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INHIBITION OF COPPER-MEDIATED LOW DENSITY LIPOPROTEIN PEROXIDATION BY QUINOLINE AND INDOLINONE NITROXIDE RADICALS

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Abstract—Quinoline and indolinone nitroxide radicals are known to be efficient scavengers of oxygen-centred (rate constants (k) between 10^3 and 10^5/M/sec) and carbon-centred radicals (almost diffusion-controlled rate). In this study, the relative effects of these compounds in protecting low density lipoprotein (LDL) from oxidation induced by copper have been investigated. The extent of lipid peroxidation was assessed by monitoring the increased conjugated diene formation, the altered surface charge of the apolipoprotein B and the generation of aldehydic breakdown products of oxidized LDL. All the nitroxides inhibited LDL peroxidation in a concentration-dependent manner. The corresponding hydroxylamines of the nitroxides were also studied and were shown to inhibit lipid peroxidation to almost the same extent as the parent nitroxide. The data indicates that this class of nitroxide radicals (and their reduced hydroxylamine forms) are effective lipophilic antioxidants with the quinoline nitroxide being more efficient than the indolinone nitroxides.

Key words: oxidized LDL; conjugated dienes; free-radical scavenger; nitroxides

Nitroxide radicals such as tetramethyl-piperidine and pyrrolidine nitroxides, oxazolidine nitroxides and their derivatives have been applied extensively in a variety of biochemical studies. These include uses as spin labels [1], probes of oxygen concentration [2] and activities akin to those of superoxide dismutase [3] and catalase [4]. Addition of the nitroxides to mammalian cellular systems has revealed protective effects against ionizing radiation [5], blocking DNA scission, and from hydrogen peroxide-induced damage [6].

In this paper, the antioxidant activity of the nitroxide derivatives (Fig. 1) of the quinoline (**1**) and indolinone types (**2–6**) has been studied. Indolinone nitroxides have been shown in chemical systems to scavenge effectively aroyloxyl [7], phenoxyl [8], alkyl radicals [9, 10] (the latter at a nearly diffusion-controlled rate), and to react with alkoxyl and peroxyl radicals with estimated rate constants (k) between 10^3 and 10^5/M/sec [10, 11]. Recent studies in biological systems have revealed their effectiveness as chain-breaking antioxidants during the oxidation of linolenic acid mediated by cytochrome *c* and *tert*-butylhydroperoxide [12]. In this work, we have investigated the relative abilities of indolinone and quinoline nitroxide radicals and their associated hydroxylamines to protect human LDL‡ against oxidation. The results show that these classes of nitroxides act as chain-breaking antioxidants in inhibiting the oxidation of LDL mediated by copper in a concentration-dependent manner.

MATERIALS AND METHODS

Chemicals. The nitroxides **1–6** and hydroxylamines **4a**, **6a**, were synthesized, respectively, according to the literature: 1,2-dihydro-2,2-diphenyl-4-ethoxy-quinoline-1-oxyl (quinoline nitroxide, **1**) [13]; 1,2-dihydro-2,2-diphenyl-3*H*-indole-3-one-1-oxyl (2-phenyl-3-keto derivative, **2**); 1,2-dihydro-2,2-diphenyl-3*H*-indole-3-phenylimino-1-oxyl (2-phenyl-3-phenylimino derivative, **3**); 1,2-dihydro-2-ethyl-2-phenyl-3*H*-indole-3-phenylimino-1-oxyl (2-ethyl-3-phenylimino derivative, **4**); 1,2-dihydro-2-methyl-2-phenyl-3*H*-indole-3-one-1-oxyl (2-methyl-3-keto derivative, **5**); 1,2-dihydro-2-ethyl-2-phenyl-3*H*-indole-3-one-1-oxyl (2-ethyl-3-keto derivative **6**); 1-hydroxy-1,2-dihydro-2-ethyl-2-phenyl-3*H*-indole-3-phenylimino (2-ethyl-3-phenylimino hydroxylamine, **4a**); 1-hydroxy-1,2-dihydro-2-ethyl-2-phenyl-3*H*-indole-3-one (2-ethyl-3-keto hydroxylamine, **6a**) [14]. The identity and purity of the compounds were checked by thin layer chromatography and by mass spectroscopy on a Carlo Erba QMD 1000 spectrometer. Melting points were measured on an Electrothermal Melting Point Apparatus. Methanol (HPLC Grade) was obtained from Rathburn Chemical Ltd (Walkerburn, U.K.). All other reagents were of the highest quality and obtained from the Sigma Chemical Co. (Poole, U.K.) or from BDH Ltd (Dagenham, U.K.).

