when it was combined with SMase-α-tocopherol-enriched-LDL or SMase-LDL, showed inhibition effects. Thus, it appears that probucol can act as a pro-oxidant or an antioxidant, depending on the condition. The reasons for these complex and suprising effects are unknown.

A


Control
Control cells
100 µm
100 µm
Native LDL
SMase LDL
SMase- LDL+ probucol
100 µm
100 µm
100 µm
α- toc-LDL
SMase α-toc-LDL
SMase α-toc-LDL + probucol
100 µm
100 µm
100 µm

B

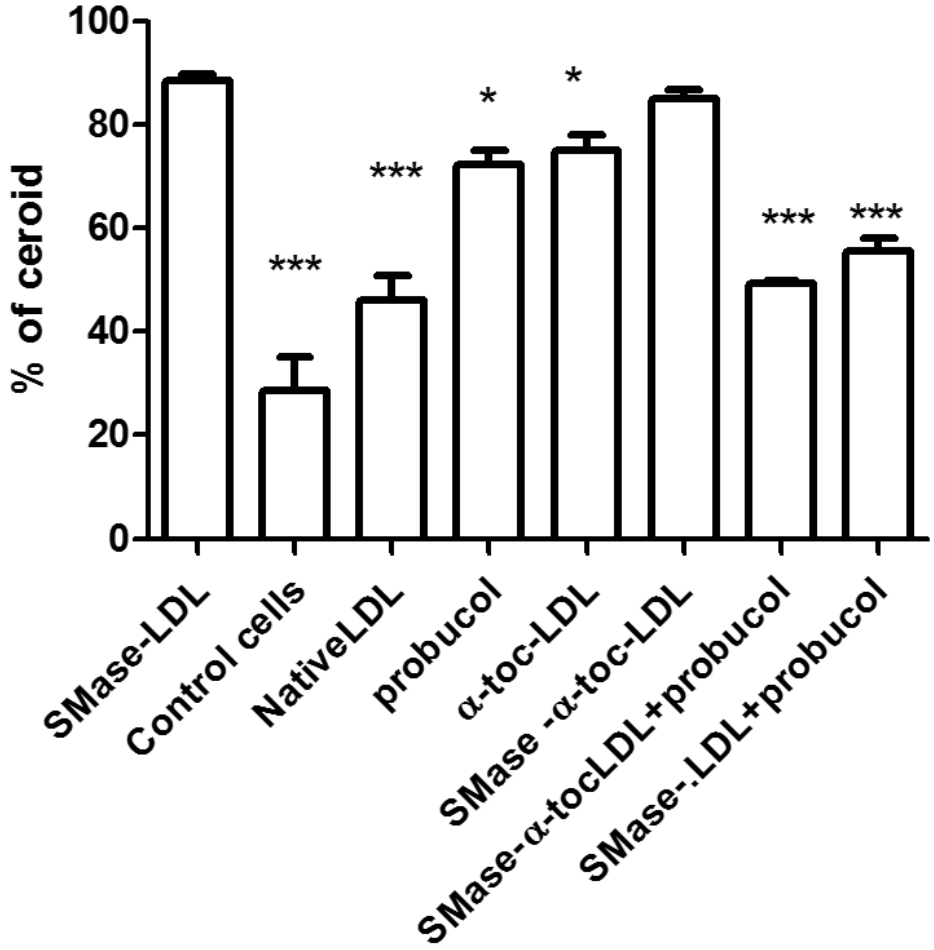


Figure 5.4 Measuring ceroid formation in macrophages with or without probucol.

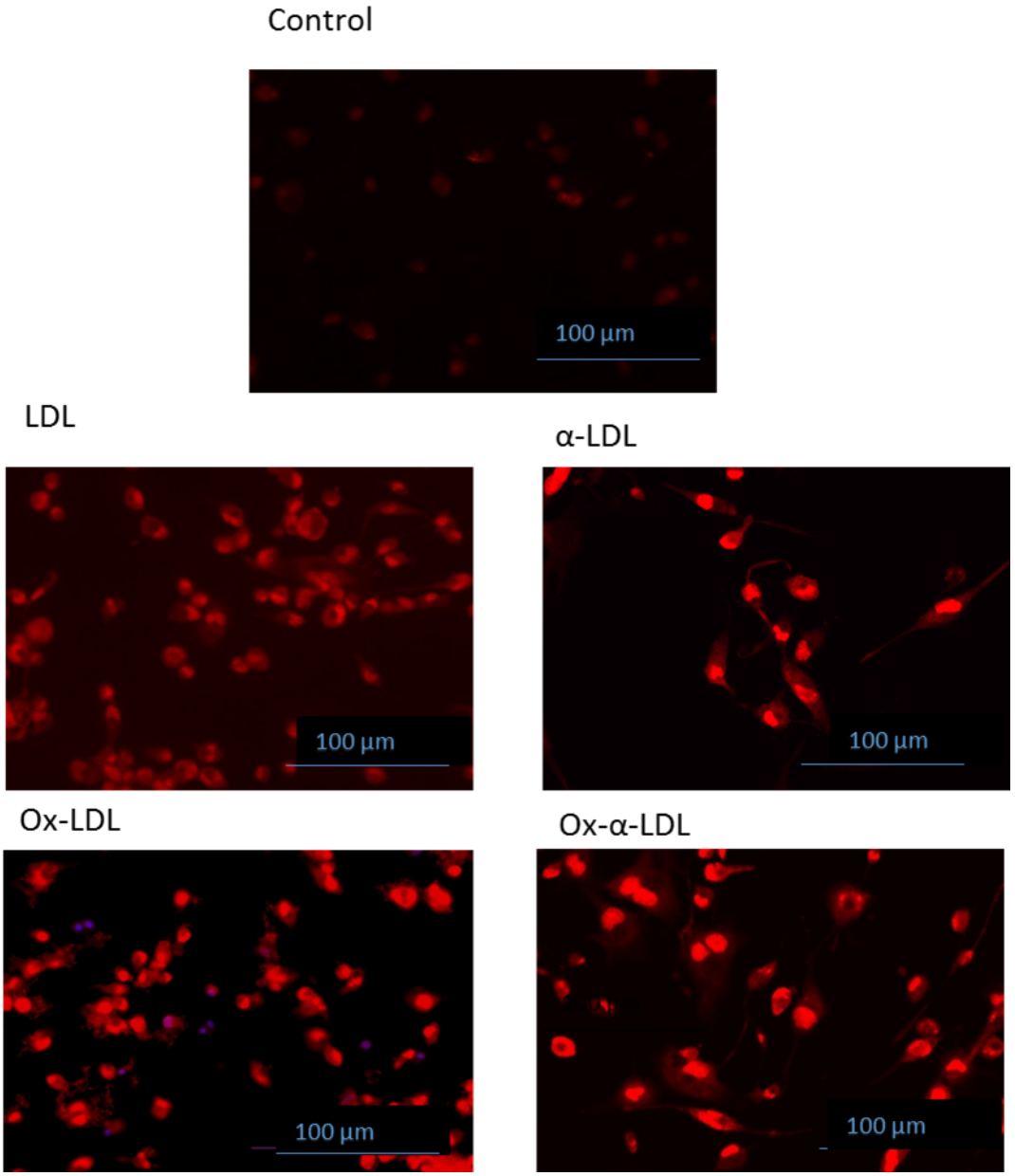
(A)THP-1 macrophages were grown on coverslips in 6 well sterile tissue culture plates. Wells were treated with SMase-LDL and SMase α-tocopherol enriched LDL (200 µg protein/ml) for 24 h and after washing were further incubated for 7 days in fresh RPMI containing 10% (v/v) lipoprotein-deficient serum, either alone or with 5 µM probucol. After 7 days, cells were treated with ethanol and xylene, followed by Oil Red O staining, and examined by light microscopy to demonstrate ceroid. (B) The bar chart shows the intracellular ceroid accumulation in macrophages. A one-way ANOVA followed byTukey's posthoc tests were significant (*P< 0.05, ***P<0.001, n= 4) compared to the SMase-α-tocopherol- enriched LDL.

5.3.4 Monitoring of ROS production with the use of DHE

I investigated the production of ROS by macrophages in the presence of oxidised LDL and if this would be decreased by antioxidants.

Dihydroethidium (DHE) can be used to measure superoxide and hydrogen peroxide production, in intact macrophages by forming a fluorescent product (Zhao et al., 2012). The aim of this study was to investigate antioxidants either individual or combination, to reduce ROS production in macrophages induced by oxidised LDL, which was produced by incubation with Cu^{2+} (5 µM). There was a significant increase in reaction oxygen specious produced by the macrophages in the presence of α-tocopherol-enriched native LDL,oxidised LDL or oxidised α-tocopherol-enriched LDL, but not native control LDL. This study also evaluated the intracellular ROS formation induced by ox-LDL after incubating the cells with tempol (10 µM) only and untreated cells as a control and LDL and LDL enriched with α -tocopherol by Cu^{2+} (5 µM) in the presence or absences of tempol (Figure 5.6 and 5.7). ROS formation increased in the cells which were treated with oxidised LDL and oxidised LDL enriched with α-tocopherol, while in the presence of tempol or probucol there was significantly decreased generation of ROS. This suggests tempol and probucol can scavenger free radicals produced by the cells from oxidised LDL with or without additional α-tocopherol.

A


Control
100 µm
LDL
100 µm
α-LDL
100 µm
Ox-LDL
100 µm
Ox-α-LDL
100 µm

B

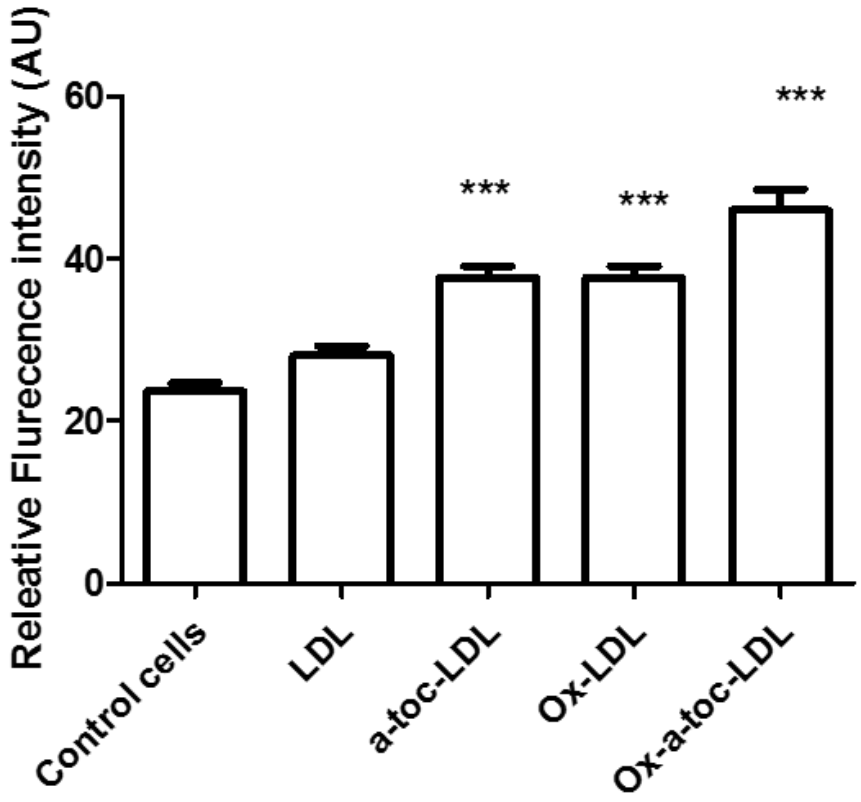
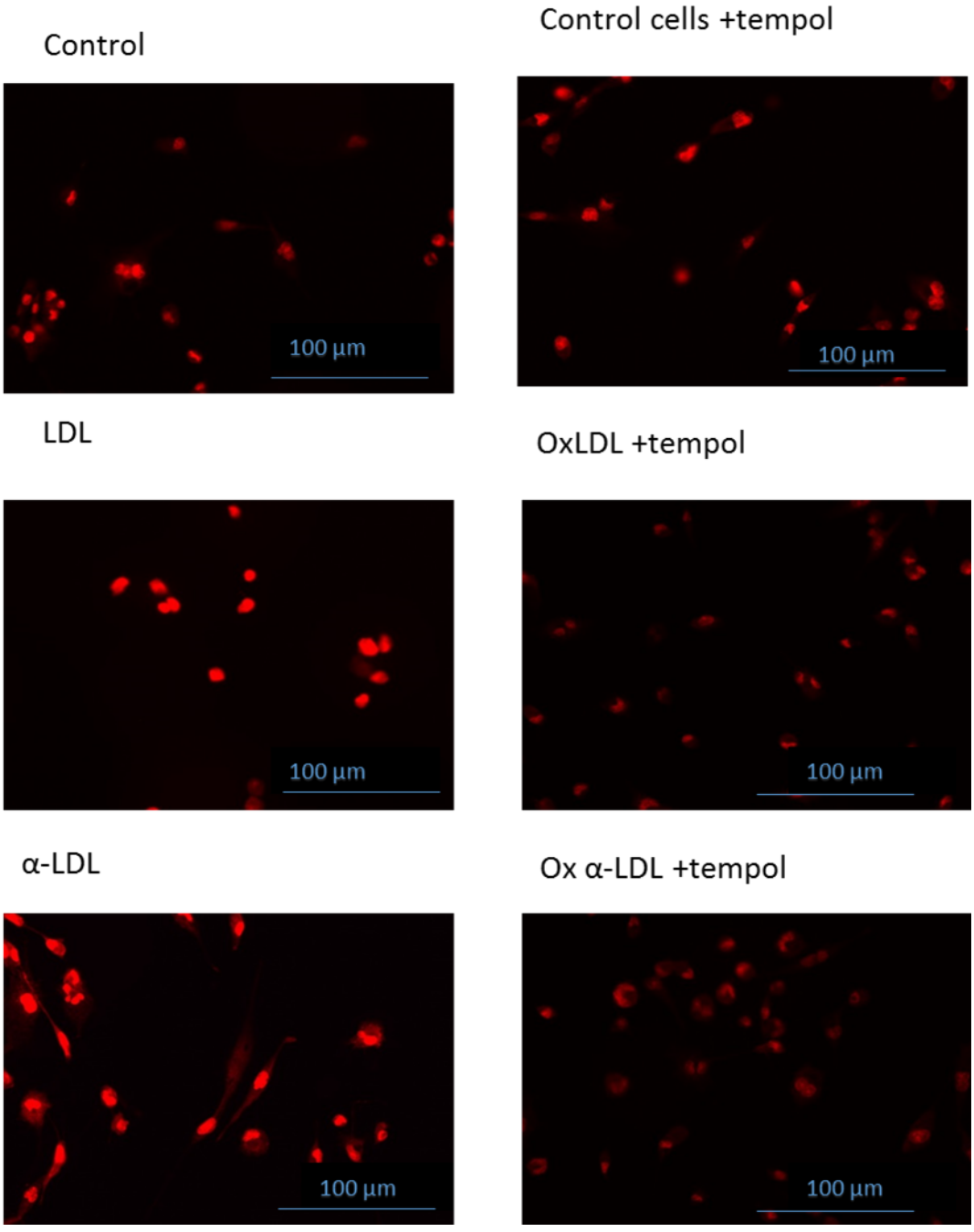


Figure 5.5 ROS formation in macrophages.

