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Atherosclerosis: Oxidative Modifications of Low Density Lipoproteins

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Abbreviations:

Cu,ZnSOD	Copper, zinc superoxide dismutase
ECSOD	Extracellular superoxide dismutase
Fe^{2+}	Iron (II)
Fe^{2+}-O_2	Perferryl ion
GPx3	Plasma glutathione peroxidase
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HPLC	High-performance liquid chromatography
LDL	Low density lipoprotein
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid hydroperoxide
LPO	Lipid peroxidaton
MnSOD	Maganese Superoxide dismutase
NADH	Nicotinamide adenine dinucleotide – reduced form
$NO^{\bullet}$	Nitric oxide
$[NO^{\bullet}]_{ss}$	Nitric oxide steady-state concentration
$^1O_2$	Singlet oxygen
$O_2^{\bullet-}$	Superoxide
PUFA	Polyunsaturated fatty acid
α-TOH	α-tocopherol

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ABSTRACT

Atherosclerosis is the major source of morbidity and mortality in the developed world. It histologically defined by the archetypal plaque which contains a central lipid core and is often hypocellular. There are currently three hypotheses for the onset of atherosclerosis: response-to-injury, response-to-retention, and oxidative modifications. All three hypotheses possess different initiating events, but have a common underlying redox theme involving low density lipoprotein (LDL). Subsequently, we focus on the oxidative modification theme since the cornerstone of its argument involves LDL oxidation. The oxidative modification hypothesis states that LDL in its native/natural form is not atherogenic, rather chemical variations cause LDL to serve as a ligand for the "scavenger receptor" pathway in macrophages. This theory asserts that oxidation of LDL gives rise to numerous proatherogenic events including the activation of macrophages; leading to the production of "foam cells". Biologically, lipid peroxidation (LPO) can be initiated by a variety of oxidants; however studies show that the oxidants superoxide ($O_2^{\bullet -}$) and hypochlorous acid (HOCl) are particularly important in the development of atherosclerosis. Based on this hypothesis it is believed that inhibition of LPO and thus oxidative modification of LDL is critical to preventing and delaying atherosclerosis. Superoxide dismutase is an enzymatic antioxidant that is capable of preventing LPO and atherosclerosis by removing $O_2^{\bullet -}$ from the system. Similar both nitric oxide and α-tocopherol are non-enzymatic anti-oxidants that can work independently or synergistically to inhibit LPO. Subsequently, we also examine over-expression of plasma glutathione peroxidase as a viable means to avert atherogenesis *in vivo*.

INTRODUCTION

What is Atherosclerosis?

Atherosclerosis is derived from the Greek words athero (meaning gruel) and sclerosis (meaning hardening) and is in fact, the major source of morbidity and mortality in the developed world. The accumulation of cholesterol deposits within macrophages occuring in both large and medium-sized arteries typifies atherosclerosis.

Normally, the arterial wall consists of three well-defined concentric layers that surround the arterial lumen. Each layer has a distinctive composition of cells and extracellular matrix. The layer immediately adjacent to the lumen is the intima, the middle layer is known as the media, and the outermost layer is the arterial adventitia. A continuous layer of endothelial cells lines the luminal surface of the artery [1]. These endothelial cells regulate a variety of processes including vascular tone, thrombosis, and leukocyte trafficking through the artery to name a few.

The histological development of an atherosclerotic lesion, commonly referred to as plaque, is divided into early, developing, and mature/advanced stages. The archetypal plaque contains a central lipid core, which is often hypocellular and may include cholesterol crystals. The lipid core is separated from the arterial lumen by a fibrous cap and myeloproliferative tissue that consists of extracellular matrix and smooth muscle cells [2].

Atherogenic Hypotheses

There are currently three hypotheses about the development of atherosclerosis that are conceptually distinct and yet intrinsically related by a common theme of redox reactions involving low density lipoproteins (LDL). These three general hypotheses are: response-to-injury, response-to-retention, and oxidative modification.

Response-to-injury

This hypothesis, **Figure 1**, proposes that the first step in atherosclerosis is “endothelial denudation”. Endothelial denudation refers to the loss of endothelial cells along the intima. This

loss leads to a slue of compensatory responses that alter the normal vascular homeostatic properties of the artery [3]. An injury would consequently lead to increased endothelial permeability and deposition of both leukocyte and LDL into the sub-endothelial space. Recruited leukocytes release cytokines, vasoactive agents (such as nitric oxide ($NO^{\bullet}$)), and growth factors that further promote a proinflammatory response that is depicted by recruitment of macrophages into the arterial wall. The macrophages subsequently pick up LDL to form "foam cells", which is considered to be the hallmark of early atherosclerotic lesions.

