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To the Graduate Council:

I am submitting herewith a thesis written by Jyotsna Muthuswamy entitled "Vascular smooth muscle cell intracellular pst." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Michael B. Zemel, Major Professor

We have read this thesis and recommend its acceptance:

Whelan, Haughton

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting here with a thesis written by Jyotsna Muthuswamy entitled "VASCULAR SMOOTH MUSCLE CELL INTRACELLULAR CALCIUM RESPONSE TO LOW DENSITY LIPOPROTEIN: AN EX-VIVO INDEX OF ARTHEROGENICITY." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Michael B. Zemel, Major Professor

We have read this thesis
and recommend its acceptance:

Jay Whelan

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Associate Vice Chancellor and
Dean of the Graduate School

VASCULAR SMOOTH MUSCLE CELL INTRACELLULAR CALCIUM RESPONSE TO LOW DENSITY LIPOPROTEIN: AN EX-VIVO INDEX OF ATHEROGENICITY

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Jyotsna Muthuswamy
May 1997

DEDICATION

This thesis is dedicated to

My Parents, **Mrs. Uma Muthuswamy** and **Mr. S. Muthuswamy**,

My Grandmother, **Mrs. B. Kamala**,

and My Brother, **Mr. M. Gopinath**.

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ABSTRACT

Exposure of cultured vascular smooth cells [VSMCs] to LDL stimulates a transient intracellular free calcium ($[Ca^{2+}]i$) response, while in vitro oxidation of low density lipoproteins [LDL] produces marked increases in the magnitute of $[Ca^{2+}]i$ elevation compared to native LDL [N-LDL]. Increased $[Ca^{2+}]i$ exerts both trophic and contractile effects in VSMCs, and increased LDL oxidation may thereby contribute to atherosclerotic diseases. This may explain, in part, the increased atherogenicity of oxidized LDL [OX-LDL]. Complications in diabetes have been proposed to result, in part, from increased oxidative stress and OX-LDL has been demonstrated in vivo in atherosclerotic lesions. Consequently, the ability of LDL, to stimulate $[Ca^{2+}]i$ responses in cultured VSMCs, may provide a useful index of atherogenicity.

To evaluate this concept, the present study examined (i) the effect of a 12 week randomized, double-blind, placebo-controlled cross-over trial of α-tocopherol [400mg] in 12 healthy adult males, on the ability of their subsequently isolated LDL to increase $[Ca^{2+}]i$ in cultured VSMCs; and [ii] whether LDL isolated from persons with diabetes [8 type I diabetic patients] would stimulate a greater $[Ca^{2+}]i$ response in cultured VSMCs compared to healthy controls.

LDL was isolated in the presence of butylated hydroxytoluene [BHT] and the extent of oxidation was determined by quanititating thiobarbituric acid reactive substances [TBARS]. LDL 20μg from each subject then was used to stimulate fura-2 loaded rat VSMCs [10^6 cells/ml] and $[Ca^{2+}]i$ responses were measured flourometrically.

Although in vitro OX-LDL elicited a greater $[Ca^{2+}]i$ response than N-LDL, α-tocopherol supplementation was without effect on either VSMC $[Ca^{2+}]i$ or TBARS. Thus α-tocopherol supplementation appears to have conferred no protection in healthy individuals using this ex vivo atherogenicity index. However, in patients with higher oxidative stress, as in persons with diabetes, there was a 55% increase in TBARS [$P<0.0001$] and an approximate four-fold increase in the $[Ca^{2+}]i$ response to their LDL compared to healthy controls [444nM vs 104nM $[Ca^{2+}]i$ response in persons with diabetes and controls, respectively; $p<0.025$].

Thus the ability of LDL to stimulate $[Ca^{2+}]i$ in cultured VSMCs may serve as a useful ex vivo index of atherogenicity in clinical trials as well as in patients with higher oxidative stress, such as smokers and persons with diabetes.

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CHAPTER 1

INTRODUCTION

There is increasing evidence that oxidatively modified LDL is involved in the pathogenesis of atherosclerosis [1-3]. Lipid-laden foam cells, which are derived from blood monocytes, are found in early fatty streaks as well as in advanced atheroma [4]. Using monoclonal antibodies, it has been demonstrated that some of the lipid in foam cells and in the acellular matrix of the atheroma is oxidized LDL (OX-LDL) [5]. It is well known that all of the major cell types in the arterial walls (macrophages, endothelial cells and smooth muscle cells) involved in atherosclerosis are capable of oxidizing LDL in vitro [6-8].

The excessive risk of atherosclerosis both in insulin-dependent and non-insulin dependent persons with diabetes is well documented [9,10]. Atherosclerosis-related complications remain the leading cause of morbidity and mortality in persons with diabetes [11]. However, the mechanisms by which metabolic abnormalities of diabetes contribute to the development of atherosclerosis are unclear [9,11]. It has been postulated that the complications of diabetes result from oxidative stress resulting from hyperglycemia [12]. In vitro studies have shown that glucose can produce free radicals which can result in peroxidation of low density lipoproteins (LDL) [12,13]. In addition OX-LDL has been demonstrated in both atherosclerotic and diabetic animals and humans [14-18], and antioxidant supplementation has been shown to inhibit progression of atherosclerosis as well as retard oxidative modification of LDL [19-21].

All-rac-α-tocopherol is the most active and abundant isomer of the vitamin E family [22, 23]. It is the principle lipid soluble chain breaking antioxidant in tissues and plasma, and it is the predominant antioxidant in the LDL particle [23, 24]. Lipid peroxidation of LDL particles appears to start only after depletion of all the endogenous lipophilic antioxidants, with α-tocopherol acting as a major line of defense [25, 26]. Preliminary work has suggested that supplementation with vitamin E in humans decreases the susceptibility of LDL to oxidation, using in vitro techniques [27-29]. The potential use of α -tocopherol to inhibit atherogenesis is supported by recent epidemiological studies which demonstrate an inverse relationship between intake of α -tocopherol supplements and risk of cardiovascular disease [22, 30].

It has been shown that in cell cultures OX-LDL is capable of causing generalized cellular activation including increased intracellular calcium $[Ca^{2+}]i$, increased DNA synthesis [31-33] and increased collagen production [34]. Moreover, in vitro oxidation of LDL markedly enhances its ability to elicit an increase in intracellular calcium $[(Ca^{2+})i]$ in cultured vascular smooth muscle cells [VSMCs] [35, 36]. Many cellular activities involved in atherosclerosis, including VSMC contraction, migration and proliferation are characterized by increased $[Ca^{2+}]i$ [35, 37]. Two key mechanisms of atherogenesis in VSMCs, primarily proliferation and cell contractility [37-39], are mediated in part via mobilization of $[Ca^{2+}]i$ [39]. Low concentrations
[1 - 10 μg/ml] of LDL stimulate a significant dose dependent increase in $[Ca^{2+}]i$ and contraction in VSMCs [32], while OX-LDL stimulates substantially more pronounced increases in VSMC $[Ca^{2+}]i$ and contractility than does native LDL [N-LDL] [32, 36, 39].

This observation provides a novel approach for the evaluation of the potential atherogenicity of LDL isolated during clinical trials. It would be expected that antioxidant manipulations directed at reducing LDL oxidation would inhibit increases in VSMC $[Ca^{2+}]i$. LDL isolated from antioxidant treated subjects would not be expected to elicit as great a $[Ca^{2+}]i$ response in cultured VSMCs as LDL isolated from control subjects. Further, based on evidence for the prevalence of elevated levels of peroxidized LDL in persons with diabetes with atherosclerosis, it would be expected that LDL isolated from persons with diabetes with atherosclerosis would elicit elevated $[Ca^{2+}]i$ responses in VSMCs, compared to healthy individuals.

Consequently, the present study was undertaken to evaluate the effect of orally supplementing normal subjects with all-rac-α–tocopherol, on the ability of their subsequently isolated LDL to increase $[Ca^{2+}]i$ in VSMCs. In order to determine if the existence of elevated levels of peroxidized LDL <u>in vivo</u> in susceptible populations (in persons with diabetes with atherosclerosis) would behave like LDL oxidized <u>in vitro</u>, by increasing $[Ca^{2+}]i$ in VSMCs, the present study also determined whether LDL isolated from persons with diabetes with atherosclerosis is capable of eliciting a greater $[Ca^{2+}]i$ response in cultured rat VSMCs compared to LDL isolated from healthy controls.

CHAPTER 2

REVIEW OF LITERATURE

2.1 ROLE OF OXIDIZED LOW DENSITY LIPOPROTEINS - [OX-LDL] IN ATHEROGENESIS

Atherosclerosis is a degenerative disease of the large and medium sized arteries [40]. It is primarily characterized by the focal thickening of the intima, in association with fatty deposits which leads to the reduction in the luminal diameter of the arteries [14, 41]. There is mounting evidence that arterial intima - media thickness might represent an early atherosclerotic lesion [41]. Histologically, descriptions of the classic atherosclerotic lesions emphasize cellular proliferation, primarily of macrophages and smooth muscle cells [SMCs], accumulation of lipids, primarily cholesterol, connective tissue matrix components, calcium and cell debris [2, 14, 41]. The cholesterol that accumulates appears to be primarily derived from plasma lipoproteins [14, 15]. There is now adequate clinical and experimental evidence to substantiate the "cholesterol hypothesis" attributed to atherosclerosis, according to which there is a continuum of increasing risk for the development of atherosclerosis when plasma cholesterol levels exceed ~160-180 mg/dl [2]. However, the molecular and cellular mechanisms associated with the link between hypercholesterolemia and atherogenesis and its sequelae remain unclear [2, 40]. Further, at any given level of hypercholesterolemia there is considerable variation in the clinical development of cerebrovascular and cardiovascular disease, both caused primarily by atherosclerosis [1, 43-45].

Epidemiological and clinical evidence implicate increased levels of low density lipoprotein [LDL] - cholesterol with atherosclerosis [46-47]. LDL is the major cholesterol carrying lipoprotein (contains 60-70% of total serum cholesterol). LDL, which consists of free and esterified cholesterol, phospholipids, triglycerides, apo B-100 proteins and lipid soluble antioxidants, delivers cholesterol to the peripheral cells by means of a receptor-mediated endocytosis [38, 48]. Several studies have focused on the role of this cholesterol delivering lipoprotein as a pivotal factor in the evolution of atherosclerotic lesions [47]. Since the receptor-mediated pathway for the delivery of cholesterol to the cells is down-regulated by an increase in cellular cholesterol, this classical LDL-receptor pathway cannot contribute to the accumulation of cholesterol as seen in the atherosclerotic lesions. Hence it was speculated that parameters other than LDL concentrations could modulate the initiation of atherosclerotic lesions. The concept that modification of LDL is a prerequisite for cellular uptake and accumulation of cholesterol in the atherosclerotic lesions originated with observations made by Goldstein and his coworker in 1979 [38]. They demonstrated that macrophages in culture were converted into lipid laden foam cells only in the presence of chemically modified LDL, but not with native LDL (N-LDL). The major chemical modification that lead LDL to be taken up avidly and internalized by macrophages was recognized to be oxidation [38, 47].

In the presence of a given level of plasma LDL cholesterol, there is reasonable variation in the development of atherosclerotic lesions. One basis for this variation is attributed to the biologic response of cells in the arterial wall to LDL. Arterial wall - LDL interaction studies demonstrate that post-secretory modifications in the structure of

lipoproteins, and especially oxidative modification of LDL, accelerated development of atherosclerotic lesions [14].

2.1.1. PATHWAYS OF LDL METABOLISM IN ATHEROGENESIS

In vivo studies reveal that two-thirds or more of the removal of LDL from plasma is mediated via the N-LDL receptor and that this removal occurs predominantly in the liver [1, 49-50]. This elimination occurs through the classical N-LDL receptor [apo B/E receptor], first described by Brown and Goldstein [51]. Since these receptors are saturable and down-regulated, the apo B/E receptor pathway cannot generate lipid laden foam cells. This suggests the existence of an alternate pathway, independent of the N-LDL receptor pathway, facilitating arterial uptake of LDL and giving rise to fatty streaks and foam cells seen in the atherosclerotic plaques [14].

The following observations indicate a likelihood of an alternate pathway leading to foam cell genesis. First, formation of atherosclerotic plaque entails a focal accumulation of lipids, macrophages, smooth muscle cells, T-lymphocytes and extracellular matrix proteins in the intima of arteries. Macrophage-derived foam cells and subsequently atheroma develop in patients with familial hypercholesterolemia and Watanabe heritable hyperlipidemic (WHHL) rabbits, although both are receptor negative, i.e. both do not possess the apo B/E receptor. This suggests that lipid loading occurs via an alternate pathway [50, 52]. Second, monocyte-derived macrophages are precursors of foam cells and are seen in early atherosclerotic lesions. However, in vitro studies have demonstrated that macrophages in culture cannot be converted into foam cells by incubation with even

very high concentrations of N-LDL. This may be explained by the relatively limited number of saturable and down-regulated receptors for N-LDL expressed on the monocyte/macrophage cell membrane [53-54].

Foam cells develop in vivo when plasma levels of LDL are elevated [14]. Yet, circulating N-LDL is rapidly cleared by the liver. Goldstein and Brown [1979] first postulated that the circulating LDL must undergo initially some kind of post secretory modification to participate in foam cell formation [38]. They showed that chemical modifications of LDL, such as acetylation, led to macrophage uptake at a rate 3-10 times faster than N-LDL. This uptake occurred via a novel receptor termed "acetyl LDL receptor." Other similarly chemically modified forms of LDL, such as acetoacetylation and malondialdelyde conjugated LDL, are recognized by the same receptor and have been shown to lead to the formation of cholesteryl esters [7, 55-56]. Macrophages demonstrate specific cellular receptors for the modified forms of LDL collectively called "scavenger receptors" [7, 14]. They differ from the N-LDL receptor in that they do not bind N-LDL and are not down regulated [14].

However, these chemical modifications have not been identified in vivo [1-2, 14]. A biologic modification that maybe analogous to these modifications has been reported in vivo [1, 14, 56]. In vivo studies demonstrated that incubation of LDL with cultured endothelial cells or smooth muscle cells (SMCs) or metal ions converts it to a form that is taken up rapidly by macrophages through the "scavenger receptors" [7, 56]. The most plausible and biologically relevant modification of LDL has been described as oxidation [3, 15, 44, 57-58]. Oxidized LDL has been identified in vivo in atherosclerotic lesions [2,

14]. Hoff and his coworker [59] assessed the functional properties of LDL oxidized in the presence of copper [in vitro] and LDL isolated from minced aortic plaques [in vivo]. Both demonstrated increased electrophoretic mobility, increase in cholesterol to protein ratio and an increased reactivity with monoclonal antibodies to epitopes of malondialdehyde-modified proteins. Also an increased uptake of the oxidatively modified LDL by the macrophages and subsequent lipid loading leading to foam cell formation was reported.

Other modifications of LDL, which are taken up by the macrophages and are demonstrated in vivo, include self aggregation, complex formation with a wide range of macromolecules such as proteoglycans and various matrix proteins, and immune complex formations leading to macrophage uptake via Fc receptor pathways [1-2, 60].

2.1.2. MECHANISM OF LDL MODIFICATION

Despite vast interest in the oxidative processes associated with LDL that may be involved in leading to atherogenesis, the specific nature of involvement is poorly understood. A detailed understanding of the mechanism(s) of oxidative pathways could lead to the development of specific inhibitors, and an understanding of the malfunctioning of the oxidative pathways could help devise preventive stratergies [2].

In vivo and in vitro studies have repeatedly shown to involve (i) free radicals, (ii) cell systems and (iii) transition metals in the oxidative modification of LDL. The exact mechanism of oxidation has not yet been eluciated, but several possible pathways have been proposed [2, 41, 48, 61].

(a) Free Radicals: Chemistry and Free Radicals in Biological Systems

A free radical, in electron orbital terms, can be defined as a molecule or atom with an unpaired electron in its outer orbit [62-63]. The oxygen molecule, as it occurs naturally, qualifies as a radical: it possesses two unpaired electrons, each located in a different Pi orbital. These two electrons have the same spin quantum number and are therefore located in a parallel spin configuration [63-65]. If oxygen attempts to oxidize another molecule by accepting a pair of electrons from it, both these electrons must be of antiparallel spin in order to fit into the vacant spaces in the Pi orbitals. In accordance with Pauli's Principle, a pair of electrons in an atomic or molecular orbital would not meet this criterion, since they would have opposite spins [62-63, 65]. Therefore during oxidation by oxygen, there is a restriction on electron transfer, and hence, oxygen accepts its electrons one at a time. Subsequently, oxygen reacts only sluggishly with many non-radicals. This is advantageous to aerobic organisms because it slows down oxygen reactions. Yet, this one electron transfer can lead to the formation of a molecule or an atom with an unpaired electron, or a free radical [65].

In biological systems the route for molecular oxygen metabolism involves its complete reduction to H_2O. During the one electron reduction, several free radicals can be formed. Once the reduction of oxygen has occurred, by-products, called reactive oxygen species [ROS], are generated, the first of which is the superoxide anion.

$O_2 + e^-$	$\rightarrow$	O_2	Superoxide radical
$O_2 + H_2O$	$\rightarrow$	$HO_2^{\cdot} + OH^-$	Hydroperoxyl radical
$HO_2 + e^- + H$	$\rightarrow$	H_2O_2	Hydrogen Peroxide

$$H_2O_2 + e^- \rightarrow \cdot OH + OH^-$$ Hydroxyl radical

Under normal conditions, 95% to 98% of molecular oxygen consumed by cells is finally reduced to H_2O by the addition of four electrons. It is the remaining 2% to 5% that are reduced by a univalent pathway giving rise to reactive free radicals even during daily homeostatic oxidative stress [63, 65, 66]. There are numerous organic free radicals, but the number of oxygen-derived free radicals that occur in biological systems is limited. The most prevalent forms in biological systems include superoxide anion (O_2), hydroxyl radical ($HO^{\cdot}$), perhydroxyl radical (HO_2), hydrogen peroxide (H_2O_2), peroxyl radical ($ROO^{\cdot}$), alkoxyl radical ($RO^{\cdot}$,) and singlet oxygen ($^1O_2$). All of these have been shown to be involved in LDL oxidation, although controversies exist regarding their mechanisms of action [61, 62, 66].

Oxygen is an integral part of aerobic life, yet its metabolism has ominous metabolic consequences. The oxidant by-products of normal metabolism, i.e. free radicals, are not only involved in the pathogenesis of degenerative diseases (e.g. cardiovascular and cerebrovascular disease and cancer) and aging, but also play a pivotal role in normal biological functions [66]. Free radicals are involved in supporting life processes, such as mitochondrial respiration [67], prostaglandin synthesis [68], platelet activation [66-67], and leukocyte-phagocytosis [69]. Free radicals cause extensive damage to lipid membranes, organelles and even the DNA [66]. This obviously represents a paradox. It is this paradox in free radical chemistry that has stimulated researchers, and health-care professionals, especially those interested in preventive medicine [65]. There is now increasing emphasis as well as evidence for an important role for oxygen free radicals in

coronary artery disease [61]. In the arterial intima, free radicals can be generated by endothelial cells, SMCs and macrophages [2, 6, 70]. These free radicals can attack LDL in the subendothelial space, causing oxidation [61]. Free radical attack on LDL can result in oxidation of cholesterol and the apolipoprotein, and peroxidation of the fatty acyl chains in the phospholipids, triglycerides and cholesterol ester fractions [63].

(b) Effect of Transition Elements on LDL Oxidation

Transition elements are all metals [63]. From a radical view point, the most important feature is their variable valency, which allows them to undergo changes in thier oxidation state, involving one electron [71]. In biological systems, iron and copper are the most likely redox-active candidates that can participate in the activation of oxygen-related reactions, due to their abundance [63, 71].

Hochstein and Ernster [73] were the first to demonstrate the requirement for a metal in lipid peroxidation [72]. Numerous in vitro studies have utilized Cu^{2+} and Fe^{2+} to oxidize LDL to study the possible mechanisms of OX-LDL in atherosclerosis, while metal chelators are often used to suppress progression of LDL oxidation. Iron-chelating compound desferrioxamine has been used in vitro to suppress production of hydroxyl radicals.

