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Invited Commentary

Molecular Mechanisms of the Copper Dependent Oxidation of Low-density Lipoprotein*

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There is little doubt that oxidative modification of low-density lipoprotein (LDL) is an important process during atherogenesis. This conclusion has been derived in a relatively short period of time since the initial descriptions of LDL oxidation with a significant contribution from Professor Esterbauer and colleagues. In this short overview, we have described the mechanisms by which copper promotes LDL oxidation focussing on the importance of lipid hydroperoxides in this process. These mechanisms are discussed in the context of the ongoing debate as to relevance of metal dependent LDL oxidation *in vivo* and as a model reaction for assessing antioxidants.

Keywords: Copper, lipid hydroperoxides, low density lipoprotein

INTRODUCTION

The evidence that oxidised LDL (oxLDL) mediates pathological events important in atherosclerosis has been reviewed extensively.[1–3] The oxidative hypothesis for atherosclerosis is based upon a number of lines of evidence. For example material isolated from macrophage derived from cells and atherosclerotic arteries is biologically and chemically similar to LDL which has been oxidised *in vitro*. More recently it has been shown that partially oxidised LDL present in the circulation (which is more electronegative than native LDL) or within the vessel wall (termed minimally modified LDL) can be isolated. These LDL fractions contain elevated levels of peroxides and can mediate some of the initial processes which are involved in atherogenesis.[2] It is fair to say that without a controlled and simple procedure to prepare oxLDL many of these studies could not have been undertaken and it is striking how often copper dependent LDL oxidation is used to fulfil these requirements. Hermann Esterbauer and his co-workers contributed more in this respect than most in

* This brief review is dedicated to the memory of Prof. Dr. Hermann Esterbauer, a pioneer in the field.

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characterising the biochemistry of oxLDL and describing the reaction between copper ions and LDL.[3–6] A survey of the literature using the keywords copper and LDL, yields approximately 600 citations. The reports listed demonstrate the widespread use of this model reaction ranging from identification of risk factors for atherosclerosis, assessing antioxidant therapies, to gaining insights into metal dependent oxidation reactions. Here we will focus on the impact of this model reaction system on the field of atherosclerosis research with particular emphasis on the mechanisms by which copper ions mediate oxidative modification of LDL.

CHARACTERISTICS OF REACTION BETWEEN COPPER AND LDL

The seminal characterisation of the oxidation of LDL by copper appeared in Free Radical Research Communications in 1989.[4] These studies gave the original detailed description of the copper dependent oxidation of LDL. This particular protocol has been reproduced in numerous laboratories throughout the world and is remarkable for its consistency and ease of application. An example of this type of experiment is shown in Figure 1. Such data is classically divided operationally into the following phases: (1) an initial lag phase in which the rate of conjugated diene formation is relatively low, (2) a propagation phase in which oxidation proceeds at its greatest rate and (3) termination phase in which the absorbance increase at 234 nm ceases and then increases but at a much slower rate.

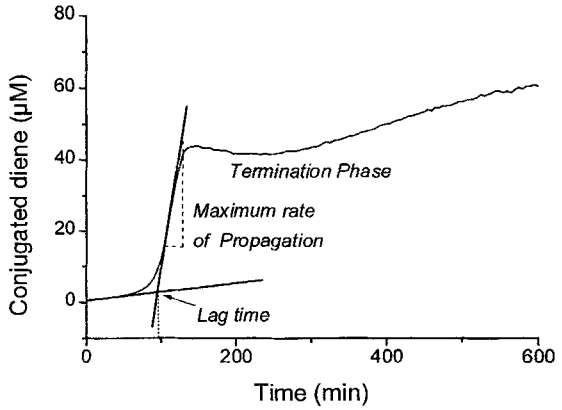

FIGURE 1 Copper Dependent LDL oxidation – Formation of conjugated dienes. A typical time profile for conjugated diene formation is shown illustrating how the lag time and maximal rate in the propagation phase are determined. The lag time is defined as the time at the point of intersection of the two tangents. The maximum rate of propagation being defined as the slope during the propagation phase. Conjugated dienes formation is followed by monitoring the absorbance increase at 234 nm versus a blank consisting of LDL without addition of copper. Reactions are initiated by addition of copper (0–10 μM) to LDL (50–100 μg/ml) in PBS at 37°C.