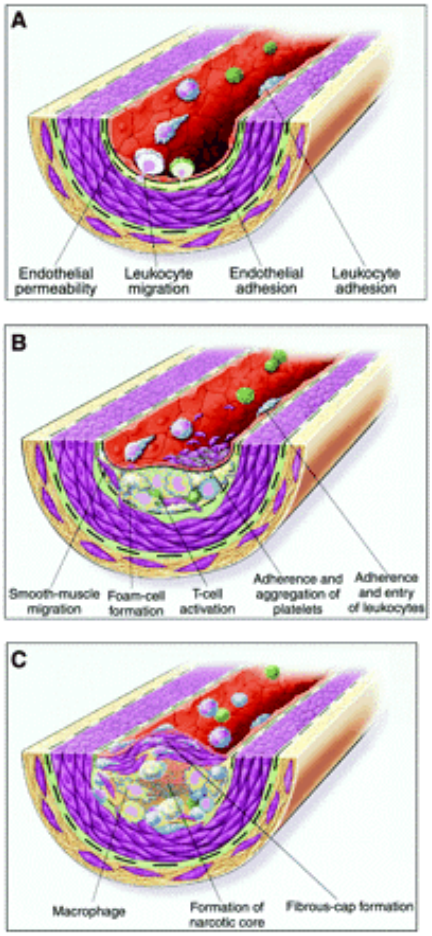

Figure 1 – Response-to-injury hypothesis of atherosclerosis [4]. A: Endothelial injury is characterized by enhanced permeability and LDL deposition. Followed by leukocyte adhesion and transmigration. **B**: Intermediate stages characterized by foam cell development. **C**: Advanced atherosclerosis shows a fibrous cap and necrosis of the lesion core.

Recently, Ross (1999) has demonstrated that endothelial "desquamation" is not common and that an intact endothelial cell layer covers the atherosclerotic lesion [4]. It was further shown that even normal segments of artery have higher entry rates of LDL than the rate of LDL accumulation; suggesting that the atherogenic lipoprotein entry into the arterial wall is not dependent on endothelial dysfunction/injury [5]. Rather an endothelial injury is capable of inducing atherogenesis but atherosclerosis in not dependent upon an injury.

Response-to-retention

This hypothesis, **Figure 2**, is based upon the supposition that lipoprotein retention is the "inticing event" for atherosclerosis [6,7,8]. Within this model, mild hyperlipidemia causes lesion

development at arterial bifurcation sites, since these sites possess apolipoprotein B-retentive molecules, such as biglycan and decorin [6]. It is believed that lipoprotein lipase activity is required to reach the subendothelial space [9]. This theory indicates a strong relationship between the retention of LDL and atherosclerosis; however it fails to demonstrate how the preservation of LDL along the vessel induces atherogenesis.

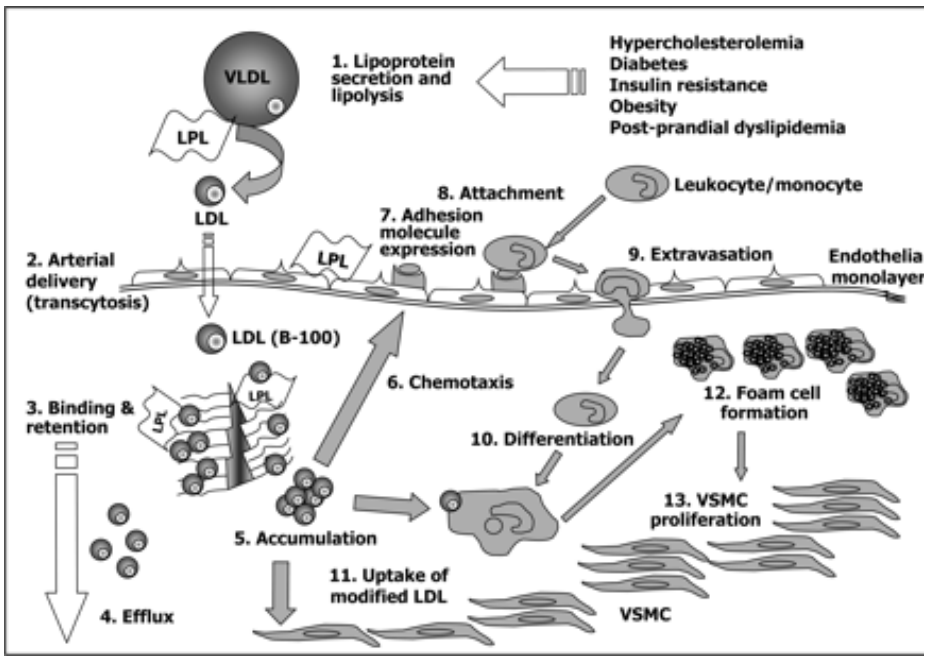

Figure 2 – Response-to-retention hypothesis of atherosclerosis [6]. Hyperlipidemia causes lesion development in bifurcation sites and is characterized by local synthesis of biglycan and decorin (apolipoprotein B-retentive molecules). The cartoon shows the initial stages of delivery, retention, and efflux of arterial LDL.