In vivo copper ions may arise from damage to ceruloplasmin within the arterial wall, and iron could arise from the breakdown of hemoglobin during cell death in advanced lesions [73-74]. The heme protein-oxidized mixtures can by themselves potentiate another mechanism for oxidative damage in advanced lesions, in that they can peroxidize LDL [75]. Iron catalysis have been shown to induce oxygen-derived free

radical generation and subsequent lipid peroxidation. This has been reported to occur during cardiopulmonary bypass in humans. Menasche' et al [73] reported that desferrioxamine (iron chelator) administered intravenously to cardiopulmonary bypass patients resulted in decreased susceptibility of LDL to peroxidation as assessed by TBARS, compared to control patients. The researchers suggest that desfferioxamine could be clinically useful for limiting the iron-mediated free radical damage and the iron dependent abnormalities of oxidative metabolism.

(c) Cells and Transition Elements in LDL Oxidation

Several potential sources of oxidant(s) have been suggested in the initiation of formation of oxidatively modified LDL [1, 40, 76]. In vitro studies have demonstrated that all the three major cell types in the artery wall, namely smooth muscle cells [77], endothelial cells [78], and monocyte-macrophages [78], are capable of oxidatively modifying LDL. LDL has been oxidized also in the presence of transition metals alone [40, 63, 76]. Cell induced oxidative modification of LDL can be mimicked by incubating LDL in a serum free medium, in the presence of sufficiently high concentrations of copper or iron [1, 79]. LDL oxidized in the presence of transition metals is physiochemically and biologically indistinguishable from cell-oxidized LDL [14, 40, 76]. It has been shown that whether the oxidation of LDL was carried by incubation under appropriate conditions with cultured cells or by autooxidation catalyzed by metal ions in the absence of cells, oxidative modification of LDL leads to recognition by scavenger receptors [80-81]. Scavenger receptors have now been cloned and sequenced [82].

The normal arterial wall contains endothelial cells and SMCs, whereas atherosclerotic lesions in addition contain macrophages and T-lymphocytes [80]. Incubation of LDL with endothelial cells, SMCs, monocyte/macrophages and lymphocytes have all been shown to induce oxidation of LDL [2, 6, 70]. Henricksen and coworkers [83] demonstrated that when OX-LDL was incubated with cultured endothelial cells for 12 to 18 hours, it was modified to a form that was taken up avidly by macrophages which are known to have scavenger receptors. As the macrophages accumulate, cholesterol and cholesteryl esters are deposited which can subsequently lead to the formation of foam cells. Henriksen et al [83] and Heinecke et al [84] showed that SMCs in culture also induced similar modifications of LDL. Monocytes [2], monocyte derived macrophages [62, 80] and macrophages in culture [62, 80], all lead to oxidative modifications of LDL [2]. Although the above mentioned cell culture systems have been shown to oxidize LDL, the oxidant(s) relevant for LDL oxidation have not been delineated clearly [1, 80].

Superoxide anion radical is a cell derived oxidant, which has been shown to mediate LDL oxidation by SMCs and monocytes [62, 85]. The mechanisms of production of superoxide radicals by these cells have been suggested to involve the autooxidation of extracellular thiol compounds, with the cells functioning merely as reductants of disulfides such as cysteine present in the culture medium [86-87]. Sparrow et al [87] described the cellular oxidation of LDL in a media containing transition metal ions occurred via the cell-dependent appearance of thiol in the medium. Heinecke et al [86] reported similar findings. Superoxide dismutase [SOD] inhibits cell mediated oxidation [40]. SOD also chelates metals in a redox-inactive form and thereby inhibits cell free copper-induced

oxidation of LDL [14]. SOD catalyzes breakdown of $O_2^{\cdot}$ to H_2O_2 [14, 40]. The role of superoxide in the endothelial and macrophage mediated oxidation of LDL has been controversial [40]. Studies indicated that the superoxide anion radical produced by the cells cannot oxidize LDL directly [40, 62, 88]. It is speculated that the superoxide radical might react with the nitric oxide radical [No·] to form the peroxynitrite anion [$ONOO^-$], which decomposes into the hydroxyl radical [OH·], a potent oxidant [62, 88].

Nitric oxide and peroxynitrite have been suggested to be potential oxidants relevant in the endothelial cell and macrophage mediated oxidation of LDL [88]. However, nitric oxide also has been viewed as a protector rather than a stimulator of LDL oxidation, as inhibitors of nitric oxide synthase increased LDL oxidation by macrophages [89]. Candipan et al [90] used L-arginine therapy, a precursor of nitric oxide, and demonstrated that the restoration of nitric oxide activity could induce regression of the pre-existing atherosclerotic lesions in rabbits. Similar findings were reported by Bolton et al [91] who demonstrated that induction of nitric oxide synthase down regulated LDL oxidation.

Lipoxygenase-derived products have been implicated in LDL oxidation by endothelial cells [61] and macrophages [62]. 15-Lipoxygenase oxidizes LDL in vitro and may be involved in the cell mediated oxidation of LDL by the generation of free radicals by the cellular lipoxygenases [92]. Sparrow et al [92] demonstrated that incubation of LDL with pure soybean lipoxygenase can mimic most of the changes induced by incubation of LDL with endothelial cells and hence lipoxygenase inhibitors might help retard or reverse the atherosclerotic process. However, Sparrow and coworkers [95]

have demonstrated that lipoxygenase inhibitors exhibit antioxidant properties, and that this antioxidant activity is responsible for the inhibitory effect of LDL oxidation, rather than the inhibition of lipoxygenase activity itself.

The presence of functional, extracellular myeloperoxidases has been identified in macrophage rich and acellular lipid-rich areas of human atherosclerotic lesions [96]. Neutrophils and monocytes can synthesize and release myeloperoxidases [40, 96]. Although it is not known if they can oxidize LDL in vivo, it has been demonstrated that the myeloperoxidase-derived oxidants hypochlorite [97] and tyrosyl radicals [98] can convert LDL to a form taken up by macrophages. Nalini et al [99] suggested the involvement of myeloperoxidases in the oxidation of LDL by peroxidases, which has been suggested as a model for in vivo oxidation. The generation of intermediate radicals such as phenoxy radicals has also been suggested to be involved in this mechanism. It was demonstrated that probucol but not vitamin E inhibited this oxidation.

Controversies persist as to which oxidant(s) initiate LDL oxidation in the arterial walls. However, there is consensus that redox active transition metal ions are required for cells in culture to mediate LDL oxidation [40]. This consensus is based on observations that cell mediated oxidation of LDL in culture does not occur when devoid of metal ions and that the addition of chelators in culture inhibited any oxidation of LDL [40, 84]. Since large doses of desferroxamine, a strong metal chelator, only partially inhibited the macrophage mediated LDL oxidation, but completely inhibited the SMCs mediated LDL oxidation, this postulated absolute requirement of transition metals may not hold true for LDL oxidation mediated by macrophages [80] and monocytes [100]. However,

Steinbrecher et al [101] and Morel et al [102] independently showed that oxidative modifications of LDL in culture was absolutely dependent on low concentrations of copper or iron in the media, as any modification was completely inhibited by the addition of ethylenediamine tetraacetic acid [EDTA] or other metal chelators. In vivo atherosclerotic lesions have demonstrated detectable amounts of transition metals which can be involved in lipid peroxidation [103].

2.1.3. PATHWAYS OF LDL OXIDATION

BASIC CHEMISTRY OF LIPID PEROXIDATION

In a peroxide free lipid system, the INITIATION of peroxidation occurs by an attack of a species that is primarily capable of abstracting hydrogen atoms [e.g. peroxyl and hydroxyl radicals]. Hydrogen abstraction is facilitated by the presence of double bonds in the carbon side chain. Hence in LDL, the polyunsaturated fatty acid [PUFA] side chains are most susceptible to attack [104]. If the fatty acid side chain is designated L-H [L for lipid], the reaction can be written as follows:

$$\underline{\text{INITIATION}} \quad LH \xrightarrow{\text{initiator}} L^{\cdot}$$

The fate of the carbon centered [$L^{\cdot}$] radical generated from the PUFA side chain, is most likely to undergo molecular rearrangement to conjugated diene structures. This is followed by reaction with oxygen to give peroxyl radicals [104-105]. This reaction can be represented as:

$$\underline{\text{PROPAGATION}} \quad L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$

The peroxyl radical [$LOO^{\cdot}$] can damage proteins, deplete antioxidants, and attack an adjacent PUFA side chain, hence propagating lipid peroxidation. Lipid peroxides [LOOH] are thus generated [105].

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$$

Once initiated, peroxidation of lipids can accelerate in an autocatalytic manner.

$$\underline{\text{BRANCHING}}\ LOOH \rightarrow LO^{\cdot} + HO^{\cdot}$$

$$2LOOH \rightarrow LOO^{\cdot} + LO^{\cdot} + H_2O$$

Peroxidation will terminate when the substrate is exhausted [e.g. PUFA and/or oxygen]. Lipid peroxidation proceeds in an autocatalytic manner until radical termination reactions consume a significant percentage of the propagating peroxyl radicals [104].

$$\underline{\text{TERMINATION}}\ LO^{\cdot} + LO^{\cdot} \rightarrow \text{NON RADICAL PRODUCTS}$$

$$LOO^{\cdot} + LOO^{\cdot} \rightarrow \text{NON RADICAL PRODUCTS}$$

LH - unsaturated fatty acid

$HO^{\cdot}$ - hydroxyl radical

$L^{\cdot}$ - allyl radical

$LO^{\cdot}$ - alkoxyl radical

$LOO^{\cdot}$ - peroxyl radical

LOOH - hydroperoxide

Oxidation of LDL induces a wide range of changes that alter its metabolism in many ways e.g. increased uptake of scavenger receptors, and enhanced cholesterol accumulation leading to foam cell formation [105]. All of these changes are mediated by a

common initiating step which involves lipid peroxidation or the peroxidation of PUFA in the lipid molecule [104-105].

Overall, the oxidation process of LDL can be described to occur in three phases: [i] an initial lag phase when endogenous LDL antioxidants [e.g. vit E] are depleted; [ii] a second propagation phase, which involves the oxidation of unsaturated fatty acids to lipid hydroperoxides [62, 106]. These phospholipid hydroperoxides then are hydrolyzed to lysophospholipids and fatty acid hydroperoxides, either by a phospholipase A_2-activity which is intrinsic of apo-B [106], or by the platelet-activating factor acetylhydrolase associated with LDL [107]; [iii] consequent to the propagation reactions, once the LDL molecule contains fatty acid lipid hydroperoxides, there is a dramatic augmentation in the number of free radicals, accompanied by an extensive fragmentation of its PUFAs, generating a complex array of shorter chain-length fragments. This is referred to as the decomposition phase, wherein the hydroperoxides are converted to a broad spectrum of reactive intermediates, like aldehydes (e.g. malondialdehyde, 4-hydroxynonenal) and ketones [62].

The oxidatively modified LDL has now conferred on it an array of biological properties which have been postulated to probably enhance its atherogenic potential [14, 40, 44].

2.1.4. CHARACTERISTICS OF OXIDATIVELY MODIFIED LDL

The following are characteristics of oxidatively modified LDL:

- Reduction in the amount of PUFA [especially linoleic (18:2) and arachadonic (20:4) acids], concurrent with an increase in lipid peroxides;
- Fragmentation of apo B100;
- Increased negative charge and electrophoretic mobility;
- Increased hydrated density and decreased particle size;
- Increased lysophosphatidyl choline content;
- Increased content of oxidized form of cholesterol-oxysterols;
- Decreased cholesterol-protein and phospholipid-protein ratio; and
- Increased uptake through "scavenger receptor" and, reduced uptake by LDL receptor.

The above summarized characteristics of OX-LDL [2, 40, 61-62] have been suggested to contribute to its atherogenic properties.

2.1.5. BIOLOGICAL EFFECTS OF OXIDATIVELY MODIFIED LDL

Biological effects of oxidatively modified LDL include:

- Increased uptake by "scavenger receptors" leading to lipid accumulation and "foam cell" formation;
- Monocyte chemoattractant;
- Inhibition of macrophage migration;
- Cytotoxicity;
- Stimulation of endothelial-leukocyte adhesion;

- Inhibition of endothelium derived relaxation factor;
- Stimulation of tissue factor expression; and
- Initiation of oxidation of N-LDL.

All of the above mentioned "biological effects" [15, 40, 44, 108] have been potentiated in leading to foam cell formation.

2.1.6. EVIDENCE FOR THE EXISTENCE OF OX-LDL IN VIVO

1. Antibodies against "models" of OX-LDL, such as malondialdehyde or 4-hydroxynonenal-conjugated LDL, or against OX-LDL itself [e.g. 0x5, antibody for OX-LDL] have demonstrated immunostaining in the aorta of rabbits with atherosclerosis [1, 14, 40]. Further, LDL isolated from aortic lesions of Watanabe Heritable hyperlipidemic [WHHL] rabbits demonstrate cross reactivity with antibodies specific to malondialdehyde [3, 14, 16].
2. LDL isolated from aortic atherosclerotic lesions have physiochemical and biological properties similar to in vitro OX-LDL [14, 40].
3. Auto-antibodies in plasma of rabbits [normal as well as WHHL rabbits] as well as humans react with in vitro OX-LDL [14, 16].
4. Treatment with antioxidants has been shown to prevent progression of atherosclerosis in animal models [66].
5. LDL isolated from persons with atherosclerosis and/or diabetes has demonstrated elevated levels of lipid peroxidation compared to healthy individuals [40, 45, 47].

LDL oxidized in vitro in the presence of transition metals or cells exhibits characteristics similar to OX-LDL isolated from atherosclerotic lesions [72, 75].

2.1.7. POTENTIAL MECHANISMS THAT COULD CONFER ATHEROGENIC PROPERTIES TO OX-LDL

OX-LDL shows several chemicophysical modifications [53]. As a consequence of oxidation, OX-LDL is no longer recognized by the apo B/E receptor [14, 40]. With extensive oxidation of LDL, there is degradation of its PUFAs, generating a complex array of short chain fragments which are highly reactive intermediate-short chain aldehydes and ketones [76]. Some of these aldehydic products covalently link to apo B. Much of this conjugation involves the E-amino groups of lysine residues in apo B and fragmentation of apo B occurs [109]. The lysine residues of apo B are required for recognition and interaction of LDL with its apo B/E receptor. Since there is a masking of the lysine residues in the OX-LDL molecules, they are no longer metabolized through the LDL receptor pathway and an alternate pathway involving the "scavenger receptor" begins to metabolize OX-LDL, subsequently leading to the formation of "foam cells" [109-110]. Biological effects of OX-LDL based on the characteristics of OX-LDL, together provide mechanisms that render OX-LDL atherogenic. Some of these mechanisms include:

(a) OX-LDL Demonstrated Enhanced Uptake by Macrophages Leading to Intracellular Cholesterol Accumulation

Interest in studying OX-LDL stemmed from observations by Henriksen and coworkers [56] who first demonstrated that oxidative modification led to its enhanced

uptake by cultured macrophages, via the scavenger receptors, leading to the formation of foam cells. The modifications in the apo B of LDL leading to recognition by the scavenger receptor for metabolization has been described previously. The ability of OX-LDL to cause cholesterol ester accumulation has been proposed to occur in part due to the inability of macrophages to degrade OX-LDL as readily as N-LDL, thus leading to the accumulation of undegraded OX-LDL [111-112]. The cholesterol in the LDL is oxidized to form oxysterols, which by themselves exert several atherogenic properties, including cytotoxicity [113] and modulation of intracellular cholesterol metabolism [114]. Oxysterols also can interfere with a range of intracellular enzymes [2], for example, inhibiting the normal cellular processing of ingested lipids and proteins, and possibly even leading to accumulation of toxic intermediates within the cell. Oxysterols have been described as established indicators of oxidation in tissues [115].

(b) OX-LDL is Chemotactic for Circulating Monocytes and Inhibits Mobility of Tissue Macrophages

One of the earliest events in atherosclerosis is the adherence of the circulating monocytes to the endothelial cells [40, 116]. The adherence is followed by the migration of monocytes into the intima. This adhesion and penetration are influenced by chemotactic factors [2, 40].

Cushing et al [117] demonstrated that minimally modified LDL [MM-LDL] was chemotactic for circulating monocytes and stimulated the production of monocyte chemotactic protein - 1 [MCP-1] by SMCs and endothelial cells. MCP-1 further lead to differentiation of monocytes to macrophages, which in turn further oxidized MM-LDL to

severely OX-LDL, which stimulated again the MCP-1, thereby leading to a viscous cycle gathering even more OX-LDL and macrophages. Once the macrophages are trapped within the arterial walls, they participate actively in oxidizing LDL and these autocatalytically expand the atherosclerotic lesions [118]. Macrophage derived foam cells are the hall marks of early atherosclerotic lesions [118-120].

Quinn et al [3] described the chemotactic activity of OX-LDL to reside in the lipid component of the LDL molecule. Much of the chemotactic activity was attributed to lysophosphatidyl choline [LPC]. During LDL oxidation up to 40% of LDL's phosphatidyl choline is hydrolyzed to LPC [120]. The fatty acid that is removed is from the 2nd position. This removal is believed to be the result of phospholipase-A_2 activity that is intrinsic to the LDL molecule [120-121]. Cyulski et al [122] reported that the LPC in OX-LDL stimulated production of monocyte adhesion molecules by endothelial cells. Extracts of whole aorta also have demonstrated chemotactic activity [76].

In addition to attracting monocytes, OX-LDL inhibits migration of resident tissue macrophages, possibly by trapping the monocyte-derived macrophages in the sub endothelial space, thereby preventing their escape back into circulation [1, 40]. Quinn and his coworkers [3] described the release of chemotactic activity from cultured endothelial cells for the mouse peritoneal macrophages and showed that OX-LDL inhibited the chemotactic response of the macrophage. Tissue macrophages, normally, after it has accumulated in localized cellular debris, would exit and help remodel the damaged tissue space. But OX-LDL inhibits macrophage chemotaxis [1, 3]; in other words, it inhibits the mobility of tissue macrophages. Thus OX-LDL in addition to favoring cholesterol

accumulation and foam cell formation, through its chemotactic activity which is described to be predominantly through LPC, also plays a role in the recruitment and retention of monocyte/macrophages in the intima [1-2, 40].

Two macrophage phenotypes were reported by Vladimir et al [123], at the site of the atherosclerotic lesions. One phenotype was representative of macrophage of monocytic origin. The other phenotype consisted of small cells that were localized in the superficial as well as deep layers of fatty streaks, surrounded by foam cells. This suggested the possibility of macrophage proliferation within the lesion.

(c) OX-LDL is Cytotoxic

Accumulation of OX-LDL in the intima might lead to the disruption of various cellular processes. Aldelydes, LPC and oxysterols formed during unregulated oxidation of LDL lipids are cytotoxic [15, 40, 119]. Cathcart and coworkers [78] demonstrated the cytotoxicity of OX-LDL for endothelial cells, and Reid and coworkers [124] described similar cytotoxicity for macrophages. Morrel et al [102] and Hessler et al [125] reported that OX-LDL but not N-LDL was cytotoxic. These may be particularly relevant to lesion progression, endothelial loss and denudation of the artery wall leading to cell death.

Coffey et al [105] described the cellular and molecular mechanisms by which OX-LDL induced cell injury, as a sequence involving lipid hydroperoxide-induced, iron-mediated formation of alkoxyl, lipid and peroxyl radicals. They identified the potent toxic moiety of OX-LDL as 7β-hydroperoxy cholesterol. These oxysterols have been demonstrated in human carotid atherosclerotic lesions and are reported to be cytotoxic. Sticko et al [121] reported that exposure of LDL to copper resulted in a rapid

accumulation of peroxides and LPC. LPC stimulated SMC DNA synthesis in culture and this mitogenic activity occurred very rapidly. Previously, LPC has been demonstrated as the most potent cytotoxic component of OX-LDL [15, 40]. Bjorkerud et al [126] reported a dual effect of OX-LDL: one with a strong growth promoting effect and another leading to the induction of cell death by apoptosis, based on the degree of oxidative modification. This is compatible with the notion that OX-LDL not only plays a role in atherogenesis, but also could lead to the development of structures typical of the atherosclerotic lesions, with focal excessive growth alternating with necrosis.

(d) OX-LDL is Immunogenic and Can Elicit Autoantibody Formation

Another atherogenic property of OX-LDL is its immunogenicity. Malondialdehyde-modified LDL [MDA-LDL] and 4-hydroxynonenal modified LDL [4-HNE-LDL] have been shown to stimulate formation of autoantibodies [127-128]. OX-LDL in the proximity of macrophages in the lesions may act as an antigen and induce autoimmune antibodies [129]. MDA-LDL and 4HNE-LDL stimulate autoantibody formation and the immune complexes of LDL aggregates are taken up avidly by the macrophages via Fc receptors [128-129]. Antigen-antibody complexes involving such autoantibodies and LDL have been demonstrated in the plaques of the artery wall [2, 40].