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‡ Abbreviations: LDL, low density lipoproteins; BHT, butylated hydroxytoluene; TEMPO, 2,2,6,6-tetramethyl-piperidine-1-oxyl; REM, relative electrophoretic mobility; DMF, dimethylformamide; SCE, saturated calomel electrode; TBARS, thiobarbituric acid reactive substances.

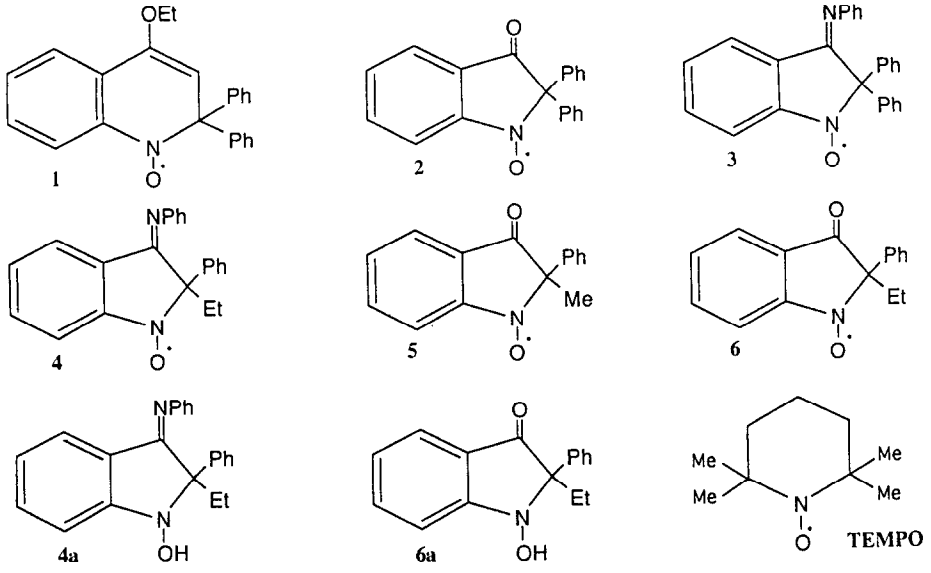


Fig. 1. Structures of nitroxides and hydroxylamines. (Me, methyl; Et, ethyl; Ph, phenyl; NPh, phenylimino.) 1,2-Dihydro-2,2-diphenyl-4-ethoxy-quinoline-1-oxyl (**1**); 1,2-dihydro-2,2-diphenyl-3*H*-indole-3-one-1-oxyl (**2**); 1,2-dihydro-2,2-diphenyl-3*H*-indole-3-phenylimino-1-oxyl (**3**); 1,2-dihydro-2-ethyl-2-phenyl-3*H*-indole-3-phenylimino-1-oxyl (**4**); 1,2-dihydro-2-methyl-2-phenyl-3*H*-indole-3-one-1-oxyl (**5**); 1,2-dihydro-2-ethyl-2-phenyl-3*H*-indole-3-one-1-oxyl (**6**); 1-hydroxy-1,2-dihydro-2-ethyl-2-phenyl-3*H*-indole-3-phenylimino (**4a**); 1-hydroxy-1,2-dihydro-2-ethyl-2-phenyl-3*H*-indole-3-one (**6a**); 2,2,6,6-tetramethyl-1-piperidine-1-oxyl (TEMPO).