Oxidative modification

The basis of this theory, **Figure 3**, is that LDL in its native/natural state is not atherogenic, rather chemical variations cause LDL to serve as a ligand for the "scavenger receptor" pathway in macrophages [10, 11]. The chemical variation, in general, refers to redox modifications of poly unsaturated fatty acids (PUFAs) composing LDL. This theory asserts that oxidation of LDL gives rise to numerous proatherogenic events including the activation of macrophages; leading to the production of "foam cells". These proatherogenic events include: aiding the recruitment of circulating monocytes to the intimal space, inhibiting macrophages from leaving the intima, enhancing LDL uptake, and cytotoxicity that leads to loss of endothelial integrity [12].

Even though macrophages are the predominant cell type that stimulates foam cell production, early observations showed that incubation of LDL and macrophages alone did not give rise to foam cells. Rather an oxidation of LDL must occur to initiate atherosclerosis. Atherosclerotic lesions have been reported to contain hydroxyoctadecaenoic acids (a hydroxyl product of linoleic acid) [13], oxo-octadecaenoic acids [14] and F_2-isoprostanes (a secondary radical product of arachidonic acid) [15]. The presence of these compounds indicates that the oxidation of LDL polyunsaturated fatty acids is occurring. It is believed that the initial chemical change, *via* oxidation, of LDL acts as a ligand to the macrophages leadings to not only their activation but also atherosclerosis.

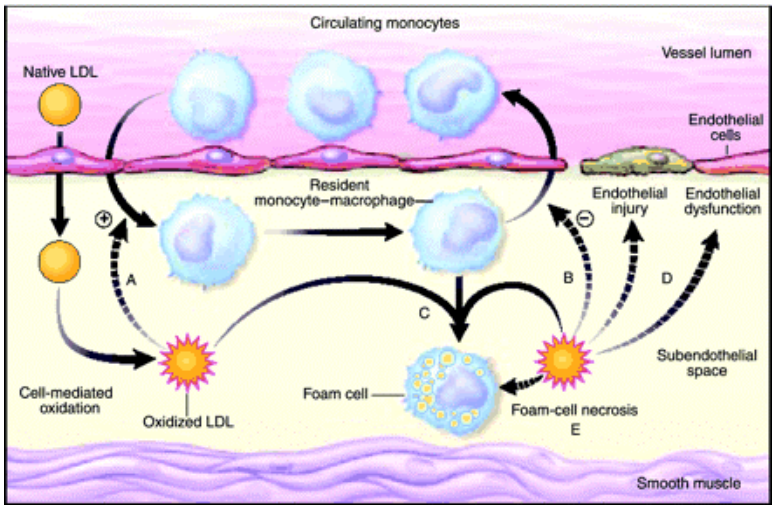

Figure 3 – Oxidative modification hypothesis for atherosclerosis [11]. LDL is entrapped in the subendothelial space where it is subject to oxidative modifications. This modification stimulates monocyte chemotaxis (**A**), prevents monocyte departure (**B**), and encourages foam cell formation (**C**). This process encourages endothelial injury (**D**), which leads to foam cell necrosis (**E**).

As previously mentioned each hypothesis is distinct, particularly in their initiating events, however all three possess common features such as a component of inflammation and an important role for LDL. The oxidative modification hypothesis is ubiquitous in the fact that it emphasizes oxidative events and redox reactions as a prominent atherogenic element. These redox events appear to be a link between lipid infiltration into the endothelial and fatty streak formation. The following will accentuate the role of LDL in atherosclerosis by discussing lipid peroxidation of LDL and mechanisms of inhibition *via* both enzymatic and non-enzymatic antioxidants. We propose that over-expression of plasma glutathione peroxidase will be capable of removing lipid hydroperoxides in LDL and in turn prevent atherosclerosis as proposed by the oxidative modification hypothesis.

OXIDATIVE MODIFICATION OF LDL

Oxidative Stress

Oxidative stress is a disruption in the cellular pro-oxidant antioxidant balance [16]. As the balance shifts towards pro-oxidants, potential damage in the form of oxidized DNA, proteins, and lipids can occur. Lipid peroxidation (LPO) can be defined as the oxidative deterioration of lipids containing two or more carbon-carbon double bonds. The propensity of PUFAs to undergo LPO is due to the *bis*-allylic methylene hydrogens, which are more susceptible to hydrogen abstraction by oxidants than fully saturated lipids [17].

