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Antioxidant and Pro-oxidant Effect of the Thiolic Compounds *N*-acetyl-L-cysteine and Glutathione against Free Radical-induced Lipid Peroxidation

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The antioxidant ability of thiol compounds has been the subject of much of the current research about oxidative stress. The direct scavenging of hydroxyl radicals by thiols has been suggested as their protection mechanisms. Nevertheless, the interaction of thiols with reactive radicals can generate thiyl radicals, which, in turn, may impart a pro-oxidant function. The purpose of this study has been to establish the effect of the thiol compounds *N*-acetyl-L-cysteine (NAC) and glutathione (GSH) against the peroxidative processes involving membrane lipids. The results obtained support the ability of NAC and GSH to suppress the 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH)-dependent or to enhance the Fe^{2+}/H_2O_2-dependent oxidative actions. The evaluation of thiobarbituric acid reactive substances (TBARS) production, the study of the influence of oxidants on membrane fluidity and the measurements of the changes in the fluorescence of bilayer probes, such as 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA), have shown the antioxidant and pro-oxidant effects of both NAC and GSH. Also their dependence on the nature of the radicals generated by the oxidative systems used has been shown. The use of ESR spectroscopy has allowed us to establish the ability of these compounds to scavenge the AAPH-derived radicals, to determine the formation of thiyl radicals in the iron-mediated oxidation and to evaluate the enhanced production of hydroxyl radicals by NAC and GSH.

Keywords: Liposomes; *N*-acetyl-L-cysteine; Glutathione; ESR; Spin trapping; Free radicals

Abbreviations: AAPH, 2,2′-azobis-(2-amidinopropane) dihydrochloride; BBE, bovine brain extract II; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPH-PA, 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid; ESR, electron spin resonance; G, gauss; GS•, glutathionyl radical; GSH, glutathione; GSSG, oxidised glutathione; hfc, hyperfine coupling constants; HRP, horseradish peroxidase; I_{VH}, intensity obtained with an analyser oriented perpendicular; I_{VV}, intensity obtained with an analyser oriented parallel; L•, lipid radical; LDL, low density lipoproteins; MDA, malondialdehyde; MLVs, multilamellar vesicles; NAC, *N*-acetyl-L-cysteine; •OH, hydroxyl radical; P, polarisation; POBN, α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone; ROS, reactive oxygen species; RS•, thiyl radical; SUVs, small unilamellar vesicles; TBARS, thiobarbituric acid reactive substances; TRIS, 2-amino-2-(hydroxymethyl)-1,3-propanediol

INTRODUCTION

The balance between pro-oxidant and antioxidant mechanisms is essential for the life of aerobic systems and oxidative stress happens when the antioxidant capability of the cellular systems is overwhelmed, with the consequent imbalance between antioxidants and pro-oxidants.[1–3] Besides this endogenous oxidative stress that can be associated with many pathological states,[4–9] there can be an exogenous oxidative stress influenced by agents such as radiation, trauma, drug activation or oxygen excess.[10–12] In any case, the uncontrolled overproduction of pro-oxidants will result in a free radical damage to cellular structures and to their molecular constituents like proteins, lipids, DNA and carbohydrates and in a possible alteration of cell function.[13–16]

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It has been well established that the oxidative stress mainly affects the membranous systems of aerobic cells. The overexposure of biomembranes to reactive oxygen species (ROS) results in progressive structural and functional degenerative changes. Cytotoxic effects include metabolic changes, effects on membrane-associated enzymes and alterations in the physical properties of bilayer phospholipids and, consequently, in the dynamic properties of the bilayers such as fluidity and permeability.[17–19]

Antioxidant compounds have the ability to behave as scavengers of free radical species or to act as chain-breaking substances and they can remove chemically or catalytically the pro-oxidant species generated as the result of oxidative stress.[20–22] They can be natural or synthetic compounds, hydrophobic or hydrophilic molecules, enzymes or vitamins,… etc. There are quite a number of references in the literature about the antioxidant ability of thiolic compounds.[23,24] There are some sulphur-containing compounds, with antioxidant properties, that have been used in antioxidant therapy, such as *N*-acetyl-L-cysteine (NAC), penicillamine, mercaptopropionylglycine and dihydrolipoate.[25–27] NAC, for example, besides being an oxygen radical scavenger, is a precursor for the biosynthesis of glutathione (GSH) and can be used as an antidote in paracetamol intoxication. The role of GSH is pivotal in protecting aerobic cells against free radical damage since it functions both as substrate or cofactor of antioxidant enzymes and as a good radical scavenger.[28,29] Nevertheless, the interaction of GSH with reactive radicals would generate the glutathionyl radical ($GS^\bullet$), the fate of which in biological systems has been the subject of much research in the areas of radiation sensitivity and biochemical toxicology.[30–32]

$$R\text{–}SH + {}^\bullet OH \rightarrow R\text{–}S^\bullet + H_2O \quad (1)$$

The reactions of the thiyl radicals in biological systems are complex and involve the subsequent generation of excited species, but the outcome of thiyl radical production is likely to be the accumulation of oxidised glutathione (GSSG), which is also involved in interactions with other endogenous antioxidants.[33]