Isolation of LDL. Fresh human blood was obtained by venipuncture from volunteers (with informed consent), taken into acid citrate dextrose anti-coagulant pH 6.4 in the presence of 0.1 mM EDTA and the plasma separated after centrifugation for use in isolation of LDL. The LDL was then separated by equilibrium density ultra-centrifugation at 16° on a Beckman L-70 centrifuge using a fixed-angle rotor working at 150,000 *g* according to a modified method of Chung *et al.* [15]. The lipoprotein sample was dialysed for 5 hr in a 5-fold volume of PBS, pH 7.4, 10 mM containing 10 μM EDTA at 4°. After dialysis, the LDL was filtered through a sterile 0.22 μm diameter pore and used within 2–3 weeks after preparation. Protein concentration was determined by the modified procedure of Markwell *et al.* [16] using bovine serum albumin as a standard. In all experiments LDL was used at a final concentration of 0.125 mg LDL protein/mL.

The effects of the nitroxides and their hydroxylamines on the suppression of copper-mediated oxidative modification of LDL was assessed by measuring the conjugated diene formation during LDL peroxidation, by measuring the extent of formation of aldehydic breakdown products of lipid peroxidation using the thiobarbituric acid assay and by measuring the electrophoretic mobility of oxidized LDL.

Measurement of the suppression of copper-mediated LDL oxidation by nitroxides and hydroxylamines. LDL protein in PBS (0.125 mg/mL) was oxidized by incubating at 37° with 3 μM $CuSO_4$ for 2 hr (unless otherwise stated). The nitroxides and hydroxylamines were prepared as a 0.6 mM stock solution in methanol and added as methanol solution (maximum 1.6% v/v) prior to addition of copper. The reaction was stopped after 2 hr by the addition of BHT (final concentration, 300 μM).

Conjugated diene formation. The effects of the nitroxides on the oxidation of LDL was determined by continuous monitoring of the increased conjugated diene hydroperoxide formation by difference spectroscopy [17]. The oxidation of 0.125 mg/mL LDL in PBS was initiated by addition of 3 μM copper in the presence of 1 μM nitroxide compounds. The LDL oxidation was determined by recording the increase in the absorbance from 220 to 300 nm at 5 min intervals during a 3 hr period [18] on a Beckman DU65 spectrophotometer fitted with Quant 1 software and linked to an IBM PC/2. The increased absorption at 234 nm during copper-mediated oxidation indicates the formation of conjugated diene hydroperoxides of the fatty acids, and can be directly measured by recording the UV spectra [17]. The oxidizability of the LDL was expressed as the time to reach half maximal absorbance for each experiment and was measured as described by Jessup *et al.* [19].

Thiobarbituric acid assay. The extent of lipid peroxidation was also measured by determining the percentage inhibition of aldehydic breakdown products using the thiobarbituric acid assay [20]. To 0.5 mL of the incubated samples, 0.5 mL of trichloroacetic acid [10% (w/v)] was added, followed by 0.5 mL of thiobarbituric acid [0.75% (w/v) in 0.1 M HCl]. The samples were heated for 20 min at 95°, followed by cooling and centrifugation. The absorbance of the pink chromophore of the