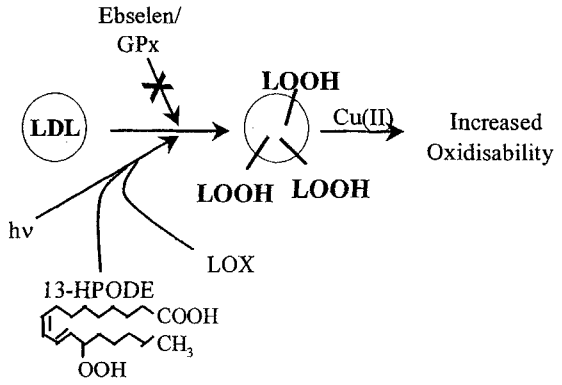

FIGURE 2 Evidence implicating a central role for Lipid hydroperoxides in copper dependent oxidative modification of LDL. Treatments which increase the lipid hydroperoxide concentration in LDL (e.g. lipoxygenase mediated insertion, photo-oxidation (hν), addition of 13-HPODE) enhance the rate of copper dependent oxidation of LDL. Accordingly, treatments which lower the LDL lipid hydroperoxide concentration (e.g. reduction by glutathione peroxidase or ebselen) inhibits copper dependent LDL oxidation.

COPPER AND LIPID HYDROPEROXIDES

The involvement of lipid hydroperoxides (LOOH) in copper dependent LDL oxidation is not disputed and a substantial body of evidence shows that LOOH are intimately required for oxidation to occur (Figure 2). For example, reduction of endogenous or seeding lipid hydroperoxides, to the corresponding hydroxides, either by the glutathione peroxidase mimic, ebselen or triphenylphosphine treatment inhibits

Cu^{2+} mediated oxidation of LDL or simple lipid based systems.[7,8] Alternatively, increasing the lipid peroxide content of LDL by either lipoxygenase dependent insertion,[9–11] addition of fatty acid peroxides,[9,10] or *in situ* generation by photo-oxidation,[12] enhances the rate of Cu(II) mediated oxidation.

The "Lag Phase"

One corollary of the hypothesis that oxidation of LDL contributes to the development of atherosclerosis is that the lower the resistance of plasma LDL the greater the chance an individual has of developing the disease. Copper-dependent oxidation of LDL remains one of the few simple and direct methods available to test this hypothesis. Table I shows the risk factors identified for atherosclerosis through use of copper mediated LDL oxidation system. The mechanism by which copper oxidises LDL becomes important, therefore, in order to understand why plasma LDL is more oxidisable in patients at a higher risk of developing atherosclerosis. The operational parameter defined by most investigators using this method is the "lag phase" (see Figure 1) which represents the intrinsic resistance of a given sample of LDL to oxidation. To understand this the characteristics of the oxidation process must be briefly described. There are several factors which govern the oxidisability of LDL including the lipid composition and size of the particle, the concentration of chain breaking antioxidants and the levels of seeding or endogenous lipid hydroperoxides (LOOH).[3,9,10,22–26]

TABLE I Identification of risk factors for atherosclerosis in man using copper dependent oxidative modification of LDL

Risk factor	*References*
Coronary artery disease	13,14
Hypertension	15,16
Insulin-dependent diabetes mellitus (with poor glycaemic control)	17–19
Low Vitamin E	3,20,21

Lag Phase and Antioxidants

It was demonstrated that the transition from the lag to the propagation phase occurs as the endogenous antioxidants to LDL (e.g. α-tocopherol, ubiquinol-10) are consumed.[3,4,27,28] It was quickly realised, therefore, that this assay could be used to assess the efficiency of antioxidants towards inhibiting the oxidation process, with an increased lag time indicating an antioxidant effect. Thus, since 1989 numerous studies have used this oxidation system to test the efficacy of various natural and synthetic antioxidants which potentially could be used as anti-atherosclerotic agents. An example is shown in Figure 3, where the lipophilic peroxyl radical scavenging antioxidant butylated hydroxy toluene (BHT) (which has been shown to prevent atherogenesis in animal models of the disease[29]) is shown to inhibit LDL oxidation mediated by copper ions.