Thus, although GSH and others thiols are often considered to be cellular antioxidants, there are references in the literature suggesting the implication of thiol-derived radicals in the oxidative damage of biological systems.[34] Thus, it has been shown the cellular oxidation of low density lipoproteins (LDL), involved in the pathogenesis of atherosclerosis, by thiol production in media containing transition metal ions and the ability of thiyl radicals to abstract hydrogen atoms from polyunsaturated fatty acids. Also, the oxidation of thiols to form sulphur-centred radical species ($GS^\bullet$), when transition metals are present, may impart a pro-oxidant function by means of the reduction of these metals to catalytic states capable of reacting with H_2O_2 to generate ${}^\bullet OH$.[35,36] In this way, the oxidative metabolism of GSH would enhance, rather than diminish, the production of ${}^\bullet OH$ and would cause damage to membrane lipids and to DNA.

The present paper studies the effect of the thiolic compounds GSH and NAC on the iron-catalysed generation of ${}^\bullet OH$ by means of the Fenton reaction. The results are compared with those obtained when the oxidation of the bilayer lipids is achieved with the participation of the water-soluble azo-compound 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH). The use of electron spin resonance spectroscopy (ESR) has allowed us to establish the ability of these compounds to scavenge the AAPH-derived radicals, to determine the formation of thiyl radicals in the iron-mediated oxidative processes, and to evaluate the enhanced production of ${}^\bullet OH$ by NAC and GSH.

MATERIALS AND METHODS

Materials

The lipidic substrate used in this study was a bovine brain extract (BBE, obtained from Bioiberica S.A.) which mainly contains phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, cholesterol and phosphatidylserine in a 29, 27, 17, 11 and 9%, respectively. All experiments were performed in a 20 mM Tris–HCl buffer solution (pH 7.4), prepared from 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) (Boerhinger Mannheim) and chlorhydric acid (Panreac). Milli-Q water (generated by a Millipore system with a resistivity of 18 MΩ) was used to prepare all solutions.

AAPH and Fe^{2+}/H_2O_2 were used as oxidants. AAPH was purchased from Polysciences (Warrington, PA) and dissolved in the buffer solution. The Fe^{2+} solutions were prepared from $Fe(NH_4)_2(SO_4)_2{\cdot}6H_2O$ (Probus) using Milli-Q water. Hydrogen peroxide was obtained from Probus and dissolved in the buffer. Iron, AAPH and hydrogen peroxide solutions were prepared immediately before use and maintained in amber glass vials.

NAC and GSH were purchased from Sigma Chemical. Stock solutions of the thiolic compounds were prepared in the buffer solution indicated and stored at 0°C in amber glass vials.

Horseradish peroxidase (HRP) (type VI) and the spin traps α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (4-POBN) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were purchased from Sigma Chemical and dissolved in Milli-Q water to perform ESR experiments. The fluorescent probes 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl) phenylpropionic acid (DPH-PA)

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and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Molecular Probes and Sigma Chemical, respectively. Stock solutions of DPH-PA and DPH were prepared in *N*,*N*-dimethylformamide and tetrahydrofuran, respectively. The spin traps and the fluorescent probes were stored at −20°C in amber glass vials under nitrogen. All other reagents were of analytical grade.

Liposome Preparation

Multilamellar or unilamellar liposomes were prepared depending on the nature of the assay to be carried out.

Multilamellar vesicles (MLVs) were obtained by vortexing a BBE dried lipid film with 20 mM Tris–HCl buffer, pH 7.4, at 45°C for 30 min. After hydration, the dispersion of MLVs was frozen and thawed ten times using liquid N_2 and sonicated in a bath sonicator for 60 min in all cases except for thiobarbituric acid reactive substances (TBARS) measures, in which case a MLV vesicles suspension, 90 min bath-sonicated, was used. To prepare liposomes for the DPH-PA fluorescence assay, the lipid and the DPH-PA solutions were dried together and the following steps, carried out to obtain unilamellar vesicles, were conducted away from direct light. The lipid concentration in the dispersion of MLVs was 2 mg ml^{-1} if not otherwise is indicated.

Small unilamellar vesicles (SUVs) for fluidity, fluorescence of DPH-PA and ESR measurements, were prepared from the suspension of MLVs by extrusion, under pressure, through 400, 200 and 100 nm pore-sized polycarbonate filters for 7, 7 and 12 times, respectively (extruder device from Lipex Biomembranes, Canada).