(A) Control LDL and LDL enriched with α-tocopherol LDL was oxidised by Cu^{2+}(^{5} µM) at pH 4.5 for 24 h.THP-1 macrophages were grown on coverslips in 6 well sterile tissue culture plates. Wells were treated with oxidised LDL or oxidised α-tocopherol enriched LDL (50 µg protein/ml) for 24 h. Untreated cells were taken as control and after washing, cells were further incubated for 15 minutes with 10µM DHE in fresh PBS in a non-CO_2 incubator. Cells were washed with PBS, followed by staining the coverslips with DAPI, and examined by fluorescence microscopy to demonstrate DHE fluorescence.(B) The bar chart shows the fluorescence intensity of the macrophages. A one-way ANOVA followed by Tukey's posthoc tests were significant (***P<0.001, n= 3) compared to the control.

A


Control
Control cells +tempol
100 µm
100 µm
LDL
OxLDL +tempol
100 µm
100 µm
α-LDL
Ox α-LDL +tempol
100 µm
100 µm

B

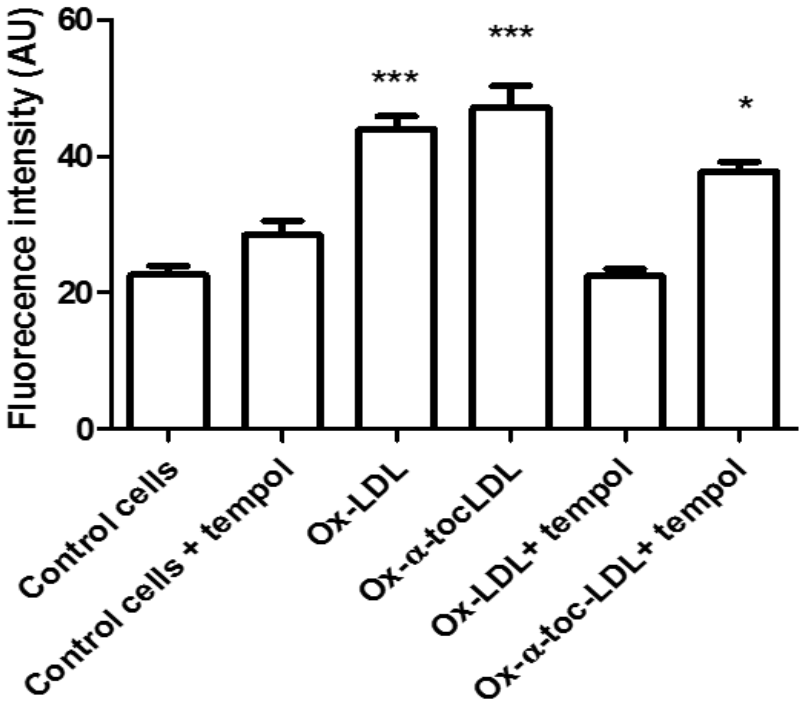
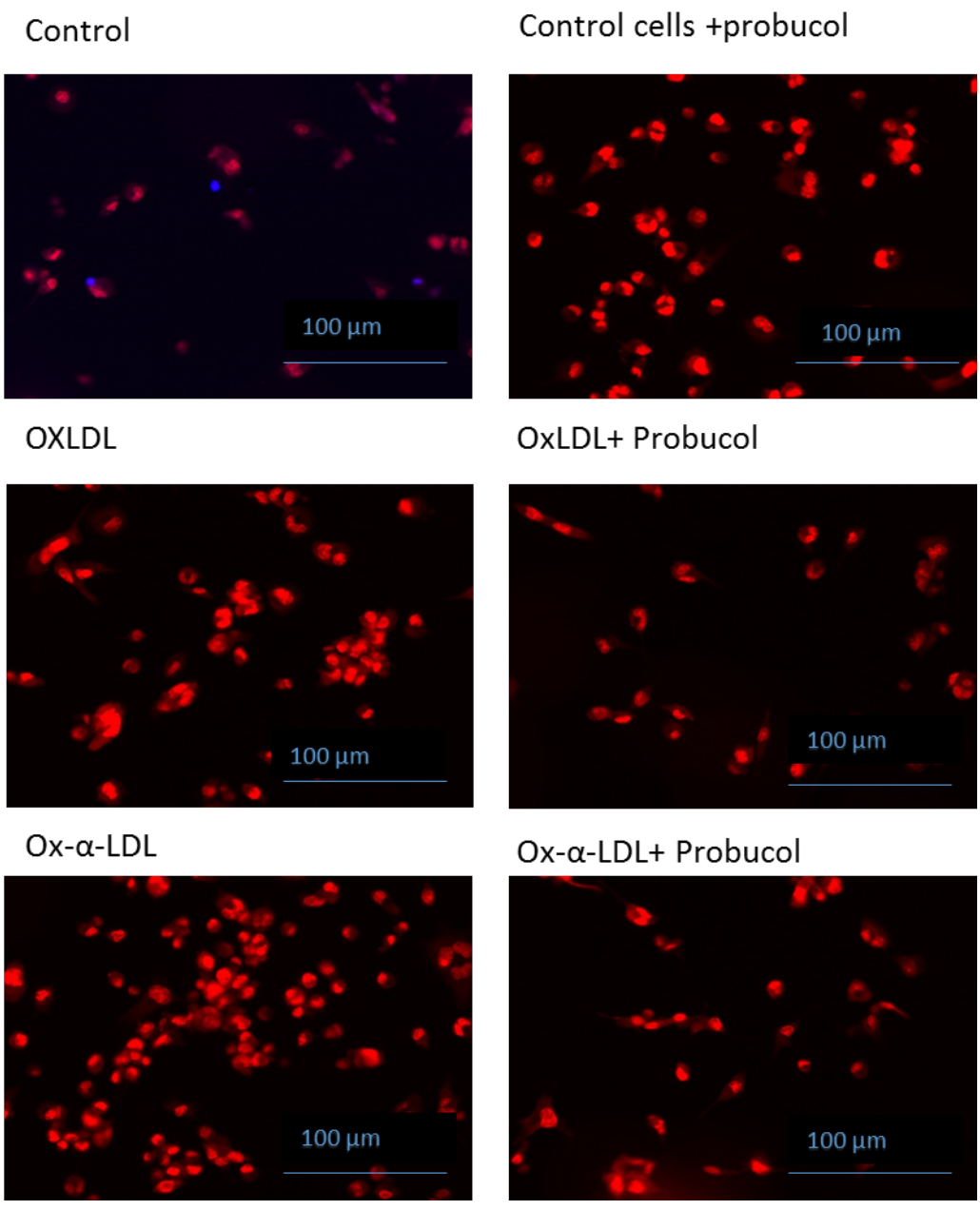


Figure 5.6 Detection of ROS formation in macrophages with or without tempol

(A) Control LDL and α- tocopherol enriched LDL was oxidised by Cu^{2+}(5 µM) at pH 4.5 for 24 h. THP-1 macrophages were grown on coverslips in 6 well sterile tissue culture plates. Wells were treated with oxidised LDL or oxidised α-tocopherol enriched LDL (50 µg protein/mL) for 24 h either alone or with 10 µM tempol. Untreated cells were taken as control and after washing, cells were further incubated for 15 minutes with 10µM DHE in fresh PBS in a non-CO_2 incubator. Cells were then washed with PBS, followed by staining the coverslips with DAPI and examined by fluorescence microscopy to demonstrate DHE fluorescence. (B) The bar chart shows the fluorescence intensity of the macrophages. A one-way ANOVA followed by Tukey's posthoc tests were significant (*P< 0.05, ***P<0.001, n= 4) compared to the control.

A


Control
Control cells +probucol
OXLDL
OxLDL+ Probucol
Ox-α-LDL
Ox-α-LDL+ Probucol
100 µm
100 µm
100 µm
100 µm
100 µm
100 µm

B

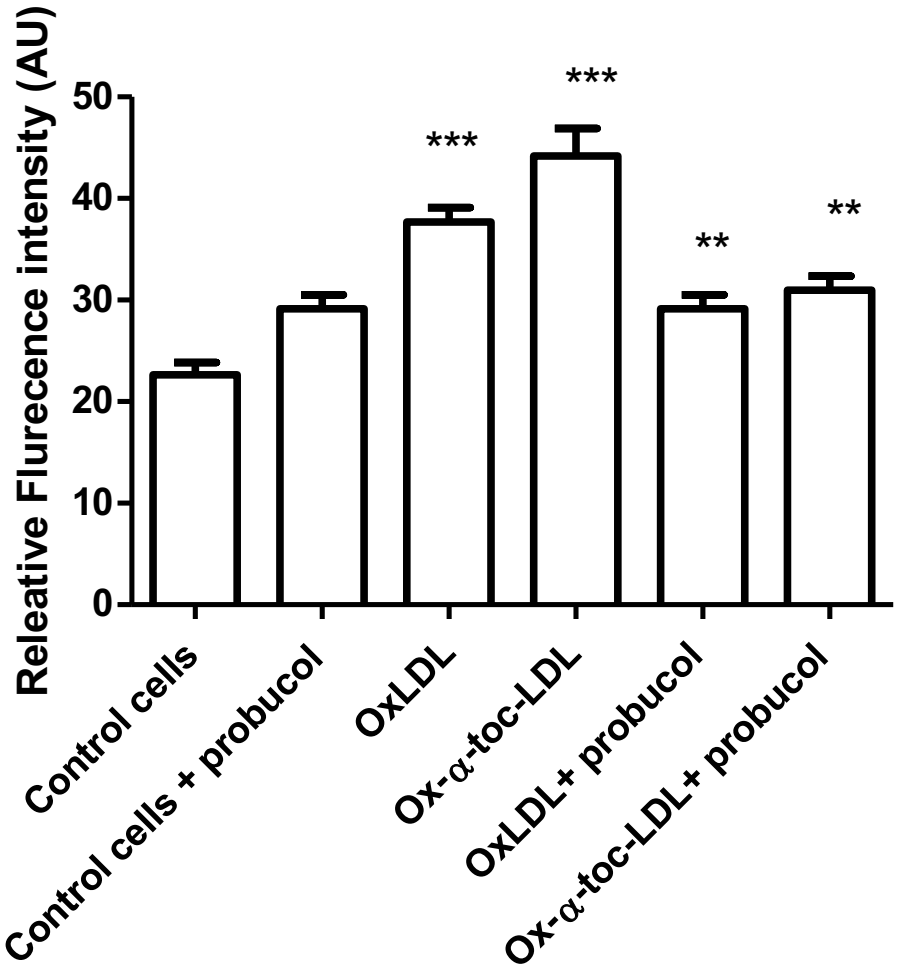


Figure 5.7 Detection of ROS formation in macrophages with or without probucol

(A) Control LDL and α -tocopherol-enriched LDL was oxidised by Cu^{2+}(5 µM) at pH 4.5 for 24 h. THP-1 macrophages were grown on coverslips in 6 well sterile tissue culture plates. Wells were treated with oxidised LDL or oxidised α-tocopherol- enriched LDL (50 µg protein/mL) for 24 h either alone or with 5 µM probucol. Untreated cells were taken as control and after washing, cells were further incubated for 15 minutes with 10µM DHE in fresh PBS in a non-CO_2 incubator. Cells were washed with PBS, followed by staining the coverslips with DAPI and examined by fluorescence microscopy to demonstrate DHE fluorescence. (B) The bar chart shows fluorescence intensity of the macrophages. A one-way ANOVA followed by Tukey's posthoc tests were significant (***P<0.001, n= 4) compared to the control.

5.3.5 Effect of α-tocopherol and tempol on apoptosis of macrophages

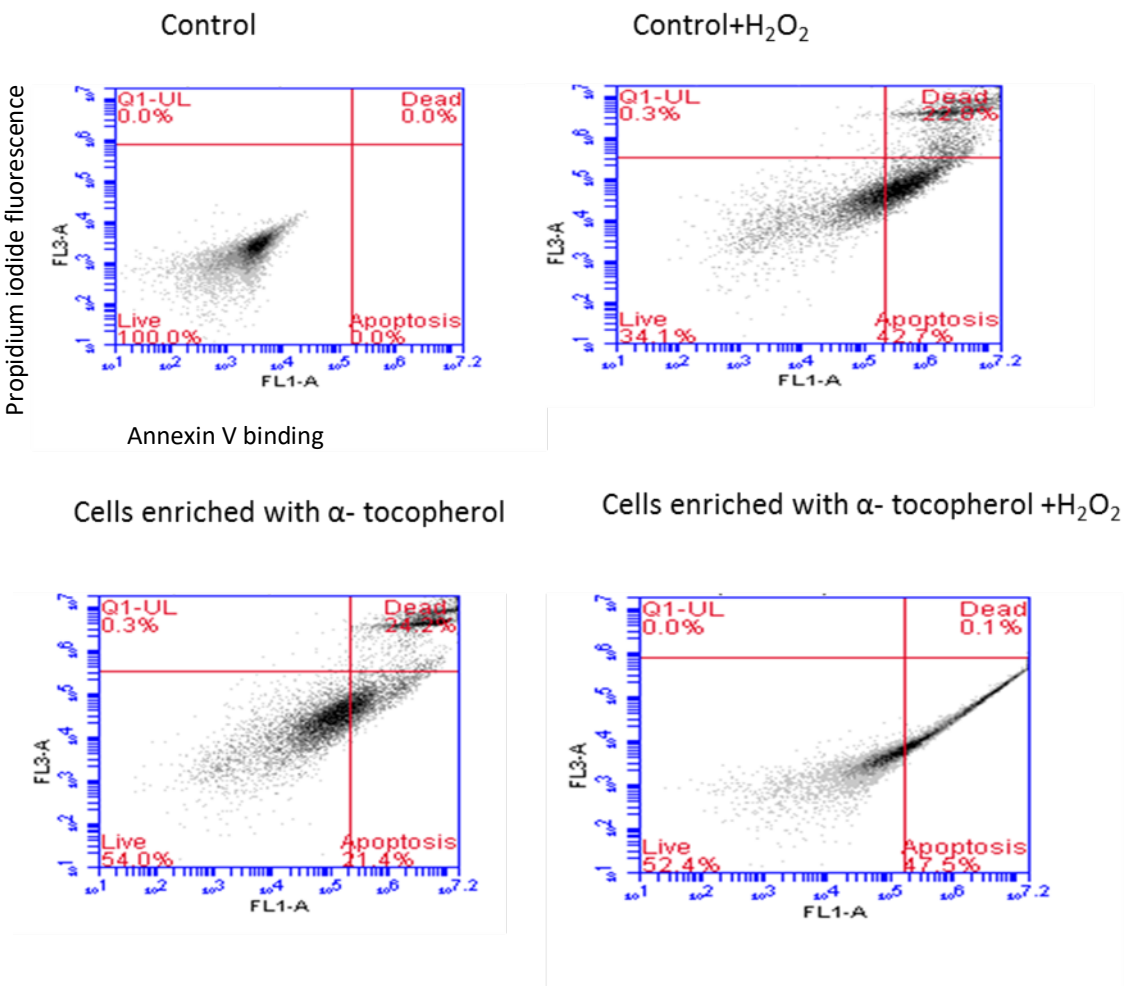
Macrophages are an important cell type in atherosclerotic lesions, and some of these cells become apoptotic, mainly in advanced lesions. Although they persist for a comparatively long time in a physiological situation, their death produced by such stimuli as cytokines, microbial invasion, or endoplasmic reticulum or ROS stress is frequently detected in pathological situations (DeVries-Seimon et al., 2005, Pan et al., 2010). Consequently, there is substantial clinical significance in reviewing macrophage cell death caused by ROS. Cell death is classically described either as apoptosis or necrosis. Since the consequences of apoptosis and necrosis are quite different for an entire organism, the investigation of the cell death mode has considerable clinical significance. Apoptosis followed by the phagocytosis of the apoptotic cells or cell fragments does not normally lead to inflammation, whereas necrosis causes inflammation (Lin et al., 2010). The objective of this study was to demonstrate if α-tocopherol and tempol protect cells from apoptosis and necrosis induced by oxidative stress (100 µM H_2O_2) by using the Annexin V and FITC .

