Peroxynitrite and atherosclerosis

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Introduction
The oxidative modification of low-density lipoprotein (LDL) within the artery wall is thought to be a key event in the formation of an atherosclerotic lesion [1]. Modification in vitro can be achieved by initiating peroxidation reactions within the lipid phase of the LDL particle [2]. Agents that can accomplish this form of modification include copper ions, haem proteins, organic peroxyl radicals and some types of cell in serum-free culture including macrophages, smooth muscle cells and endothelial cells. The processes that contribute to the modification of LDL in vivo, however, are poorly understood. It has become clear that lipid-mediated modification of LDL requires both the generation and the breakdown of lipid hydroperoxide groups. One current idea is that the enzyme lipoxygenase is able to insert hydroperoxide groups into unsaturated fatty acid side chains of the LDL particle [3]. This hypothesis requires the presence of agents that are able to promote the breakdown of peroxide, once inserted. Possible candidates for this role include transition metal ions (e.g. copper and iron), either free or bound in the prosthetic group of proteins (e.g. haem proteins such as haemoglobin).

An alternative hypothesis is that peroxide formation and breakdown occur as a single process. Free radical-initiated peroxidation is such a process. The abstraction of a hydrogen atom from an unsaturated fatty acid leads to a chain reaction within the LDL particle [4]. This reaction is mediated by the lipid peroxyl radical and can result in the modification of LDL without the requirement for transition metal ions. The initiation of this reaction in vivo would require the formation of a highly reactive free radical oxidant such as the hydroxyl radical. Hydroxyl radicals can be generated in vitro from chelated iron(II) and hydrogen peroxide in a reaction known as the Fenton reaction. The relevance of this reaction in vivo remains controversial. More recently, another possible route for hydroxyl radical formation has been described. This involves the interaction of nitric oxide and superoxide. Nitric oxide is generated by the vascular endothelium and acts to modulate vascular tone [5]. It achieves this by trig-

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; REM, relative electrophoretic activity.
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...triggering smooth muscle relaxation within the artery wall. Superoxide is generated at low levels by most cell types due to 'leakage' of partially reduced forms of oxygen from the respiratory process [6]. Under certain conditions, such as in the stimulated macrophage, superoxide is generated in greater amounts by the enzyme NADPH oxidase.

The product of the reaction between nitric oxide and superoxide is peroxynitrite [7]:

\[ \text{NO}^+ + \text{O}_2^- \rightarrow \text{ONOO}^- \] (1)

We have shown that the simultaneous generation of both nitric oxide and superoxide leads to the production of an oxidant with hydroxyl radical-like reactivity [8]. We have also shown that the simultaneous generation of these two free radicals can result in oxidative modification of LDL [9]. Peroxynitrite can be synthesized from the reaction between hydrogen peroxide and nitric acid as follows:

\[ \text{H}_2\text{O}_2 + \text{HNO}_2 \rightarrow \text{ONOOH} + \text{H}_2\text{O} \] (2)

Peroxynitrite synthesized in this way has been shown to generate an oxidant with similar reactivity to the hydroxyl radical [10] and has also been shown to be able to initiate lipid peroxidation [11]. We show here that chemically synthesized peroxynitrite is also able to modify LDL, a result consistent with the hypothesis that the simultaneous generation of nitric oxide and superoxide exerts its oxidative effect through the production of this compound.

**Materials and methods**

LDL was prepared from human plasma by the method of Chung et al. [12]. Peroxynitrite was synthesized by mixing hydrogen peroxide and sodium nitrite in hydrochloric acid followed by rapid addition of sodium hydroxide [13]. Peroxynitrite is stable when frozen as the anion at high pH. Electrophoretic mobility of LDL was measured using the 'Paragon' system supplied by Beckman. Relative electrophoretic mobility (REM) was calculated as the ratio of the distance migrated by the sample to that of an untreated control. Vitamin E was measured by normal phase h.p.l.c. using a LiChrospher 100 CN (5 \( \mu \)m) column (Merck) and heptane/propan-2-ol (99:1) as the mobile phase [14]. Macrophage uptake of modified LDL was measured by monitoring the incorporation of \[^{14}\text{H}\]oleate into cholesteryl ester by THP-1 macrophages. All other incubations were performed in phosphate-buffered saline (PBS) in the presence of diethylenetriaminepenta-acetic acid (DTPA) to inhibit any oxidation that may occur due to the presence of contaminating metal ions.