Lag Phase and Lipid Hydroperoxides

Simple lipid based systems such as phosphatidyl choline liposomes, are also oxidised by copper ions. These lipid systems contain no detectable levels of antioxidants, and the time course of

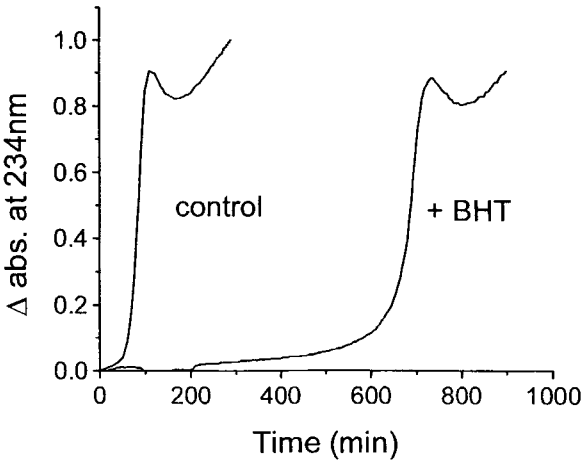

FIGURE 3 Inhibition of copper dependent LDL oxidation by BHT. LDL(75 μg/ml) oxidation was promoted by the addition of copper (10 μM) to control LDL or LDL containing BHT (10 μM). BHT inhibited oxidation as indicated by the increase in lag time (from approx. 50 min to approx. 650 min).

oxidation is similar to that seen with copper dependent oxidation of LDL (Figure 1).[30] Therefore, the presence of a lag phase is not simply attributable to chain breaking antioxidants, but is an intrinsic property of the lipid peroxidation process itself.

Operationally, the transition from the lag phase into the propagation phase can be considered as a shift from one rate limiting step to another. The observed rate of the oxidation process will be determined by the slowest reaction. Thus, the increase in rate of oxidation observed going from the lag to the propagation phase represents a shift in the rate limiting step between two different reactions. This shift occurs as the lipid peroxide concentration increases and is consistent with the sequence of reactions shown in scheme 1. As oxidation is progressing the lipid peroxide, and hence the lipid peroxyl radical concentration is increasing (Scheme 1, reactions 1–3). Peroxyl radicals can themselves initiate lipid peroxidation reactions, thereby increasing lipid hydroperoxide levels and reforming peroxyl radicals (Scheme 1, reactions 4–5). This makes the reaction autocatalytic in nature.[28,31] However, a maximum rate is seen (i.e. the propagation phase), and this is likely to be due to peroxyl radical termination reactions (Scheme 1, reaction 6). The square dependency of the rate of this termination process on the peroxyl radical concentration, will mean that an increase in the rate of peroxyl radical formation will be accompanied by a large increase in the rate of peroxyl radical termination. Thus the maximum observed rate in the propagation phase is likely to be limited by the rate of bimolecular termination of peroxyl radical (Scheme 1, reaction 6). In the lag phase, the LOOH and peroxyl radical concentration is relatively low, and thus the termination rate is negligible. At this stage of the reaction the rate limit is the rate of initiation i.e. the interaction between copper ions and LOOH (Scheme 1, reaction 1).

