Antioxidant drugs and the inhibition of low-density lipoprotein oxidation
Emma S. R. Green, Chris E. Cooper*, Michael J. Daviest and Catherine Rice-Evans
Free Radical Research Group, Division of Biochemistry, UMDS-Guy's Hospital, Thomas's Street, London SE1 9RT,
*Division of Biomolecular Sciences, King's College London, Campden Hill Road, London W8 7AH and
†Department of Chemistry, University of York, York YO1 5DD, U.K.

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/monocytes [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:

\[ \text{HX[Fe}^{II}] + \text{H}_2\text{O}_2 \rightarrow \text{X[Fe}^{IV}=\text{O}] + \text{H}_2\text{O} + \text{H}^{+} \]  

(1)

Electron paramagnetic resonance (e.p.r.) evidence suggests that the tyrosine phenoxyl radical thereby formed appears to react subsequently with oxygen to give a tyrosine peroxyl radical [19, 20]. These species are located on the surface of the protein,
and are therefore available to initiate lipid peroxidation. As a consequence of these observations, we have studied metmyoglobin-and ferryl myoglobin-induced peroxidation of LDL. Both metmyoglobin and, to a greater extent, ferryl myoglobin cause an increase in the electrophoretic mobility of LDL, consistent with a decrease in the positive charge, and oxidation of the LDL (Figure 1). The relative electrophoretic mobility of LDL, altered by reaction with metmyoglobin and ferryl myoglobin, was reduced in the presence of the hydroxamate drugs (at 10 \( \mu \)M concentrations). For LDL that had been altered by reaction with metmyoglobin, the relative electrophoretic mobility was reduced by 13%. For LDL altered by ferryl myoglobin, the extent of reduction varied depending on the drug added: desferrioxamine caused a 38% reduction, NMHH a 58% reduction, and NMBH a 46% reduction.

LDL oxidation was also monitored using the thiobarbituric acid assay, which detects the aldehydic breakdown products of lipid peroxidation. After 90 min incubation, the extensive peroxidation mediated by metmyoglobin resulted in an increase, over the controls, of 5.5, 8.1 and 3.6 nmol thiobarbituric acid-reactive substances per mg LDL protein for the three different LDL preparations, LDL5, LDL6 and LDL13, respectively. Differences between LDL preparations result from the well-documented variation in the susceptibility of LDL isolated from different donors to oxidation [21, 22]. Even more extensive peroxidation was detected with ferryl myoglobin, generating an increase, over the controls, of 40.0, 40.5 and 22.7 nmol thiobarbituric acid-reactive substances per mg LDL protein for the three different LDL preparations, LDL5, LDL6 and LDL13, respectively.

**Can hydroxamate drugs quench the ferryl myoglobin radical species?**

Low temperature e.p.r. spectroscopy investigations of the trihydroxamate desferrioxamine and the monohydroxamate NMBH were undertaken in order to determine whether hydroxamates can directly quench the ferryl myoglobin radical species. On reaction of metmyoglobin with hydrogen peroxide, a signal with features in common with that previously assigned to the ferryl myoglobin radical species is observed (Figure 2a) [23, 24]. In the presence of desferrioxamine (Figure 2b), this signal is replaced with that assigned to the desferrioxamine nitroxide radical signal on the basis of a splitting of 8 G that is characteristic of the nitroxide splitting [25]. That the second signal is due to the desferrioxamine nitroxide radical is confirmed on detection of an identical signal on reaction of desferrioxamine and potassium ferricyanide (Figure 2c). This suggests that desferrioxamine can scavenge the haem protein radical species, by its hydrogen-donating activity [15, 26, 27].

On reaction of metmyoglobin and hydrogen peroxide in the presence of NMBH, a radical signal is observed (Figure 3b) that is different from that generated in the absence of NMBH (Figure 3a), but identical to that generated on reaction of NMBH and potassium ferricyanide (Figure 3c). The identification of the NMBH nitroxide radical, formed on reaction of ferryl myoglobin radical with NMBH, proves that NMBH can also quench the haem protein radical. Desferrioxamine and NMBH are therefore acting as hydrogen-donating drugs, and not as iron chelators, in this system.

Further evidence for the ability of hydroxamates to act as antioxidants and reduce ferryl myoglobin formation is obtained from visible spectroscopic investigations. Addition of hydrogen peroxide to metmyoglobin results in changes in the redox state of the haem protein which can be interpreted using the Whitburn algorithms [28]. The presence of the hydroxamates before addition of hydrogen peroxide results in a reduction in both the rate and extent of ferryl myoglobin formation, as previously reported [15].
Can the hydroxamate drugs inhibit myoglobin-induced lipid peroxidation of LDL?

The monohydroxamates NMBH and NMHH were investigated, in comparison with desferrioxamine, for their protective effects against myoglobin-induced lipid peroxidation of LDL.

Metmyoglobin-induced lipid peroxidation of LDL was reduced by the inclusion of the hydroxamate drugs at the time of addition of the metmyoglobin to the LDL. After 1.5 h of incubation, for all of the LDL samples studied, oxidation was inhibited by 70% in the presence of desferrioxamine and by 90% in the presence of 100 μM of either NMHH or NMBH. When present at the lower concentration of 10 μM, desferrioxamine inhibited oxidation by 30%, NMHH by 58% and NMBH by 48%.

Ferryl myoglobin-induced lipid peroxidation of LDL was also reduced by the presence of the hydroxamate drugs at the time of addition of the metmyoglobin and hydrogen peroxide to the LDL. For all the LDL samples studied oxidation was inhibited by 92% in the presence of 100 μM desferrioxamine and by 96% in the presence of 100 μM either NMHH or NMBH. When present at 10 μM, desferrioxamine inhibited oxidation by 60%, and NMHH or NMBH by 90%.

In contrast, when the drugs (all 100 μM) were added 30 min after the reaction between LDL and ferryl myoglobin was initiated, and the samples left to incubate for a further hour (i.e. the same total incubation time as before), all of the drugs reduced lipid peroxidation significantly but to a much lesser extent (desferrioxamine by 10%, and NMHH and NMBH by 25%).

Thus, it would appear that desferrioxamine, which has three hydroxamate groups, is less efficient than the monohydroxamates both at inhibiting metmyoglobin- and ferryl myoglobin-mediated lipid peroxidation of LDL, and as a chain-breaking antioxidant when added to peroxidizing lipids.

Evidence for iron release

Low temperature e.p.r. can distinguish between high-spin iron (III) haem and high-spin chelatable iron [29]. High-spin iron (III) haem in metmyoglobin is visible with an e.p.r. line at a g-value of 6, whereas high-spin chelatable iron (III) is visible with a signal at a g-value of 4.3. The concentration
Low temperature e.p.r. spectra observed on reaction of (a) metmyoglobin (20 µM) with hydrogen peroxide (25 µM), (b) metmyoglobin with hydrogen peroxide in the presence of NMBH (100 µM), (c) NMBH with excess potassium ferricyanide, and (d) metmyoglobin control (no hydrogen peroxide).

E.p.r. temperature = 100 K. Samples frozen 5 s after peroxide addition.
gation phase of peroxidizing lipids in LDL, as shown by their inhibition of the metmyoglobin-mediated peroxidation of LDL. Importantly, due to their iron chelation properties, they also have the potential to inhibit the formation of hydroxyl radicals, which may be generated on disruption of the haem protein by the oxidized LDL. Since the hydroxamates possess the dual activities of antioxidant and iron-chelating properties, these new compounds are excellent candidates for the protection of LDL against ferryl myoglobin-mediated lipid peroxidation.

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