In this study, 100 µM H_2O_2 was used to induce apoptosis and we examined the antioxidant effect of α-tocopherol alone or in combination with tempol (10 µM) on THP-1 macrophages apoptosis. The α-tocopherol levels in the cells were increased by incubation with FCS enriched in α -tocopherol. We measured cell death by flow cytometry using annexin V binding, which measures the externalisation of phosphatidylserine and thus apoptosis, and propidium iodide binding to DNA, which measures membrane permeabilisation . An increase of both annexin V binding and propidium iodide binding measure secondary necrosis, i.e. membrane

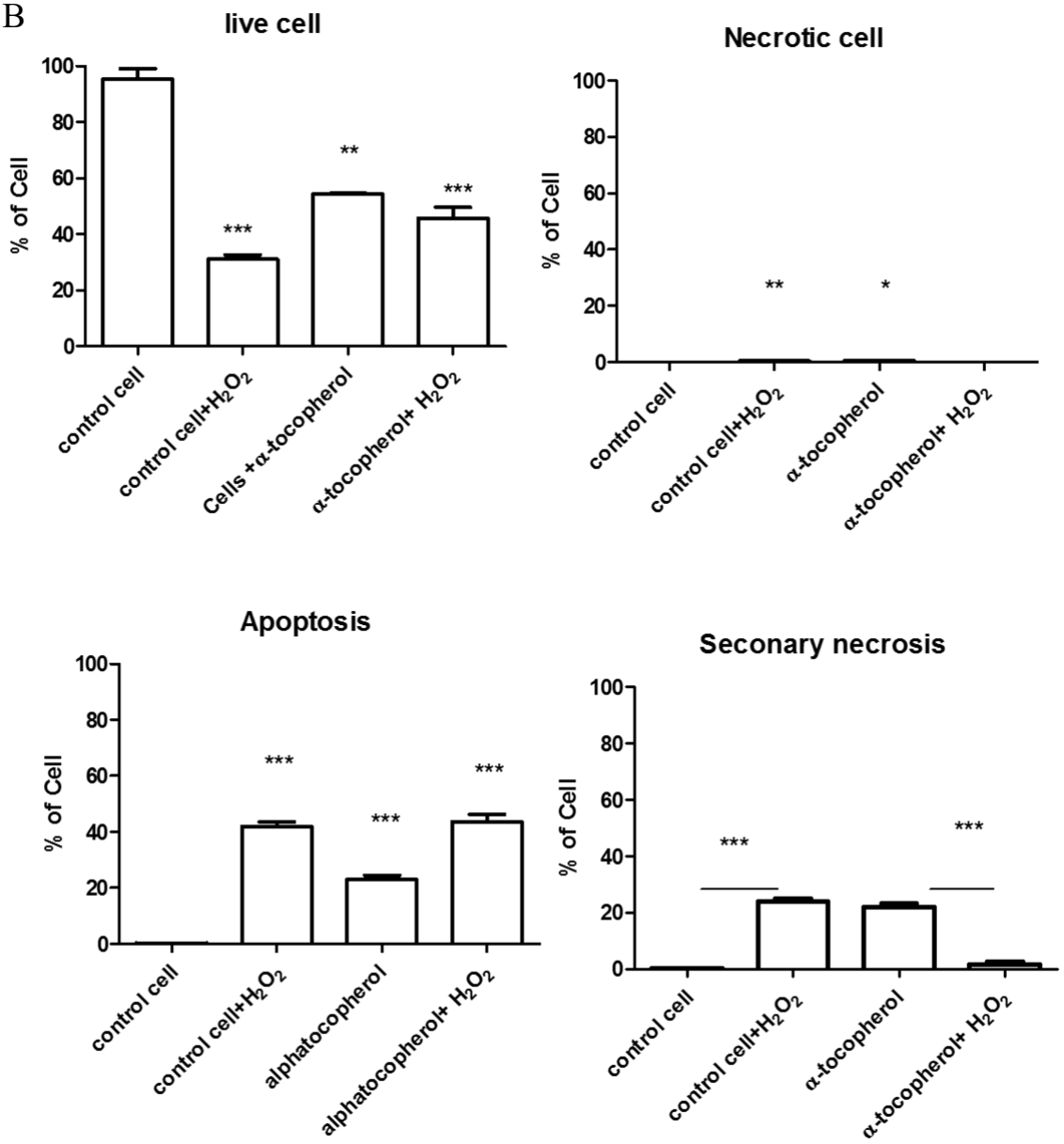
permeabilisation following apoptosis. A plots were employed for gating cells and to recognise each change in the scattering features of the cells. Annexin V FITC-A vs Propidium Iodide-A plots from the gated cells show the populations conforming to viable (annexin V--PI--, lower left), apoptotic (annexin V+ PI--, lower right), dead (annexin V+ PI+, upper right) and necrotic (annexin V--PI+, upper left).

There was a significant increase in apoptosis and secondary necrosis during treatment of normal cells with H_2O_2 (100 μM) (Figure 5.8). Suprisingly, there was an increase in apoptosis and secondary necrosis when the cells were treated with α-tocopherol (Figure 5.8). Treatment with α-tocopherol and H_2O_2 increased apoptosis,but decreased secondary necrosis compared to cells incubated with α-tocopherol alone . There was no significant difference between control cells and cells treated with tempol, in terms of live, apoptotic and secondary necrotic cells except that there was a small increase in necrotic death percentage (Figure 5.9). Tempol had the ability to protect cells,with or without α-tocopherol treatment, almost totally from the toxic effect produced by hydrogen peroxide (Figure 5.8), α-tocopherol treatment increased the percentage of primary necrotic cells in the presence of tempol.

A

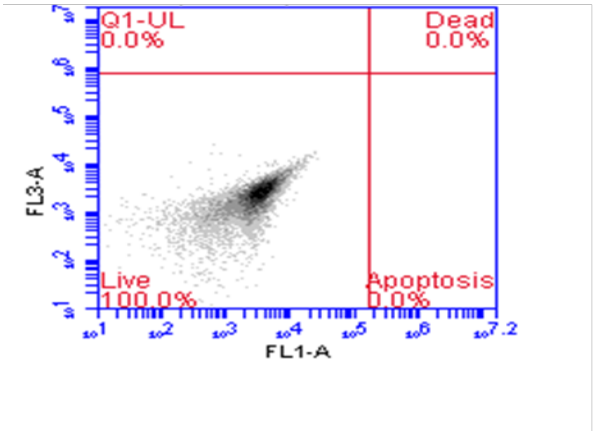

Control
Control+H2O2
Propidium iodide fluorescence
Q1-UL
0.0%
Dead
0.0%
Live
100.0%
Apoptosis
0.0%
FL3-A
FL1-A
Q1-UL
0.3%
Dead
22.0%
Live
34.1%
Apoptosis
42.7%
Annexin V binding
Cells enriched with α- tocopherol
Cells enriched with α- tocopherol +H2O2
Q1-UL
0.3%
Dead
24.2%
Live
54.0%
Apoptosis
21.4%
Q1-UL
0.0%
Dead
0.1%
Live
52.4%
Apoptosis
47.5%

B

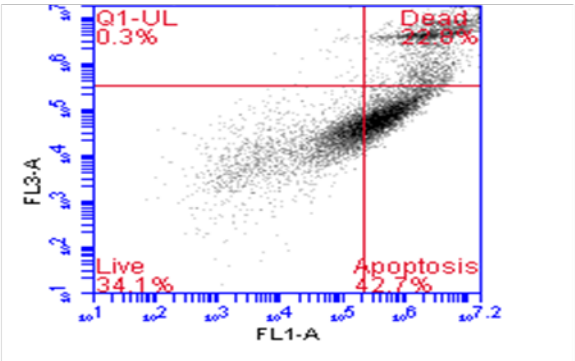

live cell
% of Cell
100
80
60
40
20
0
***
**
***
control cell
control cell+H2O2
Cells +α-tocopherol
α-tocopherol+ H2O2
Necrotic cell
% of Cell
100
80
60
40
20
0
**
*
control cell
control cell+H2O2
α-tocopherol
α-tocopherol+ H2O2
Apoptosis
% of Cell
100
80
60
40
20
0
***
***
***
control cell
control cell+H2O2
alphatocopherol
alphatocopherol+ H2O2
Seconary necrosis
% of Cell
100
80
60
40
20
0
***
***
control cell
control cell+H2O2
α-tocopherol
α-tocopherol+ H2O2