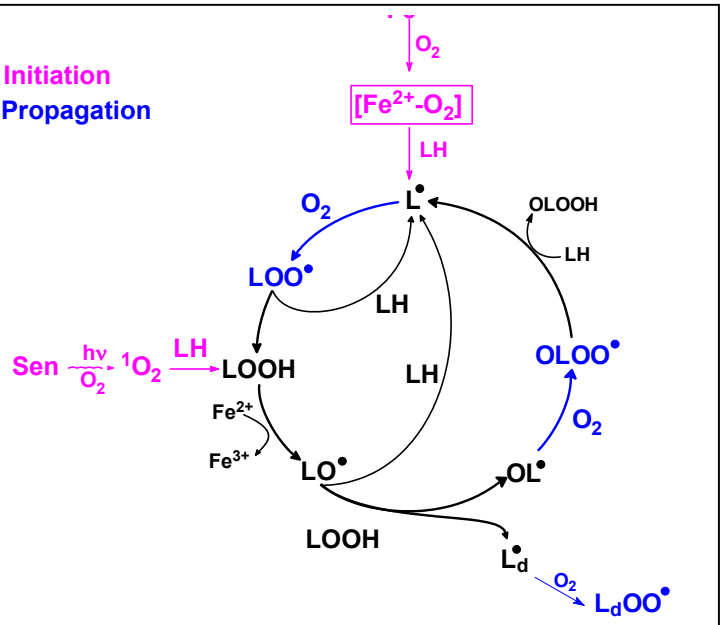

Figure 4 – Lipid peroxidation cycle.

Lipid peroxidation is an important research topic due to its involvement not only in atherosclerosis but with other conditions including inflammation, ischemia/reperfusion, and vascular disease [18,19]. **Figure 4** illustrates the lipid peroxidation cycle with perferryl ion (Fe^{2+}-O_2) initiation. LPO can however be initiated by an assortment of strong oxidants (**Rxn 1**) including hydroxyl radical, peroxyl radicals, and singlet oxygen ($^1O_2$). For the example shown in **Figure 4**, iron (II) (Fe^{2+}) reacts with oxygen to form the perferryl ion (Fe^{2+}-O_2). This perferryl ion abstracts a hydrogen from the unsaturated lipid (LH), forming a lipid carbon-centered radical ($L^{\bullet}$) that can reversibly react with oxygen (**Rxn 2**) forming a lipid hydroperoxyl radical ($LOO^{\bullet}$). The $LOO^{\bullet}$ can propagate further by abstracting a hydrogen atom from another PUFA (**Rxn 3**) giving rise to the formation of a lipid hydroperoxides (LOOH) and a new carbon-centered

radical. The reduction of the O-O bond of the lipid hydroperoxides by ferrous iron in a Fenton-like reaction (**Rxn 4**) generates an alkoxyl radical ($LO^{\bullet}$) that is capable of initiating a new peroxidation reaction [20].

$$LH + \text{oxidant} \rightarrow L^{\bullet} + \text{oxidant-H} \quad \text{(initiation)} \quad \text{(Rxn 1)}$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad \text{(propagation cycle)} \quad \text{(Rxn 2)}$$

$$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH \quad \text{(propagation cycle)} \quad \text{(Rxn 3)}$$

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + {}^{-}OH + Fe^{3+} \quad \text{(propagation cycle)} \quad \text{(Rxn 4)}$$

Lipid peroxidation can be terminated through the formation of non-radical products. Two carbon-centered lipid radicals can react with each other to from a non-radical product, **Rxn 5**. A lipid peroxyl radical could also react with a carbon-centered lipid radical to form non-radical product (**Rxn 6**). Also a peroxyl radical could get reduced by an antioxidant to form a lipid hydroperoxide and an oxidized antioxidant.

$$L^{\bullet} + L^{\bullet} \rightarrow \text{non-radical products} \quad \text{(termination)} \quad \text{(Rxn 5)}$$

$$L^{\bullet} + LOO^{\bullet} \rightarrow \text{non-radical products} \quad \text{(termination)} \quad \text{(Rxn 6)}$$

$$LOO^{\bullet} + \text{Antioxidant-H} \rightarrow LOOH + \text{Antioxidant}^{\bullet} \quad \text{(termination)} \quad \text{(Rxn 7)}$$

What is LDL?