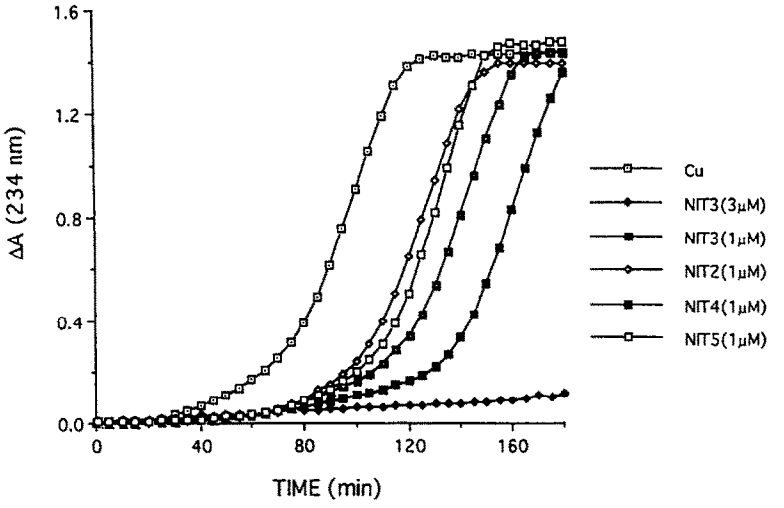


Fig. 2. Conjugated diene formation of copper-mediated oxidation of LDL in the absence and presence of nitroxides. The oxidation of LDL (0.125 mg protein/mL) was initiated by the addition of 3 μM copper in the absence and in the presence of nitroxides. The progress of the peroxidation reaction was monitored during a 3 hr period at 37°, by measuring the increased formation of conjugated dienes at 234 nm, as described in Materials and Methods. Results are an average of two experiments (difference < 17%). (⊡) Copper (3 μM), (◆) 2-phenyl-3-phenylimino (**3**) (3 μM), (■) 2-phenyl-3-phenylimino (**3**) (1 μM), (◇) 2-phenyl-3-keto (**2**) (1 μM), (■) 2-ethyl-3-phenylimino (**4**) (1 μM), (□) 2-methyl-3-keto (**5**) (1 μM).

supernatant was measured at 532 nm and corrected for the absorbance at 580 nm.

Gel electrophoresis. Oxidative modification of LDL was assessed by measuring the altered surface charge of the apolipoprotein B of the LDL. At the end of the 2 hr incubation, 15 μL samples were taken and applied to agarose gels for electrophoreses at 100 V in barbital buffer, pH 8.6 (Beckman Paragon Lipo Gel electrophoresis system). The electrophoretic mobility of the LDL samples was measured relative to the mobility of untreated LDL and expressed as the % inhibition of REM of oxidized LDL in the presence and absence of nitroxides. Lipoproteins were visualized by staining with Sudan Black B stain.

Appropriately incubated controls were carried out including nitroxides and hydroxylamines with native LDL and methanol with copper and LDL. All concentrations are expressed as final concentrations. Three different batches of LDL were used: one for the concentration–response curves (TBARS formation) and gels; one for the experiments comparing the hydroxylamines with their corresponding nitroxides and TEMPO; and one for the conjugated diene experiments.

RESULTS

Oxidation of low density lipoprotein by copper-induced stress was assessed by three methods: monitoring the increased formation of conjugated diene hydroperoxides, measuring the breakdown products of lipid peroxidation, and by assessing the consequence for the apolipoprotein B protein portion of the LDL molecule by observing the change in surface charge.

The relative abilities of the indolinone nitroxides of both the phenylimino and the keto varieties **2–5** to inhibit LDL oxidation were monitored by observing their effects on conjugated diene formation at 234 nm (Fig. 2). The data show that incorporation of the nitroxides (1 μM) delays the time to the commencement of oxidation (the lag phase), the phenylimino derivatives being more effective than the keto ones, but the rate of propagation of oxidation, once the lag phase is complete, is approximately the same for all incubations. Incorporation of elevated nitroxide concentration (3 μM) (2-phenyl-3-phenylimino derivative **3**), showed complete inhibition of conjugated diene formation of the 3 hr incubation period. The time to achieve half maximal absorbance was used as an index of oxidizability [19]. In comparison with the values for oxidized LDL (88 min), the effects of the nitroxides (1 μM) are greater for the phenylimino compounds (130 min and 148 min, respectively for the phenyl and ethyl derivatives) than for the keto ones (115 min and 122 min, respectively for the phenyl and methyl derivatives) (Table 1).