TBARS Measurements

The measure of TBARS gives the amount of the peroxidation products resulting from the oxidation of the bilayer lipids. The concentration of malondialdehyde (MDA) and related compounds was determined by the formation of a pink chromophor (λ_{max} 532 nm), due to their reaction with thiobarbituric acid at high temperature.[37]

MLVs were incubated at 37°C with NAC or GSH prior to the addition of the oxidising agent. Lipid peroxidation was initiated by adding AAPH or Fe^{2+}/H_2O_2 solutions and 0.5 ml aliquots of each incubate were withdrawn at 15, 120 and 240 min and mixed with 2 ml of 0.375% thiobarbituric acid solution, 15% of trichloroacetic acid and 0.25 M HCl. The mixtures were heated at 95°C for 15 min and then centrifuged. The absorption of the supernatant was measured at 532 nm in a Beckman DU 640 spectrophotometer and the MDA concentrations were calculated using an ε value of $1.56 \times 10^5\ M^{-1}\,cm^{-1}$.

Membrane Fluidity Evaluation

Membrane fluidity was studied by measuring the fluorescence polarisation of the lipophilic probe DPH, which is specific for the hydrocarbon core. SUVs (2 mg lipid ml^{-1}) were incubated for 12 h with the thiolic compounds (NAC or GSH), prior to the addition of the oxidant system. Lipid peroxidation was initiated by adding AAPH (10 mM) or Fe^{2+}/H_2O_2 (0.1/1 mM) to the suspensions. After 2 h of incubation at 37°C, aliquots of 500 μl of each incubate were diluted in 25 mM Tris–HCl buffer (pH 7.4) to a final volume of 2.5 ml and, then, 5 μl of a solution containing the fluorescent probe DPH (1 mM) was added. The samples containing 0.40 mg lipid ml^{-1} (0.57 mM) and 2.3 μM DPH were incubated, in the dark, for 1 h at 45°C. The lipid/DPH molar ratio in the incubates was of 250:1. The suspension was transferred to a controlled temperature cell, with continuous stirring.

The changes in fluorescence polarisation (P) were calculated from the emission intensities, obtained with an analyser whose polarisation axis was oriented parallel (I_{VV}) and perpendicular (I_{VH}) to the direction of the vertical excitation polariser, according to the following expression (Eq. (2)):

$$P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}} \tag{2}$$

The emission intensity measures were carried out at 5°C intervals, from 20 to 50°C, in a Kontron SFM 25 spectrofluorimeter, at excitation and emission wavelengths of 360 and 430 nm, respectively. The polariser used was composed of two HNP'B perpendicular polaroid filters. The position of the polarisers was controlled with a computer by specific software. The decrease in the probe mobility into the membrane core is indicated by an increasing value of fluorescence polarisation.[38]

Fluorescent Intensity Assay

The fluorescent intensity assay with DPH-PA[39] was used to evaluate the effect of the thiolic compounds, NAC and GSH, on the lipid peroxidation. An aliquot of 2.5 ml of the SUVs suspension, containing 2 mg of lipid ml^{-1} (2.84 mM) and 8.12 μM DPH-PA, was incubated at 37°C for 5 min with continuous stirring and away from light. Then, NAC or GSH were added to the cuvette and incubated for 5 min and, finally, the peroxidation was initiated by addition of three different concentrations of Fe^{2+}/H_2O_2 or AAPH. Controls were prepared in the same way, but without any oxidant system nor thiolic compound. The volume of the oxidant and thiolic compounds solutions added to liposomes never surpassed the 1% of the

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total volume of incubates. Incubates were diluted 28 times prior to the fluorescence measures.

The decay in the DPH-PA fluorescence intensity was monitored for 60 min in a SFM 25 spectrofluorimeter (Kontron Instruments), at excitation and emission wavelengths of 360 and 430 nm, respectively. The data were converted to relative fluorescence values (F_{rel}), by dividing the fluorescence intensity at a given time by the fluorescence intensity at 0 min. The percentage of oxidation was calculated using Eq. (3),

$$\%\text{oxidation} = 100 \times \frac{(F_{rel})_{control} - (F_{rel})_{antiox}}{(F_{rel})_{control} - (F_{rel})_{ox}} \qquad (3)$$

where $(F_{rel})_{control}$, $(F_{rel})_{antiox}$ and $(F_{rel})_{ox}$ are the relative fluorescence values for the control, for the sample containing the oxidant system and the thiolic compound and for the sample containing the oxidant system, respectively, at 60 min. It has been assigned the 100% of oxidation to the sample with the lowest oxidant concentration.