Herttuala et al [130] reported that both humans and rabbit atherosclerotic lesions contain IgG [autoantibodies against epitopes of OX-LDL] that recognize epitopes characteristic of OX-LDL. These autoantibodies were found to be more prevalent in the sera of individuals with atherosclerosis than in healthy individuals [130-131]. Subclasses of IgG [IgG1 and IgG3] were identified by Mironova et al [132] and these were described

as the predominant isotopes that were specific for OX-LDL. These two sub-classes also demonstrated highest binding for the Fc receptor.

(e) OX-LDL Adversely Affects Coagulation Pathways and Vasomotor Activities

OX-LDL has been described to be involved in cardiovascular disease through mechanisms that are not involved in atherogenesis directly. For example, LPC in OX-LDL has been demonstrated to inhibit endothelial cells to release the endothelium derived relaxing factor [EDRF] in response to agonists, thus leading to impaired vasorelaxation and increased platelet aggregation [132, 134]. OX-LDL has been shown to be involved in the stimulation of procoagulant activity on the surface of human monocyte macrophages by an increase in thrombolplastin activity [135] or by the expression of tissue factor by aortic endothelial cells or monocytes [136].

(f) OX-LDL Demonstrates Alteration of Gene Expression in the Arterial Cell Walls

The involvement of OX-LDL in the atherogenic process is further demonstrated by alterations in the gene expression induced in the arterial cells. For example, OX-LDL [i] in cultured bovine aortic endothelial cells inhibits the expression of the platelet derived growth factor [PDGF] [137]; [ii] in cultured human monocyte derived macrophages inhibits the secretion and expression of PDGF-B chain [138]; [iii] in cultured marine peritoneal macrophages inhibits expression and secretion of Tumor necrosis factor [TNF-α] [139]; [iv] in cultured human aortic endothelial cells, minimally modified LDL stimulates the expression and secretion of the macrophage-colony stimulating factor, the granulocyte macrophage-colony stimulating factor, and the granulocyte-colony stimulating factor, which facilitates the adherence of monocyte to the endothelium [140-141]; and [v]

in cultured human aortic endothelial cells and SMCs stimulates the transcription and secretion of monocyte chemotactic protein-1 [MCP-1]. MCP-1 mRNA has been demonstrated in situ hybridization in macrophage rich lesions of plaques in human and rabbit-aortas, but not in normal aorta [141]. Thus it is likely that OX-LDL in the intima can affect the ability of the cells to produce various chemotactic factors, growth factors and cytokines, all of which can contribute to the atherosclerotic process [137-141].

DIFFERENCES IN THE ATHEROGENIC POTENTIAL OF LDL SUBFRACTIONS

LDLs are a heterogenous population of particles that vary in size, density, electric charge and composition [142-143]. LDL particles are distributed as a continuum over a density range of 1.019 to 1.063g/ml. Within this continuum, multiple subpopulations of LDL particles have been identified, which vary in their physiochemical and biological functions [144-145]. On structural and metabolic basis, LDL has been grouped into 3 major subclasses [142]: [i] light, large LDL [d=1.02-1.03g/ml] -- Pattern A; [ii] intermediate LDL [d=1.03-1.04g/ml] -- Intermediate Pattern; [iii] small, dense LDL [d=1.04-1.06g/ml] -- Pattern B.

Studies reveal that coronary heart disease patients exhibit elevated levels of small, dense LDL subfractions [146-147]. Data from the St. Thomas Atherosclerosis Regression Study [STARS] revealed that the prevalence of small, dense LDL particles [d-1.04-1.063g/ml] correlated significantly with coronary artery segment luminal diameter [148]. Reports from the Physician's Health Study proposed the prevalence of small, dense LDL subfractions as a strong, prospective, independent predictive factor of myocardial

infraction and stroke [149]. Chapman et al [144] reported that in patients with combined hyperlipidemia, the atherogenic lipoprotein phenotype was represented by a predominance of small, dense LDL. Sevanian et al [142] found that small, dense LDL subfractions were more susceptible to oxidation and hence individuals with a predominance of this pattern B phenotype of LDL would be at a greater risk for atherosclerosis. They reported that populations with Pattern B contain lipid peroxides at levels approaching the threshold required for radical propagation reactions. The researchers postulated that the presence of elevated levels of small, dense LDL subfractions may contribute to a pro-oxidant state that encourages oxidative reactions in vascular complications [142]. Chait et al [150] found that increased atherogenic risk associated with Pattern B was, in part, due to increased concentrations of small, dense LDL subfractions, that were relatively more susceptible to oxidation. Demuth et al [151] reported that the small, dense LDL subfractions carried an electronegative charge in vivo. This subfraction was shown to be cytotoxic to endothelial cells in culture. The increased incidence of atherosclerosis in men compared to women correlated with the increased prevalence of small, dense LDL particles in men [152].

Some of the properties which confer greater atherogenic potential to the small, dense subfraction include: poor binding to LDL receptor [153], prolonged circulation time in plasma [142, 152], low resistance to oxidative modification [154-155] and high binding affinity to extracellular matrix proteins in the arterial wall [144].

OX-LDL INDUCED ATHEROSCLEROSIS-HYPOTHESIS

Atherosclerotic plaques are located generally at the lesion-susceptible branch sites or bifurcations where fluid mechanical sheer stress is low [156]. Researchers have suggested two possible categories of mechanisms by which lipoproteins may be atherogenic [40, 44]: [i] The Endothelial Infiltration Hypothesis; and [ii] The Endothelial Injury Hypothesis.

(i) Endothelial Infiltration Hypothesis

According to this hypothesis, endothelial leak may result from two processes [i] elevated levels of circulating LDL concentrations may cause the junction between the adjacent cells to separate, thus allowing LDL into the subendothelial spaces, or [ii] LDL may reach the subendothelial spaces via specific high affinity receptors on the endothelial cells. Once LDL enters the subendothelial space it can be modified oxidatively and therefore can be taken up avidly for "foam cell" formation [40, 44, 76].

(ii) Endothelial Injury Hypothesis

According to this hypothesis, alterations in the endothelial permeability might contribute to atherosclerosis [157]. Evidence indicates that mechanical [158], chemical [156-157] and viral injury [156] to the endothelial layer, all can promote atherosclerosis. High concentrations of OX-LDL have been shown to cause damage to endothelial cells in culture [159]. Cytotoxicity of OX-LDL also has been conceived to induce functional changes in the endothelial cells, that favor the penetration of monocytes or the movement of LDL into the subendothelial space. There is now sufficient evidence that oxidatively modified LDL occurs in circulating plasma [157-158]. Gerrity [160] and Faggiotto et al

[161] identified loss of endothelial cells from the surface of established fatty streaks. They suggested that the cytotoxicity of OX-LDL may be sufficient to lead to such denudation.

Although these two hypothesis by themselves can explain the genesis of altherosclerosis and foam cell formation, a UNIFIED HYPOTHESIS [1] linking events in the endothelial injury and infiltration hypothesis seems like a more probable in vivo applicable hypothesis. A schematic representation of the unified hypothesis linking events in the above mentioned two hypotheses is described in Figure 1.*

According to the unified hypothesis, once the LDL enters the subendothelial spaces, it is taken up avidly by the macrophages, subsequently leading to the formation of 'foam cells.' Although the endothelial injury hypothesis and the endothelial infiltration hypothesis describe differently the possible initiating events in atherogenesis, there is consensus on the final event, i.e., 'foam cell' formation and cell necrosis. The link between the two hypotheses further substantiates the 'oxidation' hypothesis of atherosclerosis.

Recently, research has focused on the 'foam cell' hypothesis, and there has been increasing interest on the molecular events involved in OX-LDL induced atherosclerosis. Several studies have also focused on the possible role of antioxidants in inhibiting atherosclerosis [14,29]. The protective role of antioxidants has been demonstrated in several animal models [40], and the possible protective roles of antioxidants are also being investigated in human clinical trials.

* All figures and tables may be found in the appendices.

2.2. CALCIUM HOMEOSTASIS AND LOW DENSITY LIPOPROTEINS

The nexus between elevated levels of LDL and risk for developing atherosclerosis is well established [40, 44]. The potential role of OX-LDL as a critical factor in lesion development in the atherosclerotic process has been documented in both experimental and human atherosclerosis [162, 163]. There is consensus that atherosclerotic lesion development from fatty streaks to "fibrous plaques" and "complicated lesions" are characterized by accumulation of lipids in macrophages and other arterial cells, smooth muscle cell migration and proliferation, extracellular deposition of connective tissue matrix, VSMC necrotization, calcification of elastic fibers and progressive mural accumulation of calcium [61].

Although the precise mechanisms by which OX-LDL initiates and propogates the plaque formation are not defined, recently, on the basis of several lines of evidence, it has been hypothesized that calcium homeostasis may be disrupted during atherogenesis and that this defect could be involved in initiating the atherogenic process [61, 165].

2.2.1. ROLE OF CALCIUM IONS IN THE PATHOGENESIS OF ATHEROSCLEROSIS

Several observations imply the involvement of calcium in atherosclerosis. Firstly, Blumental and coworkers [164] reported that calcium plays an important role in the early stages of lesion development. To get an idea of the pathogenetic significance of calcium accumulation in altherosclerosis, Grun et al [165] examined atherosclerosis in spontaneously hypertensive rats. In the healthy arterial segments the contents of calcium

amounted to 1.24 ± 0.08g/kg dry tissue weight. In the very early lesions [stage 1] the calcium content was elevated by a factor of 7.8 compared with normals and [at stage 2 and stage 3] there were 12.8 fold and 29 fold increases in calcium content over controls, respectively. Thus, calcium accumulation appears to be an early event in lesion development long before cell death and final necrotic "calcification" become visible. Secondly, Strickberger et al [166] reported that in the arteries of cholesterol fed rabbits a five fold increase in intracellular calcium compared to controls. Thirdly, the calcium dependent processes associated with atherogenesis are amongst others, morphological changes, migration, proliferation and matrix production of VSMCs, $[Ca^{2+}]i$ overload with subsequent necrotization, calcification of cell debris, calcification of elastic fibers, secretion of biological products [e.g., collagen, elastin, chemoattractants, proteoglycans, and growth factors], and platelet aggregation which are all initiated and regulated by cytosolic calcium [167-171]. Finally, experimental and clinical studies indicate that calcium antagonists are, as a group, antiatherogenic [172-173]. Research in the 1980's demonstrated that there was at least a five fold increase in the plasma membrane calcium transport in the aortic wall of rabbits with experimental atherosclerosis [174-176]. Treatment with calcium channel blockers [CCBs] indicated suppression of such experimental atherosclerosis [171, 175]. The calcium antagonist nifidipine established antiatherogenic effects in rabbits fed a diet rich in cholesterol [171], while several other calcium antagonists [verapamil, diltiazem, nitrindipine, nisoldipine and felodipine] normalized blood pressure and prevented atherosclerosis in rats [171, 173]. These findings encouraged research on the evaluation of CCBs-associated antiatherosclerotic

effect in humans [174, 177]. Loaldi et al [178] reported reduction in new lesions as well as retardation in the progression of pre-existing stenosis in patients treated with nifidipine. Similar protective effects have been demonstrated for verapamil and diltiazem [177]. Litchen et al [179], Yoshida et al [46] and Blumenthal et al [164] have all demonstrated that calcium antagonists were effective for prevention of atherosclerosis. Isradipine, another CCB, also was demonstrated to possess antiatherogenic properties by Skepper et al [182]. Jukeman et al [174] based on the REGRESS [Regression Growth Evaluation Statin Study] trial reported that CCBs may have beneficial effects on the evolution of coronary atherosclerosis in patients treated with lipid lowering drugs. Recently, Roistaczer et al [172] reported that calcium channel antagonists possess lipid antioxidant properties, and that this property might contribute to their role in atherosclerosis. Similar findings have been previously reported [183-184].

Therefore, if calcium is an important mediator in atherosclerosis, and if LDL can be a vital contributor to atherosclerotic heart diseases, the interrelationship between LDLs and calcium homeostasis can probably explain the cellular events linking LDLs and atherosclerosis [61].

2.2.2. EVIDENCE FOR THE ACTION OF LDLs ON CELL CALCIUM

(a) Indirect Evidence

$[Ca^{2+}]i$ plays a significant role in regulating a wide range of activities in different types of cells [61, 177]. Hence $[Ca^{2+}]i$ has been considered as an important second messenger [37]. Elevations or alterations in the homeostasis of $[Ca^{2+}]i$ can be stimulated

either by ligand-receptor binding and subsequent activation of a second messenger system or through depolarization of excitable cells [61]. Changes in $[Ca^{2+}]i$ can mediate migration, proliferation and matrix production of VSMCs, muscle contraction, secretion of biological products, platelet aggregation and blood cell activation [61, 173].

(b) Direct Evidence

The use of fluorescent probes that can measure the changes in $[Ca^{2+}]i$ have provided a direct evidence for the mobilization of $[Ca^{2+}]i$. Fluorescent calcium ion indicators [such as Fura 2,1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2 [2'-amino-5' methylphenoxy]-ethane-N,N,N',N'-tetra-acetic acid and guin 2, have been used to provide such direct evidence for the involvement of $[Ca^{2+}]i$ [185]. It was first demonstrated by Block et al [186] and Knorr et al [187] using quin 2, that cellular activation induced by LDL was mediated by an increase in $[Ca^{2+}]i$.

DIRECT AND INDIRECT EVIDENCES FOR THE ROLE OF $[Ca^{2+}]i$ IN THE ATHEROGENIC POTENTIAL OF OXIDIZED LOW DENSITY LIPOPROTEINS

Sachinidis et al [31] reported that LDL at concentrations [1 to 30 μg/ml], much lower than those physiologically occurring in blood [3-4 x 10^3μg/ml], caused a concentration dependent increase in the contractile response in aortic rings, an increase in $[Ca^{2+}]i$ and a biphasic change in intracellular pH [pHi] in rat VSMCs. LDL was isolated from normolipidemic subjects and cumulative addition of 1-7μg/ml resulted in a fast concentration-related contractile response of the aortic ring, both in the presence and absence of an intact endothelium. No additional increase in contractile response was

observed with LDL concentrations of 15 and 30 μg/ml. When contractile responses were investigated with 15μg/ml LDL, either in the absence of extracellular calcium or in the presence of the calcium channel blocker verapamil, LDL induced contractile responses were weaker than those generated in the presence of extracellular calcium. These findings were consistent with the results from $[Ca^{2+}]i$ measurements obtained on exposure of LDL to rat VSMCs.

It was suggested that the $[Ca^{2+}]i$ mediated responses may be, at least in part, due to the influx of calcium from the external medium into the VSMCs. The researchers also proposed the possibility of the involvement of the phosphatidyl inositol [PI] system in the LDL stimulated $[Ca^{2+}]i$ responses.

In 1990, Sachindis et al reported that LDL at concentrations [1-15 μg/ml] demonstrated increased $[Ca^{2+}]i$ responses and also induced increased DNA synthesis. These cellular mechanisms may contribute to LDL involvement in atherosclerosis [32].

LDL [density 1.019-1.063g/ml] was isolated from eight normolipidemic individuals and experiments were conducted with each of the samples. LDL [7μg/ml] stimulated a rapid rise in $[Ca^{2+}]i$ from a basal value of 118nM to a peak of 240nM at 15 seconds and returned to resting levels within 1.5 minutes. The LDL stimulated elevations in $[Ca^{2+}]i$ were less pronounced in the absence of extracellular calcium, as previously reported [31]. Addition of 1-3μg/ml of LDL to VSMCs demonstrated a dose dependent mitogenic effect on VSMCs, i.e. enhanced DNA synthesis. Further augmentations of LDL concentrations did not demonstrate any enhanced proliferative effect of LDL on VSMCs. Similar dose dependent aortic ring contractions were initiated with increasing LDL doses [1-7 μg/ml].

These responses were reduced by the calcium channel blocker verapamil or by the presence of EGTA in the extracellular medium.

Based on the above observations, it was proposed that the LDL mediated elevations in $[Ca^{2+}]i$ can be partly due to mobilization of $[Ca^{2+}]i$ and partly due to the influx of extracellular calcium. Since $[Ca^{2+}]i$ mobilization in VSMCs is thought to involve the PI system, the authors [32] suggested that LDL evoked $[Ca^{2+}]i$ responses to occur via the PI system. The calcium influx from the extracellular environment was proposed as stimulated either by voltage operated or receptor operated channels activated by the LDL receptor combinations. However, it was not clear whether these responses occurred via a pathway dependent or independent of the classical LDL receptor [32].

In other experiments, Scahinidis and his coworkers [33] demonstrated that the LDL induced elevations in $[Ca^{2+}]i$, biphasic change in pHi (a rapid acidification followed by a prolonged alkalinization) and increased contractile responses were independent of the classical LDL-receptor. To investigate whether the LDL stimulated rise of $[Ca^{2+}]i$ was via the classical LDL-receptor pathway, the researchers preincubated VSMCs with a range of concentrations of $IgGC_7$ [monoclonal antibodies to specific cell surface LDL receptors] before the VSMCs were exposed to LDL. $IgGC_7$ at any concentration neither abolished nor attenuated the LDL induced $[Ca^{2+}]i$ responses. In both receptor negative human cultured fibroblasts from normocholesterolemic individuals and from patients with familial hypercholesterolemia, LDL [7μg/ml] induced elevations in $[Ca^{2+}]i$ responses and a biphasic change in pHi. However, these responses were attenuated in the absence of extracellular calcium. Since $[Ca^{2+}]i$ mobilization in VSMCs is thought to occur via the PI

system, the authors examined the effect of LDL on the phosphatidyl inositol-turnover in VSMCs and fibroblasts. LDL had no effect on the phosphatidyl inositol-turnover, contrary to other researchers.

Since recent evidence indicates that the oxidative modification of LDL increases its atherogenic potential, Weisser et al [35] compared the effects of N-LDL and LDL oxidized in vitro on cultured rat VSMCs. The researchers reported more pronounced contractile responses of rat aortic rings and markedly greater $[Ca^{2+}]i$ responses elicited in rat VSMCs by OX-LDL compared with N-LDL. Their results thus provide evidence for an increased biological activity of OX-LDL compared to N-LDL on a cellular level.

Weisser et al [36] proposed that at least some of the processes and cellular events leading to atherosclerosis are among others regulated by alteration in $[Ca^{2+}]i$. The researchers compared the effects of in vitro OX-LDL and N-LDL on rat VSMCs. The extent of LDL oxidation was assessed by thiobarbituric acid reactive substances [TBARS]. Their findings indicated that the levels of TBARS were significantly higher in OX-LDL compared to N-LDL, and OX-LDL elicited markedly more pronounced $[Ca^{2+}]i$ responses in VSMCs.

Based on the observation that in vitro OX-LDL elicited more pronounced $[Ca^{2+}]i$ elevations and also stimulated greater contractile actions in rat aortic rings compared to N-LDL, Weisser et al [39] investigated the possible mechanisms that could contribute to the differences in $[Ca^{2+}]i$ responses between N-LDL and OX-LDL.

Lysolecithin is a component of OX-LDL formed during the oxidative modification of LDL. Lysolecithin is found in much higher concentrations in OX-LDL, and it produced

a dose dependent increase in $[Ca^{2+}]i$ in VSMCs. Therefore, it was suggested that lysolecithin might be responsible for the OX-LDL induced influences on alterations in calcium metabolism.

Locher et al [88] reported that lysolecithin concentrations were 10-fold elevated in in vitro OX-LDL, compared to N-LDL. They reported that, in addition to causing elevations in $[Ca^{2+}]i$ lysolecithin, also induced increased $[^3H]$-thymidine incorporation. However, elevations in $[Ca^{2+}]i$ stimulated by lysolecithin remained unaffected when VSMCs were preincubated with nifedipine or the intracellular calcium antagonist TMB-8. But, there was no $[Ca^{2+}]i$ response in the presence of EGTA in the extracellular medium.

Although it is popularly accepted that LDLs are the major risk factor for atherosclerosis, many individuals who develop atherosclerosis have normal LDL levels [189]. Fisher et al [190] and Krauss [191] reported that human LDLs are comprised of discrete subfractions which vary in size, density and chemical composition and that subjects with similar LDL levels may demonstrate variations in the distribution of the subfractions. Several researchers have described the predominance of the small, dense LDL subfractions as a risk for atherogenesis [146-148]. DeGraaf [192] and others [146-147] have demonstrated that the small, dense subfraction also exhibit an enhanced susceptibility to in vivo oxidation.