The effects of the nitroxides on the extent of lipid peroxidation were also assessed by detecting the extent of formation of secondary metabolites of lipid peroxidation products using the thiobarbituric acid assay [20]. The average absorbance of TBARS in the absence of inhibitor was 3.529/mg LDL protein. Figure 3 shows a typical concentration–response curve (0–10 μM) for one of the nitroxides used (2-phenyl-3-phenylimino derivative, **3**) where almost maximum inhibition of copper-induced lipid peroxidation assessed after 2 hr incubation was achieved with concentrations $\geq$6 μM nitroxide. The con-

Table 1. Oxidizability of copper-mediated LDL peroxidation in the absence and presence of 1 μM nitroxides **2–5**

Nitroxide added	Oxidizability (Time to half maximal absorbance in min)
None	88
2-phenyl-3-keto (**2**) (1 μM)	115
2-phenyl-3-phenylimino (**3**) (1 μM)	130
2-phenyl-3-phenylimino (**3**) (3 μM)	⩾180
2-ethyl-3-phenylimino (**4**) (1 μM)	148
2-methyl-3-keto (**5**) (1 μM)	122

LDL (0.125 mg protein/mL), pH 7.4, was incubated for 3 hr at 37° with 3 μM copper and 1 μM nitroxide when present and increased conjugated diene formation was measured by difference spectroscopy at 234 nm as described in Materials and Methods.

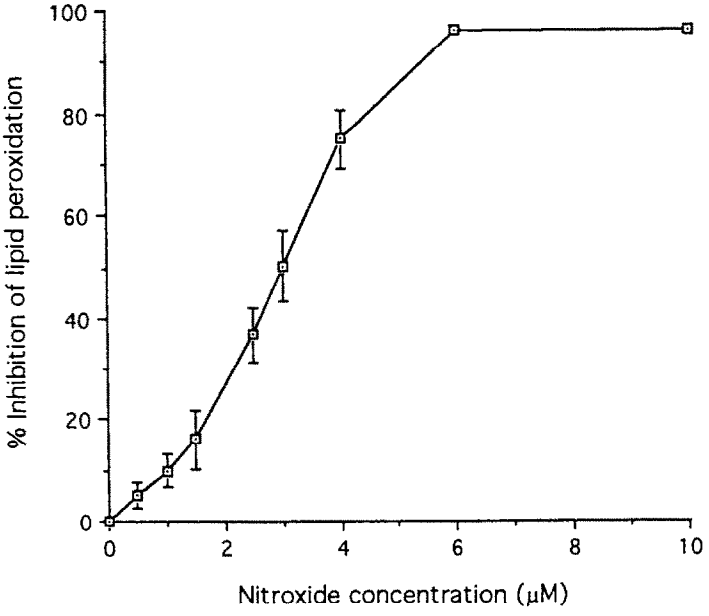


Fig. 3. Concentration–response curve showing percentage inhibition of copper-mediated LDL oxidation by 2-phenyl-3-phenylimino nitroxide (**3**). LDL (0.125 mg protein/mL) at pH 7.4 was incubated at 37° with 3 μM copper for 2 hr in the presence of a range of concentrations of the nitroxide (**3**). The extent of peroxidation was measured using the thiobarbituric acid assay as described in Materials and Methods. Results are means ± SD. N = 3.

centration of nitroxide giving 50% inhibition of lipid peroxidation, defined as IC_{50}, was determined from the corresponding concentration–response curves of the nitroxides **1–5**. All the indolinone nitroxides (**2–5**) gave similar concentration–response curves, which is also reflected in the similarity between the IC_{50} values (3.06 ± 0.24, N = 12) compared with the more effective quinoline nitroxide (**1**) with an IC_{50} value of 2.23 ± 0.56, N = 3, ($P < 0.001$ = ***** estimated with a Student's *t*-test).