ESR Study

The spin-trap method uses an addition reaction of free radicals to diamagnetic spin traps to produce relatively stable spin-trap-free radical adducts[40] and the identity of a free radical can be determined from the hyperfine coupling constants (hfc) of the spin adduct. The spin trap DMPO was used for the detection of the hydroxyl radicals generated by the Fe^{2+}/H_2O_2 oxidising system (DMPO/$^{\bullet}$OH). Moreover, since DMPO also traps thiyl radicals,[30,31,41] such as those generated from the oxidation of NAC (NAC$^{\bullet}$) or GSH (GS$^{\bullet}$), it was also used to identify the action mechanism of these thiolic compounds when Fe^{2+}/H_2O_2 was used as oxidant. The spin trap POBN was used for the detection of carbon centred radicals[42,43] when the peroxidation process was initiated with AAPH or with Fe^{2+}/H_2O_2/ascorbic acid.

The reaction was initiated by the addition of a small aliquot of the oxidant system to a 20 mM Tris–HCI buffer solution (pH 7.4) containing the spin trap (500 mM DMPO or 300 mM POBN), the thiolic compound and the liposomal suspension (2 mg ml^{-1}).

To obtain the ESR spectra, the incubates were diluted five times prior the measures, and an aliquot of 400 μl of each sample was transferred to a flat quartz aqueous ESR cell. ESR spectra were recorded using a Bruker ESP300E ESR spectrometer. When Fe^{2+}/H_2O_2 was used as oxidant, the measures were obtained at 0.5, 10 and 15 min after the addition of the oxidant. With AAPH, the spectra were recorded at 2, 4 and 6 h and, finally, with Fe^{2+}/H_2O_2/ascorbic acid only one spectrum was obtained at 12 h.

When the lipid peroxidation was initiated with AAPH or Fe^{2+}/H_2O_2/ascorbic acid, the ESR settings were as follows: modulation amplitude, 2.018 G; time constant, 10.24 ms; receiver gain, 2×10^5; frequency, 9.77 GHz; resolution of the field axis, 1024 points; power 10 mW; conversion time, 81.92 ms; sweep width, 60 G; and sweep time 83,8 s. On the other hand, the settings, when the oxidant system Fe^{2+}/H_2O_2 was used, were as follows: modulation amplitude, 0.72 G; time constant, 20.48 ms; receiver gain, 2×10^5; frequency, 9.77 GHz; resolution of the field axis, 1024 points; power, 10 mW; conversion time, 163.84 ms; sweep width, 60 G; and sweep time, 167.6 s.

Statistics

Results are expressed as mean ± SD. Statistical significance was examined using the Kruskal–Wallis one-way ANOVA on ranks test and the multiple comparison Kruskal–Wallis z-values due to non-homogeneity in variances. Values less than $p < 0.05$ were accepted as significant.

RESULTS AND DISCUSSION

Liposome Oxidation as Measured by TBARS Formation

TBARS production was first evaluated in liposomes samples undergoing AAPH-induced lipid peroxidation in the absence and in the presence of the thiolic compounds NAC (Fig. 1a) and GSH (Fig. 1b). TBARS levels did not show significant changes after 15 min of oxidation, neither in the presence of NAC nor GSH, independently of the concentration assayed. However, after 120 and 240 min of oxidation, it can be observed an inhibition of the TBARS production, of about 50%, in the presence of both thiolic compounds. The percentage of inhibition did not show any change with time and was independent on the thiol nature and on the thiol concentration in the sample.

When hydroxyl radicals were produced by means of the iron-mediated Fenton reaction, the effect of NAC and GSH was quite different. In this case, TBARS production increased with time and, in general, with the thiol concentration, although an irregular behaviour, with regard to the influence of the concentration, was observed when the effect of NAC was assayed. Figure 1 shows the high pro-oxidant effect of both thiols, NAC (Fig. 1c) and GSH (Fig. 1d), against the hydroxyl radical oxidative damage.

Fluidity Change Studies

Peroxidation of polyunsaturated fatty acids in biomembranes always leads to a decrease of

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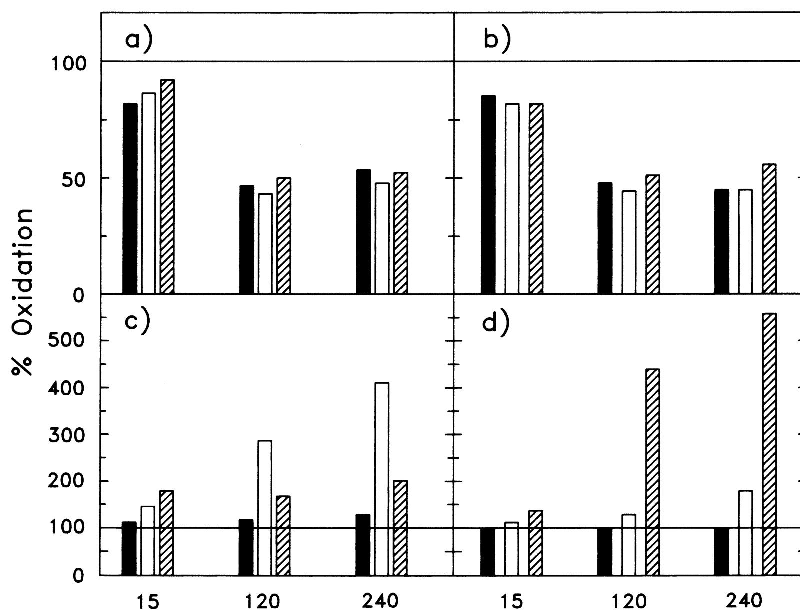