C

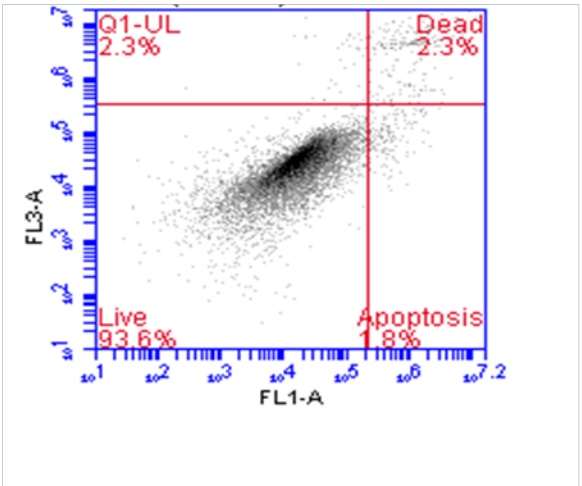
Control



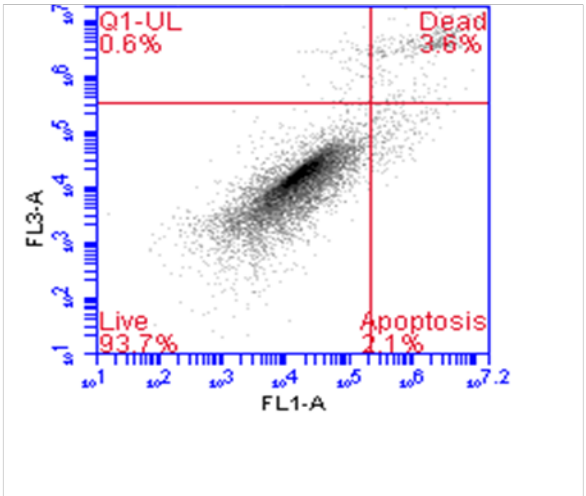
Control+H_2O_2



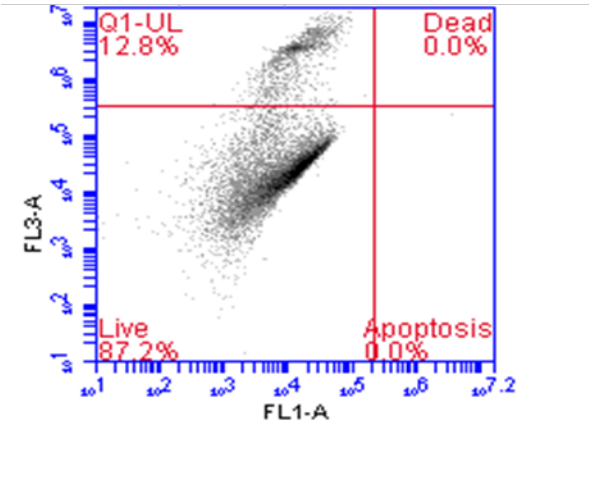
Control + tempol



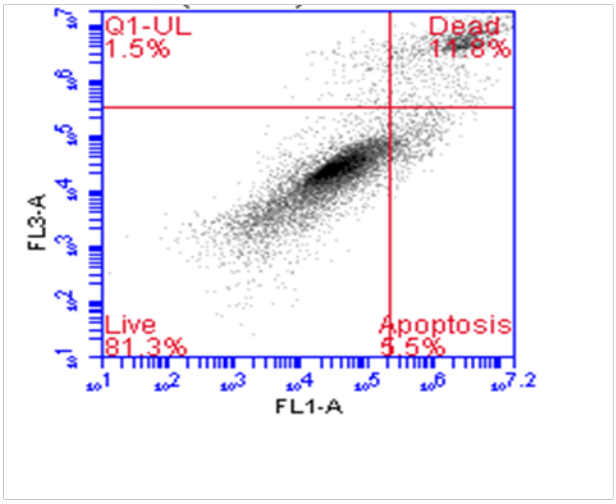
tempol+H_2O_2



α- tocopherol+ tempol



α- tocopherol +tempol +H_2O_2



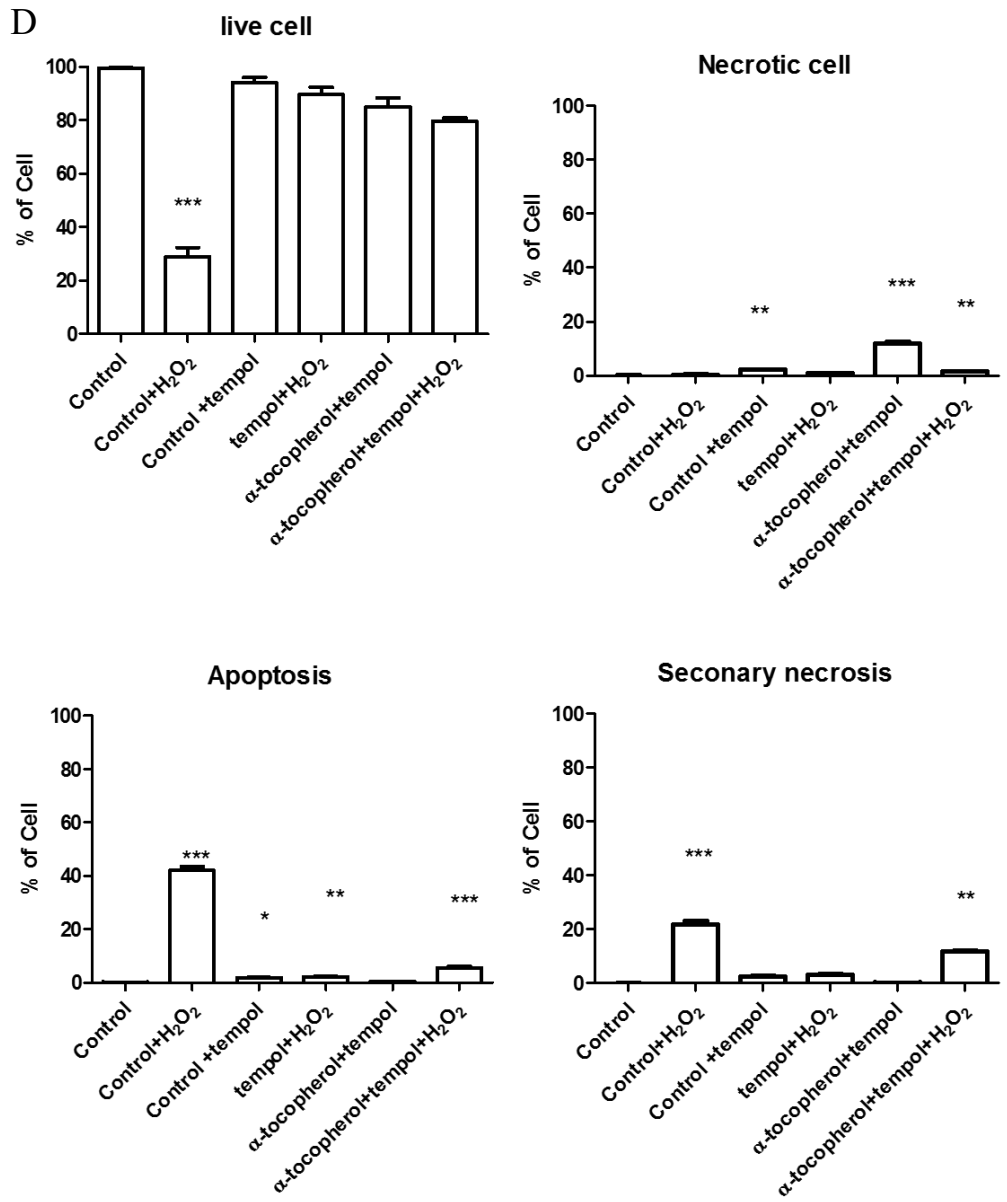


Figure 5.8. Apoptosis detection in THP-1 cells by flow cytometry.

THP-1 cells were enriched with α-tocopherol for 24 h and then were treated with H_2O_2 (100 µM) and tempol (10 µM). The quadrants were set based on the population of healthy, unstained cells in untreated samples (control) compared to treated cells. (A) Representative figures showing population of unstained live (annexin V--PI--, lower left), apoptotic (annexin V+ PI--, lower right), dead (secondary necrosis) (annexin V+ PI+, upper right) and necrotic (annexin V--PI+, upper left) cells.(B) Bar chart represents the quantification of the all death cells induced upon treatment with H_2O_2 or without α-tocopherol and tempol. (C) Repersentitve figure showing live ,apoptotic,dead and necrotic cells with tempol.(D) Bar chart showing quantative results of cell-death in the presence of tempol.Data are expressed as mean± standard error of the mean (SEM), n = three. Statistical significance was estimated with respect to the control (untreated sample) by two-way ANOVA, followed by Tukey's post hoc test (n = three,*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

5.4 Discussion

Ceroid, a final product of LDL oxidation comprising of a compound of insoluble oxidised lipids and proteins, is found both intracellularly and extracellularly within atherosclerotic lesions (Mitchinson et al., 1985). Our laboratory has previously recognised that intralysosomal ceroid is formed in human macrophages when they are incubated with aggregated LDL (Wen and Leake, 2007) and it also observed lysosomal ceroid accumulation when THP-1 macrophages were incubated with SMase-LDL. The aim of this part of the study was to investigate the effects of aggregated LDL oxidised under lysosomal conditions (which might released from macrophages) on macrophage characteristics and if antioxidants ceroid formation, ROS production and apoptosis.

In the first part from this study, when control LDL and LDL enriched with α -tocopherol and aggregated with sphingomyelinase was incubated with THP-1, the accumulation of intracellular lipid and ceroid formation, detected by oil red O, were significantly increased compared to native LDL as expected.

In the current study, tempol significantly decreased ceroid formation with SMase-LDL (but surprisingly not α-tocopherol-enriched LDL aggregated by secretory sphingomyelinase). Nitroxides are stable organic free radicals, and tempol has the greatest power of the nitroxides in protecting cells and tissues from the damaging effects of ROS (Czepas et al., 2008, Tikhonov et al., 2015). Numerous mechanisms may explain the antioxidant activity of tempol, including the oxidation of reduced transition metals (Hyodo et al., 2006, Mitchell et al., 1990a). The inhibition of lipid peroxidation by nitroxides is believed to depend on their lipophilicity and chemical structure. This might possibly help to explain why tempol inhibited atherosclerosis in apo E knock-out mice (Cannizzo et al., 2014).

Probucol decreased lipid accumulation (detected by Oil Red O) with α-tocopherol-enriched LDL aggregated by sphingomyelinase (Figure 5.2). Yamamota et al. (Yamamoto et al., 1986) observed that probucol decreased lipid accumulation in U-937 macrophage-like cells incubated with acetylated LDL. The mechanism for the decrease remains unknown.

Oxidative stress is the result of increased reactive oxygen species (ROS) or diminished antioxidant ability. ROS are chemically reactive materials that can react with cell constituents, causing cell injury (McMurray et al., 2016). The dihydroethidium (DHE) method is based on fact that dihydroethidium in the cytoplasm is colourless, but is converted to ethidium by $O_2^{\bullet-}$, which is trapped intracellularly (Saenz-de-Viteri et al., 2014). LDL oxidised under lysosomal conditions (Cu^{2+} at pH 4.5) caused oxidation stress in macrophages (Figure 5.5). Surprisingly, oxidised α-tocopherol–enriched LDL caused more oxidative stress than oxidised LDL (Figure 5.5). This, might be related to the creation of unstable α-tocopheroxyl radicals.

Probucol decreased ROS in response to oxidised LDL (Figure 5.7), which might help to explain the anti-atherosclerotic effect of probucol in some but not all animal models (Xiao et al., 2017).

In this study, hydrogen peroxide (H_2O_2) was used to induce apoptotic cell death. It has been documented that exposure of specific cell types to low concentration of H_2O_2 can cause morphological alterations associated with apoptosis, while higher doses of H_2O_2 causes necrosis (Hampton and Orrenius, 1997, Krifka et al., 2012). α-Tocopherol enrichment of macrophages increased apoptosis, necrosis and secondary necrosis itself (Figure5.8). In the presence of H_2O_2, α-tocopherol had no effect on apoptosis but decreased secondary necrosis substantially. The mechanism by which α-tocopherol itself induces apoptosis is uncertain One of the possible explanations was that α-tocopherol adversely affected membrane structure

and function in the cells. More research would be needed to identify the mechanisms involved.

Cells undergoing apoptosis from exposure to H_2O_2 showed a significant decrease in apoptosis when treated pre-incubated with tempol alone whereas there was an increase in apoptotic cells enriched with α- tocopherol treated with H_2O_2 plus tempol compared to cells that had not been enriched with α-tocopherol. The reasons for the apparently toxic effects of α-tocopherol are unknown. Furthermore, several inhibitors of apoptosis have antioxidant properties or increase cellular antioxidant defence mechanisms. We used the nitroxide tempol as a radical scavenger for trapping superoxide anions (Monti et al., 2001). Tempol greatly increased cell viability in the presence of H_2O_2, decreasing both apoptosis and secondary necrosis (Figure 5.8). This might help to explain why it has anti-atherogenic activity.Once it is delivered to the cells, α-tocopherol serves as an antioxidant, preventing free radical peroxidation and injury to cell membranes. However, α-tocopheroxyl radical (α-TO$^{\bullet}$), which might be generated during the apoptosis process, can act as a chain transfer agent of lipid peroxidation (Witting et al., 1997). Tempol might prevent the toxicity of the α-tocopherol radicals by acting as a co-antioxidant (Thomas et al., 1995).

α-toc O$^{\bullet}$ + tempol O$^{\bullet}$ $\longrightarrow$ non-radical product

The reason for the increase in primary necrosis by tempol in cells enriched in α-tocopherol is unknown (Figure 5.8). I expected to see the protective effects of α-tocopherol in the macrophages, but some toxic effects were seen. This might help to explain why the protective effects of α-tocopherol against the cardiovascular disease were not observed in the large clinical trials (Collins et al ., 2000). α-Tocopherol can sometimes inhibit the oxidation of LDL, but it has less antioxidant effect on LDL at lysosomal pH, as shown in chapter 3, and if it

increase cell death in macrophages (as shown in this chapter) this might outweigh its potential benficial effects.

Chapter 6

Effect of LDL oxidised under lysosomal conditions on nitric oxide synthesis by endothelial cells

6.1 Introduction

Nitric oxide is the supreme vasodilating molecule continuously produced by the endothelial constitutive isoform of nitric oxide synthase (eNOS or NOS III) under the stimulus of several neuro-humoral mediators, such as acetylcholine and circulating hormones, plasma constituents, platelet products and autacoids (Michel and Vanhoutte, 2010). In addition to vasodilatation, endothelium-derived NO has many pleiotropic vaso-protective, cardioprotective and anti-atherogenic properties, described in numerous review articles (Ungvari et al., 2010, Jin and Loscalzo, 2010, Bermudez et al., 2008).

Endothelial nitric oxide synthase (eNOS) is a one of a group, which contain three mammalian nitric oxide synthase ("NOS") isoforms that catalyse the oxidation of the terminal guanidino group of L-arginine to make NO and L-citrulline (Dudzinski et al., 2006, Forstermann et al., 1998, Shaul, 2002). The N-terminus of eNOS shows homology mainly to the other NOS members, while the C-terminal has important sequence homology to cytochrome P450 reductases. The NOS N-terminus binds with tetrahydrobiopterin (BH4) and heme. L-arginine binds with the enzyme in the active site near heme, while an oxygen molecule is bound to the ferrous heme iron. The binding sites for tetrahydrobiopterin and heme are restricted along the border of the monooxygenase domains of the dimeric, active form of NOS. Binding of heme and tetrahydrobiopterin, may be dynamic in inducing dimerization and NOS catalytic action. The C-terminal domain connects with NADPH, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) cofactors (Stuehr, 1997). Both N- and C-terminal domains are connected by a small sequence that binds calmodulin, an allosteric effector that is essential for full NOS activity. The complete NOS functions as a monooxygenase/ reductase enzyme and has the ability to produce a number of molecules besides NO, most remarkably

superoxide anions. In all NOS catalysis reactions, electrons inside the reductase domain flow from NADPH to FAD and finally to FMN. Calmodulin is thought to enable the movement of electrons from both the reductase zone to the monooxygenase area as well as from FAD to FMN. Because electrons seem to move from the reductase field of one NOS monomer to the oxygenase area of other NOS monomer, enzyme dimerization is essential for complete enzymatic activity (Balligand et al., 2009, Forstermann et al., 1998).

Endothelial eNOS goes through a complex form of posttranslational regulatory alterations. These modifications trigger the active regulation of enzymatic activity in reaction to physiological alterations and pathophysiological environments (Dudzinski et al., 2006, Jagnandan et al., 2005, Sessa, 2004). The production of NO can be initiated or enhanced by several stimuli including shear stress (Kolluru et al., 2010b) and acetylcholine, which acts on particular receptors on the endothelial cell membrane to increase the intracellular concentration of calcium, which binds to calmodulin and causes the activation of calmodulin-binding domain of eNOS (Walch et al., 2001). This enables electron flux from the reductase to the oxygenase domains of the enzyme for NO production. On the other hand, phosphorylation of eNOS is also important for the activation of the enzyme because it facilitates the active flux of electrons from the reductase to the oxygenase domains (Sessa, 2004) Phosphorylation is a post-translational alteration, which adds a phosphate group to eNOS by the kinases, the phosphate, in turn, is removed by phosphatases (Butt et al., 2000, Fleming et al., 2001). This modification alters the activity of eNOS and different sites of phosphorylation can have opposing effects (Kolluru et al., 2010a). Thus, for eNOS, Ser1177 (or Ser1179 depending on the species) is an activation site and Thr495 is an inhibitory site. Protein kinase B activates eNOS by phosphorylating Ser1177 in response to various stimuli (Dimmeler et al., 1999). Increases in NO release induced by increases in haemodynamic shear

stress are mediated mainly through this calcium-independent pathway (Kuchan and Frangos, 1994). Shear stress is accompanied by the elevation of intracellular calcium and the subsequent liberation of NO (Muller et al., 1999). The aims and objective of this study are to investigate if LDL oxidised by iron at lysosomal pH would decrease the generation of nitric oxide by endothelial cells and decrease vasodilatation. This is based on the hypothesis that LDL oxidised in the lysosomes of macrophages might become extracellular when the macrophages die by apoptosis or necrosis and the plasma membrane and lysosomal membranes lyse. Endothelial cells might be exposed to this oxidised LDL at their subendothelial surfaces. In order to achieved endothelial cells to LDL oxidised under lysosomal conditions and measure endothelial-depedent or endothelial-independent vasodilatation by a myography instrument, as well as to examine whether the type of endothelial cell affects the response to oxidised LDL.

Objectives

1. To see if incubating rat aortic rings in a myograph with LDL oxidised by iron at lysosomal pH would inhibit vasodilatation.
2. To see if oxidised LDL caused gross changes to the aorta by histology and immunohistochemistry.
3. To see if oxidised LDL increased ROS production from the aortic rings or cultured endothelial cells, using DHE staining.
4. To see if oxidised LDL increased the phosphorylaytion of eNOS by western blotting and immunocytochemistry.

6.2 Methods

To test the effects of LDL oxidised under lysosomal conditions, the following techniques were used.

Exp.6.3.1: Rat aortic rings were incubated in a myograph with oxidised LDL, as described in chapter 2.

Exp.6.3.2 and 6.3.3: I then investigated the integrity of the aortic rings using histology and immunohistochemistry,as described in chapter 2.

Exp.6.3.4 and 6.3.5: To try to explain the findings from experiment 6.3.1, the effect of oxidised LDL, on reactive oxygen species generation by the aortic and cultured endothelial cells were investigated using dihydroethidium.

Exp.6.3.6 and 6.3.7: To try to explain the effect of oxidised LDL on nitric oxide activity, the effects of oxidised LDL on eNOS phosphorylation were investigated in aortic rings and cultured endothelial cells.

6.3 Results

6.3.1 Effect of oxidised LDL on the relaxation of aortic rings

Atherosclerosis involves endothelial dysfunction and modifications in vascular reactivity, which can be studied by wire myography. The process allows *vitro* recording of the transverse isometric pressure developed by a vessel segment in response to various pathophysiological factors. I investigated the effect of LDL oxidised under lysosomal conditions (which might be released from lysed macrophages in atherosclerotic lessions) on the vasodilatation of rat aortic rings in a myograph.