The effects of the nitroxides on suppression of the change in surface charge on the protein portion of the LDL induced by copper were monitored by electrophoresis after 2 hr incubation. The nitroxides **2–5** prevented the increase in electrophoretic mobility of oxidized LDL in a concentration-dependent manner to approximately similar extents (Fig. 4). The values were evaluated from gels such as that shown in Fig. 5 for the 2-ethyl-3-phenylimino derivative (**4**), where the progressive decrease in

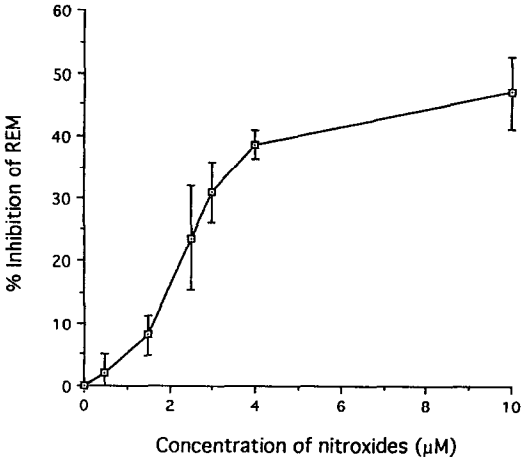


Fig. 4. Percentage inhibition of REM of oxidized LDL in the presence of varying concentrations of indolinone nitroxides **2**–**5**. LDL (0.125 mg protein/mL) was oxidized for 2 hr at 37° with 3 μM copper in the absence and presence of the nitroxides and samples applied to agarose gels as described in Materials and Methods. The percentage inhibition of REM by the nitroxides is concentration dependent and was calculated relative to the mobility of untreated LDL (N = 2–4).

electrophoretic mobility of oxidised LDL can be observed as the nitroxide concentration increases.

The two hydroxylamines (2-ethyl-3-phenylimino hydroxylamine **4a**, 2-ethyl-3-keto hydroxylamine **6a**), studied in comparison with their corresponding nitroxides (2-ethyl-3-phenylimino derivative **4**, 2-ethyl-3-keto derivative **6**) and the commercial nitroxide TEMPO (used for comparison), also inhibited the oxidative modification of the LDL. In these experiments, 1 μM hydroxylamines was sufficient to suppress the formation of aldehydic breakdown products of lipid peroxidation by approximately 50%. The LDL used in this experiment was from a different donor to the one used for the determination of conjugated dienes and it is well-known that the endogenous antioxidant status of LDL varies from individual to individual [21]. No major differences were observed between the nitroxide derivatives and their hydroxylamines. The 2-ethyl-3-phenylimino nitroxide **4** was found to be a more efficient inhibitor of lipid peroxidation (57.6 ± 4%, N = 3) than the 2-ethyl-3-keto nitroxide **6** (48.1 ± 6%, N = 3) as assessed by measuring the percentage inhibition of lipid peroxidation by using the thiobarbituric acid assay. TEMPO, which is a widely used piperidine nitroxide along with its many derivatives in lipid peroxidation studies, inhibited LDL peroxidation to a lesser extent.

It has been suggested that antioxidants, under certain conditions can act as prooxidants when acting as hydrogen-abstractors [22]. There was no evidence for the nitroxides acting as prooxidants in our case since experiments incubating LDL with nitroxides and hydroxylamines (up to 10 μM) for 2 hr showed no oxidation of the LDL.

DISCUSSION

The susceptibility of LDL to oxidation is dependent on its content of peroxyl radical scavenging