Weisser et al [189] studied the effects of the three LDL subfractions [pattern A, B and Intermediate Patter Phenotypes] on $[Ca^{2+}]i$ responses in rat VSMCs. The small, dense subfraction elicited markedly higher $[Ca^{2+}]i$ response and this subfraction also was more prone to in vitro oxidation [189]. To further investigate the mechanisms involved in the

stimulation of $[Ca^{2+}]i$ by the LDL subfractions, VSMCs were incubated with calcium antagonists nifedipine, diltiazem and verapamil in separate experiments. These antagonists did not exert any affect of the elevations in $[Ca^{2+}]i$. Neither did TMB-8, the inhibitor or $[Ca^{2+}]i$ mobilization exert any influence on rises in $[Ca^{2+}]i$. The elevations in $[Ca^{2+}]i$ seemed to be caused by an influx of calcium ions from the extracellular space. These conclusions were confirmed by the results obtained in the absence of external calcium. The researchers also demonstrated the lack of involvement of the LDL receptor on the $[Ca^{2+}]i$ responses elicited by the LDL subfractions as similar responses were obtained in the receptor negative fibroblasts isolated from patients with familial hypercholesterolemia [189].

Hughes et al [193] investigated the effect of LDL on $[Ca^{2+}]i$ homeostasis in human VSMCs. LDL stimulated a concentration dependent rise in $[Ca^{2+}]i$ and this elevation was reduced in a calcium free EGTA medium. Dihydropyridine, a calcium channel antagonist, did not affect the $[Ca^{2+}]i$ responses. Further, depolarization with K^+ either in the presence or absence of calcium channel antagonist [BAY k 8644] did not demonstrate detectable effect on $[Ca^{2+}]i$ responses. This suggests the negligible contributions of the voltage operated calcium channels to calcium influx. Their study failed to demonstrate any action of pertusis toxin on LDL-induced $[Ca^{2+}]i$ elevations in saphenous vein cells [193], although LDL has been reported to induce phosphatidyl inositol-turnover by an action involving a pertussis toxin sensitive G protein [189].

2.2.3. POSSIBLE PATHWAY FOR LDL-MEDIATED $[Ca^{2+}]i$ RESPONSES

The precise pathway of calcium transport that LDL stimulates is a matter of critical interest. Presently, there is no convincing evidence that LDL acts directly via the classical LDL receptor. Sachinidis et al [33] demonstrated that the LDL induced $[Ca^{2+}]i$ rise was independent of the receptor, while Morita et al [194] showed that apo B-100 alone can induce a rise in $[Ca^{2+}]i$. The involvement of the receptor in other cell types has also been controversial [195-196]. It has been proposed that the rise in $[Ca^{2+}]i$ in VSMCs can be mediated through entry of calcium from the extracellular space via L-type calcium channels, Na^+/Ca^+ exchanger and the ligand operated calcium channels [61]. However, there have not been consistent evidence for their involvement [189].

Binding of LDL to its receptor on the plasma membrane in VSMCs has been postulated to lead to the activation of the pathway through stimulation of phospholipase C, which leads to the production of IP_3 which can induce the initial phase of calcium release from the sarcoplasmic reticulum [32, 194]. In smooth muscle cells, this initial rise in $[Ca^{2+}]i$ has been shown to lead to a second phase of calcium influx across the sarcolemma [31, 33, 189].

When smooth muscle cells were incubated with high levels of LDL for 1-3 days, the cells responded with a significantly faster calcium exchange kinetics in both intracellular and extracellular compartments. This may lead to the accumulation of calcium within smooth muscle cells, as seen in the atherosclerotic vessels [169, 197]. This work suggests that the cellular membrane system may be modified by LDL [Sarcolemma].

However, it does not explain the precise pathway or mechanism within the membrane system [169].

There have been several reports on the $[Ca^{2+}]i$ release in VSMCs to have occurred in a phasic fashion: the initial release of $[Ca^{2+}]i$ due to its mobilization from intracellular stores and the second phase of calcium mainly due to calcium entry from extracellular spaces [31, 33, 189]. These findings are in accordance with data suggesting the involvement of the PI pathway [32, 194] as well as findings that indicate that calcium channel blockers attenuated the action of LDL on VSMCs [189].

2.3. LIPIDS AND ATHEROSCLEROSIS IN DIABETES MELLITUS

Diabetes mellitus is associated with a host of complications, including those related to the involvement of the arterial wall of both large vessels [macroangiopathy] and small vessels [microangiopathy] and of the peripheral nervous system [neuropathy [198-200]. Hyperglycemia is a distinguishing feature of diabetes and it appears to be a likely candidate for the poor cardiovascular outlook in persons with diabetes [201]. Atherosclerosis is the most frequent complication of diabetes [200, 202]. Epidemiological studies have demonstrated that premature onset and rapid progression of atherosclerosis are characteristic features [200, 203]. Elevated levels of cholesterol has been associated with increasing vascular risk in diabetes [204-205]. Cardiovascular events are known to be the leading causes of morbidity and mortality in both Type I and Type II persons with diabetes [20, 206].

In insulin dependent diabetes mellitus [IDDM], total cholesterol and low density lipoprotein cholesterol levels are elevated and these correlate significantly with reduced arterial endothelial function, i.e. flow mediated vasodilation is impaired as assessed by measures of brachial artery diameter [207]. High blood glucose levels can contribute to impairment of endothelium-dependent relaxation, also a feature in atherosclerosis [201, 207]. Endothelial dysfunction has been considered to be an important early event in atherosclerosis [207].

Dyslipideamia is more prevalent in non-insulin dependent diabetes mellitus [NIDDM] than in IDDM, and the major abnormality is hypertriglyceridemia, often associated with low and high density lipoprotein cholesterol [207-211]. In diabetic populations the relationship between dyslipidaemia and vascular risk appears to be strong [209-210]. Hypertriglyceridaemia in diabetes has been shown to contribute to atherosclerosis, in part by accumulation of remnant particles [208, 209, 211]. The role of remnant particles in altherosclerosis has been reported by in vitro studies demonstrating foam cell formation when OX-LDL was incubated with macrophages [209, 212]. Prevalence of remnant particles has been correlated with progressive atheroma [212].

2.3.1. MODIFIED ATHEROGENIC LDL IN DIABETES

Several studies have revealed that LDL from persons with diabetes unlike LDL from healthy subjects, when incubated with cells cultured from unaffected, healthy human aortic intima, caused a significant increase in their cholesterol content, i.e. produced a direct atherogenic effect [198]. Therefore, like atherogenic sera, sera from persons with

diabetes possesses atherogenic properties, with respect to intracellular cholesterol accumulation [198, 200, 213]. On average, sera from persons with diabetes increased intracellular cholesterol content by 75% [200]. Further, lipoprotein deficient sera [deprived of all lipid particles - LDL, HDL, VLDL] lost its atherogenic potential entirely [213]. Amongst the lipoproteins, the LDL fraction [d 1.019-1.063g/ml] was reported as the most predominant lipoprotein possessing atherogenic properties. It was notable, that nonatherogenic diabetic sera did not induce cellular cholesterol accumulation [207, 213]. Hence, it is apparent that LDL is the primary and major contributor to the atherogenic potential of diabetic sera [213]. Since N-LDL did not influence intracellular cholesterol accumulation, it is possible to conclude that in persons with diabetes, LDL may be modified to participate in the atherogenic process. Further, Sobenin [198] reported some in vivo modified atherogenic LDL subfractions. LDL of persons with diabetes has been reported to be represented by small, dense, glycosylated, desialylated and more electrongative subfractions [198, 217]. These properties are similar to the characteristics described for OX-LDL in vivo in plasma and lesions of atherosclerotic patients [217]. Characteristic features of diabetic LDL that can confer atherogenic properties can be characterized as follows:

(a) Non-enzymatic Glycosylation [NEG] of LDL

NEG is a process that can affect many proteins upon exposure to elevated glucose concentrations [107, 117]. NEG of proteins results in the formation of a stable bond between a glucose molecule and a protein amino group [198, 215]. Hyperglycemia in diabetes can induce an increase in glycosylated products. Protein glycosylation is elevated

in persons with diabetes and thus is used as an index for long-term glucose control [216]. LDL from persons with diabetes was characterized by an increase in NEG, as assessed by fructosyl lysine content by 25% compared to healthy controls [198, 213]. Hence NEG has been considered as an in vivo atherogenic modification of LDL in persons with diabetes [198].

Experiments have demonstrated that there is an increase in accumulation of cholesteryl esters in macrophages exposed to lipoproteins isolated from persons with diabetes [217-218]. The uptake of glycosylated LDL by human monocyte-derived macrophages has been shown to be directly proportional to the degree of glycosylation [219]. Glycosylated LDL also has been demonstrated in platelet aggregation [220]. Marishita et al [221] reported hypercoagulability as a factor in diabetes which might contribute to increased cardiovascular morbidity.

Metabolic alterations associated with the glycosylation of LDL include altered metabolism of glycated apo B leading to diminished recognition of LDL by the classical LDL receptor [222], increased residence time in circulation [223] thereby leading to retardation in the plasma clearance of LDL, increased uptake of LDL by macrophages and generation of foam cells [213], enhanced thrombin-induced platelet aggregation [212], and an increase in the rate of free radical production, possibly causing damage to both lipid and protein moities of LDL [198, 225].

As a consequence of glycosylation, oxidation of LDL has been shown to be promoted in vitro [12, 214] and in vivo [198] by the generation of free radicals. The contribution of free radicals and peroxides to protein alterations during glycosylation has

been termed as autoxidative glycosylation. Autoxidative glycosylation is a mechanism which is capable of producing free radicals, leading to fragmentation of proteins and oxidation of associated lipids during glycosylation reactions [12, 218]. In the case of LDL when the lipoprotein is exposed to glucose, the lipid moiety has been reported to peroxidize [214]. Glycosylated LDL has been demonstrated in persons with diabetes with micro or macrovascular disease [198]. Autoxidative glycosylation of LDL has been implicated as a possible contributory factor to diabetic tissue damage [12].

(b) Oxidized LDL in Diabetes

In recent years, the central tenet for the study of atherosclerosis has been influenced by the hypothesis that lipoproteins can become modified in vivo, possibly by free radical mediated oxidation and this might initiate atherosclerotic lesion development or worsen its course [215, 226]. Amongst others, diabetes has been viewed as a strong risk factor for atherosclerosis. Hence it would be logical to consider potential ways by which diabetes may complicate or accelerate lipoprotein oxidation [215]. Lipoprotein fractions with elevated levels TBARS, an index of lipid peroxidation, have been demonstrated in diabetic animals and humans [228, 229]. There is evidence that diabetes is accompanied by enhanced lipid peroxidation or lipoprotein oxidation and therefore, hyperglycemia [a characteristic feature of diabetes] and accelerated oxidation may be interlinked [214].

If hyperglycemia is the major cause for the complications in diabetes, then it would be reasonable to postulate increased plasma and tissue glucose as sources of increased oxidation apparent in diabetes [11-12]. The demonstration that glucose can oxidize when

catalyzed by trace amounts of transition metals, by generating free radicals, hydrogen peroxide and reactive ketoaldehydes supports the concept that glucose can contribute to oxidative stress [12, 215]. Transition metals may also participate in the formation of glycosylated proteins, which are by themselves a source of oxygen free radicals [11, 216]. Hence it has been postulated that the etiology of the complications of diabetes involves oxidative stress, possibly as a result of hyperglycemia [12, 214-215]. Oxidative stress has been well documented as a phenomenon in atherosclerosis [14, 40]. Hence, it is apparent that oxidative stress can predispose persons with diabetes to atherosclerosis. This may explain in part, premature and accelerated atherosclerosis seen in diabetes.

The predominant pathways by which hyperglycemia may contribute to oxidative events are by enhancing free radical production and/or by forming glycated products that can propagate free radical events [11]. Oxidative stress can be amplified by a viscious cycle of metabolic stress, tissue damage and cell death, leading to increased free radical production and compromised free radical inhibitory and scavenger systems, which further exacerbate oxidative stress [11, 216].

The potential role for oxidized lipids in the pathogenesis of diabetes is supported by findings that demonstrate that LDL oxidation in vitro is accelerated in the presence of glucose [214]. Hunt et al [12] demonstrated that when incubated with high glucose and cupric ions, human LDL yielded higher levels of TBARS than LDL exposed to copper without glucose. Similar findings were reported by Sakuri et al [230] with glycosylated LDL and iron. Increased iron storage and increased urinary excretion of iron have been reported in poorly controlled diabetes [231]. Kawamura et al [232] reported increased

lipid peroxides conjugated dienes and TBA reactivity in LDL incubated with glucose compared with controls. These effects were inhibited by superoxide dismutase. Oxidized lipids have been demonstrated in plasma and tissue of rats made diabetic with streptozotocin [229]. Evidence for the role of OX-LDL in diabetes is further supported by the in vivo occurrence of elevated lipid peroxide levels of LDL in plasma of persons with diabetes [17-18, 223]. Gallon [234] reported elevated TBARS [plasma malondialdehyde] in both Type I and Type II persons with diabetes. Bowie et al [235] reported that glycated LDL was more susceptible to oxidation and that the esterified/free cholesterol ratios correlated positively to oxidation. Griesmacher et al [18] demonstrated that free radicals were involved in enhanced lipid peroxidation in diabetes. Zadeh et al [17] also documented elevated levels of lipid peroxides in diabetes. Perriello et al [223] suggested that suppression of lipid oxidation could reduce hyperglycemia. Sobenin et al [198] reported properties of LDL in persons with diabetes to possess characteristics similar to OX-LDL in vivo. OX-LDL levels have been reported to be greater in persons with diabetes with established atherosclerosis than in persons with diabetes without atherosclerosis. In a control, population with persons without diabetes, there were hardly any detectable amounts of lipid peroxides [236]. Lipid oxidation products were reported in plasma as well as in the tissues of persons with diabetics and were particularly elevated in persons with poorly controlled diabetes with atherosclerosis [228, 236]. Oxidized lipid levels also correlated positively in persons with diabetes with hypertriglycerideamia than those without hypertriglycerideamia and healthy controls [237]. The cytotoxicity

associated with oxidized LDL has been suggested to be involved in the tissue injury associated with diabetes [11, 194].

The atherosclerotic potential of LDL in diabetes is often discussed in terms of protein glycosylation and peroxidation [12, 198]. Both glycosylation and peroxidation have been shown to occur concomitantly in LDL modified by glucose in vitro and under hyperglycemic conditions in vivo [11, 198, 215]. Oxidative damage to proteins in diabetes has been attributed to include free radicals generated by autoxidation reactions of sugars and sugar adducts to proteins and by autoxidation of unsaturated lipids in plasma and membrane proteins [12].

(c) Desialylated LDL [Sialic Acid - Poor LDL]

Sobenin et al [238] reported the existence of a sialic acid-rich and sialic acid-poor subfractions in LDLs isolated from persons with diabetes. The proportion of desialylated LDL was significantly higher in populations with diabetes versus the control populations [198, 238]. Desialylated LDL has been identified as a diabetic LDL modification that exists in vivo [198]. In vitro sialic acid-poor LDL stimulated lipid accumulation in monocyte/macrophages and SMCs [239-240].

Sialic acid-rich LDL was nearly identical to N-LDL from healthy persons. Reduced sialic acid levels were reported in LDL from atherosclerotics as well as in persons with diabetes [213, 238]. Sialic acid-poor LDL demonstrated reduction in the content of neutral lipids [especially esterified cholesterol] increased intracellular cholesterol content [198, 238] and elevated levels of lysolecithin [238]. Further, this

fraction was glycosylated and hence it was postulated that desialylation and glycosylation could produce a synergetic effect on LDL atherogenecity in diabetes [198].

Additional characteristics of the modified LDL fraction in diabetes include [i] a predominance of the small, dense LDL subfraction; [ii] increased electronegative charge; and [iii] increased eletrophoeretic mobility [198, 241-244]. All of these characteristics of LDL in persons with diabetes are strongly associated with atherosclerotics as well.

Hypertriglyceridaemia is commonly seen in NIDDM, and has implications for vascular risk [211, 244]. Hypertriglyceridaemia has been known to be associated with an alteration in the distribution of particles within the LDL density range, with a predominance of small, dense particles [198, 204]. A possible explanation for this alteration in LDL density distribution may be the lipid exchange promoted by hypertriglyceridaemia, which involves transfer of triglycerides to LDL via cholesterol ester transfer protein [204]. Other lipid profiles associated with hypertriglyceridaemia include low levels of HDL-C. This could again predispose persons with diabetes to atherosclerosis [211, 245]. Ginsberg [211] reported that the increased free fatty acid transport in plasma often seen in persons with diabetes was due to elevated levels of plasma triglycerides and reduced levels of HDL-C. Similar findings were reported by Brien et al [246]. Berthezene [247] reported alterations in lipid profiles could lead to accelerated altherosclerosis in NIDDM patients. Virella et al [217] demonstrated that the different types of modified LDL detected in persons with diabetes, including glycated LDL, advanced glycation end product-modified LDL, OX-LDL and glycoxidized LDL were immunogenic, inducing the formation of autoantibodies and immune complexes. The

immune complexes formed with LDL and anti-LDL have been isolated from persons with diabetes and persons with atherosclerosis. Also the exposure of macrophages to the LDL immune complexes resulted in an overexpression of LDL receptors in the macrophages, which further promoted persistent influx of LDL into the cells and excessive cholesterol accumulation [217].

2.4. ANTIOXIDANTS IN ATHEROSCLEROSIS

For several years it has been suggested and evidence continues to accumulate that oxidation is involved in the pathogenesis of many diseases, including cancer, arthritis, cardiovascular and cerebrovascular diseases [2, 4, 40]. Particularly, studies on the pathogenesis of atherosclerosis have gained importance with atherosclerosis contributing to mortality in a large proportion of the Western population [1, 40, 53, 57]. Further, research on lipid metabolism has contributed to the formulation of the hypothesis that the oxidative modification of LDL is the key step in the genesis of the atherosclerotic lesions [2, 58]. The "oxidation hypothesis of atherosclerosis" has been supported by a number of in vivo findings demonstrating the involvement of OX-LDL in the phenomenon of "atherosclerosis" [16, 131, 162]. As a corollary of the oxidation hypothesis of atherosclerosis, it has been widely proposed that antioxidants can inhibit LDL oxidation and may act as antiantherogens [40, 47]. Fortunately, the body has a complex antioxidant defense system against free radicals, which have been primarily known to contribute to oxidative stress [40, 61]. If not for the quick response of these protective antioxidant

defense system, multiple chain reactions of free radicals, generated rapidly within seconds, could cripple and destroy cellular functions [61, 66].

It is believed that native tissue antioxidant systems that protect against naturally occurring free radicals consist of [i] enzymes [e.g. superoxide dismutase, catalase, glutathione peroxidase and glutathione-s-transferase] [66, 85, 86, 248], most of these naturally occurring enzymes deactivate free radicals by using them to generate safer chemical reactions [66], and [ii] nonenzymatic antioxidant free radical scavengers [e.g. B-carotene, vitamin E, vitamin C, and glutathione] [249-250]. These enzymes and antioxidant systems together represent a synergistic, multilevel defense system against free radical injury [66]. In both tissues and plasma, the balance between free radical production and multilevel defense system, "Pro" and "Anti" oxidant defense mechanisms, are very critical, as the protective systems could be overwhelmed, particularly if the levels of any components of the defense mechanisms were deficient or inadequate [66, 251-252]. Amongst these protective mechanisms, the antioxidant vitamin E has been widely studied with regard to protection of the OX-LDL induced atherosclerosis. Experimental and epidemiological data have reiterated the important role of vitamin E in the protection of low density lipoprotein induced oxidative damage [249, 251, 254-255].

2.4.1. ANTIATHEROGENIC POTENTIAL OF VITAMIN E

The tocopherols [vitamin E] represent a group of selected lipid-soluble alcohols present in oils of food like cereals, nuts and legumes [256]. Alpha tocoherol [α-tocopherol] is the most active and abundant isomer of the vitamin E family [241, 257]. It

is the principle lipid-soluble, chain breaking antioxidant in the tissues and plasma [24, 257-258]. In humans, 90% of the total serum vitamin E is contained in lipoproteins [250]. It is transported in plasma by lipoproteins, particularly LDL and HDL secreted by the liver [249-250]. Vitamin E protects the lipids in the lipoproteins and it is the predominant antioxidant in the LDL particle [24, 260-261].

One of the first reactions in the oxidative modification of LDL is the peroxidation of polyunsaturated fatty acids [PUFAs] [262-263]. The PUFAs contained in the LDL are degraded to a great variety of aldehydes [e.g. malondialdehyde, 4-hydroxynonenal, propanal, hexanal, 2,4-alkadienals] and other products [24, 261, 265]. Reactive oxygen species together with the PUFA degradation products that are present in both serum and arterial wall lipid medium have been proposed to drive the inflammatory processes propagating events in the pathogenesis of atherosclerosis [265-267].