FIGURE 1 Thiobarbituric acid reactive substances (TBARS) measurements in time course of lipid peroxidation generated by BBE MLVs (2 mg lipid ml^{-1}) with 10 mM AAPH (a,b) or 0.1 mM Fe^{2+} and 1 mM H_2O_2 (c,d). The concentrations of NAC (a,c) and GSH (b,d) in the incubates were 1.25 (■), 2.5 (□), and 3.75 mM (▨) A 100% oxidation percentage was assigned to MDA content, at each time, in the oxidized samples without thiols (5.1, 9.8 and 11.6 nmol ml^{-1} with AAPH and 7.36, 8.2 and 9.4 with Fe^{2+}/H_2O_2 at 15, 120 and 240 min, respectively). Absorption measurements were carried out at 532 nm and room temperature.

membrane fluidity. Diphenylhexatriene (DPH), as a lipid probe inserted in the hydrophobic core of the lipid bilayer, was used to determine membrane fluidity by the fluorescence polarisation method. Polarisation (P) changes were determined at different temperatures, from 20 to 50°C and the values obtained at 37°C are shown in Fig. 2. Statistical comparison of the results after the application of Kruskal–Wallis one way ANOVA on ranks test and the multiple comparison Kruskal–Wallis z-value test showed the significances with respect to the oxidised sample without thiols given in Fig. 2.

When liposomes were oxidised (bar B), in the absence of the thiolic compounds, membrane rigidity, expressed as the degree of fluorescence polarisation (P), showed a significant increase with AAPH (Fig. 2a, $p < 0.05$) but not with Fe^{2+}/H_2O_2 (Fig. 2b, $p > 0.05$), with regard to the non-oxidised sample (bar A). The incubation of liposomes either with NAC or GSH, before to AAPH addition, gives decreases in P values, which were in all cases significantly different ($p < 0.05$) with respect to those obtained for the oxidised sample without thiols (Fig. 2a, B). The decreases in P observed in the presence of NAC and GSH are dose dependent. Moreover, the differences between the control (Fig. 2a, A) and the thiol-containing samples (Fig. 2a, C–F) neither were significant, as the values of P in the presence of thiols became similar to that corresponding to the non-oxidised sample. The results obtained showed that, at the same concentration, GSH and NAC displayed a similar behaviour (bars C and E or D and F).

The incorporation of the thiolic compounds to the samples undergoing Fe^{2+}/H_2O_2 oxidative attack increased the membrane rigidity, independently of the nature of the thiol, and in a concentration-dependent manner (Fig. 2b, C–F). Nevertheless, only the changes observed for samples containing the thiolic compounds at 3.75 mM (bars D and F) were statistically significant with regard to the oxidised sample (bar B). The differences between the control (bar A) and the samples containing thiols (bars C–F) were always significant. Moreover, P values were higher as the thiol concentration increased and both thiols display the same behaviour against the fluidity changes resulting from lipid peroxidation processes.

There are references in the literature showing decreases in the membrane fluidity associated to any free radical attack to the membrane lipids.[18,19,44] The data obtained when liposomes were exposed to oxidising conditions, as presented here, are consistent with the reported observations with regard to the polarisation changes in the presence of pro-oxidant species. The changes in the membrane fluidity could be explained by the enhanced cross-linking between the membrane lipid moieties, which results from lipid peroxidation.[44] Then, the behaviour of the thiolic compounds will depend on the oxidising conditions. In AAPH-induced lipid peroxidation, the mean values of P decreased in the presence of NAC or GSH, with regard to the damaged sample, so it could be concluded that the thiolic compounds are able to inhibit the progress of AAPH-induced lipid peroxidation and to avoid the decrease of membrane fluidity. However, in the iron-mediated Fenton