$Cu^{2+} + LOOH \longrightarrow Cu^{+} + LOO\bullet$ ....Reaction 1

$Cu^{+} + LOOH \longrightarrow Cu^{2+} + LO\bullet$ ....Reaction 2

$LO\bullet + O_2 \longrightarrow OLOO\bullet$ ....Reaction 3

$LOO\bullet/OLOO\bullet + LH \longrightarrow LOOH/OLOOH + L\bullet$ ....Reaction 4

$L\bullet + O_2 \longrightarrow LOO\bullet$ ....Reaction 5

$LOO\bullet + LO\bullet$
$LOO\bullet + LOO\bullet$
$LOO\bullet + OLOO\bullet$
$OLOO\bullet + OLOO\bullet$
Termination ....Reaction 6

SCHEME 1 Reactions describing copper mediated lipid peroxidation.

The termination phase starts when one of the substrates for the peroxidation process e.g. polyunsaturated fatty acids (LH) or oxygen are consumed. A transitory decrease in absorbance at 234 nm is seen followed by an increase (Figure 1). The decrease has been attributed in part to the formation of LOOH decomposition products such as aldehydes and ketones which have different absorbance maxima than conjugated dienes,[3] and the latter increase due to the formation of 7-oxocholesterol, a cholesterol oxidation product containing a conjugated diene moiety.[32]

Such a sequence of reactions also explain why very low molar equivalents of the metal are capable of inducing 10–20 times the amount of lipid peroxidation products (see Figure 1). This result is consistent with copper acting as a catalyst in the oxidation of LDL and this was subsequently shown to be the case[4,5,7,9,30] with cupric ions (Cu^{2+}) initially being reduced by a lipid peroxide and then the resulting cuprous ions (Cu^{+}) being oxidised by another lipid peroxide. Since this is a one electron redox cycle the products derived from the lipid peroxides are lipid peroxyl or alkoxyl radicals which can then serve to propagate the lipid peroxidation reaction.[31]

REDUCTION OF Cu^{2+} BY LIPID HYDROPEROXIDES

Some elements of the scheme described above were initially difficult to substantiate, and the

premise that LOOH can reduce Cu^{2+} ions was challenged on the basis of unfavourable thermodynamic calculations[3] (the oxidation of Cu^{+} by LOOH is not disputed and, in fact, is thought to represent the oxidative arm of the redox cycle). This has led to the proposition that other reductants, endogenous to LDL are responsible for reduction of Cu^{2+} to Cu^{+}. Candidates include α-tocopherol and amino acid residues on apoB. Alpha-tocopherol, regarded as the principal antioxidant in LDL has been shown to reduce Cu(II) to Cu(I) with the concomitant formation of the α-tocopheroxyl radical.[33,34] Oxidation can then proceed either by LOOH dependent oxidation of Cu(I) to Cu(II) and/or by so called tocopherol mediated peroxidation.[34,35] However, this latter process will only become significant under conditions of low radical flux[36,37] and when reducing agents such as ascorbate that are capable of regenerating α-tocopherol from the α-tocopheroxyl radical, are not present.[38]

Another recently proposed reductant for Cu(II) in LDL is apoB and the previously observed saturation kinetics of Cu(II) dependent LDL oxidation have been interpreted as representing a finite number of Cu(II) binding sites on apoB.[5] In agreement Kuzuya *et al.* have reported that a large proportion (~80%, equivalent to Cu : LDL molar ratios of ~13) of added Cu(II) is bound to apoB.[39]

However, more recent studies using copper dependent oxidation of lipids in the absence of any α-tocopherol or protein suggest that LOOH, in the presence of oxidisable fatty acids, can in fact directly reduce Cu^{2+} to Cu^{+} further emphasising the important role of LOOH in copper dependent oxidative modification of LDL. This process is summarised in Figure 4. Furthermore the kinetics of oxidation are saturable in the absence of protein, implying that copper can bind to lipid hydroperoxides. This also suggests that although protein may chelate added copper ions, amino acids are not an absolute requirement for copper oxidation of LDL to occur.