It has been reported that on an average, one LDL particle contains 6 α-tocopherol molecules and 1000 molecules of PUFA [268]. It has been suggested that 1 molecule of vitamin E can protect roughly 3000 PUFA molecules against free radical mediated injury [250]. Esterbauer et al [1991] reported that vitamin E content of LDL demonstrated a tendency to increase with its PUFA content [262].

"Interception" has been described as the domain for the activity of chain-breaking antioxidants like the tocopherols which react with peroxyl radicals, and the term "antioxidant" is used in a restricted sense to denote just this activity. α-tocopherol, as the predominant antioxidant present in the LDL particle, blocks the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals [24, 257]. This activity of chain

breaking antioxidants is important because the free radical-mediated oxidation is amplified by a chain mechanism, i.e. a single hit of the initiating radical can induce the oxidation of many molecules and thus amplify the oxidative damage [269]. The evidence that α-tocopherol can block LDL oxidation has been demonstrated in vitro by the observation that oxidation promoted by transition pro-oxidant metals was inhibited by α-tocopherol. Vitamin E appears to be especially promising as it is carried directly in the LDL particles [66, 263]. Supplementation with vitamin E has been reported to increase vitamin E levels in LDL by as much as 2.5 fold and reduce lipid peroxidation in LDL by 40% [66]. Reaven et al [270] demonstrated that long-term supplementation with large doses of vitamin E alone, but not B-carotene or vitamin C, increased the resistance of LDL to oxidative damage.

2.4.2. MECHANISM OF ACTION OF α-TOCOPHEROL IN THE INHIBITION OF LIPID PEROXIDATION IN LDL

Much attention has been devoted to the inhibition of lipid peroxidation in LDL by both endogenous and exogenous antioxidants [66, 250, 251, 271]. Because vitamin E is the major antioxidant carried by LDL, it has received much attention as a suppresser of LDL lipid peroxidation and as an epidemiological marker for ischaemic heart disease [271]. α-tocopherol [α-TOC] is biologically and chemically the most active form of vitamin E [250]. Minor lipid soluble antioxidants associated with LDL include γ-tocopherol, carotenoids, retinol and ubiquinol-10 [$CoQ_{10}H_2$] [271], and the water soluble

LDL-associated antioxidant is vitamin C. However, α-tocopherol is the predominant and most active of them all.

The initiaton of LDL lipid peroxidation is often attributed to the hydroxyl radical [$HO^{\bullet}$]. In biological systems the $HO^{\bullet}$ is very reactive and potentially severely damaging as it can extract an electron from almost any organic molecule in its vicinity [251]. This can further initiate radical or non-radical processes that may lead to biochemical changes that can promote diseases [251, 273]. These $^{\bullet}OH$ attack enzymes and other proteins and, in particular, PUFA moeities of lipoproteins and of membrane phospholipids [251]. $^{\bullet}OH$ radicals can be formed in abundance from $O_2^{\bullet-}$ radicals and H_2O_2 that are available in aerobic living organisms. Divalent cations such as iron or copper can catalyze reactions involving $O_2^{\bullet-}$ or H_2O_2 leading to the formation of HO.

$$O_2^{\bullet-} + Fe^{3} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^{-} + HO + Fe^{3+}$$

Attack by $HO^{\bullet}$ on lipid PUFA [LH] can lead to the formation of the initial carbon centered radical [$L^{\bullet}$]. The L can in turn react with molecular oxygen to produce a lipid peroxyl radical [$LOO^{\bullet}$]. The $LOO^{\bullet}$ can attack an adjacent PUFA side chain and propagate this reaction with the generation of lipid peroxides [LOOH] and regenerate the $L^{\bullet}$. These reactions can occur as a viscious cycle in absence of α-TOC, as follows:

$$LH + HO^{\bullet} \rightarrow L + H_2O$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \rightarrow \underline{\text{Chain Reaction}}$$

In the presence of the chain breaking, peroxyl radical scavenging lipid antioxidant, α-TOC, the chain reaction can be terminated.

$$LOO^{\bullet} + \alpha\text{-TOC-H} \rightarrow LOOH + \alpha\text{-TOC}^{\bullet} \quad [\alpha\text{-tocopheroxyl radical}]$$

The intervention of α-TOC results in the formation of the α-TOC$^{\bullet}$. α-TOC is believed to be regenerated from the α-TOC$^{\bullet}$ by a system that involves ascorbic acid and glutathione [253, 271-272].

The fate of the lipid hydroperoxide is vital, as its formation in the membrane with its -OOH group in the hydrophobic region will lead to the disruption of the membrane as the polar function seeks to move out into the nonpolar environment [251]. This may lead further to the activation of the endogenous phospholipase A_2, so that the peroxidized fatty acid is cleaved from the membrane phospholipid. The peroxidized free fatty acid then can be reduced to its hyroxy derivative by a reaction catalyzed by glutathione peroxidase.

$$2\ LOOH + 2\ GSH \xrightarrow[\text{Peroxidase}]{GSH} 2\ LOH + H_2O + GSSG$$

The role of selenium dependent glutathione peroxidase is important here as lipid hydroperoxides may otherwise undergo iron-catalyzed degradation into radical species.

$$LOOH + Fe^{3+} \rightarrow LOO^{\bullet} + Fe^{2+} + H^{+}$$

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + Fe^{3+} + OH^{-}$$

2.4.3. HOW AND WHEN THE ANTIOXIDANT α-TOC CAN BECOME A PRO-OXIDANT

Bowry et al [271] reported that α-TOC can either behave as an antioxidant or prooxidant in LDL. It was reported that in the absence of an effective co-antioxidant, α-TOC acted as a prooxidant and that this activity was due to the action of the α-tocopheroxyl radical [α-TOC$^{\bullet}$] with the LDL's PUFA. Bowry et al [272] demonstrated that after, but not before, the consumption of the LDL-associated antioxidants vitamin C and ubiquinol-10 [$CoQ_{10}H_2$] [coantioxidants], the peroxidation of LDL's lipid proceeded by a free-radical process even in the presence of greater than 90% of the original α-TOC.

The researchers found that the type of activity exhibited by α-TOC in LDL depended on the time taken for α-TOC to be consumed. It was noted that in the presence of a rapid flux of peroxyl radicals relatively little lipid hydroperoxides [LOOH] were formed in LDL in the brief period of α-TOC consumption, whereas in the presence of a comparatively mild flux of initiating peroxyl radicals there was a rapid formation of LOOH, that was faster during rather than following the period of α-TOC consumption [271, 275]. Similarly, the rapid oxidation of LDL with high concentrations of copper ions produced little LOOH in a brief period of α-TOC consumption, while low concentration of copper or iron in a culture medium causes a faster rate of α-TOC consumption, in the presence of the transition metals and α-TOC rather than after the consumption of α-TOC [272]. In the latter case, more than 50% of the PUFAs in the LDL lipids were oxidized

before α-TOC was depleted, indicating that substantial LOOH formation occurred in the presence of α-TOC containing LDL.

In the absence of α-TOC, the PUFA of LDL can react with a peroxyl radical, yielding lipid hydroperoxides.

$$\text{L-H} + \text{LOO}^{\bullet} \rightarrow \text{L}^{\bullet} + \text{LOOH}$$

$$\text{L}^{\bullet} + \text{O}_2 \rightarrow \text{LOO}^{\bullet}$$

The above chain reactions can be terminated by α-TOC with the generation of α-TOC by scavenging the peroxyl radical.

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

According to the prooxidant theory attributed to α-TOC, α-TOC can act like a prooxidant when the $\alpha\text{-TOC}^{\bullet}$ is formed during the chain breaking action of α-TOC. The usual fate of the $\alpha\text{-TOC}^{\bullet}$ is to trap a second peroxyl radical and thus destroy two peroxyl radicals:

$$\alpha\text{-TOC}^{\bullet} + \text{LOO}^{\bullet} \rightarrow \text{non-radical products.}$$

It is hypothesized that during the time interval between the formation and disposal of the $\alpha\text{-TOC}^{\bullet}$, even a radical as unreactive as $\alpha\text{-TOC}^{\bullet}$ would find "something to do" and that is, the $\alpha\text{-TOC}^{\bullet}$ can attack a PUFA containing lipid [L-H] in the LDL and regenerate $\text{L}^{\bullet}$, which can further propagate chain reactions.

$$\alpha\text{-TOC}^{\bullet} + \text{L-H} \rightarrow \alpha\text{-TOC} + \text{L}^{\bullet} \text{ [Prooxidant]}$$

$$\text{L}^{\bullet} + \text{O}_2 \rightarrow \text{LOO}^{\bullet} \text{ [chain reaction]}$$

Thus for prevention of LDL oxidation <u>in vivo</u>, it is essential to reduce α-TOC by reagents that would yield radicals which are incapable of continuing the peroxidation chain [104,

271]. Bowry et al [271-272] have proposed that this could be achieved under normal circumstances by two endogenous antioxidants, vitamin C [in plasma] and $CoQ_{10}H_2$ [in LDL] which can prevent the peroxidation of LDL containing α-TOC$^{\bullet}$, for e.g. with ascorbic acid, the reaction would proceed as follows [256].

$$\alpha\text{-TOC}^{\bullet} + \text{ASCH}^{-} \rightarrow \alpha\text{-TOC} + \text{ASC}^{\bullet}$$

ASCH^{-} - endogenous vitamin C, ascorbate

ASC$^{\bullet}$ - ascorbic acid radical

Halliwell [104] reported that the α-TOC$^{\bullet}$ can abstract hydrogen from PUFA, but this potential was, however, much less than that of the LOO radicals. It was suggested that the α-TOC$^{\bullet}$ can be recycled to α-TOC by any ascorbic acid present in the LDL suspension medium <u>in vitro</u>.

$$\text{a-TOC}^{\bullet} + \text{ascorbate} \rightarrow \alpha\text{-TOC} + \text{semidehydro ascorbate}$$

This further substantiates the protective role of coantioxidants in the a-TOC mediated prooxidation cycle. Similar protective action has also been demonstrated by $CoQ_{10}H_2$.

$$CoQ_{10}H_2 + \text{a-TOC}^{\bullet} \rightarrow CoQ_{10}H + \text{a-TOC}$$

2.4.4. POTENTIAL ANTIOXIDANT ROLE OF VITAMIN-E

Several animal studies have been conducted to test the hypothesis that antioxidants delay atherosclerosis. It was in the 1940's that the "antioxidant" theory of vitamin E function came to be investigated [273]. Beginning in 1948, several anecdotal reports on the beneficial effects of vitamin E on human atherosclerosis appeared [274]. It has been reported that there were peroxides in the adipose tissue of animals fed diets that were

deficient in vitamin E and supplementation with synthetic antioxidants prevented vitamin E deficiency in them [275].

Decreased atheromatous lesion formation in vitamin E-fed [1% of vitamin E in diet] rabbits compared to controls have been described by Wilson et al [276]. Willimas et al [277] in experimental atherosclerotic rabbits demonstrated that dietary vitamin E inhibited atherogenesis and there was no evidence of lipid peroxides in the arterial lesions after supplementation. In Watanabe heritable hyperlipidemic [WHHL] rabbits dietary d, l-α-tocopherol [0.5%/wt/wt] supplementation resulted in an increased serum vitamin E concentration and an increased resistance to copper mediated oxidation of LDL. Also, there was retardation in the formation of atherosclerotic lesions [277]. Smith and Kummerow [278], using restricted ovulatory hens that develop hyperlipidemia and subsequent aortic intima thickening, demonstrated that when compared to controls, those fed with 100mg of vitamin E per kilogram of feed not only had low levels of plasma peroxides but also had less aortic intima thickening. In another study, Wojcicki et al [279] described in hypercholesterolemic mongrel rabbits a 25% reduction in aortic atherosclerotic lesions when the animals were fed 10mg per kilogram of vitamin E per day. In a randomized trial, Verlangieri et al [280] reported a 54% lesion reduction in monkeys fed 108mg α-tocopherol per day over a period of three years as assessed by carotid Doppler Studies. Damacker et al [281] in WHHL rabbits that were treated for 24 days with vitamin E [0.1%] reported a two-fold increased resistance of LDL to oxidation, compared to control animals. Loss of endothelium dependent arterial relaxation, which develops early in atherosclerosis, was attenuated in male New Zealand white rabbits that

consumed diets containing moderate amounts of α-tocopherol [1000mg/kg chow] rather than those fed with very high amounts of α-tocopherol [10,000 mg/kg chow]. Morel et al [282], in cholesterol fed New Zealand white rabbits, demonstrated that treatment with vitamin E and vitamin C inhibited lipid oxidation as indicated by TBARS and conjugated dienes, but did not attenuate atherosclerosis as measured by plaque thickness. Lofont et al [283] reported that in rabbits that were treated with oral α-tocopherol, the incidence of restenosis [which results largely from initimal smooth muscle cell proliferation] after angioplasty was markedly inhibited compared to the untreated control group.

Although it would be necessary to consider limitations and exert caution before extrapolating results of animal studies to humans, there is, however, a promising note that vitamin E possesses properties of antioxidant nature that could help prevent LDL oxidation and retard atherosclerosis. Suggestive results have been provided by cross-cultural surveys that dietary antioxidants are associated with reduced ischemic heart disease [284]. Descriptive studies have shown that intake of antioxidant rich foods could offer protection against cardiovascular diseases. Studies in the United Kingdom have described an inverse relationship between the consumption of fresh fruits and vegetables and prevalence of atherosclerotic disease [283-286].

In the 25-year follow up of the Seven Country Study, it was notable that factors other than serum cholesterol levels were important in coronary heart disease prevention. The authors postulated that the epidemiological differences in coronary heart diseases were attributable to the dietary factors that influenced LDL oxidation, such as consumption of fresh fruits and vegetables, vegetables containing phytonutrients including

tocopherols, carotenoids, flavoids, phenols, catechins, quercetin and also green tea, onions and red wine [287]. In a cross-cultural observational study of twelve populations, an inverse association was reported between vitamin E levels and rates of cardiovascular disease mortality [288]. Gey et al [289] reported similar findings in a 16 European Population Cross Cultural Study. Results of the EURAMIC Study also revealed an inverse correlation between myocardial infraction and vitamin E intake [290].

In a case-control study, vitamin E was independently and inversely related to the risk of angina pectoris even after adjustment for other risk factors [291]. Vitamin E supplementation in human subjects resulted in a reduced susceptibility of LDL to oxidation [27-28, 292]. Dieber-Rotheneder et al [27] demonstrated that in human subjects supplemented with α-tocopherol for three weeks, there was a prolonged lag phase and the α-tocopherol levels correlated significantly with the duration of the lag phase. An inverse relationship between vitamin E levels and myocardial infraction has been suggested [293]. An inverse relationship between vitamin E and atherosclerosis has suggested that elevations in the serum levels of vitamin E may be protective [294]. However, Kok et al [295] reported no association between serum vitamin E levels in frozen serum samples and cardiovascular disease mortality in a nested case-control, nine year follow-up study in Western Netherlands. A potential limitation of this study would be the possible instability of vitamin E in serum samples after nine years of storage. No consistent association between serum vitamin A, vitamin E or selenium and death from coronary artery disease was reported from another nested case-control study of Finnish men. Again, samples were stored for seven years and hence this allows for the instability of vitamins over this

long period of storage to affect their results [268]. Hense et al [296] in a nested case-control study [MONICA] in a group of 2023 men and 1999 women reported no correlation between serum vitamin E concentration and risk of myocardial infraction. Carpenter et al [297] estimated α-tocopherol contents in samples of normal and atherosclerotic lesions. They reported that the ratio of α-tocopherol to cholesterol was consistently lower in the atherosclerotic lesions rich in macrophage foam cells. They suggested that the oxidative activity in the lesion may lead to significant oxidation of LDL, probably after α-tocopherol had been depleted. Miller et al [298] supplemented individuals who attended a hyperlipidaemic clinic with 300 mg/day dl-α-tocopherol acetate for 9 weeks. Their results indicated a positive correlation between the antioxidant activity and α-tocopherol content of LDL in the supplemented group compared to the non-supplemented group.

Data from prospective cohort studies, although limited, have to a large extent documented a protective role for dietary antioxidants in cardiovascular disease. The largest of the prospective cohort studies is the Nurses' Health Study [299]. In this study of 87,245 women aged 34 to 59 years, the daily consumption of vitamin C, vitamin E and B-carotene were assessed by using a food frequency questionnaire. Follow-up data were obtained eight years later, and the risk of coronary artery disease among those in the highest quintile of intake of the vitamins was compared with those in the lowest quintile, after adjustment for age, smoking and other cardiovascular risk factors. The results of this study indicated a decreased risk of coronary heart disease among women in the highest

quintile of vitamin E consumption compared to the lowest. No apparent benefit was reported with B-carotene or vitamin C in this study.

The Health Professionals' Follow-up Study evaluated nutrient antioxidant intake in 39,910 U.S. men aged 40 to 75 years, who had not been diagnosed with coronary artery disease or associated risk factors including diabetes mellitus and hypercholesterolemia [300]. Results revealed that men who consumed at least 100mg of vitamin E daily for two years demonstrated relatively less risk for coronary events. B-carotene but not vitamin C demonstrated some protective effects.

Losonczy et al [301] reported that the data from the "Established Populations from Epidemiologic Studies of the Elderly" revealed that vitamin E supplementation reduced risk from all causes of mortality including coronary events in a population of 11,178 persons aged 65-105 years. The benefits of vitamin E supplementation were more pronounced in the vitamin E supplemented group than in the combination vitamin [vitamin E and vitamin C] supplemented group and in those who did not take any supplements.

Although observational studies can provide results after controlling for the effects of known confounding variables, when searching for small to moderate effects, the number of uncontrolled confounding variables in observational studies can affect interpretation of results that may be as large as the likely risk reduction [66]. Randomized clinical trials, in which investigators allocate subjects at random to either treatment or placebo groups, can yield more reliable data as the process of randomization can even out both known and unknown confounding variables [249].

Jialal and Grundy [29] in a 12-week placebo controlled trial focused on the time course of LDL oxidation and its correlation with α-tocopherol levels. After α-tocopherol supplementation, the investigators showed a significant prolongation of the lag-phase, along with reduction in the rate of oxidation. In another study in which subjects received α-tocopherol supplementation for only seven days, similar findings were reported [28]. In a trial with six, healthy, non-smoking volunteers, 1000mg α-tocopherol supplementation demonstrated decreased LDL oxidation, and α-tocopherol prevented the cytotoxic effects of LDL on endothelial cells [254]. Reaven et al [302] studied the relative effects of natural R,R,R-and synthetic all-racemic-alpha tocopherol, in a healthy population of 15 men and women who were fed equal dosages of 1600mg/day for a period of eight weeks. The study was conducted in a double-blinded, randomized fashion. Their results revealed that α-tocopherol levels of both forms in LDL strongly correlated with all measures of LDL oxidation and that at this dosage, supplementation of either form of α-tocopherol, offered equal antioxidant protection to LDL. Contrary results were reported by Traber et al [303] in patients with abetalipoproteinemia.