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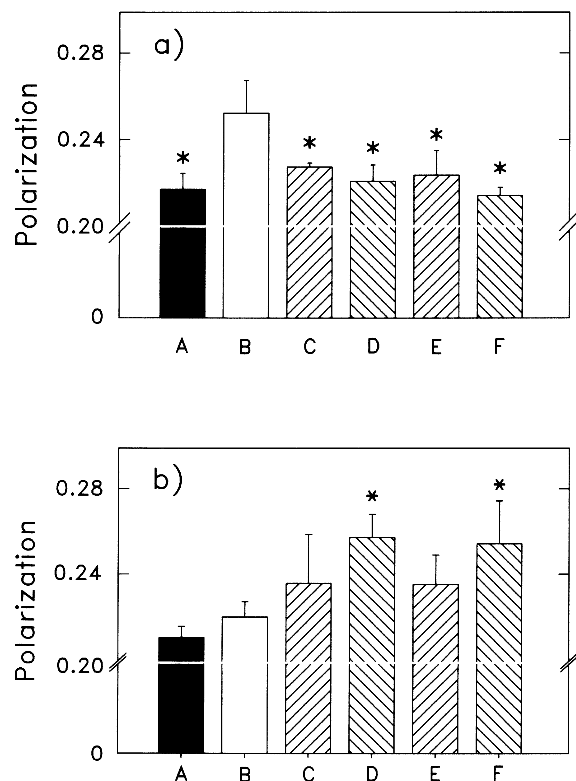

FIGURE 2 Influence of NAC and GSH in the bilayer fluidity changes induced by AAPH (a) or by Fe^{2+}/H_2O_2 (b) lipid peroxidation. 10 mM AAPH or 0.1 mM Fe^{2+} and 1 mM H_2O_2 were added, at 37°C, to 2 mg lipid ml^{-1} SUVs dispersions, incubated with different amounts of the thiols for 2 h, to initiate lipid peroxidation. The diluted incubates containing 0.4 mg lipid ml^{-1} (0.57 mM) and 2.3 μM DPH were incubated, in the dark, for 1 h at 45°C. The lipid : DPH molar ratio in the incubates was of 250 : 1. The fluorescence polarisation, P, was calculated according to Eq. (2). (A) control, (B) oxidant, (C) oxidant + 1.25 mM GSH, (D) oxidant + 3.75 mM GSH, (E) oxidant + 1.25 mM NAC and (F) oxidant + 3.75 mM NAC. The polarisation values in the figure corresponds to the mean values ± SD ($n = 4$), in each group. The polarisation values, which were significantly different ($p < 0.05$) from the oxidised sample without thiols (B) are indicated by an asterisk.

generation of hydroxyl radicals, the mean values of P were higher than those corresponding to the non-oxidised and oxidised samples, independently of the nature of the thiolic compound assayed. This result points out the ability of thiols to enhance the lipid peroxidation in the presence of hydroxyl radicals and to act as pro-oxidant molecules.

Evaluation of the Fluorescence of DPH-PA

The fluorescent intensity assay described by Arora *et al.* [39] was used to corroborate that the antioxidant and pro-oxidant effects of both NAC and GSH were dependent on the way by which radical species were generated.

The control SUVs that did not contain any added oxidant and thiolic compound had very stable fluorescence intensity values over the course of the assay (more than 90% after 60 min). Addition of AAPH to the SUVs, in the absence of the thiols, caused a gradual drop in the fluorescence intensity of DPH-PA, being the intensity value of 20% after 60 min when 10 mM AAPH was added to SUVs containing 2 mg ml^{-1} lipid and 8.12 μM probe. On the other hand, when 0.1/1 mM Fe^{2+}/H_2O_2 were added to SUVs containing the same amount of lipid and probe, a rapid drop in the fluorescence intensity of DPH-PA was observed over the first 5 min of the assay (70% of the initial value). This rapid decrease in the fluorescence intensity was followed by a more gradual loss of the signal for the remaining 55 min of the assay period (67% of the initial value at the end of the experiment).

The antioxidant efficiencies of NAC and GSH were evaluated by their degrees of inhibition of the decay in fluorescence intensity of the extrinsic probe DPH-PA. Analysis of the action of the thiolic compounds on induced lipid peroxidation in the SUVs revealed distinct effect when AAPH or Fe^{2+}/H_2O_2 were used as oxidants. Figure 3 shows the antioxidant effects of both thiols on AAPH induced lipid peroxidation. It can be observed that the percentage of oxidation increased with the concentration of AAPH in the system in a dose dependent manner. The inhibition of AAPH oxidative stress by each thiolic compound was very strong and almost independent on the three concentrations of thiols tested. Moreover, the ability of both thiols to inhibit AAPH induced peroxidation was very similar.