**Results**

The oxidative modification of LDL has been characterized extensively and is associated with many changes to the structure and composition of the lipoprotein [2]. One such change is an increase in the net negative charge of the lipoprotein particle. This has been shown to occur by reaction of lysine residues of the LDL-associated apoprotein (apoB) with reactive aldehydes formed during the oxidation of LDL lipid [15]. The effect on REM of incubating LDL with increasing concentrations of peroxynitrite is shown in Figure 1. Incubation of LDL with peroxynitrite resulted in an increase in the electrophoretic mobility of the lipoprotein particle. When 800 \( \mu \)M peroxynitrite was used, the REM of the LDL was 3.5. This can be compared with an REM of between three and five that is achieved when LDL is incubated overnight with 100 \( \mu \)M Cu(II) ions.

Incubation of peroxynitrite-modified LDL with phorbol myristate acetate (PMA)-stimulated

![Figure 1](image-url)

**Figure 1**

The effect of peroxynitrite on the electrophoretic mobility of LDL

LDL (0.2 mg/ml) was incubated in PBS containing DTPA (100 \( \mu \)M) with peroxynitrite for 20 min at 37°C and the electrophoretic mobility of LDL was measured. REM was calculated as the ratio of the electrophoretic mobility of the sample to that of an untreated control. Experimental points represent the mean ± S.E.M. (n = 3)
THP-I macrophages resulted in a dramatic increase in olate incorporation into cholesteryl ester. The response was several times greater than that observed with native LDL and was blocked by polyinosinic acid, an inhibitor of scavenger-receptor-mediated uptake.

Oxidation of LDL results in the destruction of many of the components of the LDL particle. A group of small molecules, collectively termed antioxidants, inhibit oxidation and are destroyed in the process. The most abundant and probably the most important of these is vitamin E. Consumption of vitamin E is usually associated with oxidation of LDL lipid. Figure 2 shows the effect of increasing peroxynitrite concentration on the vitamin E content of LDL. Peroxynitrite clearly results in the consumption of this antioxidant. The depletion of vitamin E is rapid, occurring within the first 30 s of the start of the reaction, at 37°C (data not shown). The concentration of peroxynitrite required to deplete vitamin E is approximately an order of magnitude less than that required to change the REM. The vitamin E content was approximately halved by 50 µM peroxynitrite.

As mentioned above, vitamin E retards the oxidation (and hence the modification) of LDL lipids. It is likely, therefore, that depletion of vitamin E by peroxynitrite will render LDL more prone to oxidation by agents such as copper ions. To test for this, LDL was pre-incubated with peroxynitrite, and the subsequent increase in conjugated dienes that occurred upon the addition of copper ions was monitored. Conjugated dienes are formed during the oxidation of unsaturated fatty acids within the LDL particle and can be monitored continuously by measuring the change in absorbance at 234 nm [16]. The addition of copper to LDL results in the rapid oxidation of the lipid component of the particle. This rapid oxidation, however, is preceded by a lag period, the length of which has been used as an indication of the intrinsic propensity of the LDL particle to become oxidized. The length of this lag time is shown as a function of peroxynitrite concentration in Figure 3. Pre-incubation of LDL with peroxynitrite decreases the lag period of copper-dependent oxidation in a concentration-dependent manner. At concentrations of peroxynitrite above 100 µM, no further decrease in lag time was observed. A comparison of Figures 2 and 3 indicates a relationship between vitamin E content and susceptibility to oxidation of LDL after incubation with peroxynitrite.

**Figure 2**

**The effect of peroxynitrite on the vitamin E content of LDL**

LDL (0.2 mg/ml) was incubated in PBS containing DTPA (100 µM) with peroxynitrite for 20 min at 37°C. Samples were extracted into hexane, and vitamin E was measured by h.p.l.c. Experimental points represent the means ± S.E.M. (n = 3)

**Figure 3**

**The effect of peroxynitrite on the lag time of copper-dependent oxidation of LDL**

LDL (0.2 mg/ml) was pre-incubated with peroxynitrite for 20 min at 37°C in the cuvette of a spectrophotometer. Copper (50 µM) was added and the absorbance was monitored continuously at 234 nm. The time course of copper-dependent oxidation exhibits a lag phase. The length of this phase, the 'lag-time', is plotted against the original peroxynitrite concentration.
Conclusions
The interaction of peroxynitrite with LDL results in an increase in electrophoretic mobility of the particle, together with a loss of vitamin E. The concentration dependence of these two processes, however, differs greatly. Although not conclusive, these data suggest that modification of LDL occurs as a result of free radical processes within the lipid compartment of the LDL particle. There are many precedents in the literature for this mechanism of oxidative modification. For example, incubation of LDL with copper ions results in oxidation of the LDL lipid. This process generates aldehydes that interact with lysine residues of apoprotein B, thus increasing the net negative charge of the particle. Vitamin E acts to retard the oxidative modification of LDL by scavenging lipid peroxyl radicals and, during this process, vitamin E is consumed [2]. At low concentrations of peroxynitrite (Figure 2), modification of LDL is unable to occur, presumably because lipid oxidation is inhibited by the presence of vitamin E. At higher concentrations of peroxynitrite, however, the antioxidant capacity of LDL is overwhelmed and modification of the lipoprotein occurs. The fact that LDL is modified by peroxynitrite in the presence of DTPA indicates that this process does not depend on the presence of transition metal ions.

