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QUINOLINIC AMINOXYL PROTECTS ALBUMIN AGAINST PEROXYL RADICAL MEDIATED DAMAGE

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A study of peroxyl radical-mediated bovine serum albumin oxidation in the presence of the quinolinic aminoxyl 1,2-dihydro-2,2-diphenyl-4-ethoxy-quinoline-1-oxyl (QAO) was carried out in order to test its efficiency as a protein antioxidant. Albumin oxidation was induced by the *tert*-butylhydroperoxide/PbO_2 system. The extent of protein oxidation, measured by monitoring the formation of carbonyl groups, was considerably reduced in the presence of QAO. ESR measurements were carried out to confirm the consumption of the nitroxide during oxidation and its incorporation in the protein. The data obtained indicate that the quinolinic aminoxyl function can be used as an effective antioxidant in biological systems.

KEY WORDS: Albumin, protein oxidation, quinolinic aminoxyl, antioxidants, free radicals.

Abbreviations: Bu^tOOH: *tert*-butylhydroperoxide; BSA bovine serum albumin; QAO: 1,2-dihydro-2,2-diphenyl-4-ethoxy-quinoline-1-oxyl; TEMPO: 2,2,6,6-tetramethylpiperidine-1-oxyl; DNPH: 2,4-dinitrophenylhydrazine; TCA: trichloroacetic acid; QINO: quinolinic quinoneimine-N-oxide.

INTRODUCTION

There is an increasing body of evidence that free radical damage contributes to the aetiology of many chronic health problems such as cardiovascular and inflammatory diseases, atherosclerosis and cancer[1] and in many instances an enhanced antioxidant status seems to diminish oxidative stress in these free-radical related diseases.[2] Considerable interest exists in the possibility that albumin may play an important protective role in the oxidant-antioxidant balance *in vivo*.[3] However, free radicals may cause the fragmentation of proteins[4] or modification of their aminoacid residues,[5,6] and consequently render them more susceptible to enzymatic hydrolysis.[7,8,9] In fact, it has been found that radical damaged bovine serum albumin (BSA) was

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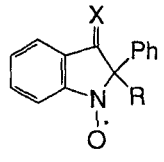
endocytosed and degraded up to 2.5 fold more rapidly than native BSA, but some radical damaged BSA was inefficiently catabolized and accumulated by macrophages thus contributing to the development of atherosclerosis.[10]

Aminoxyls such as the tetramethyl-piperidines and -pyrrolidines which have been widely used as spin labels in polymers,[11] biological systems[12] and as MR imaging contrast agents[13] also possess antioxidant properties. In fact they have been shown to prevent DNA[14] and phospholipid damage.[15] Their activities akin to superoxide dismutase[16] and catalase[17] make them interesting mimics of these enzymes. Furthermore, secondary amines applied as food additives are good antioxidants and it is already commonly accepted that they work through the intermediate formation of their corresponding aminoxyls.[18] The ability of aminoxyls to penetrate cell membranes[19] makes them attractive compounds for highly sensitive methods in the study of lipid oxidation kinetics and as membrane antioxidants in biological systems.[20,21] The antioxidant property of aminoxyls like 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO) and 4-hydroxy-TEMPO was attributed to their efficiency in trapping carbon-centered radicals, a process which is almost diffusion controlled.[22–25]

Recently, several indolinic (**1**) and quinolinic (**2**) aminoxyls (Figure 1) were synthesized in our laboratory and their antioxidant efficacy was compared with TEMPO. These studies were carried out on lipid systems consisting of linolenic acid micelles[26] and low-density lipoproteins (LDL).[27] The results obtained showed that the newly synthesized aminoxyls were very efficient in preventing lipid peroxidation. Perhaps the greater antioxidant efficacy is based on the more extended reactivity of these aminoxyls since they not only react with carbon-centered radicals but also with oxygen-centered radicals such as alkoxyl[28] and peroxyl radicals.[29] In this study we investigated the effect of the quinolinic aminoxyl 1,2-dihydro-2,2-diphenyl-4-ethoxy-quinoline-1-oxyl (QAO; Scheme 1) on peroxyl radical-mediated oxidative modification of bovine serum albumin (BSA). This aminoxyl was chosen due to its high antioxidant capability.[26]

MATERIALS AND METHODS

QAO was synthesised in our laboratory according to Berti *et al.*[30] Fatty acid free bovine serum albumin (BSA) and trichloroacetic acid (TCA) were obtained from Sigma; 2,4-dinitrophenylhydrazine (DNPH) and *tert*-butylhydroperoxide (Bu^{t}OOH)



X = O or NPh
R = alkyl or phenyl

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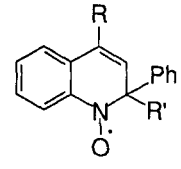


R = -OEt(QAO), -H or -C(CH$_3$)$_2$OH
R' = Me or Ph

2

FIGURE 1 General structures of indolinic (**1**) and quinolinic (**2**) aminoxyls.