Figure 4 shows, on the contrary, the pro-oxidant effect of both thiols when lipid peroxidation was induced with the Fe^{2+}/H_2O_2 system. The oxidative stress induced with Fe^{2+} ions increased, in the same way as for AAPH, with the concentration of the oxidant in a dose dependent manner. Nevertheless, at all thiol/Fe^{2+}/H_2O_2 molar ratios assayed, an increase in the rate of decay in fluorescence intensity of the extrinsic probe DPH-PA was observed. The pro-oxidant effect of NAC and GSH are also similar but, when the highest concentration of Fe^{2+}/H_2O_2 (0.2/2 mM) was used, NAC exhibits a higher pro-oxidant activity at all the concentrations tested. Moreover, the pro-oxidant effect of both thiols, when free radicals were generated with the Fe^{2+}/H_2O_2 system, increased with the amount of NAC or GSH added to the incubation media.

The results obtained with the probe DPH-PA were in agreement with those showed with the DPH probe or the TBARS evaluation studies. The changes on the fluorescence of DPH-PA showed, again, the ability of both thiolic compounds to remove the effect of AAPH radicals, but not that of the hydroxyl radicals produced by Fe^{2+}/H_2O_2 in which case an enhancement of the oxidative damage was produced.

ESR Detection of Free Radicals

ESR spectra have been used to identify the radicals generated by AAPH and Fe^{2+}/H_2O_2 and to study

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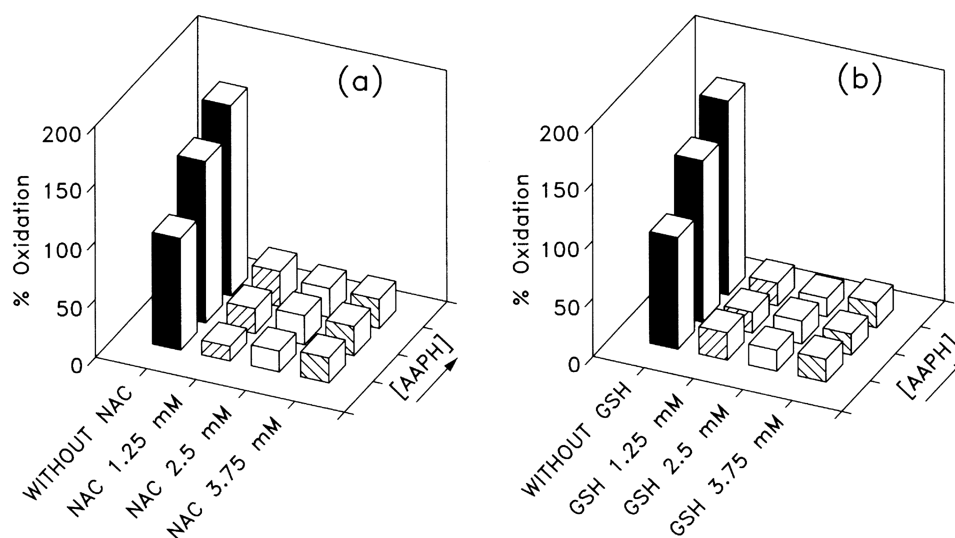

FIGURE 3 Effect of the thiols NAC (a) and GSH (b) on AAPH-induced lipid peroxidation in the SUVs at 37°C, determined by the fluorescence intensity assay of the probe DPH-PA. Peroxidation was initiated by the addition of AAPH 5, 10 and 15 mM (from ahead to behind) in SUVs containing 2.84 mM lipid (2 mg lipid ml^{-1}), 8.12 μM probe and the amounts of the thiols indicated, suspended in 2.5 ml of buffer (Tris-HCl 20 mM, pH 7.4). The percent oxidation values were calculated according to Eq. (3), using the fluorescence values at 60 min. The 100% has been assigned to the sample, without the thiols, containing the lowest concentration of AAPH (5 mM). The percentages of oxidation in the figure correspond to the mean values ($n = 4$) in each group and the coefficients of variation ranged from 1 to 8.