The low density liporotein (LDL) molecules are composed of both a protein component and a lipid component. Apolipoprotein B-100 (ApoB-100) is the major protein in both low density lipoproteins as well as very low density lipoproteins and consists of 4536 amino acid residues. On the other hand, the lipid component varies according to a person's diet. Measurements by Bowery *et al.* (1993) have shown ≈ 1450 PUFAs molecules per LDL molecule and ≈ 6 - 12 α-tocopherol (α-TOH) molecules per LDL molecule [21]. With the typical profile of PUFA in LDL, this corresponds to about 1500 *bis*-allylic positions per LDL molecule; these are highly susceptible to hydrogen abstraction by oxidants and thus initiation of oxidation. The *in vivo* mechanism of LDL oxidation remains unclear, however *in vitro* studies have shown that

Cu^{2+}, azo-initiators, lipooxygenases, macrophages, endothelial, and smooth muscle cells are capable of oxidizing LDLs [22].

Low density lipoproteins circulate in the plasma and are protect against oxidation by circulating antioxidants [23]. When LDL traverses the subendothelial space, it is no longer protected by the potent array of antioxidants in the plasma. Further studies continue to indicate that circulating LDL is relatively unoxidized; in fact oxidized LDL in plasma was a "minor fraction" [24].

Oxidants of LDL

Biologically, lipid peroxidation can be initiated by a variety of oxidants (as previously mentioned), however studies have shown that superoxide ($O_2^{\bullet -}$), and hypochlorous acid (HOCl) are particularly important in atherosclerosis.

Xanthine dehydrogenase is an iron sulfur molybdenum flavoprotein that converts hypoxanthine ($4e^-$) or xanthine ($2e^-$) to produce reduced nicotinamide adenine dinucleotide (NADH) and uric acid, **Rxn 8**.

$$\text{Xanthine} \rightarrow \text{uric acid} + \text{NADH} \qquad \text{Rxn 8}$$

During times of ischemia however, xanthine dehydrogenase can undergo protolytic cleavage or thiol oxidation leading to the conversion of xanthine dehydrogenase to xanthine oxidase. Also during ischemia, large amounts of hypoxanthine are released. Consequently, during reperfusion both substrate (hypoxanthine) and enzyme (xanthine oxidase) can then produce superoxide instead of NADH, **Rxn 9**.

$$\text{Xanthine} \rightarrow \text{uric acid} + O_2^{\bullet -} \qquad \text{Rxn 9}$$

Superoxide is a reactive oxygen species (ROS) with a single unpaired electron in its outer orbital, rather than the di-radical of ground state oxygen. Superoxide can undergo a dismutation reaction in the presence of any isoform of the superoxide dismutase enzyme to form hydrogen peroxide (H_2O_2), **Rxn 10**.

$$2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2 \qquad \text{Rxn 10}$$

Even though $O_2^{\bullet -}$ can be readily produced during early atherosclerotic conditions, it is not however believed to be the direct major initiator of LDL oxidation.

Hypochlorous acid is produced enzymatically by the reaction of myeloperoxidase with H_2O_2, **Rxn 11**.

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O \qquad \text{Rxn 11}$$

Myeloperoxidase is the only enzyme in humans known to produce HOCl and is found for example in neutrophils. Hypolchlorous acid is a weak acid ($pK_a \approx 7.5$) but a strong oxidant. Hypochlorous acid is used by the immune system to kill bacteria, however in this case apparent production can induce many redox reactions. Specifically, HOCl can react with PUFAs to form α-hydroxy and α,β-unsaturated aldehydes [25]. Also a series of secondary oxidants can be produced that are capable of oxidizing other biomolecule, such as LDL that will active macrophages and lead to foam cell formation [25].

Recent evidence has been presented suggesting that HOCl is the primary oxidant of LDL in atherosclerosis. Specifically, LDL isolated from human atherosclerotic lesions contains elevated levels of chlorotyrosine [26]. Not only do these findings demonstrate that LDL is oxidatively altered but also that HOCl is a probable participant in atherosclerosis. This premise was also confirmed by Hazell *et al*. (1996) showing antibody-based evidence that apolipoprotein B-100 is modified by HOCl in human atherosclerotic lesions [27]. This evidence demonstrates the role of hypochlorous acid in the early stages of atherosclerosis *via* oxidative modifications, however HOCl is not the only oxidant capable of oxidizing the PUFAs of LDL.

ANTIOXIDANTS AND ATHEROSCLEROSIS

Since atherosclerosis is thought to be initiated by oxidative modifications; it is therefore important to examine antioxidants defenses. An antioxidant is, a “substance that, when present at low concentrations compared to an oxidizable substrate, significantly delays or prevents the oxidation of the substrate” [28]. Antioxidants can be categorized several ways, either preventive

or chain-breaking, as well as either enzymatic and non-enzymatic. Chain-breaking antioxidants stop the propagation cycle in LPO. Here, we will examine superoxide dismutase (SOD) as a preventive antioxidant enzyme and also both $NO^{\bullet}$ and α-TOH as non-enzymatic, chain-breaking antioxidants.