were purchased from Aldrich. The oxidation of BSA was induced by incubating 5 mg of protein with 2 ml of 50 mM potassium phosphate buffer pH 7.4 containing 1 mM Bu^tOOH and 5 mg of PbO_2. Although lead dioxide (PbO_2) is insoluble, this system generates peroxyl radicals.[28] Inhibition of protein oxidation was carried out in the presence of 100–400 μM quinolinic aminoxyl. The amount of carbonyl residues in protein samples was determined by the method of Levine *et al.*[31] Specifically, the carbonyl content was evaluated on 200 μg of BSA, and calculated using a molar absorption coefficient of 22000 $M^{-1}cm^{-1}$ at 360 nm.[31]

Samples for ESR measurements were obtained by using appropriate amounts of 0.2 M stock quinolinic aminoxyl ethanol solution and adding to ESR tubes containing either ethanol or BSA (10 mg/ml) in phosphate buffer pH 7.4, to reach a final QAO concentration of 200 μM. Spectra were recorded by using a Varian E4 ESR spectrometer with a ruby placed in the ESR cavity as a reference.

RESULTS AND DISCUSSION

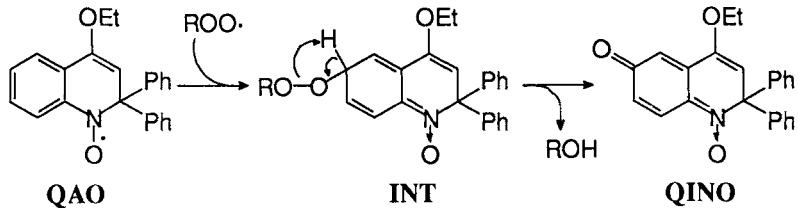
In this study the protective effect of QAO on radical mediated bovine serum albumin oxidation induced by the Bu^tOOH/PbO_2 system has been investigated. The carbonyl group content has been widely used as an index of oxidative protein damage.[31] Exposure of BSA to the $Bu^tOO\bullet$ radical generating system resulted in an increase in carbonyl residues (see Table 1). When QAO was added to the system, the oxidation of BSA was considerably reduced and a complete protection against BSA oxidation was reached when QAO was added to a final concentration of 400 μM (see Table 1). The suppression of protein oxidation by QAO may be explained by the scavenging of peroxyl radicals by the nitroxide QAO with the formation of the corresponding quinolinic quinoneimine-N-oxide (QINO), through the intermediate INT (see Scheme 1). This proposal was confirmed by comparison on thin layer chromatography with an authentic sample, and according to the reaction already described for the indolinic aminoxyls[29] (data not shown).

Furthermore, the protective effect of QAO on oxidized BSA was confirmed by ESR spectroscopy. Figure 2 shows the ESR spectra of QAO containing samples. In particular, the spectrum of the aminoxyl in ethanol in the presence of N_2 shows a

TABLE 1

Carbonyl group content of BSA after peroxyl radical-mediated oxidation and antioxidant effect of QAO. Three different sets of experiments were carried-out (I, II, III). Oxidation of BSA was performed by incubating 5 mg of protein in 2 ml of 50 mM potassium phosphate buffer pH 7.4, in the presence of 1 mM Bu^tOOH and 5 mg of PbO_2. Inhibition of protein oxidation was carried out by adding appropriate amounts of stock 0.2 M quinolinic aminoxyl ethanol solution to a final concentration of 100–400 μM. Analysis of carbonyl content was evaluated on 200 μg of BSA according to Levine *et al.*[26]

		Carbonyl Group Content in BSA (nmol carbonyl/mg protein)		
Sample	Experiment	I	II	III
BSA (Control)		1.36	1.50	1.54
BSA + Bu^tOOH + PbO_2		3.10	3.45	3.35
BSA + Bu^tOOH + PbO_2 + QAO (100 μM)		2.00	2.30	2.34
BSA + Bu^tOOH + PbO_2 + QAO (200 μM)		1.60	1.90	1.86
BSA + Bu^tOOH + PbO_2 + QAO (400 μM)		1.40	1.60	1.58



SCHEME 1 Reaction of peroxyl radical with QAO. QAO: 1,2-dihydro-2,2-diphenyl-4-ethoxy-quinoline-1-oxyl; INT: intermediate product; QINO: quinolinic quinoneimine-N-oxide.

well resolved hyperfine structure but only three broadened lines in the presence of O_2. The ESR spectrum of QAO in the presence of albumin and N_2 is composed of only one broad line. QAO injected in phosphate buffer in the absence of albumin does not give an ESR signal (data not shown) as a consequence of the insolubility of the aminoxyl in water. The presence of the broad signal in the albumin containing sample thus indicates that QAO was solubilized by binding to BSA (Figure 2). The aminoxyl spectrum was extremely sensitive to the presence of oxygen inside the solvent (Figure 2a and 2b) but only marginally when QAO was bound to the protein (Figure 2c and 2d). The consumption of QAO in the peroxyl radical generating system was revealed by the ESR spectrum of BSA-bound QAO in the absence and in the presence of peroxyl radical generating system (Figure 3). The ESR data clearly indicate that a considerable amount of QAO is consumed in the presence of $Bu^tOO\bullet$ during a short period lasting a few minutes.