LDL was oxidised by $FeSO_4$ (5 µM) for 24 h at pH 4.5 in sodium acetate buffer. Rat aortic rings with intact endothelial cells were contracted with phenylephrine (100nM) and increasing concentrations of acetylcholine and then they were washed several times by Krebs solution and incubated with oxidised LDL (50 µg protein/ml) for 60 min and then contracted with phenylephrine (100nM) and increasing concentrations of acetylcholine (10^{-9} to $3x10^{-6}$ M) were added and the relaxations measured before and after incubation with oxidised LDL (Figure 6.1). The results indicated that aortic ring subjected to incubation with oxidised LDL showed significantly less relaxation to acetylcholine. It was reduced to 34%, while the control had a normal relaxation of 95%. Relaxation to sodium nitroprusside (SNP), which is converted directly to nitric oxide,was not significantly affected, showing that the smooth muscle cells were not damaged by the oxidised LDL. In conclusion, LDL oxidised at lysosomal pH inhibited endothelial-dependent-relaxation but not endothelial-independent relaxation.

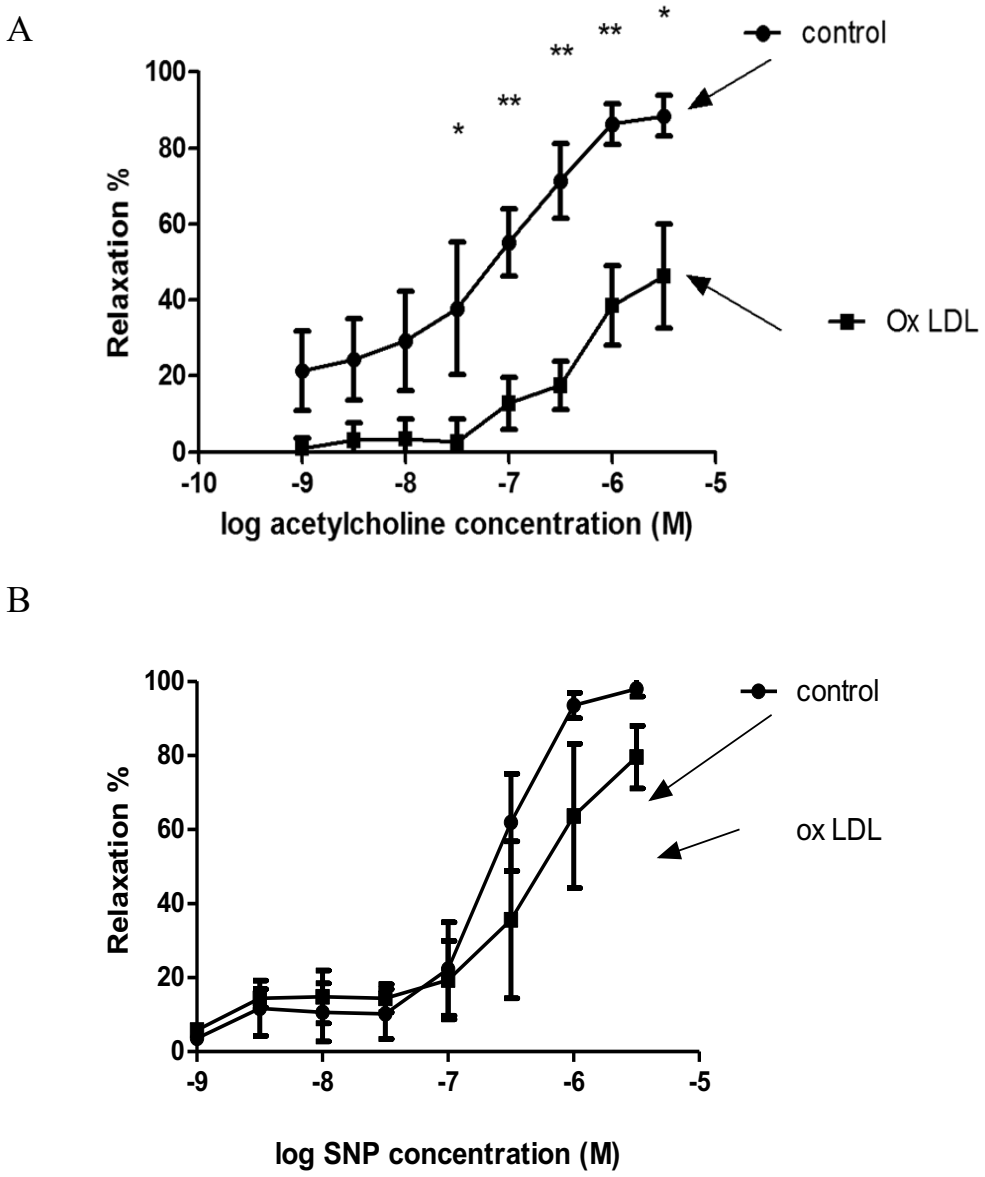


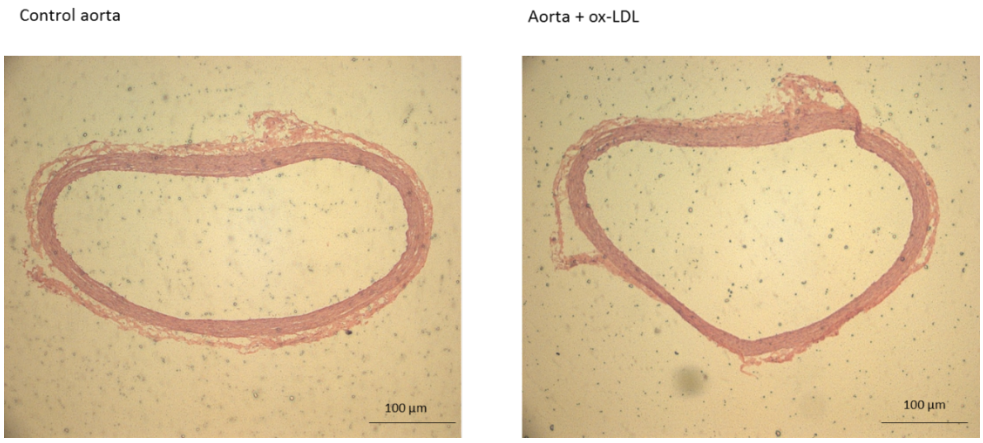
Figure 6.1. Effect of LDL oxidised under lysosomal conditions on vasodilatation.

LDL was oxidised by ferrous sulphate (5µM) for 24 hr at pH 4.5 at 37 ^{o}C. Aortae were cut into rings (2mm width) and mounted on a wire myography setup for isometric tension recording. (A) Aortic rings were incubated for 1 h with or without oxidised LDL (50 µg protein/mL) and then pre-constricted with 100nM phenylephrine and concentration-response curves to acetylcholine (1nM-3µM) were performed. Values are expressed as means ± SD (*n* = 5 independent experiments). Two way ANOVA, followed by Bonferroni post hoc test ***P<0.001 compared to control. (B) Rings were also relaxed directly using SNP.

6.3.2 Evaluation of aortic wall structure

H&E stain was used to evaluate the gross structure of the aorta after exposure to oxidised LDL in the myograph. There was no significant difference in wall thickness and lumen/outer diameter ratio of aortas incubated with or without oxidised LDL (Figure 6.2). The myography, which applied stretch forces, with or without the oxidised LDL, did not make any changes leading to gross damage in the tissue structure of the aorta.

A



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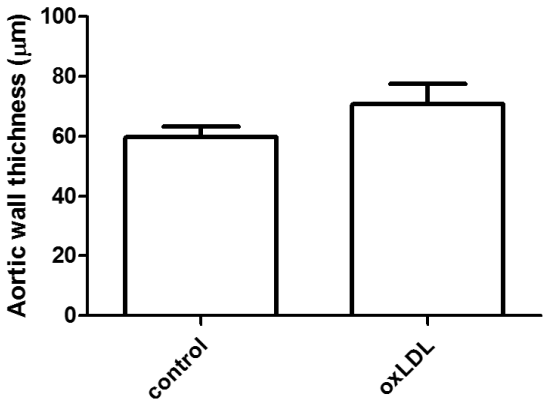


C

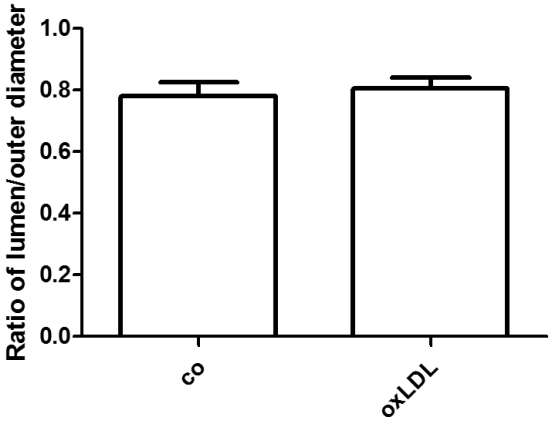


Figure 6.2. Effect of LDL oxidised under lysosomal conditions on aortic structure. (A) Aortic rings, which were mounted in the myography for 1 h with or without oxidised LDL, were fixed with 4% paraformaldehyde for 24 h, dehydrated and embedded in OTC. Histological analysis was performed on serial cross-sections (10 µm) after staining with H&E to visualize whole aorta structure. (B) The bar chart shows no significant difference in aortic wall thickness between control and aortic rings incubated with oxidised LDL, (t-test, n=5 independent experiments). (C) The bar chart shows no significant difference in the ratio of lumen/outer diameter between control and aortic rings incubated with oxidised LDL, (t-test, n=5 independents experiments).

6.3.3 Measurement of endothelial integrity of aortic rings exposed to with oxidised LDL

CD31 (also known as PECAM-1) is an endothelial marker. I investigated the prescence of this marker on aortic rings incubated with oxidised LDL using immunohistochemistry. CD31-positive cells were present in control aortic rings after being incubated in the myograph for 1 h, but when they were incubated with oxidised LDL in the myography for 1 h CD31 staining was not observed (Figure 6.3). This suggests that the oxidised LDL caused the endothelial cells to detach from the aortic rings, but this finding needs to be confirmed by using another markers of endothelial cells or by electron microscopy.

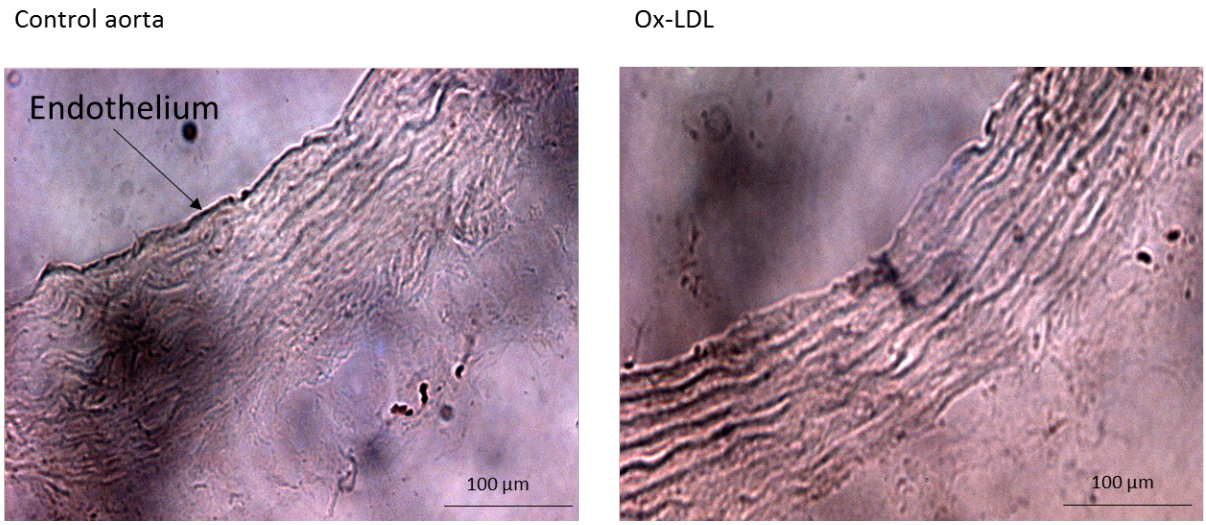
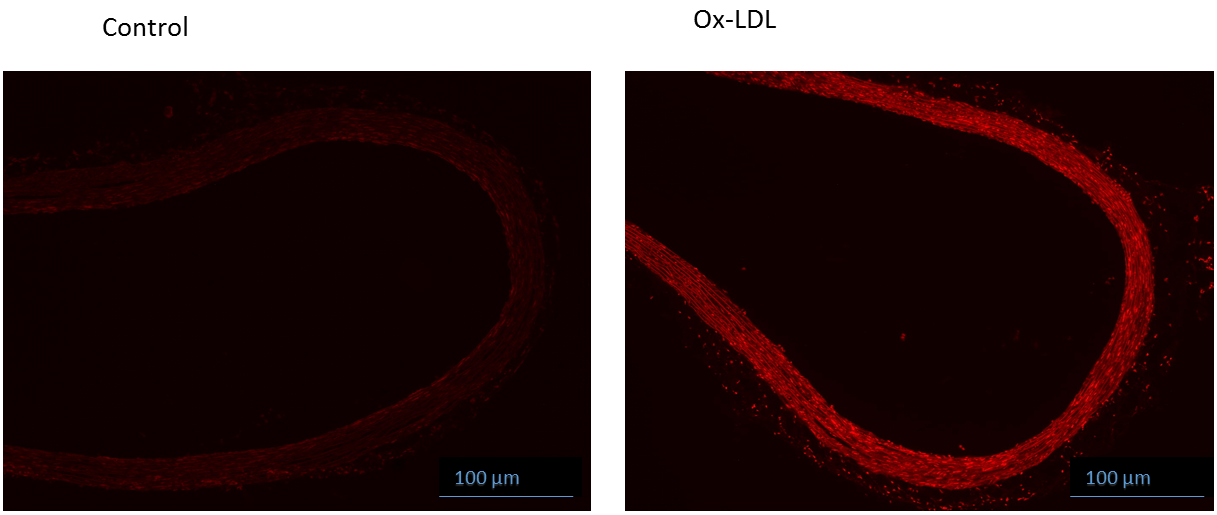


Figure 6.3. Immunohistochemical staining for CD31 in rat aortic segments.
Aortic sections were frozen in OCT embedding medium. The analysis was performed on serial cross-sections (10 µm) after staining with CD-31 primary antibody. Brightfield images were captured from immunostains of the aortic rings. CD31 staining is observed in the control section, whereas there is no staining in the aortic ring exposed to oxidised LDL (50 µg protein/ml) for 1h

6.3.4 Effect of oxidised LDL on reactive oxygen species in aortic ring

Under physiological environments, ROS concentrations control important cellular roles and signalling stimulating pathways to support vascular homeostasis. Nevertheless, when endothelial cells are challenged to certein stimuli, such as hyperlipidaemia, extreme ROS level are produced and can cause oxidative modification on proteins and nucleic acids (Wang and Zou, 2018). The aim of this study was, to detect ROS formation in aortic rings after incubation with oxidised LDL. Throughout the past decade, numerous fluorescent dyes have been commonly employed to measure cellular $O_2^{\bullet-}$ and H_2O_2, for example by dihydroethidium (DHE) (Dikalov et al., 2007, Panth et al., 2016). DHE can spontaneously permeate cell membranes and be oxidized by cellular $O_2^{\bullet-}$ to generate two red fluorescent substances, specifically ethidium (E^+), which is classically produced by a general redox reaction, and 2-hydroxyethidium (2-OH-E^+), a specific product of cellular $O_2^{\bullet-}$. The fluorescent spectra of 2-OH-E^+ (Ex 500–530 nm/Em 590–620 nm) and E^+ (Ex 520 nm/Em 610 nm) are quite similar. Therefore, exact detection of 2-OH-E^+ is a challenge (Dikalov et al., 2007, Munzel et al., 2002). In brief, tissue was incubated with DHE (10 µM) for 30 min. Fluorescence was measured with a fluorescence microscope and quantified using Image-J analysis software. The result showed oxidised LDL increased the intensity of DHE stain in the entire aorta wall compared to the control (Figure 6.4).