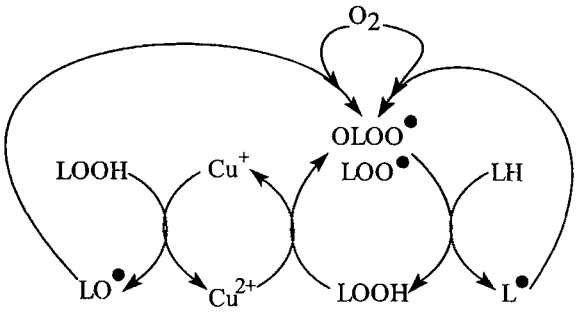

FIGURE 4 Schematic representation of the oxidation of LDL by copper. The interaction of copper with LOOH is shown forming peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals. The alkoxyl radical undergoes a rearrangement reaction and incorporates a further oxygen molecule to form a peroxyl radical ($OLOO^{\bullet}$). The peroxyl radicals can terminate by abstracting an allylic hydrogen form a polyunsaturated fatty acid (LH) thereby generating more LOOH which then contribute to the peroxide pool resulting in an acceleration in rate of peroxidation. The driving force throughout the entire course of oxidation is the redox cycling of copper between the cupric and cuprous oxidation states.

LIPID PEROXIDES ENDOGENOUS TO LDL ARE FORMED *IN VIVO* AND DECREASE RESISTANCE TO OXIDATION BY COPPER

Lipid hydroperoxides are also important in LDL oxidation mediated by other potential oxidants (e.g. haem proteins, reviewed elsewhere in this issue). The recent advent of sensitive and specific assays for lipid peroxides has allowed investigators to measure the concentration of lipid peroxide groups in lipoproteins and plasma.[12,40,41] In the most extensive study to date, a mean concentration of 240 pmol lipid peroxides/mg LDL protein was reported in a group of asymptomatic individuals which varied as much as 70 fold from donor to donor.[26] Lipid hydroperoxide concentrations in isolated LDL measured by chemiluminescence techniques, have been estimated to be around 250 pmol/mg LDL protein. These values are however, approximately 80-fold lower than the concentrations measured by either the FOX assay (ferrous-xylenol orange

assay) or iodometric techniques.[26,41,42] However, chemiluminesence methods may underestimate peroxide levels, and more recent studies suggest that the plasma concentrations of lipid hydroperoxides, in healthy individuals, are between 3–4 μM and that approximately 60–70% of these peroxides are present within the LDL fraction.[41]

It is unlikely that there is a single source of oxidants leading to the formation of lipid peroxides in native LDL. They may arise through interaction of the lipoprotein with the luminal surface of endothelial cells in those regions where nitric oxide, an anti-atherogenic agent,[43–47] levels are low. Other possibilities include the transfer of lipid peroxides from cell plasma membranes into the lipoprotein. The increased permeability of the vasculature in a lesioned area could also result in the transfer of lipid peroxides from the sub-endothelial regions to plasma lipoproteins. An example of such a mechanism is the insertion of lipid peroxides through oxidation from oxidant species such as peroxynitrite[48] or through the action of enzymes such as lipoxygenase.[9–11] Such a mechanism is summarised in Figure 5.

Furthermore, the principal carrier of lipid hydroperoxides *in vivo* has been shown to be LDL.[41] Lipid hydroperoxides may also have direct effects on cellular processes[49] some of which are pro-atherogenic. For example 13-HPODE, a lipid peroxide derivative of linoleic acid stimulates monocyte binding and entrance into the vessel wall by inducing synthesis of chemotactic factors[2,50] However, it is likely that much of the damage caused by peroxides occurs via their decomposition to radical species which can then participate in oxidative reactions that lead to modification of LDL and progression of atherosclerosis.[51] The *in vitro* system of copper dependent LDL oxidation can be therefore also be viewed as an assay for endogenous or 'seeding' lipid peroxides, which in turn relates to the degree of LDL oxidation that has occurred *in vivo*.