Enzyme Antioxidants

Many enzyme antioxidants are for the most part cell-associated proteins whose main function is to maintain the intracellular reduction state of the cell [29]. However, these enzymes are also involved in extracellular maintenance and include catalase, SODs, and glutathione peroxidases to name a few.

Superoxide dismutase

Superoxide dismutase catalyzes the reduction of superoxide ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2), **Rxn 10**. In mammals, there are three isoforms of the enzyme: copper,zinc (Cu,ZnSOD), manganese (MnSOD), and extracellular (ECSOD).

As indicated by its name, ECSOD is found outside of the cell and would likely be able to inhibit oxidation of LDL by superoxide. The enzyme is bound to hepran sulfate proteioglycans of various cells, including endothelial cells, and the matrix of connective tissue. The synthesis of ECSOD is modulated by cytokines, growth factors, vasoactive factors, and oxidants [30,31].

One of the many oxidants that are capable of oxidizing LDL and initiating atherosclerosis is superoxide. The presence of ECSOD in blood vessel would compete with LDL for $O_2^{\bullet -}$ and help diminish the occurance of LDL oxidation. Indirect evidence has shown that overexpression of ECSOD improves endothelial function in a rat hypertension model [32]. The presence of ECSOD however would not completely prevent LDL oxidation; consequently chain-breaking antioxidants are also needed.

Non-enzymatic Antioxidants

Examples of non-enzymatic, chain-breaking antioxidants during LPO are α-TOH [33] and $NO^{\bullet}$ [34].

Nitric oxide and α-tocopherol

Nitric oxide has many properties that are believed to be anti-atherosclerotic, such as inhibition of platelet aggregation, inhibition of leukocyte adhesion, inhibition of vascular smooth muscle proliferation, and $NO^{\bullet}$ can cause vasorelaxtion [35]. Since $NO^{\bullet}$ is hydrophobic it will readily diffuse into the LDL molecule. Nitric oxide can serve as a chain-terminating antioxidant by reacting with $LOO^{\bullet}$, **Rxn 12**.

$$LOO^{\bullet} + NO^{\bullet} \rightarrow LOONO \qquad k_{12} = 2 \times 10^{9}\ M^{-1}\ s^{-1}\ [36] \qquad \text{(Rxn 12)}$$

Since α-TOH is present in LDLs, we expect it to also react with $LOO^{\bullet}$.

$$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet} \qquad k_{13} = 5.9 \times 10^{5}\ M^{-1}\ s^{-1}\ [37] \qquad \text{(Rxn 13)}$$

However, there is a significant kinetic advantage for $NO^{\bullet}$ inhibition of LPO in low-density lipoprotein compared to α-TOH.

The rate of LPO inhibition is dependent on the concentration of both the anti-oxidant and $LOO^{\bullet}$:

$$\text{Rate inhibition } (NO^{\bullet}) = k_{12}\,[NO^{\bullet}]\,[LOO^{\bullet}] \qquad \text{(Equation 1)}$$

$$\text{Rate inhibition (TOH)} = k_{13}\,[TOH]\,[LOO^{\bullet}] \qquad \text{(Equation 2)}$$

To compare the rate of $NO^{\bullet}$-inhibition and the rate of α-TOH-inhibition, the concentration of $LOO^{\bullet}$ does not need to be known, **Equation 3**.

$$\text{Inhibition ratio: Rate inhibition } (NO^{\bullet}) \text{ / Rate inhibition (TOH)} \qquad \text{(Equation 3)}$$

In order to determine the rate of $NO^{\bullet}$ inhibition, we must first determine the amount of $NO^{\bullet}$ present in the lipid portion of the LDL, **Equation 4**.

$$[NO^{\bullet}]_{LDL} = K_p \times [NO^{\bullet}] \qquad \text{(Equation 4)}$$

Recent measurements by Moller *et al.* (2005) reveal that the partition coefficient (K_p) for the diffusion of $NO^{\bullet}$ from the an aqueous phase into LDL to be ≈ 3.4 [38]. Using this information,

the concentration of $NO^{\bullet}$ measured in the aqueous phase can be converted to what would be expected in LDL.

According to unpublished data by Hummel *et al.* a $NO^{\bullet}$ steady-state concentration ($[NO^{\bullet}]_{ss}$) of ≈ 72 nM is capable of inhibiting the initial burst of free radicals produced by 20 μM Fe^{2+}. The concentration of $NO^{\bullet}$ inside the LDL can be extrapolated to be ≈ 245 nM, equation 4. Also based on measurements by Hummel *et al.* the minimum $NO^{\bullet}$ concentration required to repress the increased rate of LPO is ≈ 13 nM, subsequently the minimum concentration of $NO^{\bullet}$ inside the LDL required to inhibit LPO would be ≈ 44 nM.