Jialal and Grundy [304], in a placebo-controlled, randomized, single-blinded trial with 24 healthy volunteers supplemented with dl- α-tocopherol [800mg/day] for three months, reported that vitamin E provided greater protection than in combination with ascorbate and B-carotene as indicated by measurement of TBARS and conjugated diene formations. Ricifi et al [305] reported similar results. Reaven et al [270] conducted a double-blinded, placebo-controlled randomized clinical trial. Their subjects were eight, healthy, non-smoking, mildly hyperlipidemic volunteers [five men and three women] aged

26-65 with no known medical diseases. They assessed the effectiveness of supplementation with B-carotene and vitamin E alone, in combination with each other, and with vitamin C, to protect LDL from oxidation. The duration of the study was for 11 months. The susceptibility of LDL to oxidation was measured by TBARS, formation of conjugated dienes and lipid peroxides as well as by assessment of macrophage degradation. Their results revealed that supplementation with vitamin E alone offered maximum protection against LDL oxidation. Nyyssonen et al [306] in a randomized, double-blinded, placebo-controlled study, further documented the antiatherogenic potential of vitamin E. Kleinveld et al [293] reported that α-tocopherol decreased oxidation of LDL both by increasing the lag time and by decreasing the oxidation rate. Suzukawa et al [307] reported that a low dose of α-tocopheryl acetate [150mg/day for one week followed by 300mg/day for three weeks] resulted in protection of LDL against oxidative modification. Dimitrov et al [308] have shown that in healthy adults and patients with normal lipid absorption, the fat soluble form of vitamin E was preferred for therapeutic and prophylactic use, rather than the water miscible form. In both forms, higher intakes increased plasma concentrations of vitamin E. Although, several clinical trials have demonstrated vitamin E to possess antioxidant properties, recently, Croft et al [309] reported that with elevations in vitamin E levels there was significant increase in both rate and extent of LDL oxidation as assessed by the formation of dienes and lag time of copper-induced oxidation. Boissonneault et al [310] in a in vitro study demonstrated that supplementation with vitamin E did not offer any protection against OX-LDL induced endothelial injury. Brien et al [311] noted that immunohistochemical staining in rabbits

and human atherosclerotic plaques with OX-LDL antibody [OX5] recognized the presence of OX-LDL in plaques. Staining with OX5 was more prevalent in atherosclerotic than in control segments. Their experiments revealed that OX5 staining occurred in cell-associated proteins other than in LDL, as cell-associated OX5 staining occurred even in the absence of cell-associated apo-B staining with the apo-B antibody 9A. The researchers hypothesised that atherosclerosis involves the oxidation of proteins, particularly cell-associated proteins in addition to LDL. They speculated that the antioxidant effects of antioxidants may not be solely attributable to prevention of LDL oxidation. Reports on LDL oxidation inhibiting doses of antioxidants having no atherogenic effect, might substantiate their hypothesis.

2.4.5. ROLE OF VITAMIN E IN DIABETES MELLITUS

There is ample evidence for the prevalence of modified forms of LDL in diabetes. Amongst the various modified forms, oxidatively modified LDL has been the focus of much research due to its known atherogenic properties and since atherosclerosis has been the major cause of diabetes mortality [213]. Hence it would only seem natural to research the possible benefits of antioxidants in diabetes.

Morel et al [312] reported protective effects of both vitamin E and probucol in diabetic rats. Vitamin E supplementation inhibited both oxidation and cytotoxicity of LDL, but without altering hyperglycemia. It is known that LDL is the primary transporter of vitamin E and that LDL enters the cells by a specific receptor-mediated mechanism. Kokoglu and Ulakoglu [312] reported that in persons with diabetes both LDL-cholesterol

and α-tocopherol in LDL are not sufficiently taken into the cell due to modifications in LDL. In streptozotocin-induced diabetic rats, Dovillet et al [13] found that with vitamin E supplementation there was a marked reduction in the formation of TBARS compared to controls.

Reaven et al [314] in a placebo-controlled, double blinded trial with 21 NIDDM men, aged between 50-70 studied the effects of supplementation of 1600mg/day for 10 weeks. Their results revealed that susceptibility of LDL to copper mediated oxidation as assessed by conjugated diene formation [lag time] and formation of TBARS was reduced significantly. However, protein glycation, including glycosylated hemoglobin, albumin, total plasma proteins and LDL were unaffected by vitamin E supplementation.

In another randomized, double-blinded clinical trial of 161 healthy volunteers aged 39-56 years, it was demonstrated by Takamatsu et al [315] that long-term supplementation [six years] with a low dosage of [100mg/day] α-tocopherol resulted in reduced peroxidation of LDL. Saleonen et al [316] reported a significantly strong and independent association between low vitamin E status and excessive risk of diabetes in IDDM. They suggested that free radical stress could play a vital role in the causation of IDDM. In a double-blinded, placebo-controlled, randomized trial, twenty eight persons with diabetes were supplemented with 1632 mg/day RRR- α-tocopherol acetate for eight weeks. The α-tocopherol supplemented group had significant reductions in LDL oxidizability as shown by the time-course of conjugated diene and lipid peroxide formation. However, there were no reportable changes in glycosylated hemoglobin or in glycosylated plasma proteins after α-tocopherol supplementation. Hence, Fuller et al

[317] proposed that α-tocopherol supplementation may be beneficial in reducing oxidizability of LDL in those with diabetes.

CHAPTER 3

METHODS

Introduction

Based on the above observations it appears that oxidation of LDL might contribute to the development of atherosclerosis [1-3]. Complications in diabetes have been described to result, in part, from increased oxidative stress [12-13]. Calcium homeostasis has been hypothesized to be disturbed during atherogenesis, and oxidation of LDL has demonstrated an increase in its ability to induce $[Ca^{2+}]i$ elevation in VSMCs compared to N-LDL [31]. This might explain, in part, the increased atherogenic potential of OX-LDL [31-33]. Antioxidant supplementations have been shown to inhibit LDL oxidation and also retard progression of atherosclerosis [19-21].

Previous studies with antioxidant supplementation in clinical trials measured LDL resistance to in vitro oxidation, conjugated diene formation, and TBARS [31-33]. This study sought to utilize a more biologically relevant measure for the role of OX-LDL and antioxidant supplementation in atherosclerosis. This study employed a VSMC culture system because VSMC migration, proliferation, contractility, and matrix production are key events in the pathogenesis of atherosclerosis [35,37]. Weisser et al [35] reported that OX-LDL stimulated more pronounced increases in $[Ca^{2+}]i$ responses when exposed to cultured rat VSMCs compared to N-LDL. These findings suggest a link between two key events in atherosclerosis, aberrant $[Ca^{2+}]i$ regulation and oxidation of LDL.

In order to evaluate this concept, preliminary experiments were conducted to determine whether LDL oxidized in vitro elicited increased $[Ca^{2+}]i$ responses in cultured VSMCs compared to N-LDL. After it was established that oxidation of LDL increased both TBARS and $[Ca^{2+}]i$ responses, a clinical trial with vitamin E supplementation was conducted to evaluate whether antioxidant supplementation would inhibit both LDL oxidation and the subsequent LDL induced $[Ca^{2+}]i$ response in cultured VSMCs. To further link the clinical relevance of oxidized LDL and atherosclerosis, the present study also examined whether LDL isolated from a population with diabetes clinically diagnosed with atherosclerosis exhibited elevated levels of TBARS and elicited increased $[Ca^{2+}]i$ responses compared to LDL isolated from healthy subjects.

3.1. SUBJECTS AND STUDY DESIGN

α-TOCOPHEROL SUPPLEMENTATION STUDY

SUBJECT DESCRIPTION

Twelve healthy male volunteers [residents and faculty at the University of Tennessee Medical Center, Knoxville] between the ages of 20 and 55 were selected, after giving their written informed consent. The subjects were non-smokers and did not take nutritional supplements, aspirin, or other medications. During the course of the study they consumed their usual diet, except that they abstained from alcohol and vitamin E rich foods [i.e. nuts, wheat germ, etc.]. This protocol was approved by the University Institutional Review Board.

STUDY DESIGN

Part 1: A randomized double-blinded, placebo-controlled, cross over trial of vitamin E versus placebo was conducted. The twelve week study design is summarized in Figure 1. Briefly, the study included an initial two week period to strictly adhere to the dietary and medical instructions of the study, followed by randomization to either 400mg all-rac-α-tocopherol or placebo for four weeks. There was then a second two week washout period followed by the groups crossing over to receive four weeks of the other treatment.

Blood samples were drawn from each of the subjects (placebo and treatment groups) at baseline [W_0] (where W represents week, and baseline is the period before the subjects recruited in the study were given any experimental treatment) and every week during the four week treatment period [W_1-W_4]. All blood samples were drawn at approximately the same time throughout the study period. This was followed by a two week washout period [W_4 -W_6], and blood was drawn at W_6. After the cross-over [W_6], blood samples were drawn again every week for the next four weeks [W_7-W_{10}]. LDL isolation, [Ca^{2+}]i responses to cultured rat VSMCs and TBARS were performed for every blood sample drawn during the entire course of the study [W_0 -W_{10}]. Plasma vitamin E levels were determined at baseline [W_0], at four weeks after the treatment period [W_4], after the two week washout period [W_6], and at four weeks following the cross-over of treatments [W_{10}].

Part II: Eight persons with diabetes who had atherosclerosis leading to symptomatic coronary artery disease or peripheral vascular disease were selected from the

University of Tennessee Medical Center in-patient and out-patient services. None of the patients were taking antioxidants or lipid medication. The diagnosis of cardiovascular disease was established with angiogram or doppler studies. Seven healthy controls were recruited from the hospital staff. All participants gave informed consent and the study protocol was approved by the University Institutional Review Board. Following recruitment, blood samples were drawn for analyses. All measurements of LDL and intracellular calcium responses were done by an investigator who was unaware of the diagnosis.

3.2. MATERIALS

Cells from the A7r5 vascular smooth muscle cell line were obtained from American Type Culture collection [Rockville, MD]. Dulbecco's modified eagles medium [DMEM], fetal bovine serum [FBS], and fetal calf serum [FCS] were from Gibco BRL [Gaithersburg, MD] from Sigma Chemicals Company [St. Louis, MO]. Reagents used in the isolation of low density lipoproteins, measurement of thiobarbituric acid reactive substances and protein determination was obtained from Sigma Chemical Company [St. Louis, MO].

3.3. PREPARATION OF SMOOTH MUSCLE CELLS

Vascular smooth muscle cells of rat thoracic aorta were obtained from American Type Culture Collection, Rockville, MD. The cells were grown in Dulbecco's Modified Eagles Medium [DMEM] supplemented with 5% fetal bovine serum, 5% calf serum,

10,000 IU/ml penicillin, 10 mg/ml streptomycin and 8 mg/ml tyrosin tartarate in 5% CO2, 90% humidity and 37^0 C atmosphere as previously described [318-320]. The cells were studied during passages 10-22.

3.4. INTRACELLULAR FREE CALCIUM CONCENTRATION $[Ca^{2+}]i$ DETERMINATION

The fluorescent dye Fura-2/AM [acetylmethoxy] ester, Calbiochem, San Diego, CA] was dissolved in dimethyl sulfoxide [DMSO] to yield a final concentration of 10 μM. Confluent monolayer cells were rendered quiescent by maintaining them in a 0.2% fetal bovine serum supplemented with DMEM for 24 hours. The cells were rinsed with Hank's balanced salt solution [HBSS], trypsinized and centrifuged. The pellet was suspended in N-2-hydroxyethylpiperaxine-N'-2-ethanesulfonic acid [HEPES] buffered salt solution and chilled on ice for approximately 10 minutes. The cell suspension was loaded with fura-2/AM and incubated in a shaking water bath in the dark for 20 minutes at 37^0C and then sedimented by centrifugation and resuspended at a concentration of approximately 10^6 cells/ml in HBSS [319]. Fluorescence was measured at 37^0C, while stirring cells in a cuvette placed in a Hitachi-F2000 spectroflurometer [Hitachi-F2000, Napeville, IL]. The $[Ca^{2+}]i$ levels were determined flourometrically in suspension using excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 510 nm. Excitation and emission band widths were set at 10 nm. Maximum and minimum fluorescent signals were obtained with 40 μM digitonin and pH 8.7 Tris [100 nM] and Ethylene glycol-bis [beta-amino ethylether] N,N,N^1,N^1-tetra acetic acid [EGTA] [100 mM] respectively. $[Ca^{2+}]i$ levels

were calculated using the equation of Grynkiewicz et al [185]. Peak calcium responses to LDL [20 μg protein/ml concentration] were evaluated after establishing a stable baseline [within the first two minutes of study] for each cell suspension. All the experiments were repeated three times in each subject.

3.5. PREPARATION OF LOW DENSITY LIPOPROTEINS

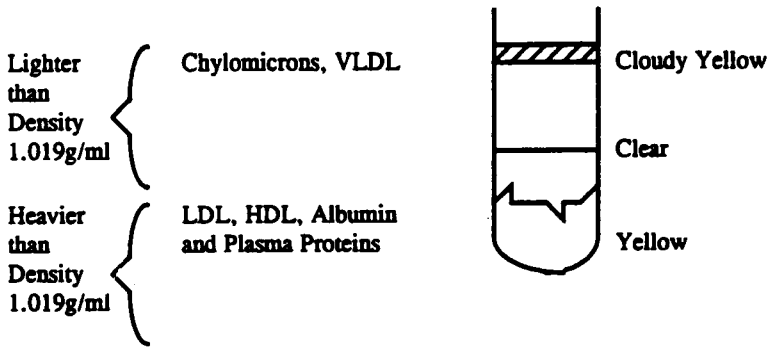
All fasting blood samples [30 ml] were drawn in the presence of 1mM ethylenediaminetetra-acetic acid [EDTA]. Plasma was isolated by centrifugation at 1500g for 30 minutes at 20^0C to 25^0C. Exposure to ultraviolet light was strictly avoided by doing all work under yellow light in the presence of an antioxidant butylated hydroxytoluene [BHT 5.0/μg/ml]. LDL was isolated by a slight modification of a density gradient ultracentrifugation method, described by Redgave et al [321]. The density salt solution was prepared using sodium chloride, sodium bromide and EDTA. The density of the salt solution was between 1.473g/ml - 1.480g/ml at pH 7.4.

To the freshly isolated plasma [density = 1.006 g/ml] was added polymethyl sulfonofluoride [PMSF], a protease inhibitor [250μM, 0.6 ml/100 ml plasma] and sodium azide, an antimicrobial agent [1.3%, 0.4 ml/100 ml plasma]. The density of the plasma was adjusted to 1.019 g/ml, when the plasma was layered with the required volume of the density salt solution based on the equation below:

$$\text{volume [ml] of density solution to be added} = \frac{x\,[1.019 - 1.006]}{[y - 1.019]} \quad \text{where,}$$

x=volume of plasma, y=density of the salt solution, 1.006=initial density of plasma, 1.019=density adjusted plasma i.e. density gradient for separation between lipoproteins.

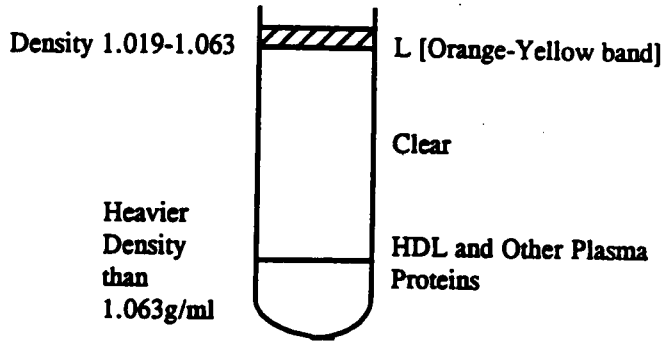
Following centrifugation in a 50 Ti rotor in a Beckman LS-50 [Beckman instruments, CA], for 20h at 45,000 RPM, at 10^0C, the sample tubes were removed carefully. The distribution of the sample after centrifugation appeared as below:



After centrifugation chylomicrons, VLDL were present at the top of tube i.e. the upper most layer, cloudy yellow in color, contained material less than density 1.019 g/ml. This uppermost fraction was carefully removed using a Pasteur pipette. To obtain LDL fraction of density 1.019 g/ml to 1.063 g/ml, the density solution was layered on the fraction [heavier than density 1.019 g/ml] at the bottom of the tube which contained HDL, LDL, albumin and other plasma proteins. Density adjustments were made in accordance with the equation:

$$\text{volume [ml] of density solution to be added} = \frac{x\,[1.063 - 1.019]}{[y - 1.063]} \quad \text{where,}$$

x=volume [ml] after removal of material lighter than density 1.019g/ml, y=density of the density solution, and 1.019g/ml to 1.063g/ml is the desired LDL density. Following density adjustments the sample was centrifuged at 45,000 RPM for 18h at 10^0C. The upper most layer consisted of the LDL fraction of density 1.019g/ml-1.063g/ml.



The freshly isolated LDL was dialyzed at 4^0C for 24h against three changes [every 8h] of 0.9% Nacl and 1mM EDTA at pH 7.4. LDL was sterilized by the use of 0.45 micrometer Millipore filters [Millipore, Millex-HV, Bedford, MA] and stored at 4^0C. All samples were utilized for experiments within three weeks of isolation.

3.6. MEASUREMENT OF THIOBARBITURIC ACID REACTIVE SUBSTANCES [TBARS]

The extent of LDL oxidation was assessed by a fluorometric determination of thiobartituric acid-reactive substances. LDL [20μl] was mixed with 1ml 12N sulfuric acid and 0.5ml 10% phosphotungstic acid was then added. Following centrifugation at 2000g for five minutes, the supernatant was discarded and the sediment was mixed with 1ml 12N sulfuric acid before centrifugation was repeated [2000g for five minutes]. The supernatant was again discarded and the sediment was resuspended in fresh thiobarbituric acid reagent [1ml of a solution of 0.67% agueous thiobartiuric acid in 0.5M Tris butter, adjusted to pH 3.2-3.8 with glacial acetic acid]. This suspension was then heated at 95^0 for 1 hour. After cooling to room temperature and centrifugation at 2000g for five minutes, the supernatant

was removed and flourometric measurement [515nm excititation, 553 nm emission] was performed in a Hitachi-F2000 spectroflurometer [Hitachi-F2000, Naperville, IL]. The standard was obtained by reacting 0.5nmol of 98% maldondialdehyde bis-[dimethylacetal] with thiobartitunic acid reagent. The intensity of fluorescence was linearly related to the malondialdehyde concentration. The concentration of OX-LDL was calculated according to the relation of the flourometric signal obtained with LDL and the standard. The lipid peroxide level was expressed in terms of melandiladehyde [35, 36, 39].

$$\text{Quantity of Maldondialdehyde in sample} = \frac{f}{F} \times \text{quantity of malondialdehyde in standard}$$

F = standard fluorescence at 0.5nmol
f = sample fluorescence

We added 1ml of TBA to 20μl of sample

$$\therefore \text{ quantity of malondialdehyde in sample} = \frac{f}{F} \times 0.5 \times \frac{1000}{20}$$

$$= \frac{f}{F} \times 25 \text{ [nmol/ml sample]}$$

All samples were measured in duplicates and measurements were completed within one week of LDL isolation.

3.7. PROTEIN DETERMINATION

LDL sample protein determinations were conducted by a photometric method described by Markwell et al [332]. 1 ml of the diluted LDL sample was mixed with 3ml of an alkaline copper reagent. The mixture was incubated at room temperature for a minimum of ten minutes. To this was added 300μl of the diluted [1:1] folin-ciocalteau

reagent. The samples were vortexed vigorously, mixing the contents of each tube immediately after addition. The tubes were incubated for 15 minutes at room temperature and read at 660nm against a reagent blank. The concentration versus absorbance curve was generated by using 0 [zero-reagent blank], 0.20, 0.40, 0.60, 0.80 and 1ml of the BSA. All standards were diluted to a final volume of 1ml. The standards were treated the same way as the samples.

3.8. α-TOCOPHEROL QUANTIFICATION

α-tocopherol quantification was done by HPLC methods as described by Catignani et al [323]. Experimental procedures were performed in the laboratory of Dr. Osmond. α-tocopherol in plasma was quantified by high performance liquid chromatography. Plasma was deproteinized with ethanol that contained the internal standard (α-tocopheryl acetate), and the lipid was extracted with hexane. An aliquot of the solvent phase was evaporated. The residue was dissolved in diethyl ether and diluted with methanol. A portion of this solution was injected into a C_{18} reversed phase chromatographic column and the absorbance of the vitamins and standards was measured at 280nm. Peak to height ratios were used for quantification of each sample.

The stock standards of α-tocopherol and tocopheryl acetate were prepared 5g/L in ethanol. The stock standards were diluted 100-fold with ethanol to prepare working standards. The concentrations of the working standards were confirmed spectrophotometrically by using their respective absorptivities in ethanol: α-tocopherol 75.8 at 292nm and α-tocopheryl acetate 43.6 at 285nm.

50μl of the α-tocopheryl acetate working standard was pipetted into a 6 x 50mm disposable glass test tube. To this 100μl of the sample was added and vortexed vigorously for 10 seconds. 100μl of hexane was then added and vortexed intermittently and vigorously for 45 seconds. The mixture was centrifuged at 800 x g for 5 minutes. Following centrifugation, 75μl of the hexane layer was transferred into a 6 x 50mm disposable glass test tube. Hexane was evaporated under a stream of air. The lipid residues were dissolved in 25μl of diethyl ether and 75μl of methanol was added with gentle mixing. With a 10μl flush of methanol, 90μl of the solution was analyzed by reversed phase HPLC. α-tocopherol levels were quantified against standard curves using peak-height ratios versus weight ratios for each vitamin.