From the results obtained in this study, we can conclude that QAO is an efficient antioxidant of free-radical induced protein damage just as it was efficient in preventing lipid peroxidation.[26,27] In this study, it has been demonstrated that QAO can be kept in solution by interaction with BSA (see ESR spectrum) which belongs to a class of proteins that non-covalently bind various types of hydrophobic ligands.[32] However it is not excluded that other soluble proteins could solubilize QAO. The protective effect of QAO against oxidation is most probably due to its ability to reach the carbon-centered and oxygen-centered free radicals which may be formed in a lipid bilayer and/or in the aminoacid side chains of proteins.

All kinds of aminoxyls may react at the nitroxide function with carbon-centered radicals inhibiting peroxidation processes and in this way these compounds act as antioxidants.[20,21,26,27] On the other hand, aminoxyls may abstract a labile hydrogen atom initiating an autoxidation chain reaction. In this case aminoxyls may exert a prooxidant effect.[26,33,34] Thus, it could be argued that those aminoxyls having a low hydrogen abstracting capability and a high radical scavenging ability could be considered as most suitable in preventing oxidation of biomolecules. The QAO aminoxyls type could therefore be proposed as a powerful new class of chain-breaking antioxidant for the study of both lipid and protein oxidation and as a promising antioxidant in anti-radical interventions, for example during organ reperfusion. As suggested it conforms to the requirements stated above. Although toxicity results are necessary on these aminoxyls, the possibility exists that these compounds could be considered as lipophilic therapeutic antioxidants since literature reports have shown that many commonly used aminoxyls such as piperidines and doxyl-stearates at concentrations as high as 100 μM have no adverse effects on cell

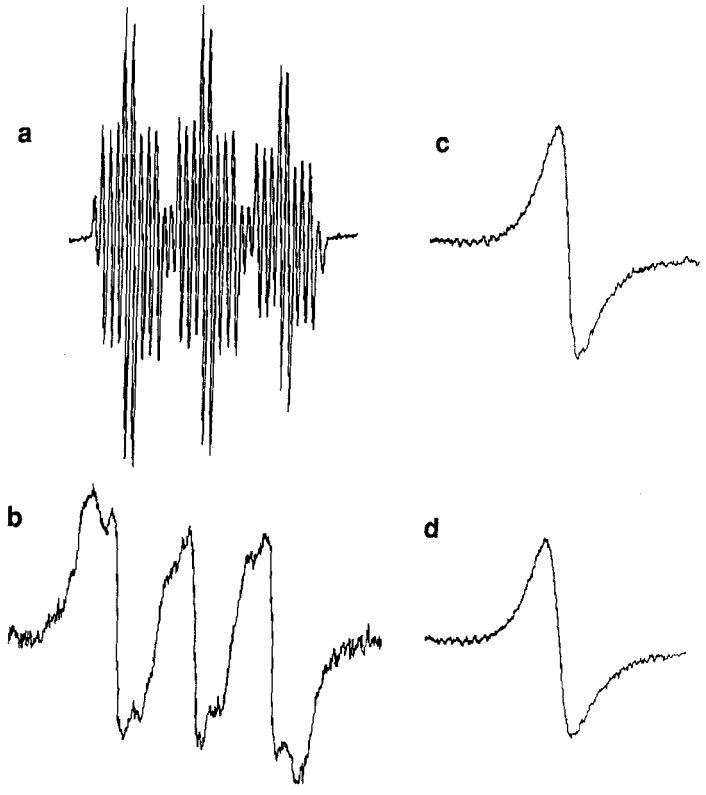


FIGURE 2 ESR spectra of QAO. Spectra (a) and (b) refer to QAO dissolved in ethanol in the presence of N_2 and O_2, respectively. Spectra (c) and (d) refer to QAO bound to albumin in the presence of N_2 and O_2, respectively in phosphate buffer pH 7.4. Concentration of QAO was 200 μM in all cases. Concentration of albumin was 10 mg/ml.

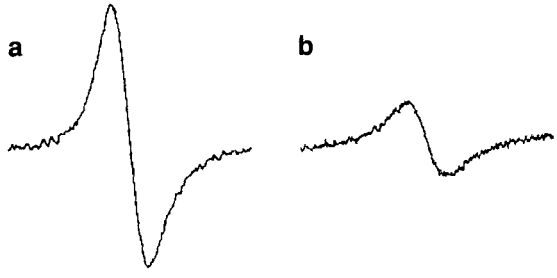


FIGURE 3 ESR spectra of albumin-bound QAO. Spectra (a) and (b) refer to albumin-bound QAO in the absence and in the presence of peroxyl radical generating system (1 mM Bu^tOOH and 5 mg of PbO_2), respectively. Concentration of QAO was 400 μM. Concentration of albumin was 10 mg/ml.

survival.[35] Some aminoxyls are tolerated remarkably well by animals,[36] although others show either toxic or anesthetic properties at high dose.[37]

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