In order to calculate the TOH concentration inside an LDL, we must first determine the mole fraction of TOH inside the LDL, **Equation 5**.

Mole fraction (TOH) = number of TOH / number of lipids (Equation 5)

The number of TOH molecules is on average ≈ 9. The total number of lipids is ≈ 3625, where ≈ 40% are PUFAs. The TOH concentration can then be calculated by multiplying the mole fraction with the effective molarity of lipids in an LDL, ≈ 3 M, **Equation 6**.

[TOH] = mole fraction (TOH) x effective molarity of lipids (Equation 6)

Subsequently, we calculate the TOH concentration inside an LDL is ≈ 7.5 mM.

Using a measured average $[NO^{\bullet}]_{ss}$ of ≈ 72 nM, we find the ratio of inhibition rates between $NO^{\bullet}$ and TOH, **Equation 3**, to be 1 ($NO^{\bullet}$): 8.8 (TOH) in favor of LPO inhibition by TOH. If the average $[NO^{\bullet}]_{ss}$ drops to ≈ 13 nM, which is the minimum concentration required to thwart LPO, we that the ratio changes to 1 ($NO^{\bullet}$): 50 (TOH). This suggests that α-tocopherol is the principle antioxidant in LDL at low ambient levels of $NO^{\bullet}$.

It should be noted that the reaction rate constant between TOH and $LOO^{\bullet}$ is ≈ 7 times higher in LDL (**Rxn 12)** than in the cell membrane. This may also be true for the rate constant between $NO^{\bullet}$ and $LOO^{\bullet}$ from cell membrane to LDL. If this rate constant is under-estimated, then the ratio would approach 1:1 at the higher levels of $NO^{\bullet}$ of our experiments.

Our average $[NO^{\bullet}]_{ss}$ is the minimum amount required to inhibit the initial surge of free radicals produced during *in vitro* LPO. Due to the vascular constriction caused by atherosclerosis

and the role of $NO^{\bullet}$ as a vasodilator, one might expect higher concentrations of $NO^{\bullet}$ at or near the *in vivo* site of oxidation. The *in vitro* LPO data suggests that a high concentration of $NO^{\bullet}$ is not required to inhibit LPO. Subsequently the *in vivo* significance of $NO^{\bullet}$ as an effective inhibitor is under-appreciated in LDL oxidation. The presence of $NO^{\bullet}$, in conjunction with α-TOH, contributes to the inhibition of LDL oxidation.

FUTURE EXPERIMENTS

Previous work by Suarna *et al.* (1995) showed that despite "adequate" levels of α-tocopherol and ascorbate, oxidized lipids still existed with human atherosclerotic plaques [39]. On the one hand, α-tocopherol is a proven chain-breaking antioxidant; however, the initial production of LOOH cannot be removed by TOH and might be sufficient to trigger atherogenesis. Assuming that the oxidative modifications hypothesis is correct, we hypothesis that the lipid hydroperoxides produced during the oxidation of LDL lipids are the specific, initial activator of macrophages, *via* the scavenger receptor. The activated macrophages then facilitate an inflammation response, culminating in the production of an atherosclerotic plaque.

Consequently, if we can prevent LOOHs from reacting with the scavenger receptor, then we can prevent atherosclerosis. Using a ligand to block the receptor is not suitable since macrophages are necessary for a standard immune response, subsequently we further hypothesize that if we can remove LOOH by over-expression of extracellular plasma glutathione peroxidase (GPx3) (**Figure 5**) prior to its interaction with macrophages, then we have succeeded, **Rxn 14**.

$$LOOH + 2GSH \rightarrow LOH + GSSG \qquad \text{Rxn 14}$$

For this to be feasible in humans, we need to express GPx3 near or at sites likely to develop plaques. We propose that this could be accomplished by using reteroviral over-expression of GPx3 in macrophages. However for this proposal to occur *in vivo*, several *in vitro* experiments regarding GPx3 expression and function need to be accomplished.

In Vitro Experiments

First we need to develop a "disarmed" GPx3 reteroviral vector for transduction into macrophages. We need to examine GPx3 expression levels, however macrophages do not grow *in vitro*, so we will use U937 leukemia cells for our model. Since U937 cells are suspension cells, we hope this will help mimic *in vivo* conditions. We will characterize expression using both a Western blot and activity assay for hydroperoxide removal.