3.9. STATISTICAL ANALYSIS

Statistical analysis was performed using SAS system [SAS Institute Inc., Cary, NC] software. ANOVA was used to determine whether there were significant changes in the α-tocopherol supplementation study i.e. [i] vitamin E levels in plasma [ii] in TBARS or [iii] in $[Ca^{2+}]i$ levels. In the study with persons with diabetes and healthy subjects, data were analyzed with the student T test and Kruskal-Wallis test for normally and non-normally distributed data, respectively. Alpha levels for all tests were set at $p<0.05$.

CHAPTER 4

RESULTS

4.1. LACK OF EFFECT OF SUPPLEMENTATION WITH ALL-RAC-α-TOCOPHEROL

Preliminary studies were conducted in our laboratory with LDL isolated from healthy volunteers. LDL isolated from each subject was divided into 2 parts; one part was retained as N-LDL and the other part was oxidized in the presence of copper sulfate. In the cultured rat VSMC system, OX-LDL elicited a marked increase in the magnitude of $[Ca^{2+}]i$ response, from a peak to baseline ratio of 2.2 with N-LDL to a peak to baseline ratio of 4.3 with OX-LDL [Figures 4A and 4B, respectively]. In vitro OX-LDL also exhibited elevated levels of TBARS compared to N-LDL [0.85±0.14 nmol/mg protein of N-LDL versus 2.2±0.18 nmol/mg protein of OX-LDL].

In the clinical trial, supplementation of 400mg vitamin E per day for four weeks resulted in a significant increase in the concentration of α-tocopherol in plasma [Table 1] indicating a high degree of subject compliance. LDL oxidation assessed by TBARS did not reveal any significant difference after supplementation with vitamin E for four weeks [Table 1]. All-rac-α-tocopherol supplementation for four weeks was also without effect on the LDL induced $[Ca^{2+}]i$ responses in cultured rat VSMCs [Table 1]. TBARS and $[Ca^{2+}]i$ peak to baseline ratios measured at each week during the four week supplementation period were unaffected by α-tocopherol supplementation [Table 2].

Our results reveal that all-rac-α-tocopherol supplementation was without effect on the status of LDL oxidation as assessed by TBARS as well as in the subsequent LDL induced $[Ca^{2+}]i$ response, in the same system that we used to conduct our preliminary experiments.

4.2. INCREASED VASCULAR SMOOTH MUSCLE $[Ca^{2+}]i$ RESPONSE TO LDL ISOLATED FROM DIABETIC VERSUS HEALTHY SUBJECTS

Our results reveal that in persons with diabetes with atherosclerotic manifestations there was a 55% higher level of oxidized LDL compared with healthy controls, as assessed by TBARS. This difference was statistically significant [1.65±0.22nmol/mg protein vs 1.087±0.14 nmol/mg protein, $p<0.0001$]. [Table 3 and Figure 5]. Although LDL from healthy subjects elicited an increase in VSMCs $[Ca^{2+}]i$, LDL from persons with diabetes with atherosclerosis elicited a four-fold greater increase as indicated by their peak minus baseline $[Ca^{2+}]i$ response values [444nM±241 vs 104±17nM, $p<0.025$]. LDL from persons with diabetes demonstrated a marked increase in the magnitude of $[Ca^{2+}]i$ response elicited. There was a peak to baseline ratio of 1.81 for LDL isolated from healthy persons and a peak to baseline ratio of 3.80 for LDL isolated from persons with diabetes [Table 3 and Figure 6A and 6B, respectively]. These findings are consistent with our preliminary experiments wherein LDL oxidized in vitro demonstrated higher levels of TBARS and more pronounced $[Ca^{2+}]i$ responses compared to N-LDL. In populations with higher oxidative stress, such as in persons with diabetes with atherosclerosis, the isolated LDLs behaved in a similar manner to that of LDL oxidized in vitro.

CHAPTER 5

DISCUSSION

5.1. LACK OF EFFECT OF SUPPLEMENTATION WITH ALL-RAC-α-TOCOPHEROL

Epidemiological studies have shown a positive correlation between LDL levels and coronary artery disease [14, 41, 157]. Moreover, oxidized LDL has a more pronounced role in the pathogenesis of atherosclerosis and subsequent coronary artery disease than does native LDL [1-2]. Smooth muscle proliferation is a key event in the genesis of lesions of atherosclerosis [38], and it has been shown that oxidized LDL stimulated more pronounced vasoconstriction than native LDL in animal models [35]. Oxidized LDL is also taken up more avidly by macrophages and as a consequence are converted to foam cells, an important step in atherogenesis [3]. It has been shown also that LDL isolated from atherosclerotic lesions is more oxidized [53]. As a consequence there has been much interest in the role of antioxidants in preventing oxidation of LDL and thereby inhibiting atherogenesis.

The goal of the present study was to test the effect of supplementation of all-rac-α-tocopherol on LDL oxidation response in VSMCs of rat aorta. Weisser et al [39] have shown that LDL causes a dose dependent increase in $[Ca^{2+}]i$, and this response is much more pronounced with oxidized LDL compared to native LDL. Hence it is possible that $[Ca^{2+}]i$ stimulation is one of the cellular mechanisms by which oxidized LDL exerts its atherogenic effects. $[Ca^{2+}]i$ is an important second messenger system involved in several

steps potentially leading to atherogenesis, and two vital mechanisms in atherosclerosis, cell proliferation and cell contractility, are triggered by changes in $[Ca^{2+}]i$ [31-32].

Several studies demonstrate that dietary supplementation of α-tocopherol results in decreased susceptibility of LDL to oxidation in vitro [29]. Grundy et al [29] have shown that oral supplementation of α-tocopherol resulted in increased resistance to in vitro oxidation of LDL particles by measuring TBARS and conjugated dienes. However, the TBARS values at 6 and 12 weeks were the same in the placebo and treated groups, before in vitro oxidation was initiated in their study. Our study had similar results, with no differences in TBARS measurement between the study groups. Apart from the lack of effect on TBARS, the LDL isolated from treated plasma had the same effect on $[Ca^{2+}]i$ response in VSMCs as compared to LDL isolated from plasma treated with placebo. Previous reports of Weisser et al [39] have shown that LDL oxidation in vitro elicited a more marked response in $[Ca^{2+}]i$ than did native LDL [36]; however, we found supplementary dietary vitamin E to be without effect in this system. There could be several reasons for this lack of effect of all-rac-α-tocopherol. One possibility is that the population studied had a low oxidative stress at baseline, since all participants were healthy, non-smoking, non-diabetic males, similar to the sample studied by Grundy et al [29]. Furthermore, in a normocholesterolemic population, it is possible that the circulating LDL is rapidly cleared by the apo B/E receptors, thereby reducing the residence time for LDL in circulation [324]. Walzem et al [324] and Millar et al [325] reported that in hypercholesterolemic individuals, the susceptibility of LDL oxidation increases with the increasing age of LDL in plasma. This increase in residence time for LDL in circulation

was attributed to the decrease of clearance of LDL by the apo B/E receptors. With an increase in residence time, LDL has been described to be more susceptible to in vitro oxidation. Chronological age and age of LDL and plasma have also been reported to be positively correlated [325]. It is of course possible that all-rac-α-tocopherol will not provide significant physiological protection as measured in this ex vivo system. Previous studies of the protective antioxidative effect of α-tocopherol have measured the resistance of vitamin E treated LDL to in vitro oxidation [27-29]. However, it is not clear how well this technique of measuring LDL protection correlated with oxidation occurring under normal physiological conditions.

Recent epidemiological studies [22, 300] have shown an association between the intake of vitamin E [100 mg/day or more] and a decreased prevalence of coronary artery disease. Accordingly, the 400 mg/day dose used in the present study should be adequate, but whether higher doses will affect induced $[Ca^{2+}]i$ responses is not clear. The four week supplementation period used also may not have been long enough, although this seems to be unlikely considering the short half life of LDL particles.

In conclusion, oral supplementation of 400 mg all-rac-α-tocopherol for four weeks failed to elicit any statistically significant effect on the oxidative status of LDL particle in a healthy male population. This lack of effect could be due to our study population which was homogenous, consisting of only healthy individuals with low oxidative stress. We believe other studies, using populations with higher oxidative stress, such as persons with diabetes or smokers, should be carried out to see if all all-rac-α-tocopherol has antioxidant effects in that population. Studies using other antioxidants, such as probucol, should be

conducted also using this method. Measuring changes in $[Ca^{2+}]i$ as a response to LDL may be a more physiologically relevant method for determination of oxidative effects than conventional in vitro methods and should be explored further.

Previous studies have reported elevated levels of TBARS and conjugated dienes in populations with high levels of oxidative stress [18]. Hence, we hypothesized that the elevated levels of lipid peroxides in these populations could induce more pronounced increases in $[Ca^{2+}]i$ response in cultured VSMCs. Therefore, as a follow up of this study, we compared a population with high oxidative stress, such as persons with diabetes diagnosed with atherosclerosis [228], with healthy controls. This follow up study could further help examine the physiological relevance of $[Ca^{2+}]i$ responses to LDL.

5.2. INCREASED VSMC $[Ca^{2+}]i$ RESPONSE TO LDL ISOLATED FROM DIABETIC VERSUS HEALTHY SUBJECTS

Data from this study demonstrate that LDL isolated from persons with diabetes with atherosclerosis is markedly more potent in stimulating $[Ca^{2+}]i$ responses in cultured vascular smooth cells compared to LDL isolated from normal subjects. This is likely due to the greater level of LDL oxidation observed in these patients compared to controls, as in vitro oxidation of LDL has already been demonstrated to increase its ability to stimulate $[Ca^{2+}]i$ [36].

In vitro studies have shown that glucose in the presence of trace amounts of transition metals can produce superoxide free radicals. Superoxide free radicals can breakdown to highly reactive hydroxyl peroxide radicals which can initiate lipid

peroxidation [12]. This may be particularly important in persons with diabetes with poor glycemic control as hyperglycemia leads to non-enzymatic glycosylation of proteins, including lipoproteins. It has been shown that glycosylated LDL is more susceptible to oxidation [230, 235]. Acordingly, free radicals produced from glucose residues in glycosylated lipoproteins may contribute LDL oxidation.

An expanding body of evidence indicates that oxidatively modified LDL is involved in the pathogenesis of atherosclerosis [1, 40]. Moreover, probucol, which inhibits LDL oxidation has been shown to inhibit the progression of atherosclerosis in animal models [19-20]. Further, the treatment of diabetic rats with antioxidants inhibits LDL oxidation and reduces cellular toxicity of LDL isolated from those diabetic rats [312]. Consistent with these observations, our data demonstrate greater LDL oxidation in persons with diabetes and a functional consequence of this more highly oxidized LDL [i.e., increased $[Ca^{2+}]i$ response]. These data support the concept that antioxidants which inhibit LDL oxidation, such as vitamin E and probucol [19, 20, 312], may have a protective effect in patients who are subject to elevated oxidative stress, such as persons with diabetes and smokers.

Our data confirm the observation of Weisser et al [39], that LDL from atherosclerotic patients is considerably more oxidized than LDL from normal subjects. Our observation that this atherosclerotic LDL also elicited a greater $[Ca^{2+}]i$ response in cultured vascular smooth muscle cells suggests a link between two key mechanisms of atherosclerosis [LDL oxidation and aberrant $[Ca^{2+}]i$ regulation]. LDL stimulates proliferation and migration of smooth muscle cells via Ca^{2+} dependent mechanisms [31, 33,

35-36, 39]. Accordingly, calcium channel blockage has been shown to retard the development of atherosclerosis in animal models [180-182], and nifedipine prevents the development of new atherosclerotic lesions in patients with angiographic evidence of mild coronary artery disease [179].

The cellular mechanism by which oxidized LDL causes an increase in intracellular calcium is unknown. LDL has been shown to cause increases in $[Ca^{2+}]i$ in fibroblasts which are LDL receptor deficient [33], indicating that these $[Ca^{2+}]i$ responses are not mediated via LDL receptors. LDL induced rise in $[Ca^{2+}]i$ in VSMCs appears to be dependent on influx from extracellular calcium. However, LDL induced activation of cells is associated also with increased phosphatidylinositol turn over [186, 187]. Scahnindis et al [128] have demonstrated that addition of isradipine to the medium prior to LDL can partially block intracellular calcium response to LDL and suppresses the expression of early growth response genes-1 in VSMCs. This suggests that proliferative effects of LDL are mediated by a calcium dependent pathway. Therefore, antiatherogenic effects of calcium channel blockers may be related to blunting of $[Ca^{2+}]i$ responses to LDL. Accordingly, we suggest that the ability of LDL to stimulate $[Ca^{2+}]i$ responses in cultured vascular smooth muscle cells may be a useful index of atherogenicity in clinical trials. TBARS assay is an <u>in vitro</u> biochemical test based on aldehyde formation [324]. TBA also can react with other substances including bile pigmets, amino acids and sugars [325-327]. This lack of specificity limits the usefulness of TBARS measurement in clinical studies. In contrast, the measurement of cultured vascular smooth muscle $[Ca^{2+}]i$

response to LDL represents a biological response of cells to LDL oxidation and, consequently may be a more useful marker for LDL atherogenicity in clinical trials.

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APPENDICES

APPENDIX A

Table 1: Effects of Supplementation with All-Rac-α-Tocopherol for Four Weeks.

Study Parameters		Mean	SEM
Plasma levels of α-tocopherol	Placebo	342	52
	α-tocopherol	780*	109
TBARS	Placebo	0.70	0.09
	α-tocopherol	0.80	0.09
$[Ca^{2+}]i$ Peak to baseline ratios	Placebo	2.98	0.85
	α-tocopherol	2.64	0.43

Means represent pooled data at W_4 and W_{10} of the vitamin supplemented groups and the placebo groups.
α-tocopherol levels expressed as μg/dL
TBARS expressed as nmol/mg protein
$[Ca^{2+}]i$ expressed as nmol

Table 2: TBARS and $[Ca^{2+}]i$ Peak to Baseline Ratios in the α-Tocopherol Supplementation Study (W_0-W_{10}).

Study Parameters	Treatment Groups	W_0 Baseline	W_1	W_2	W_3	W_4	W_5 Washout	W_6	W_7	W_8	W_9	W_{10}
TBARS	Placebo	0.76 (0.09)	0.94 (0.06)	0.70 (.0.10)	0.50 (0.08)	0.72 (0.15)		0.69 (0.07)	0.70 (0.08)	0.55 (0.08)	0.82 (0.11)	0.70 (0.09)
	Vitamin E		0.90 (0.09)	0.49 (0.09)	0.89 (0.11)	0.89 (0.08)			0.89 (0.08)	1.00 (0.09)	0.49 (0.09)	0.80 (0.08)
$[Ca^{2+}]i$ Peak to Baseline Ratio	Placebo	1.43 (0.11)	2.96 (0.09)	3.42 (0.06)	3.63 (0.08)	2.80 (0.10)		1.96 (0.11)	2.81 (0.10)	2.78 (0.06)	2.80 (0.05)	2.78 (0.08)
	Vitamin E		2.80 (0.21)	3.06 (0.42)	2.50 (0.45)	2.62 (0.21)			2.65 (0.62)	2.50 (0.45)	2.55 (0.42)	2.60 (0.45)

W_X represents weeks of study.

Table 3: TBARS and VSMC $[Ca^{2+}]i$ Responses of LDL Isolated from Healthy Subjects Versus Subjects with Diabetes.

Subject Description	TBARS		$[Ca^{2+}]i$ Peak to Baseline Ratio	
	Mean	SEM	Mean	SEM
Healthy Subjects	1.08	0.14	1.81	0.53
Subjects with Diabetes	1.65	0.22	3.80	1.49

TBARS expressed as nmol/mg protein
$[Ca^{2+}]i$ expressed as nmol
$p < 0.05$

APPENDIX B

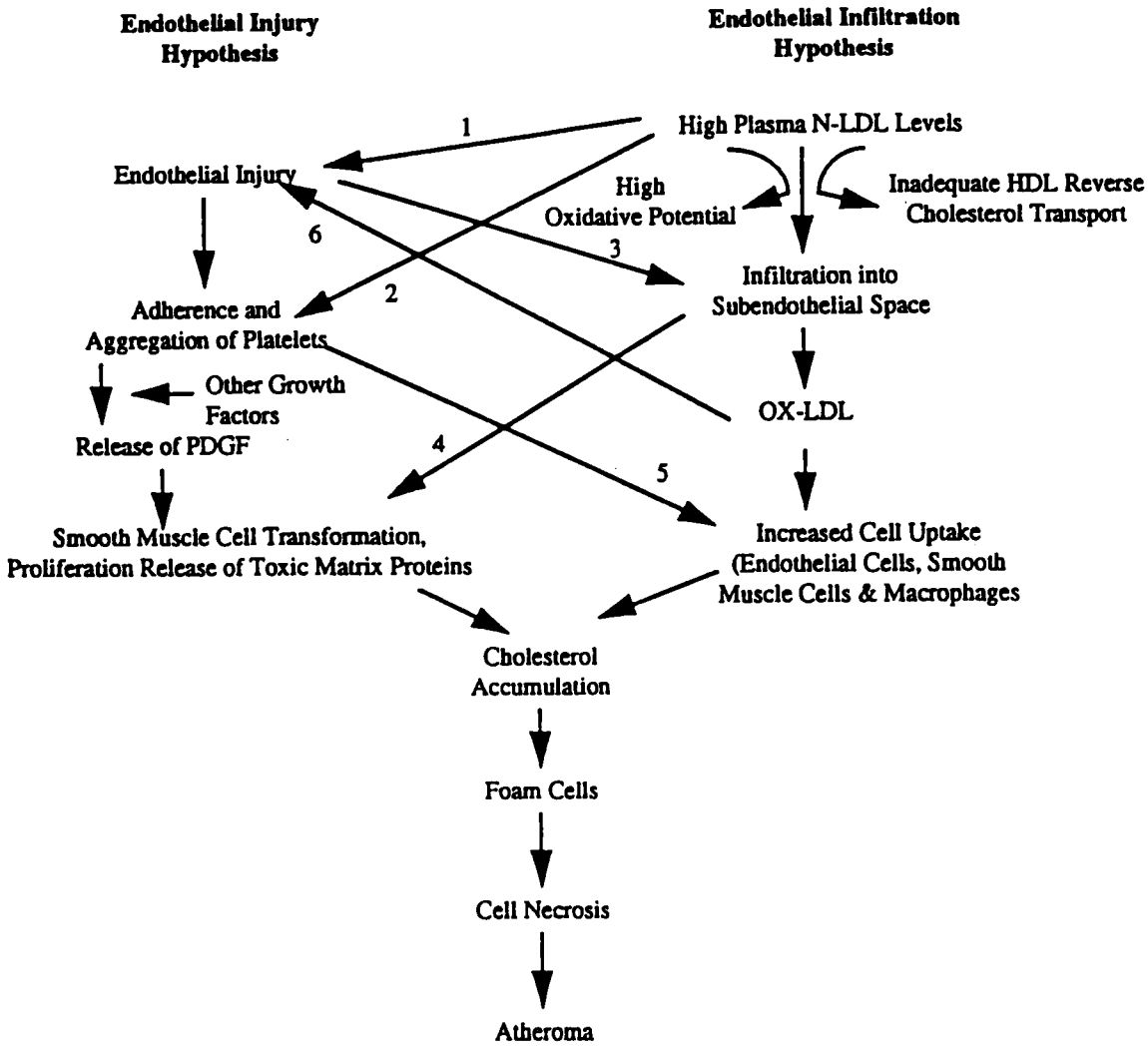
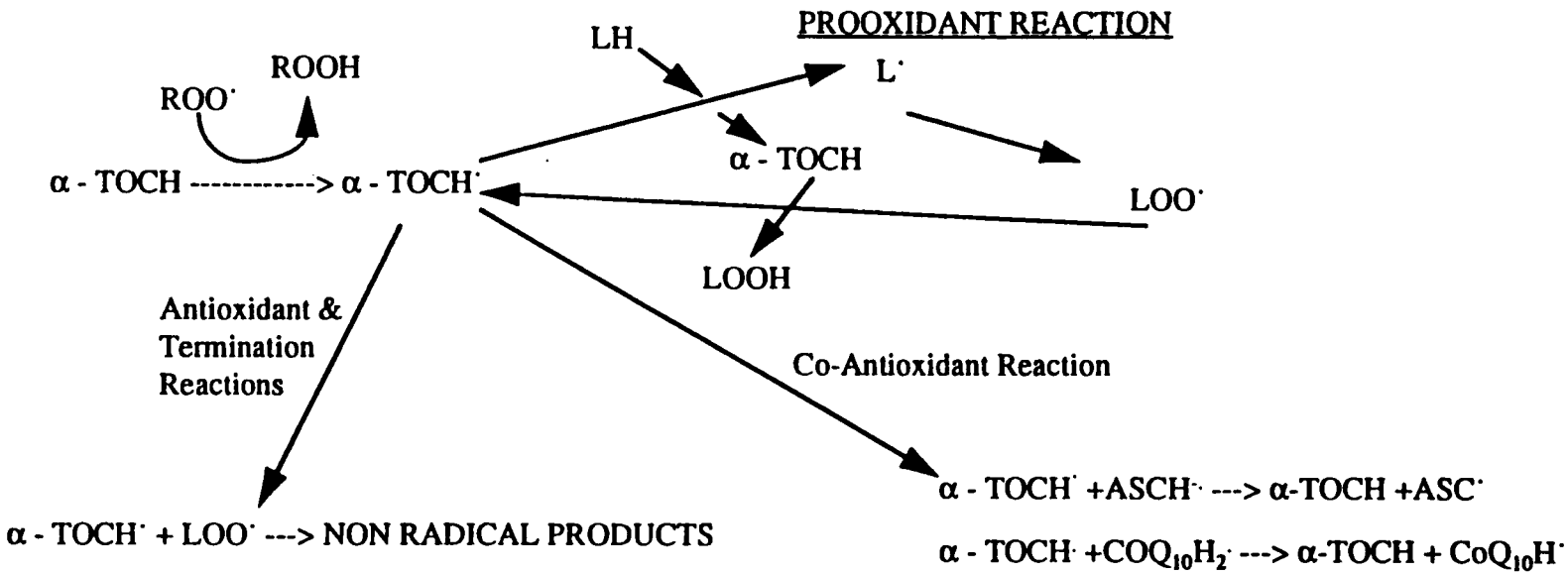