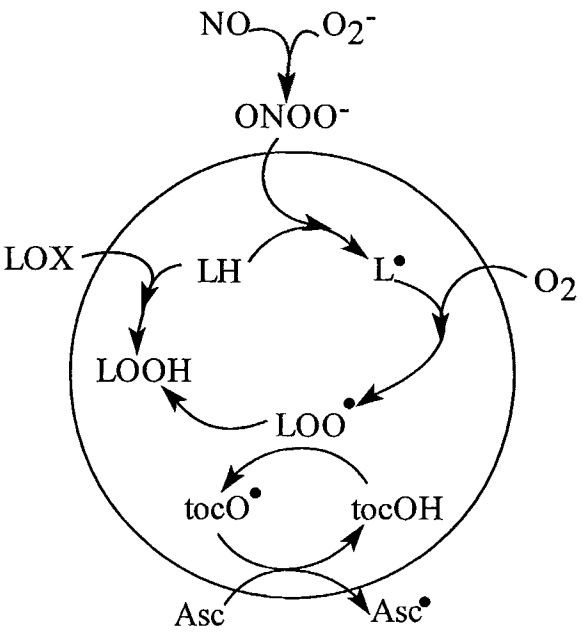

FIGURE 5 *Formation of seeding peroxides in LDL* Any oxidant e.g. peroxynitrite (ONOO) capable of initiating lipid peroxidation in LDL abstracts a hydrogen atom from a polyunsaturated fatty acid (LH) to form an alkyl radical ($L^\bullet$) which then reacts with O_2 to form a peroxyl radical ($LOO^\bullet$). The fate of this radical then depends on what is available for it to react with. In plasma LDL it is most likely to react with α-tocopherol (tocOH), which in turn forms the tocopheroxyl radical ($tocO^\bullet$). In plasma this is rapidly regenerated by ascorbate (Asc). The net result is the formation of a lipid peroxide group (LOOH) and no change in the α-tocopherol content. Direct formation of LOOH can also be mediated by enzymes such as lipoxygenases (LOX). In this way the circulating native lipoprotein can slowly accumulate lipid peroxides (LOOH) during its metabolic lifetime.

IS COPPER DEPENDENT LDL OXIDATION RELEVANT TO THE ATHEROSCLEROTIC PROCESS

The presence of metal chelating proteins in the plasma ensures that metal dependent oxidation of LDL is unlikely to occur *in vivo*. However, several lines of evidence suggest that metals may be involved in mediating LDL oxidation *in vivo*. For example, higher serum concentrations of copper ions are associated with accelerated progression of atherosclerosis and with elevated levels of auto-antibodies against oxidised LDL.[52–54] Perhaps the most compelling evidence

that free metal ions are mediators of oxidative damage in atherogenesis the detection of redox active copper and iron ions in 'gruel' samples obtained from advanced atherosclerotic lesions.[55] Recent studies suggest that these metals may be released from copper and iron containing proteins, such as caeruloplasmin and transferrin, by pro-oxidant reactions which can occur in the developing atherosclerotic lesion.[56,57] Caeruloplasmin directly has also been proposed to mediate LDL oxidation.[58] A role for metal ions in promoting oxidation reactions can be envisaged therefore, particularly when seeding peroxide levels in LDL are high.

SUMMARY

By virtue of its ease and reproducibility, the copper-LDL oxidation system has probably become the most widely used method to make oxidised LDL in the laboratory. The importance of this reaction in our current understanding of the mechanisms and efficiencies of a variety of antioxidants in preventing LDL oxidation, and hence atherosclerosis, cannot be understated. However, although it may appear a relatively simple oxidation system the mechanisms by which copper promotes LDL oxidation is still debated, a testament to the complexity of this process. In interpreting data regarding the antioxidant effect of any given compound in this system therefore, a number of possible mechanisms must be considered. For example metal chelation, peroxyl radical scavenging, and reduction of lipid peroxides are all potential antioxidant mechanisms. Professor Esterbauer and colleagues contributed much of this insight through their own contributions and also stimulated many to utilise and investigate copper dependent LDL oxidation. An enduring and consistent attribute of Hermann's work was the thorough, analytical description of biological phenomenon which has been, and still remains, so essential for the rest of us to build upon.

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