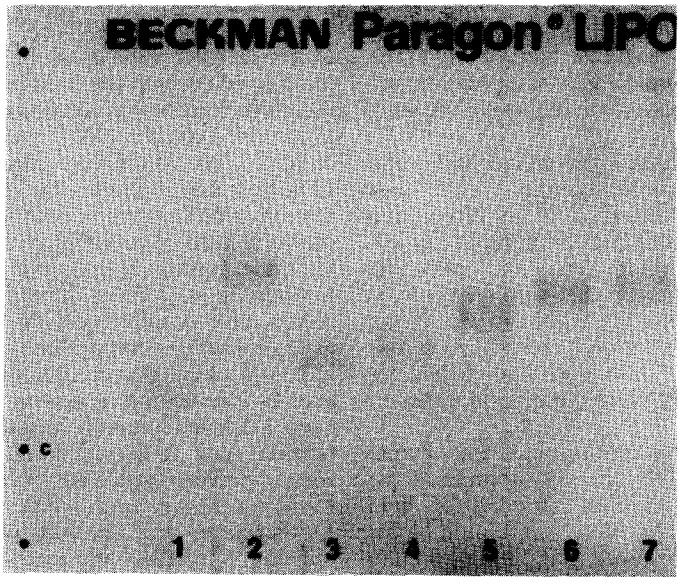
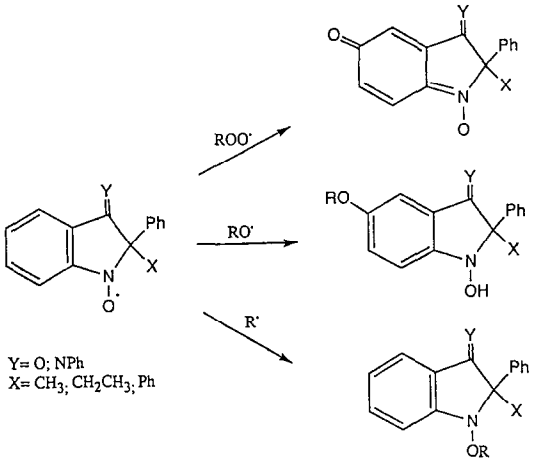


Fig. 5. The effect of 2-ethyl-3-phenylimino nitroxide (**4**) on changes in electrophoretic mobility of LDL subjected to copper-mediated oxidative stress. LDL (0.125 mg protein/mL) at pH 7.4 was oxidized for 2 hr at 37° with 3 μM copper in the absence and presence of the nitroxide and samples applied to agarose gels as described in Materials and Methods. Lane 1, incubated native LDL; lane 2, oxidized LDL; lane 3, 10 μM nitroxide during oxidation; lane 4, 4 μM nitroxide during oxidation; lane 5, 2.5 μM nitroxide during oxidation; lane 6, 1.5 μM nitroxide during oxidation; lane 7, 0.5 μM nitroxide during oxidation.



Scheme 1.

antioxidants, the polyunsaturated fatty acid content and the endogenous level of hydroperoxides [23–25]. It is well known that oxidized LDL is atherogenic and that antioxidants which inhibit LDL oxidation *in vitro* have been shown to prevent fatty streak formation in *in vivo* animal models and are associated with protection against coronary artery disease in population studies. In addition, published reports and epidemiological studies are beginning to provide confirmation that dietary peroxyl radical scavengers such as α-tocopherol, at appropriate levels, decrease the incidence of and mortality from coronary heart disease.

Previous studies [26, 27] have shown that piperidine and oxazolidine nitroxide derivatives inhibit iron-mediated lipid peroxidation in rat liver microsomes and that this is dependent on the accessibility of the nitroxide moiety to the lipid region of the membrane, thus allowing participation in the chain-breaking antioxidant activity.

We have investigated the ability of indolinone and quinoline nitroxide radicals to protect LDL from oxidation and the modification of its recognition properties induced by copper. Scavenging of oxygen-centred radicals by indolinone and quinoline nitroxides yields non-paramagnetic products of the nitroxide (Scheme 1) [10, 11]. The reactivity of the nitroxides with carbon-centred radicals gives rise to the corresponding alkylated hydroxylamines (Scheme 1) through coupling at the nitroxide function [9, 10].