Peroxynitrite treatment generates a form of LDL that is recognized by the macrophage scavenger receptor and is taken up into macrophages at a greater rate than is native LDL.

Peroxynitrite increases the susceptibility of LDL to copper-dependent oxidation at lower concentrations than those required to change the electrophoretic mobility of LDL. This is no doubt due mainly to peroxynitrite-dependent depletion of vitamin E (Figure 2). However, if peroxynitrite initiates free radical reactions within the LDL particle, it will increase the concentration of unsaturated fatty acid peroxide. It has been shown that addition of exogenous peroxides to LDL increases the susceptibility of the lipoprotein to metal ion-dependent oxidation [17].

There remains the possibility that peroxynitrite acts by direct oxidation of vitamin E and by direct modification of the apoprotein. Peroxynitrite has been shown to nitrate tyrosine residues [18] and oxidize cysteine thiols [19]. Neither of these modifications, however, would directly affect the charge of the lipoprotein.

We have shown that peroxynitrite is able to modify LDL to a potentially atherogenic form. We have also shown that peroxynitrite increases the susceptibility of LDL to oxidation by transition metal ions. The relevance of peroxynitrite as an oxidant in vivo is, as yet, controversial and it is already clear that macrophage-dependent oxidative modification of LDL is not dependent on the synthesis of nitric oxide [20, 21]. However, the data presented here and in our previous studies suggest that the interaction between different cells in the artery wall generating nitric oxide and superoxide could contribute to the oxidative modification of LDL in the initial stages of atherosclerosis.

Antioxidant drugs and the inhibition of low-density lipoprotein oxidation

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Introduction

It is well known that oxidized low-density lipoprotein (LDL) is atherogenic [1]. Current theories support the idea that atherosclerosis begins by some mechanism involving damage to the endothelium and oxidation of LDL in the artery wall. Oxidative modification of LDL alters its recognition properties allowing uptake by macrophage scavenger receptors, forming cholesterol-laden foam cells [2].

Evidence has been put forward that oxidation is mediated, in culture, by macrophages/microphages [3], smooth muscle cells [4] and endothelial cells [5]. All these cell types have been shown to generate superoxide radical. However, in general, superoxide is not very reactive. Its toxicity and that of its dismutation product, hydrogen peroxide, may be amplified by reaction with available transition metals such as iron, generating the hydroxyl radical, or with haem proteins such as myoglobin and haemoglobin, generating the oxidatively damaging ferryl radical species. The latter are more selective, and perhaps more relevant, than the hydroxyl radical in vivo.

The polyunsaturated fatty acids within LDL are protected from oxidation by α-tocopherol, present in the outer monolayer, and β-carotene and minor carotenoids present in the inner core. The susceptibility of LDL to oxidation will thus depend not only on its cholesteryl ester and polyunsaturated fatty acid content, but also on its antioxidant status [6, 7].

Biological activities of hydroxamic acids

Previous work has suggested that hydroxamate compounds are being developed as oral iron chelators [8]. A plethora of biological activities have been reported for hydroxamic acids, including the inhibition of urease [9], ribonucleotide reductase [10], lipoxygenase [12] and cyclooxygenase [13]. They are also known to function as direct acting vasodilators [11]. In light of this activity, we have synthesized a series of novel monohydroxamate derivatives [N-methylacetohydroxamic acid (NMAH), N-methylbutyrohydroxamic acid (NMBH) and N-methylhexanoylhydroxamic acid (NMHH)], and investigated their efficacy in inhibiting haem-protein-mediated LDL oxidation [14, 15] (Figure 1). An aspect of particular interest to us is the potential for myoglobin, released from its normal functional location, to oxidize LDL, and the consequences for the LDL and the haem protein.

Is the myoglobin damaging to LDL?

In the presence of low concentrations of hydrogen peroxide, myoglobin stimulates lipid peroxidation [16]. Several workers have shown that the radical species generated is akin to that designated as ferryl myoglobin [17, 18] in which the haem iron is one oxidizing equivalent above that of metmyoglobin and one oxidizing equivalent is present in the globin moiety:

\[
HX[Fe^{III}] + H_2O_2 \rightarrow *X[Fe^{IV}=O] + H_2O + H^+ \ (1)
\]

Electron paramagnetic resonance (e.p.r.) evidence suggests that the tyrosine phenoxy radical thereby formed appears to react subsequently with oxygen to give a tyrosine peroxy radical [19, 20]. These species are located on the surface of the protein,