Figure 1. Unified Hypothesis



ROO· ---> Initiating peroxyl radical

LOO· ---> Lipid peroxyl radical

ASCH·-·--> Endogenous ascorbic acid

ASC· --> Ascorbic acid radical

Figure 2. Possible Mechanisms of Action of α-TOC <u>in vitro</u>

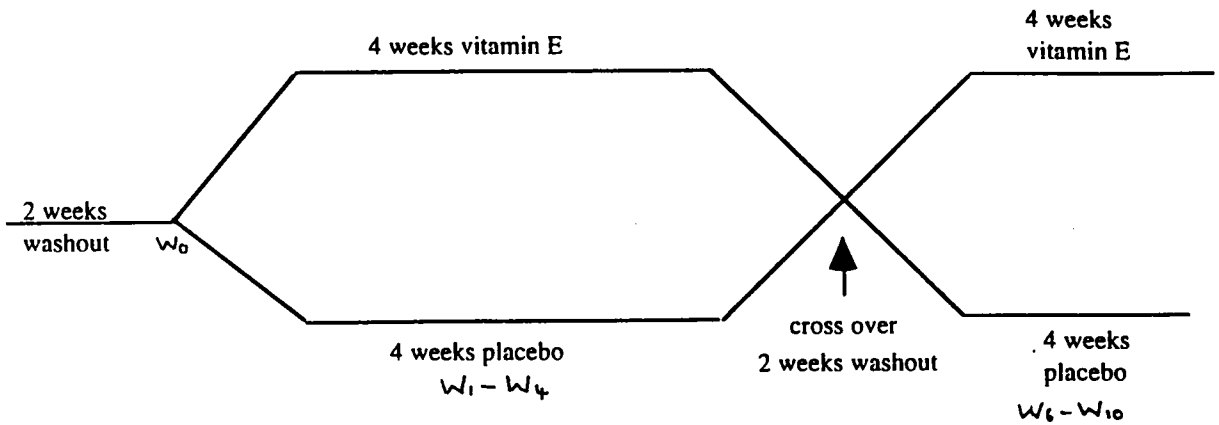
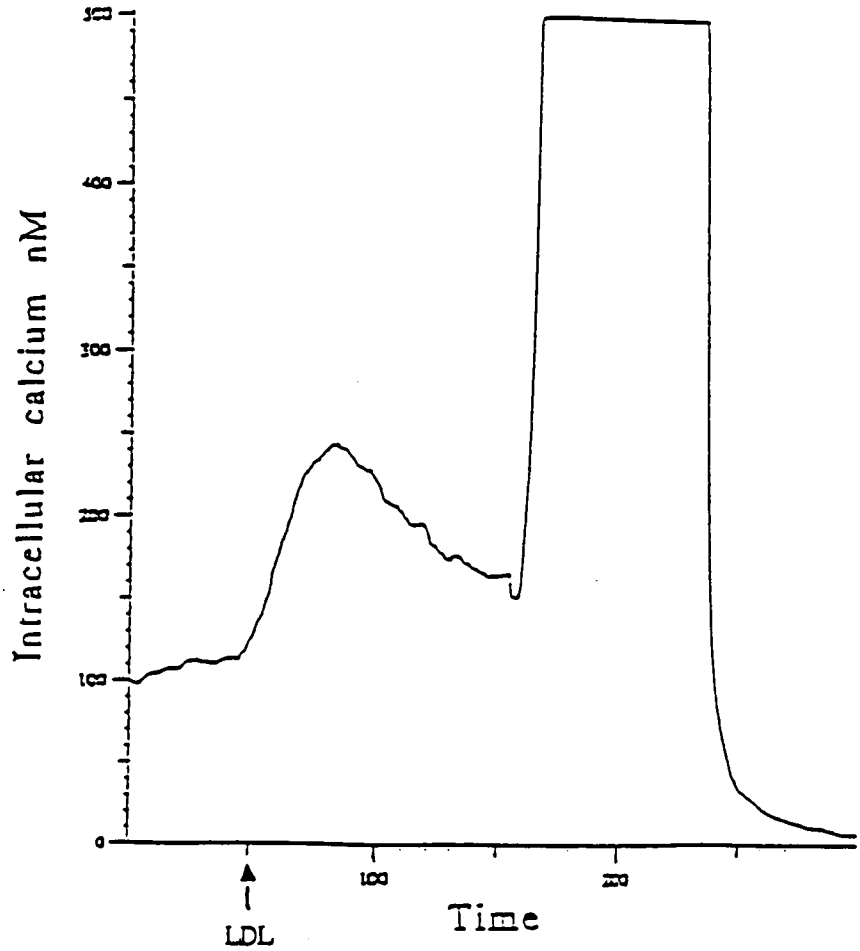
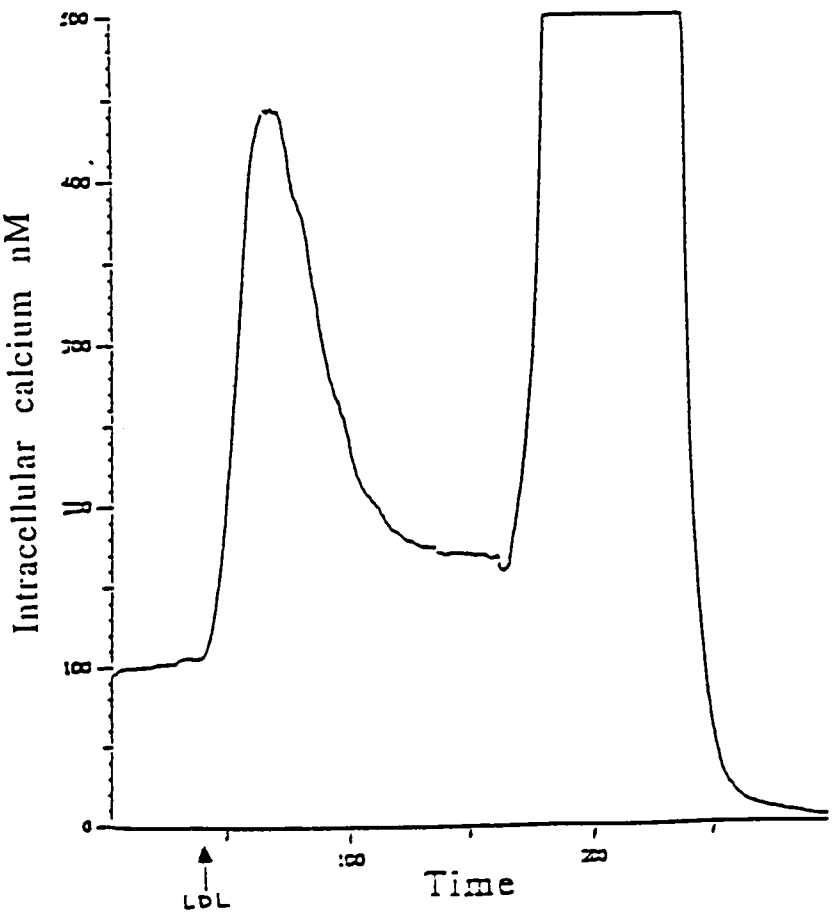


Figure 3. **α-Tocopherol Supplementation Study Design**



a.

Figure 4. $[Ca^{2+}]i$ Response in VSMCs Exposed to (a.) N-LDL and (b.) OX-LDL.



b.

Figure 4. (continued)

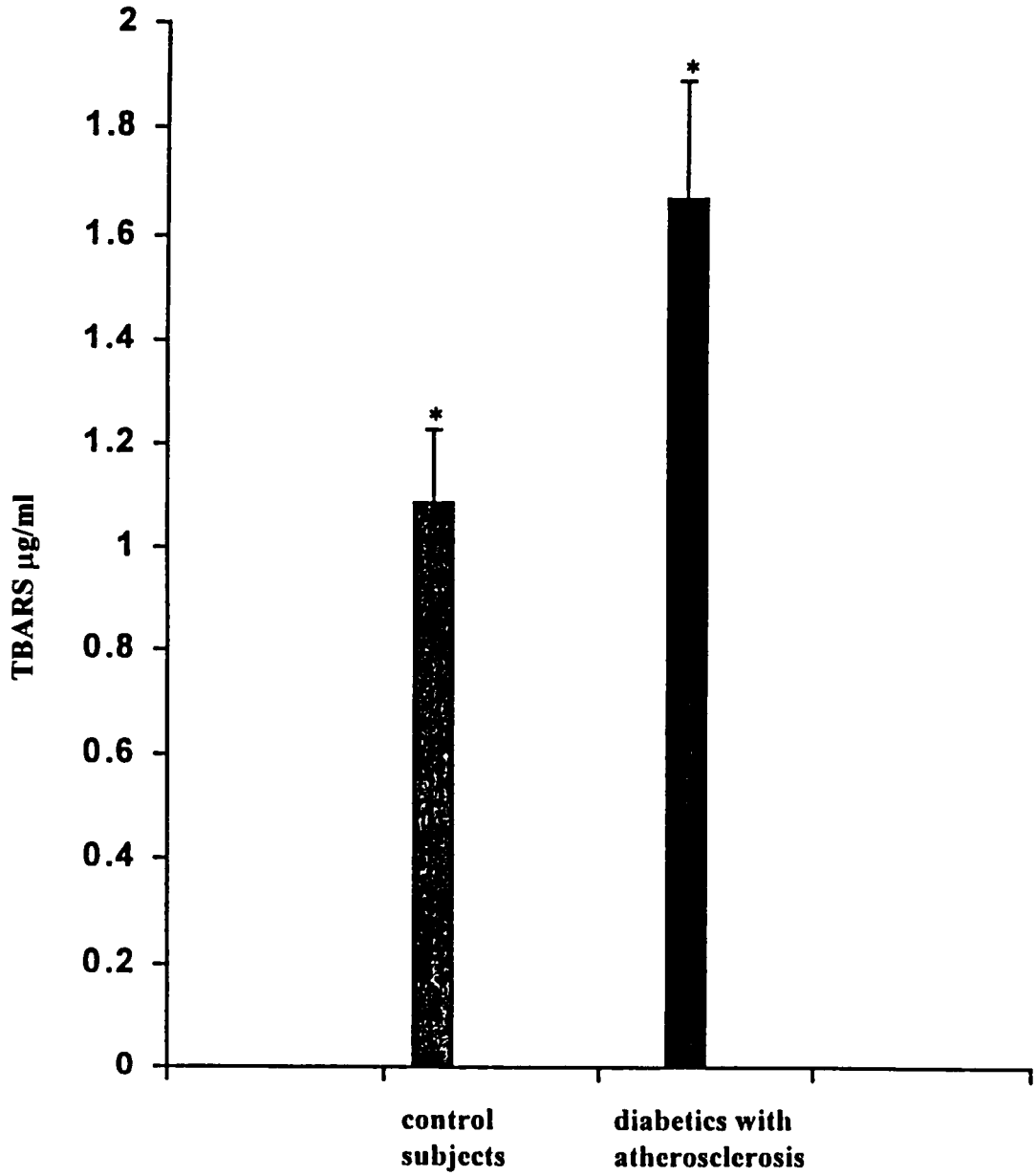


Figure 5. Levels of OX-LDL in Healthy Subjects Versus Persons with Diabetes

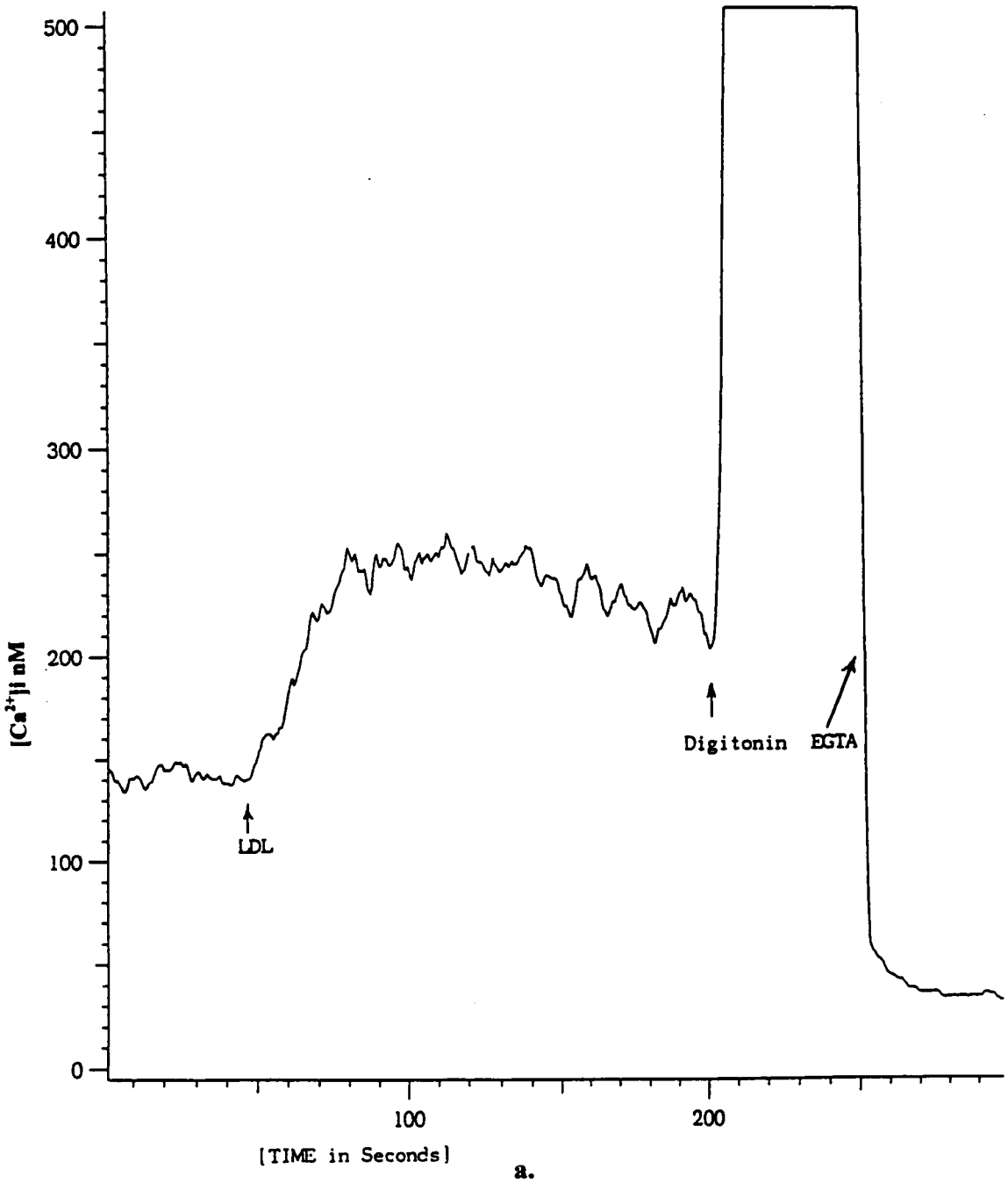
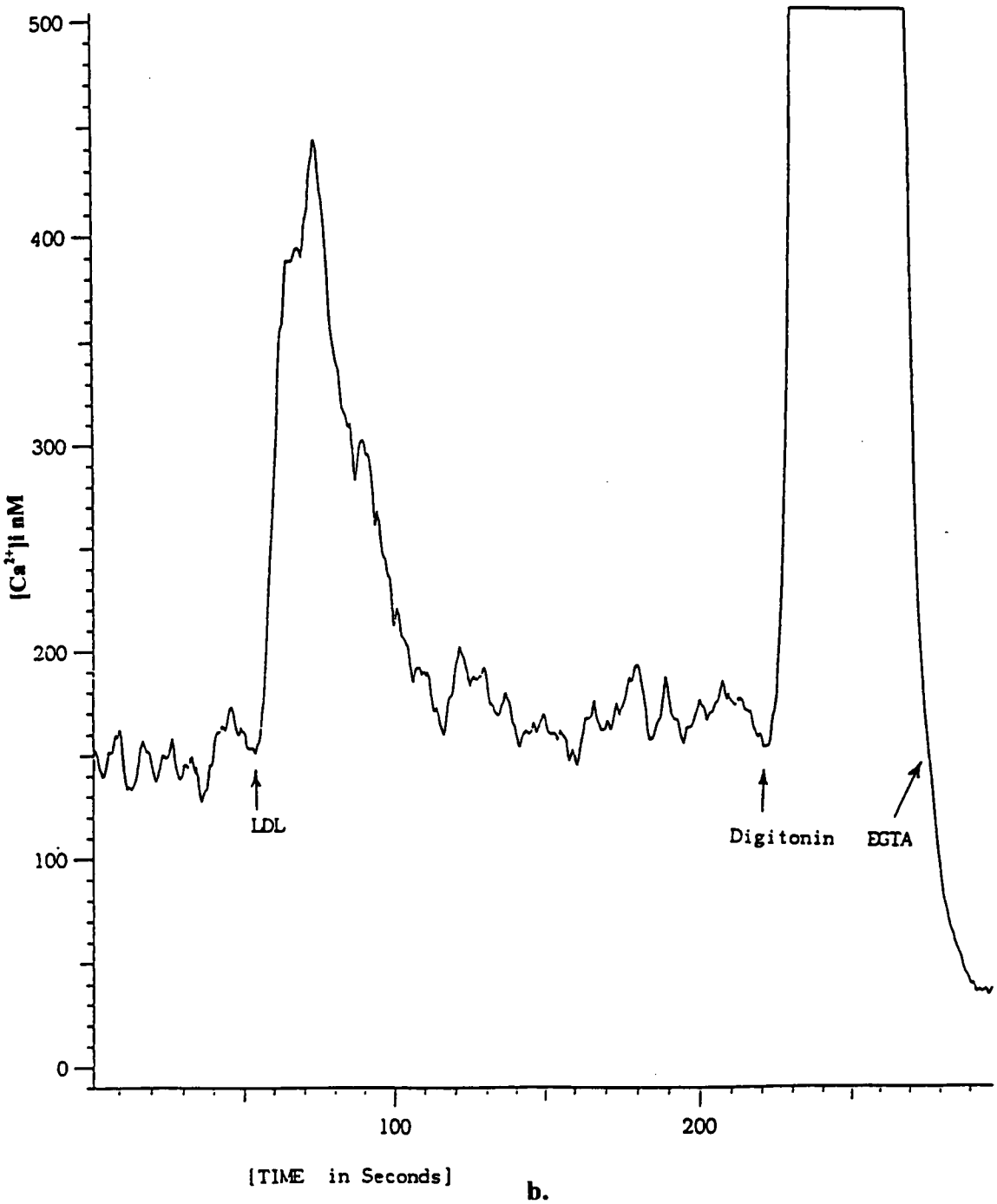


Figure 6. $[Ca^{2+}]i$ Response in VSMCs Exposed to LDL from (a.) Healthy Subjects and (b.) Persons with Diabetes Diagnosed with Atherosclerosis.



b.

Figure 6. (continued)

VITA

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