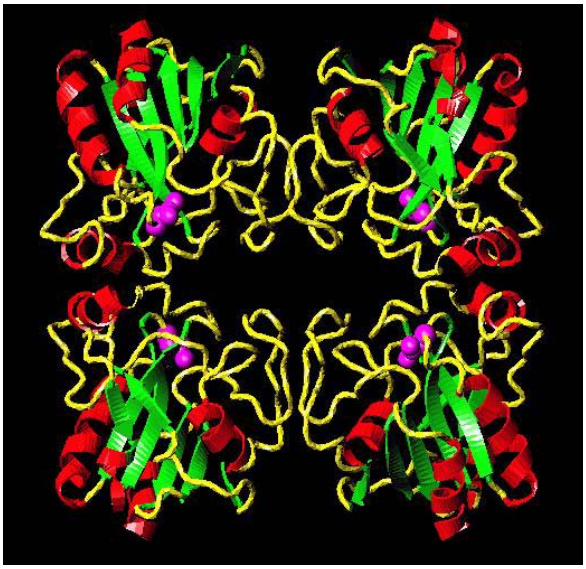

Figure 5 – Structure of plasma glutathione peroxidase.

Next, we will examine the kinetics of GPx3 removing LOOH from LDL. In these experiments, we will initiate oxidation of LDL using azo-initiators. After several minutes, the azo-initiators will fall apart and the oxidized LDL will then be isolated by centrifugation and washed in phosphate buffer solution. With a control group of oxidize LDL will quantify the present LOOH with high-performance liquid chromatography (HPLC) or the Cayman LOOH UV-Vis assay kit. With an experimental group, we will use increasing doses of GPx3 to examine the effectiveness of GPx3 to remove LOOH from LDL. The amount of LOOH after GPx3 treatments will once again be examined using HPLC or the Cayman kit.

In Vivo Experiments

Our *in vivo* model will be done in mice. We will have three experimental groups: an untreated control, an empty vector control, and expression of GPx3. All three groups will receive the same diet to ensure that any differences in the results in not due to increased intake of dietary antioxidants. Each group will have plasma removed and the macrophages will be isolated. The empty vector control group and GPx3 expressing group will be treated with titers (determined by

previous *in vitro* analysis) of their respective treatment. The mice will be followed through out there life time and be monitored for expression of GPx3, atherosclerotic plaque development, and the amount of LOOH in plasma LDL. If the model goes according to our hypothesis, we should see a delay in atherosclerotic plaque development and lower levels of LOOH in LDL of the GPx3 treated group compared to the control groups.

Toxicity and safety are a major concern when using retroviral transduction of *in vivo*. The mice will need to be monitored for any undesirable effects, such as cancers, that might develop as a result of the treatment. Also longevity of expression will need to be monitored. Macrophages have a natural turnover rate, consequently our GPx3 expression might be lost when the cell naturally dies. If macrophage turnover becomes an issue, then we could use multiple treatments over the course of a lifetime to maintain a threshold level of GPx3 in the plasma.

CONCLUSIONS

Currently there is a tremendous amount of data connecting oxidative stress and atherogenesis. The presence of reactive oxygen species in the three major hypotheses (response-to-injury, response-to-retention, and oxidative modifications) highlights its significance in proatherogenic activities. It is clear that the presence of oxidants accelerates the atherosclerotic process; however, none of the hypothesis can pinpoint a single initiating step.

The oxidative modification hypothesis emphasizes oxidative events and redox reactions as prominent and early atherogenic events. These redox events appear to be a link between lipid infiltration into the endothelial and fatty streak formation *via* the initial activation of the scavenger receptor in macrophages by oxidized PUFAs in LDL. Empirical evidence, such as the presence of F_2-isoprostanes in plaques, indicates that the oxidation of lipids is at the very least a key step in atherogenesis. Consequently, inhibition of lipid peroxidation should at least delay the pathogenesis of atherosclerosis.

Biologically, there are many oxidants that are capable of initiating LPO of LDL. In order to inhibit atherogenesis these oxidants need to be removed. Superoxide dismutase is capable of assuaging the production of superoxide that can occur during ischemic/reperfusion events when xanthine oxidase is present. The oxidation by HOCl of LDL is not preventable but the propagation of LPO can be thwarted by non-enzymatic antioxidants. Antioxidants, such as $NO^{\bullet}$ and TOH, can work synergistically as chain-breaking antioxidants in LPO.

Future atherosclerotic studies need to examine viable means of prevention. Humans are continually exposed to oxidative stresses and lipid peroxidation will always be initiated, even though we have preventive antioxidants. We are also capable of terminating the LPO cycle with chain-breaking antioxidant. However to prevent atherosclogenesis we need to thwart the interaction between LOOH and macrophages. If we can remove the LOOHs with GPx3 before it is able to activate the macrophage then we may be able to prevent atherosclerosis. Chain-breaking antioxidants are necessary to inhibit uncontrolled production of LOOHs, but they cannot repair the damage already done. Consequently, we believe that over-expression of plasma glutathione peroxidase will preclude atherogenesis *in vivo*.

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