A



B

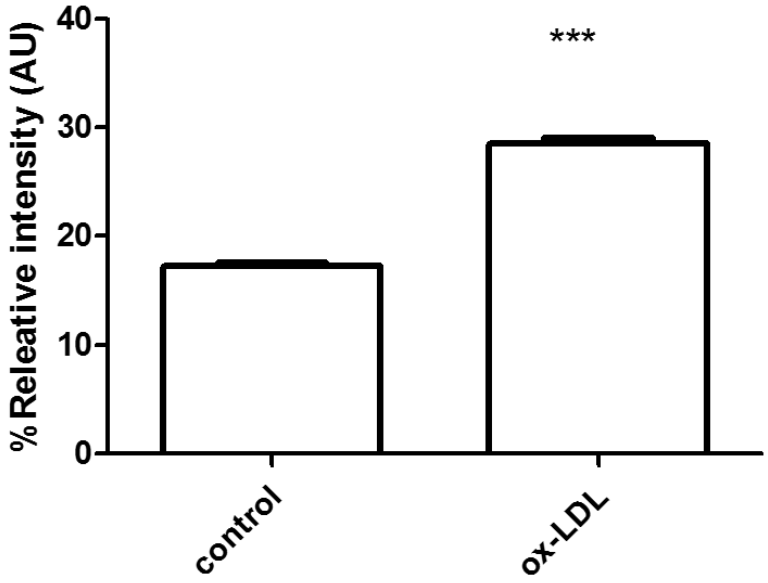
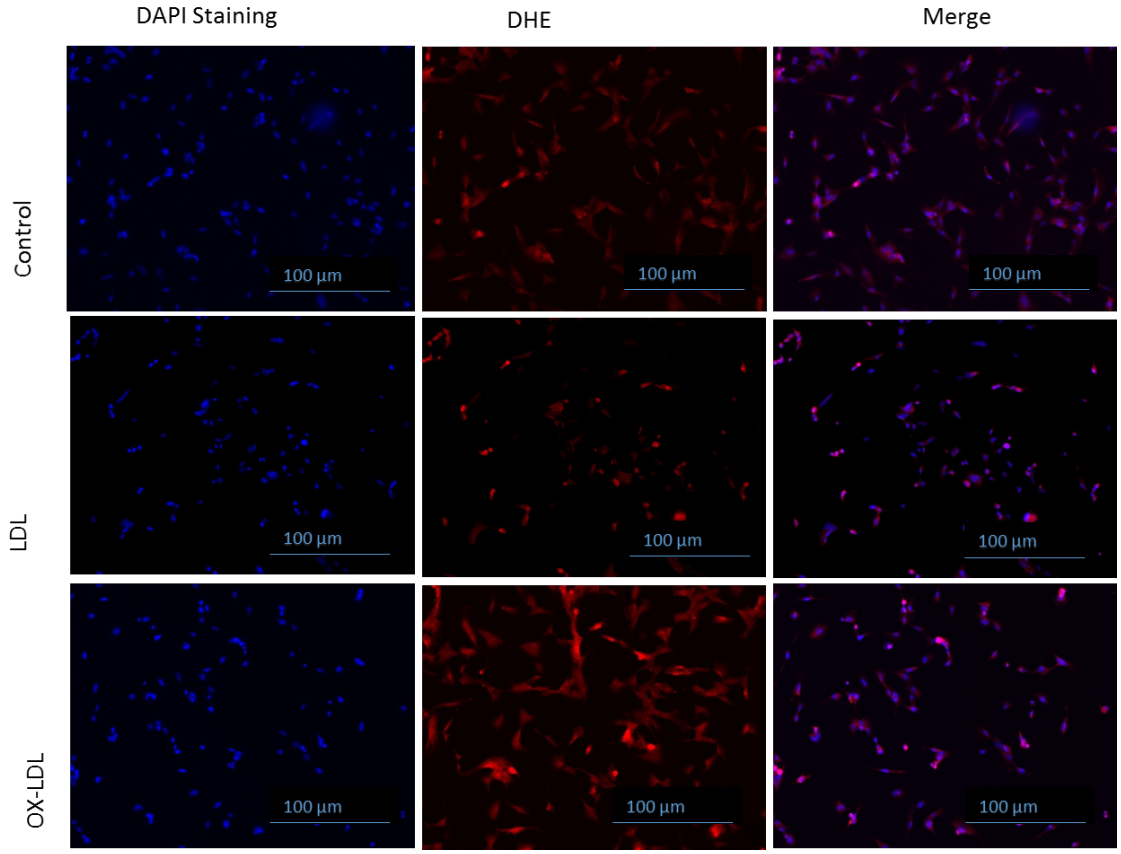


Figure 6.4 Effects of oxidised LDL on ROS formation from rat aorta.
(A) Rat aortic segments were frozen in OCT embedding medium. The analysis was performed on serial cross-sections (10 µm) after staining with DHE and taking fluorescence images of the aortic tissue. Scale bars, 100 µm. (B) Quantification of DHE fluorescence image. The bar chart show mean ± SEM of five independent experiments. Paired t-test. ***$P < 0.001$, n=5).

6.3.5 Effect of oxidised LDL on reactive oxygen species formation by cultured endothelial cells

I next investigated the effect of LDL oxidised under lysosmal conditions on cultured endothelial cells. Advanced atherosclerostic lesions have both large artery endothelial cells lining the lumen and microvascular endothelial cells in the neovessels at the base of the lesions. Human microvascular endothelial cells (HMEC) and Human aortic endothelial cells (HAEC) were incubated with LDL oxidised by iron at lysosomal pH for 24 and then washed by PBS. They were Incubated with DHE (10 μM) in PBS for 30 min and fluorescence was measured with a fluorescence microscope with wavelength 488nm and quantified using Image-J analysis software. LDL oxidised under lysosomal conditions increased reactive oxygen species generation in HMEC (Figure 6.5) and HAEC (Figure 6.6) compared to the control LDL. These results suggest that if LDL oxidised in the lysosomes of macrophages leaks out of damaged macrophages it might increase ROS generation by endothelial cells lining the lumen of the atherosclerotic lesion or in the neovessels in the base of the lesions.

A



B

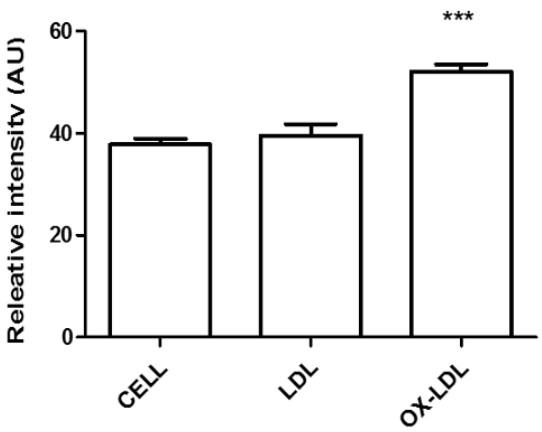
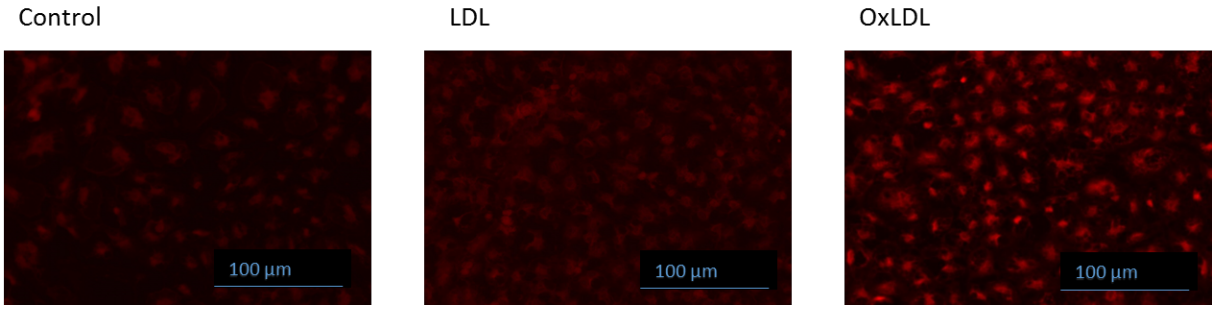


Figure 6.5. ROS detection in HMEC.

(A) Human microvascular endothelial cells were treated for 1 h with control LDL or LDL (50µg protein/ml) which had previously been oxidised with 5µM $FeSO_4$ for 24 h at pH4.5. Reactive oxygen species were measured using DHE. DAPI was used to stain the nuclei. (B) The bar chart shows the (mean ± SEM of 5 independent experiments. Two-way ANOVA, followed by Tukey's post hoc test ***P<0.001, n=5

A



B

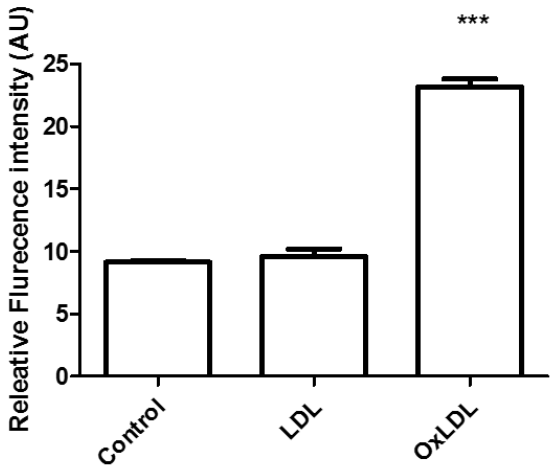


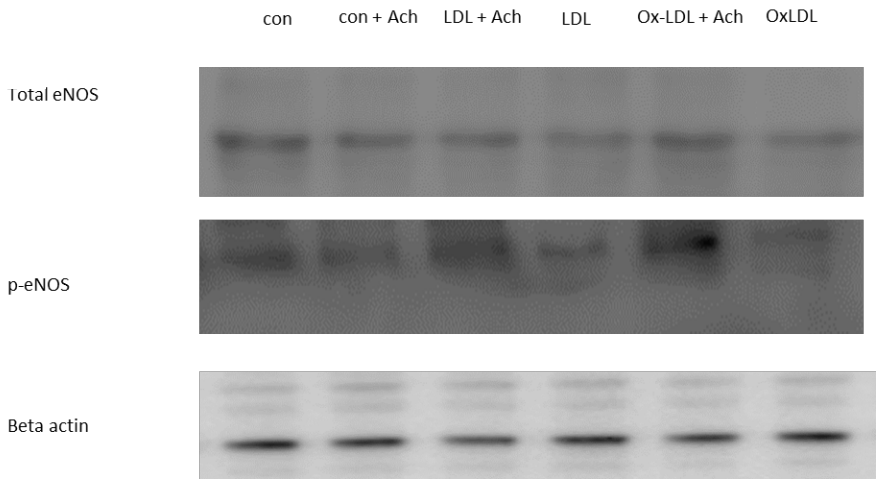
Figure 6.6 ROS detection in HAEC.

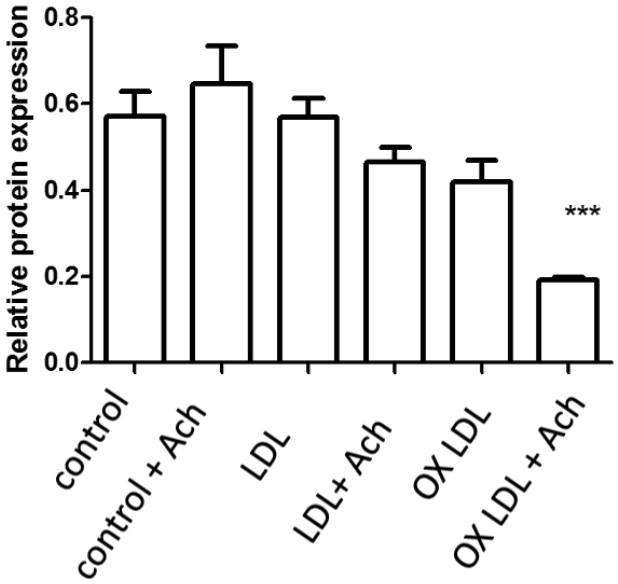
(A) HAEC were treated for 1 h with control LDL or LDL (50µg protein/ml) which had previously been oxidised with 5µM $FeSO_4$ for 24 h at pH. 4.5. Reactive oxygen species were measured using DHE. DAPI was used to stain the nuclei. (B) The bar chart shows the mean ± SEM of 5 independent experiments. Two-way ANOVA, followed by Tukey's post hoc test ***P<0.001, n=5).