Our findings suggest that both groups of indolinone nitroxides are equally effective in suppressing the formation of the breakdown products of lipid peroxidation and the altered surface charge of the apolipoprotein B. However, the 3-phenylimino derivatives (**3**, **4**) seem to be better inhibitors of the propagation of conjugated diene formation than the 3-keto derivatives (**2**, **5**). The difference in these results could be correlated to the different substituent in position 3 of the indole ring. Furthermore, the different hydrogen abstraction capacity of these derivatives could account for the differences observed, since nitroxides can potentially act as prooxidants by abstracting a labile hydrogen atom initiating an autoxidation chain reaction. Those with a high hydrogen abstraction capacity are less efficient as antioxidants and a good antioxidant is one whose hydrogen abstraction and spin-trapping capacities are well balanced. Thus, the phenylimino derivatives may be better antioxidants since their hydrogen abstraction ability is less than the keto ones (rate constants of hydrogen transfer from, dihydroacridine to 2-phenyl-3-keto nitroxide, **2** and 2-ethyl-3-phenylimino nitroxide, **4** at 60° in deareated acetonitrile are 2.5 and 0.38/M/sec, respectively). In addition, the hypothesis that phenylimino derivatives are better inhibitors than the keto ones is supported by the redox potentials of these nitroxides measured in DMF/H_2O 55:45 vs SCE. The redox potentials for the keto derivatives **2** and **5** are -0.28 and -0.26 V, respectively, while that for the phenylimino derivative **3** is -0.35 V. This shows that the latter, having a more negative value, is a weaker oxidizing agent (less hydrogen abstraction ability) therefore a better antioxidant than the two former. Also, the redox potential for the quinoline nitroxide **1** is still more negative, -0.38 V, and is therefore the least oxidizing nitroxide and the best antioxidant in the series studied.

The quinoline nitroxide **1**, with its N–O moiety present in a 6-membered ring structure conjugated with a benzene ring, seemed to be relatively more effective in inhibiting copper-mediated LDL peroxidation than all the other nitroxides bearing the N–O function in a 5-membered ring structure also conjugated to a benzene ring (indolinone nitroxides, **2–5**). It has been previously reported that piperidines (6-membered ring nitroxides) are more efficient inhibitors of lipid peroxidation in microsomal membranes than oxazolidines or pyrrolidines (5-membered ring nitroxides) [27]. This was attributed to a higher accessibility of the nitroxide moiety of the piperidines into the lipid layer which explained their higher efficacy as antioxidants. This mechanism might also explain the difference observed between quinoline and indolinone nitroxides in protecting LDL from oxidation since the ring structure bearing the nitroxide function is also different in these two classes of nitroxides. Although the study of quinoline nitroxides towards the trapping of different kinds of radicals is still under investigation, preliminary results have demonstrated that they act efficiently as chain-breaking antioxidants [12].

The possibility that the inhibition of LDL oxidation may be due to interaction of the nitroxides with copper thus hindering the metal binding to the LDL particle, may be excluded, since neither EPR nor spectroscopic evidence has been found for complex formation between nitroxides and copper ions [6]. Furthermore, no reaction was observed when reacting nitroxides and copper ions in aqueous acetonitrile.

Hydroxylamine derivatives of the nitroxides were also assessed in order to determine the relative importance of the nitroxide moiety in the radical scavenging mechanism, compared to the reduced nitroxide. In principle, the hydroxylamines tested in this study could potentially be better inhibitors of

LDL oxidation since they may have a dual action of both hydrogen donation to a lipid radical, whether alkyl, alkoxyl or peroxyl radical, thereby inhibiting the propagation of free-radical reactions, as well as the ability of the oxidized form of the hydroxylamine after hydrogen donation, the nitroxide, to possess radical scavenging properties. No difference was observed between the nitroxide derivatives and their associated hydroxylamines. Miura *et al.* [26] have previously reported, applying an oxazolidine nitroxide, that no difference between nitroxide and reduced nitroxide was observed in prevention of microsomal lipid peroxidation induced by NADPH.

Literature reports have shown that many commonly used nitroxide radical spin probes, such as piperidines and doxylstearates at concentrations as high as 100 μM have no adverse effects on cell survival, therefore are relatively non-cytotoxic [28] and that they are tolerated remarkably well by animals [29]. Although toxicity results on indolinone and quinoline nitroxides are awaited, there exists the possibility that these nitroxides or other appropriate nitroxide derivatives, might be considered as lipophilic therapeutic antioxidants.

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