6.3.6 Effect of oxidised LDL on eNOS phosphorylation in cultured endothelial cells measured by western blotting

Phosphorylation eNOS serine 1179 (S1179) enhances eNOS enzymatic reactivity and NO release (Atochin et al., 2007). The vasorelaxation activity of acetylcholine (ACh) is mediated via muscarinic receptors that causes the liberation of calcium from intracellular stores. This permits calcium-calmodulin to bind to eNOS, relocating caveolin-1 and stimulating eNOS (Gratton et al., 2000). The aim of this study was to detect whether LDL oxidised by iron at lysosomal pH leads to impaired endothelial nitric oxide synthase (eNOS) activation by acetylcholine in different types of endothelial cells. Western blotting was performed according to standard methods using antibodies specific for total-eNOS, phosphorylated-eNOS (serine 1179) and β-actin and an HRP-conjugated secondary antibody. Quantitative analysis was performed using Image J. Western blot revealed clear 145 kDa bands in both HMEC and HAEC (Figure 6.7). Acetylcholine increased phosphorylated-eNOS, especially in HAEC. Phosphorylation of eNOS was decreased considerably by oxidised LDL alone in HMEC, but not in HAEC

A
(HMEC)


con
con + Ach
LDL + Ach
LDL
Ox-LDL + Ach
OxLDL
Total eNOS
p-eNOS
Beta actin


Relative protein expression
0.0
0.2
0.4
0.6
0.8
***
control
control + Ach
LDL
LDL+ Ach
OX LDL
OX LDL + Ach

B (HAEC)

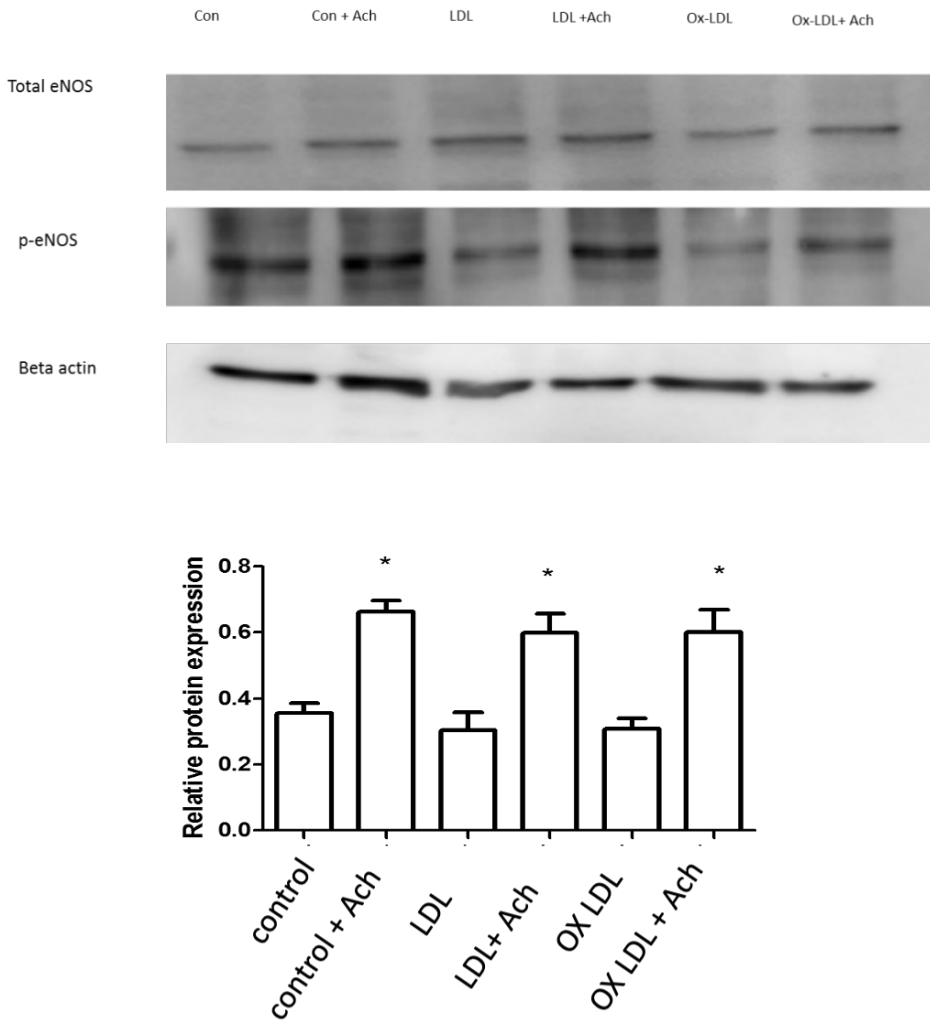


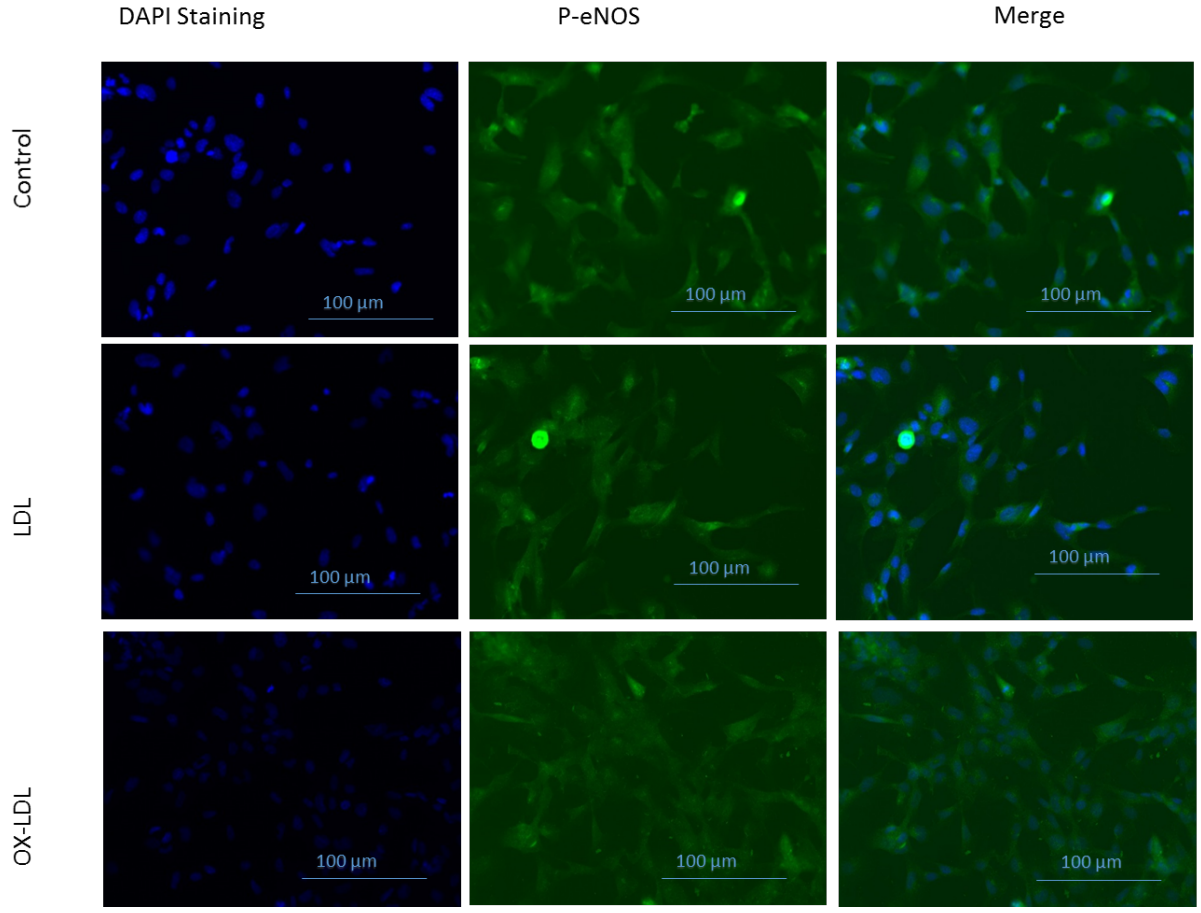
Figure 6.7 Representative western blot analysis showing effect of LDL oxidised by iron at lysosomal pH on different type of endothelial cells

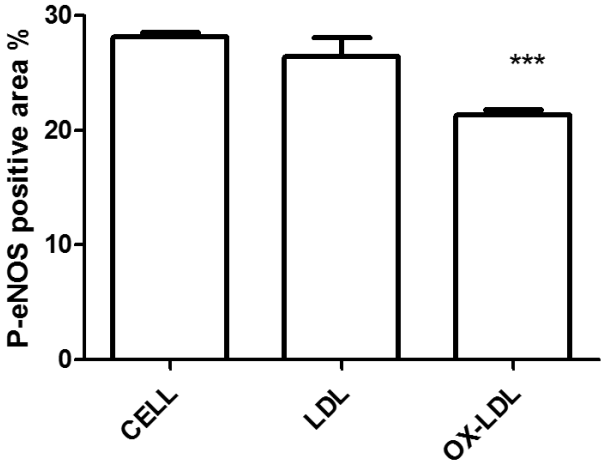
Bar charts displaying protein quantification of endothelial nitric oxide synthase (eNOS) and phosphorylated-eNOS(S1179). Endothelial cells were incubated for 24h with control LDL or LDL (50µg protein/ml) that had been oxidised by 5µM $FeSO_4$ for 24 h at pH. 4.5. Acetylcholine was added (3µM) for 8 min and the cells were then lysed and total and phosphorylated eNOS separated by SDS-PAGE followed by a polyvinylidene difluoride membrane before being probed for antibodies (western blotting). Protein levels are shown as mean ± SEM were and normalised to β-actin. The level of phosphorylated eNOS was expressed relative to the level of total eNOS. Illustrative western blots are shown. β-Actin was used as a loading control. The mean ± SEM 3 in depending experiments is shown. (One-way ANOVA, Tukey's test (**A**) HMEC (**B**) HAEC *p ≤ 0.05 ***P<0.001).

6.3.7 Effect of oxidised LDL on eNOS phosphorylation in cultured endothelial cells measured by immunocytochemistry

eNOS phosphorylation was also assessed by immunocytochemistry as well as western blotting. Endothelial cells grown on glass coverslips were washed once with PBS and then incubated at 37 °C with oxidised LDL (50 μg protein/ml) for 60 min. Control and oxidised LDL treated cells in the presence or absence of acetylcholine, permeabilised then incubated a primary antibody monoclonal antibody to phosphorylated eNOS (S1179) and then a secondary antibody and examined with an epifluorescence microscope. Figure 6.8 shows the HMEC and HAEC had phosphorylated -eNOS expression in control cells and cells treated with control LDL while the cells treated with oxidised LDL had a somewhat decreased level of phosphorylated eNOS. Figure 6.8B shows HAEC with or without LDL or oxidised LDL (50 μg protein/ml) had increased phosphorylated eNOS after incubation with acetylcholine (3 μM) for 8 min.

A
(HMEC)


DAPI Staining
P-eNOS
Merge
Control
LDL
OX-LDL
100 µm


P-eNOS positive area %
30
20
10
0
***
CELL
LDL
OX-LDL

B
(HAEC)

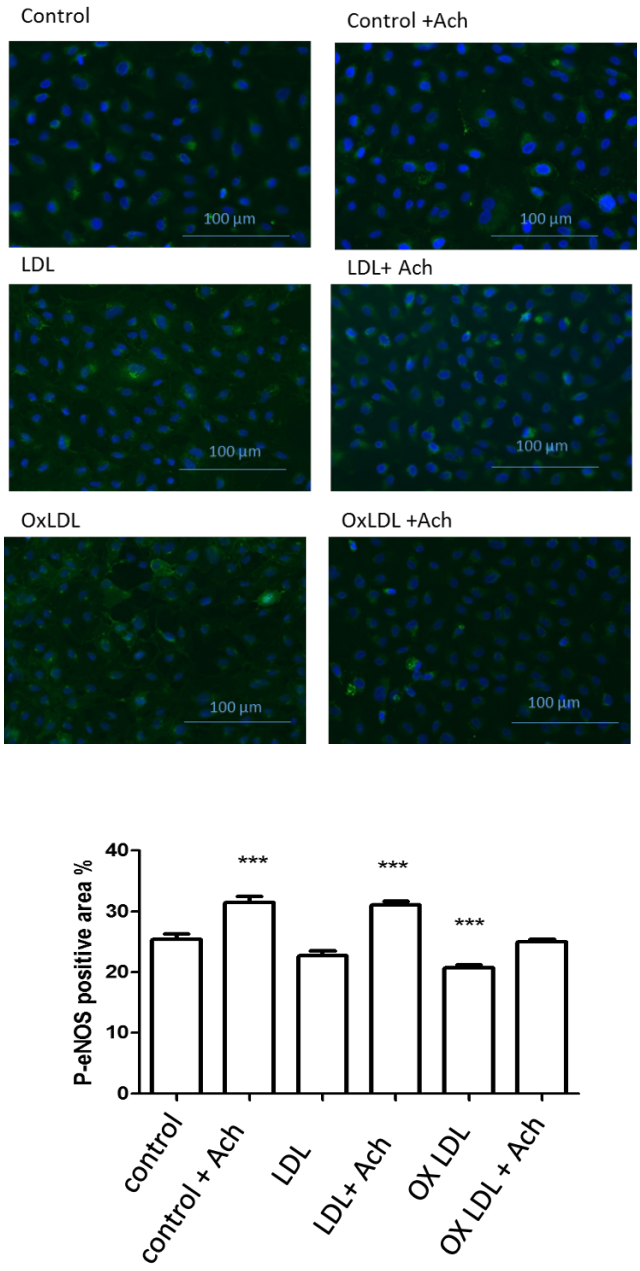


Figure 6.8 Representative eNOS immunofluorescences staining of HMEC and HAEC

(A) HMEC were treated for 1 h with control LDL (50µg protein/ml) or LDL which had previously been oxidised with 5µM Fe^{2+} for 24 h at pH. 4.5. (B) HAEC cells were treated the same as in (A) but with or without acetylcholine (3µM) for 8 min. They were immunocytochemistry stained for phosphorylated eNOS and the nucleus stained for DAPI. The mean ± SEM three independent experiments are shown. Two-way ANOVA, followed by Tukey's post hoc test ***P<0.001 test compared to the control.

6.4 Discussion

I postulated that LDL oxidised in lysosomes of macrophages would be exposed to subendothelial surfaces of endothelial cells in atherosclerotic lesions when the macrophages die and lyse. Vasodilatation is normally measured by relaxation response curves to the vasodilator acetylcholine in vessel rings, pre-contracted with a vasoconstrictor (phenylephrine). In control aortic rings acetylcholine stimulates the release of nitric oxide (NO) from the endothelial cells, which spreads within the vessel wall and leads to vasodilatation by inducing the relaxation of smooth muscle cells, while in the presence of LDL oxidised by iron there is a reduction in relaxation compared with the control (Figure 6.1A). In contrast, the endothelium-independent vasorelaxation was measured by addition of sodium nitroprusside (SNP) to pre-contracted aortic rings (Figure 6.1B) and was not significantly different between the control and oxidised LDL (control: 99.4%, oxidised LDL: 93.5% for maximum relaxation). This suggests that the effect of oxidised LDL on the aortic ring relaxation is due to its effects on the endothelium and agree with many studies, which suggested that oxidised LDL can impair endothelium relaxation (Hein et al., 2000, Kugiyama et al., 1990).

There were no significant differences in wall thickness or lumen diameter in histological sections of the aortic rings (Figure 6.2). I examined the presence of endothelium after incubation for 1hr with LDL that had previously been oxidised by iron (5 μM) for 24 hr at lysosomal pH by using CD31 as a marker for endothelial cells. Endothelial cell damage shows a key role in the pathogenesis of atherosclerosis, in which the endothelial cells are unable to regulate appropriate lipid homeostasis, resistance and inflammation (Peng et al., 2014, Sun et al., 2011, Leppanen et al., 2005, Foteinos et al., 2005, Barton et al., 1998, Szmitko et al., 2003, Hedner et al., 2000). Indicators of endothelial cells are employed by clinical

histopathologists to recognise these cells in tissue sections, the best commonly utilized marker is CD31 (Szekanecz et al., 1995). The proportion of luminal surface of aortic rings containing endothelium was inspected and suggest that the low CD31 detection results from a decreased endothelial cell number in aortic rings that had been incubated with oxidised LDL for 1hr compared to the control, but this needs to be confirmed and quantified. This agreed with several studies (Liu and Shi, 2012, Fornasa et al., 2010, Pusztaszeri et al., 2006).

While oxidised LDL is a product of oxidative stress, it can also exert pro-oxidant effects by inducing ROS generation (Heinloth et al., 2000). It is commonly found that oxidation of LDL increases its ability to increase $O_{2}^{\cdot -}$ formation; native lipoproteins produce no or merely weak effects on ROS production. Freshly prepared dihydroethidium (DHE, 10 µM) was added to the aortic rings sections for 30 min at 37 °C (Figure 6.4). There was a significant increase between aortas incubated with oxidised LDL compared to the control. In the control rings, most of the fluorescence was due to the endothelial cells, but in the rings exposed to oxidised LDL, it was mainly produced by the smooth muscles cells in the whole aorta, in agreement with other studies (Kugiyama et al., 1999, Ohara et al., 1994). The questions arise here of which components of the oxidised LDL increased ROS. It might have been lysophosphatidylcholine (LPC), aldehydic lipid peroxidation products, oxidised fatty acids produced by phospholipase A- like activity or oxysterol (Parthasarathy et al., 1985, Yokoyama et al., 1990, Esterbauer et al., 1987).

In addition to the whole aorta, two types of endothelial cells were used to detect ROS after incubation with oxidised LDL by iron for 24 hr (Figure 6.5 &6.6). The result show there was no significant difference between the controls and the cells treated with native LDL, but an increase with cells treated with oxidised LDL. Increased ROS might help to explain the reduced vasodilatation as ROS might inactivate $NO^{\bullet}$

$O_2^{\bullet -}$ + $NO^{\bullet}$ ⟶ $ONOO^-$

High level of ROS not merely modify signal transduction mechanisms but also disturb cellular processes, such as ubiquitination necessary for cyclin functions, and both protein and lipid oxidation. In these conditions, cells can be detained in all stages of the cell cycle, and then lead to apoptosis, or, in extreme cases, may lead to necrotic cell death (Boonstra and Post, 2004, Aronis et al., 2005).

To examine the endothelial dysfunction caused by oxidised LDL, we measured the phosphorylation of eNOS. Treatment of HMEC with oxidised LDL resulted in a significant decrease in the phosphorylation of eNOS in the absence of acetylcholine compared to the control cells or cells incubated to the control LDL (Figure 6.7). Oxidised LDL did inhibit the phosphorylation of eNOS in HMEC, but not HAEC, in the presence of acetylcholine as shown by western blots (Figure 6.7). Immunocytochemistry showed that oxidised LDL decreased phosphorylated eNOS in HMEC (Figure 6.8A), in agreement with the western blots.

Early atherogenesis is accompanied by weakened endothelial NO production in reaction to extracellular inducements, even though the ability for maximal NOS enzyme activation and the breakdown of NO are not affected (Katusic and Austin, 2014). As the disease develops inhibition of NO bioavailability happens, which is at least partially due to the higher block of NO by superoxide anions (Flavahan, 1992). Oxidised LDL has been reported to reduces cholesterol in the plasma membrane, changing the location of eNOS from caveolae to an intracellular membrane and to block acetylcholine-induced eNOS activation (Uittenbogaard et al., 2000, Cominacini et al., 2001). In conclusion, these results show that LDL oxidised under lysosomal conditions decreases vasodilatation in our experiments. The fundamental molecular mechanisms have not been totally clarified, but might involve a decrease in eNOS phosphorylation.

Chapter 7

General discussion

7.0 General discussion

This thesis covers a number of related possible roles of lysosomally–oxidised LDL in the field of atherosclerosis.

The main findings of the research presented in this thesis can be basically summarized as follows.

- α-Tocopherol is less antioxidant at pH 4.5 than 7.4 and can even be prooxidant with Fe^{3+} (2, 5 or 20 µM) and 2 µM Fe^{2+}.
- α-Tocopherol enrichment of LDL increased the rate of conversion of Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^{+}, which have pro-oxidant effects.
- Tempol does not inhibit the initial oxidation (possibly the cholesteryl ester core) of control or α-tocopherol-enriched LDL, but inhibits the late oxidation (possibly the phospholipid monolayer) with Fe^{2+} and Cu^{2+} but does not inhibit either the early or late oxidation of LDL by Fe^{3+} at pH 4.5. This suggests the chemistry of LDL oxidation by Fe^{2+} and Fe^{3+} is different.
- Probucol inhibited the later oxidation of control or α-tocopherol-enriched LDL by Fe^{2+} more effectively than the initial oxidation at pH 4.5.

- Incubation of human THP-1 macrophages with SMase-LDL or SMase- α-tocopherol-enriched LDL leads to intralysosomal ceroid generation, which is inhibited by the antioxidant probucol. Tempol inhibited ceroid formation with SMase-LDL but not with SMase- α-tocopherol-enriched LDL.

- α-Tocopherol enrichment of THP-1 macrophages did not protect the cells from apoptosis induced by H_2O_2.

- Endothelium-dependent relaxation, but not endothelium-independent relaxation, is impaired by LDL oxidised by iron at lysosomal pH. Therefore LDL oxidised in lysosomes when released from dying cells in an atherosclerotic lesion might adversely affect endothelial function.

Recent research has shown that atherosclerosis is not just an unavoidable development of advanced age, but also a process with several modifiable factor, such as LDL. Many studies suggest that oxidised LDL is a key factor in atherosclerotic disease (Toshima et al., 2000, Faviou et al., 2005, Meisinger et al., 2005), but this is a controversial topic. Some nutrients have antioxidant characteristics, which decreases lipid peroxidation and LDL oxidation in vitro (Basu et al., 2010). α-Tocopherol is the most plentiful type of vitamin E in plasma, and vitamin E might have numerous biological roles, including regulation of cell survival (de Nigris et al., 2000). Some research has proposed that all of the biological roles of α-tocopherol are actually a result of its antioxidant activity (Blum et al., 2008). Long-term α-tocopherol administration is related to higher proportions of heart failure and

hospitalisations for heart failure (Lonn et al., 2005), but there were no significants effects on myocardial infarctions or stroke (Yusuf et al., 2000). No particular mechanism has been suggested to clarify these observations on heart failure, however, it was proposed that α-tocopherol may have pro-oxidant properties in *vitro* models (Bowry et al., 1995, Thomas and Stocker, 2000, Miller et al., 2005). The failure of α-tocopherol supplementation to decrease cardiovascular disease (Eidelman et al., 2004) and possibly the increased heart failure in those with a high risk of cardiovascular events (Lonn et al., 2005), has led some to question the importance of oxidised LDL in atherosclerosis.

This study suggests that LDL enriched with α-tocopherol is a generally valid model to describe the molecular events occurring during lipoprotein lipid peroxidation in lysosomes of pH about 4.5 with different oxidative stresses. The concentration of iron in some lysosomes of rat liver endothelial cells has been reported to be about 16 μM (Petrat, 2002),but very widely depending on the type of cells and size of lysosome. The concentration of copper in lysosome is unknown . α-Tocopherol is probably mainly in the surface monolayer of LDL, as it contains a hydrophilic hydroxcyl group. α-Tocopherol is not antioxidant at pH 4.5 (Figure 3.4) with a low concentration (2 μM) of $FeSO_4$ while it was antioxidant with 5 or 20 μM $FeSO_4$ (Figure 3.5 and 3.6). The classical action of α-tocopherol is that of a chain-breaking antioxidant that reflects its capability to respond quickly with the chain-propagating lipid peroxyl radical ($LOO^{\bullet}$). Alternatively, α-tocopherol may directly scavenge the radical that initiates peroxidation. In both situations, the relatively nonreactive α-tocopheroxyl radical is generated. This finding agreed with other studies (Hodis et al., 2002, Stampfer et al., 1993, Hodis et al., 1995, Stephens et al., 1996, Yusuf et al., 2000), reporting that α-tocopherol appeared to have pro-oxidant properties with low levels of oxidative stress. This might have counteracted the antioxidant effect of α-

tocopherol, resulting in no antioxidant effect. There was no antioxidant effect of α-tocopherol on LDL oxidation by any concentration of Fe^{3+} or Cu^{2+} at lysosomal pH, but there was a good antioxidant effect with Cu^{2+} at pH7.4.

A pro-oxidant effectof α -tocopherol was detected under mild oxidative environments in extremely diluted (150-fold) plasma and in isolated LDL (Kontush et al., 1996). The lipoprotein's concentration of α-tocopherol and the rate of oxidation are an important marker to distinguish whether the vitamin exhibits anti- or pro-oxidant activity (Thomas and Stocker, 2000). We do not know what the pro-oxidant effects of α -tocopherol at pH 4.5 are, but propose that the hydroperoxyl radical converts α -tocopherol to its radical, which then attacks lipids.

α -toc OH + $HO_2^{\bullet}$ ⟶ α -toc O• + H_2O_2

α -toc O• + LH ⟶ α -toc OH + $L^{\bullet}$

The aim of the next part of the study was to evaluate the antioxidant and/or prooxidant activities of hydrophilic and lipophilic antioxidants in combination with α- tocopherol, which needs to be explored in more detail with a special focus on the nature of the products of LDL oxidation when oxidised by different oxidative stresses.

Tempol is a stable nitroxide radical that can freely permeate biological membranes and protect mammalian cells from oxidative stress (Bonini et al., 2002, Offer et al., 2000). It has a substantial preventative outcome related to decreased oxidative damage and stress in $Atm^{-/-}$ *mice* in vivo (Schubert et al., 2004). Tempol protected important organs, such as the heart and brain, from ischaemia-reperfusion injury and it has been active in protecting numerous aspects of oxidative stress and inflammation that underlie many of the diseases associated with ageing like atherosclerosis (Wilcox, 2010). Probucol inhibits LDL oxidation

effectively at pH7.4 (Parthasarathy et al., 1986), but did not decrease femoral atherosclerosis in patients (Walldius et al., 1994).

Adding tempol or probucol to control LDL or LDL enriched with α-tocopherol inhibited the later oxidation (maybe phospholipids) more than the early oxidation (maybe cholesteryl esters) by iron at lysosomal pH. This might be because tempol and probucol, being amphipathic reside in the surface phospholipid monolayer of LDL.

Studies which test foam cell development by the incubation of macrophages with altered monomeric LDL, like oxidised LDL formed at pH 7.4 do not completely reflect the *in vivo* environment, as the common form of the LDL in atherosclerotic plaques is recognised as an aggregated form and bound to subendothelial matrix (Boren et al., 2000). LDL captured by extracellular matrix, mainly by proteoglycans, is susceptible to hydrolysis by different enzymes such as lipases and proteases. It has been shown that secretory sphingomyelinase (SMase), is proficient in aggregating LDL to make it an atherogenic LDL type that is swiftly internalized by macrophages (Fenske et al., 2008, Deevska et al., 2009). Here in this study, tempol and probucol decreased ceroid formation when it was incubated with THP-1 macrophages after treatment with SMase -LDL or α-tocopherol-enriched SMase LDL. Tempol and probucol reduced ROS formation in the cells when incubated with LDL or α-tocopherol-enriched LDL oxidised by Cu^{2+} at pH 4.5. Tempol greatly protected macrophages against apoptosis induced by H_2O_2, whereas α-tocopherol treatment of the macrophages surprisingly increased cell death, either in the presence or absence of tempol. This may possibly relate to α-tocopherol having the ability to prevent cell proliferation in cancer cells (Neuzil et al., 2001).

Previous data caused great interest in the evaluation of antioxidants as potential anti-atherosclerotic agents. One such antioxidant is probucol. The capability of probucol to

protect against atherosclerosis in rabbits was a cornerstone of the oxidative theory of atherosclerosis, which proposed that the oxidation of LDL is an early event in, and participates in, atherogenesis (Steinberg et al., 1989). Probucol has varied pharmacological effects with respect to its therapeutic properties on the cardiovascular systems (Yamashita and Matsuzawa, 2009). However,it lowers serum HDL-C and causes QT prolongation (Guo et al., 2007). Consequently, probucol was withdrawn in most of the Western countries after statins entered the market in 1987 in the USA and in 1989 in Japan.

Although probucol totally inhibited conjugated diene formation with copper at pH7.4 (Figure 4.15), it only partially inhibited LDL oxidation by iron at pH 4.5 (figure 4.9, 4.11 &4.13). This might help to explain why it did not protect against femoral atherosclerosis in patients (Walldius et al., 1994).

Endothelial dysfunction is related to an augmented risk of cardiovascular events and a vasoconstrictor reaction to acetylcholine shows the incidence of endothelial dysfunction (Ludmer et al., 1986). As the endothelium is the main source of NO in the vasculature, damage of usual cellular function might lead to altered eNOS action and NO synthesis. Numerous evidence shows that endothelium-dependent vascular relaxation is decreased in cholesterol-fed animals (Kojda et al., 1998). Oxidized LDL relocates eNOS from caveolae by connection to endothelial cell CD36 receptors and by reducing caveolae cholesterol, leading to the interruption of eNOS stimulation (Blair et al., 1999, Feron et al., 1999). Therefore, LDL has powerful effects on eNOS's role in caveolae through activities on both membrane cholesterol stability and on the enzyme activity level. OxLDL adversely influences the endothelium by inducing the expression of cellular adhesion molecules, stimulating apoptosis after long-term exposure, and impairing vasodilatation (Kamiyama et al., 2009, Ou et al., 2006). Investigational evidence proposes that oxLDL may modify many signalling

pathway, such as PKC, MAPK, SAPK, tyrosine kinases, and the sphingomyelin/ceramide pathway, and many transcription factors, namely AP1, NF-κB, and STATs, all of which are potentially involved in atherosclerotic development (Auge et al., 2000, Napoli et al., 2000). Understanding the mechanisms of the recovery from endothelial dysfunction by antioxidants might result in effective therapeutic interventions for the prevention of CHD. LDL oxidised in lysosomes of macrophages might be exposed to endothelial cells when the macrophages die and their plasma membranes and lysosomal members rupture. Therefore, this study examined the effects of LDL oxidised at lysosomal pH on endothelium-dependent vasodilatation.

Rat aortic rings exposed to LDL oxidised by iron ions at lysosomal pH, showed reduce relaxation to acetylcholine but not reduced relaxation to sodium nitroprusside. This finding agreed with studies with other forms of oxidised LDL (Giardina et al., 2001, Galle et al., 2003). Iron–oxidised LDL increased reactive oxygen species formation by the aortic rings and cultured endothelial cells, which might help to explain the decreased activity of nitric oxide. In summary, the data presented in this thesis have identified the potentially atherogenic effects of LDL oxidised in lysosomes and the complex effects of various antioxidants on this oxidation.

7.1 Suggestions for future experiments

1. Antioxidant targeted to lysosomes could be used to see if they inhibit atherosclerosis in LDL-receptor deficient mice or WHHL rabbits. This size of the atherosclerotic lesions could possibly measured using the Vivo micro-ultrasound imaging system.
2. Different antioxidants, combined or alone would be evaluated to see if they decrease the inhibition of vasodilatation caused by LDL oxidised under lysosomal conditions using rat or rabbit aortic rings.
3. Substances extracted from plants could be used to try to inhibit LDL oxidation, under lysosomal conditions, measured by spectrophotometry and evaluating oxidative products by HPLC.
4. Antioxidants could be tested to see if they can reduce apoptosis in cultured cells, exposed to LDL oxidised under lysosomal conditions.

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