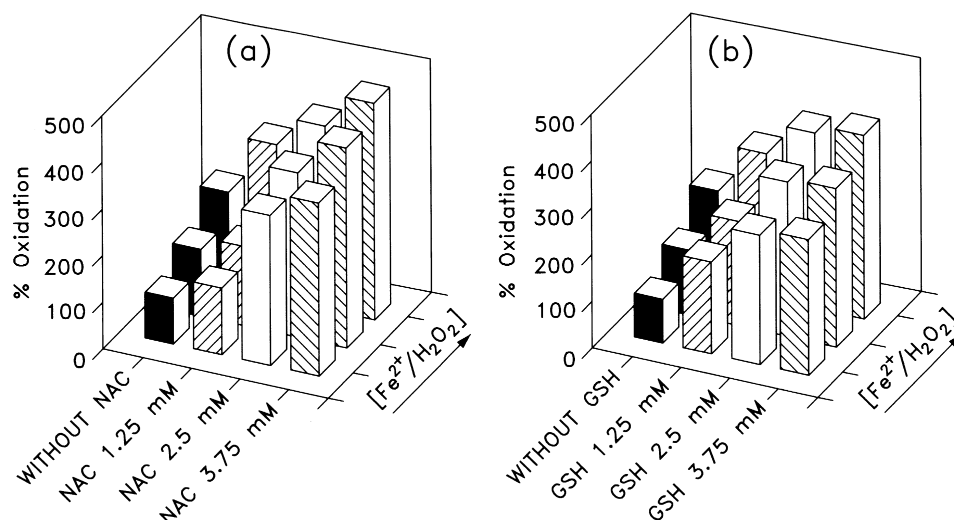

FIGURE 4 Effect of the thiols NAC (a) GSH (b) on Fe^{2+}/H_2O_2-induced lipid peroxidation in the SUVs at 37°C, determined by the fluorescence intensity assay of the probe DPH-PA. Peroxidation was initiated by the addition of Fe^{2+}/H_2O_2 0.05/0.5, 0.1/1 and 0.2/2 mM (from ahead to behind) in SUVs containing 2.84 mM lipid (2 mg lipid ml^{-1}), 8.12 μM probe and the amounts of the thiols indicated, suspended in 2.5 ml of buffer (Tris-HCl 20 mM, pH 7.4). The percent oxidation values were calculated according to Eq. (3), using the fluorescence values at 60 min. The 100% has been assigned to the sample, without thiols, containing the lowest concentration of Fe^{2+}/H_2O_2 (0.05/0.5 mM). The percentages of oxidation in the figure correspond to the mean values ($n = 4$) in each group and the coefficients of variation ranged from 3 to 15.

the effect of the thiols on the scavenging of these radicals. ESR spectra of DMPO and POBN adducts and the corresponding hfcs, using different oxidising systems, were obtained previously to identify the possible radicals generated. The ESR spectrum A (Fig. 5) was obtained when SUVs were oxidised with Fe^{2+}/H_2O_2 in the presence of the spin trap DMPO. A four-line ESR signal corresponding to DMPO/$^\bullet$OH adducts ($A_N = A_H^\beta = 14.9$ G) was detected[30] in agreement with the formation of $^\bullet$OH by means of the Fenton reaction.

The ESR spectra B and C (Fig. 5) were obtained to confirm the possible formation of NAC$^\bullet$ and GS$^\bullet$ radicals, respectively, that would account for the pro-oxidant action observed when both thiols were in the presence of the Fe^{2+}/H_2O_2 oxidative system. NAC$^\bullet$ and GS$^\bullet$ were generated in solution by the HRP enzyme reaction. Previous studies showed that DMPO could trap the radicals NAC$^\bullet$ and the GS$^\bullet$[30] generated by HRP in the presence of NAC or GSH and H_2O_2. Hyperfine coupling constants obtained from B and C spectra for DMPO/NAC$^\bullet$

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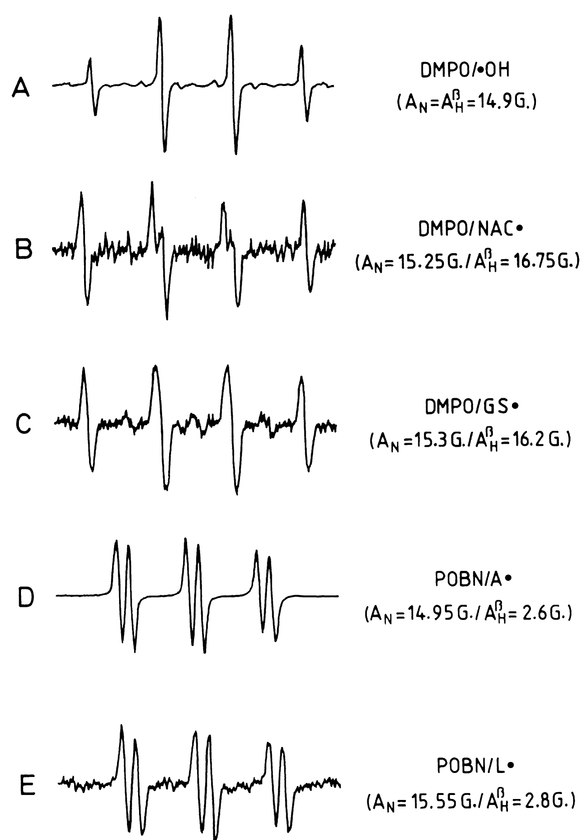
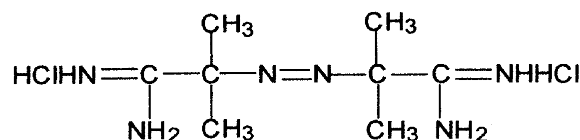


FIGURE 5 ESR spectra of DMPO and POBN adducts and hyperfine coupling constants (hfc) obtained with different oxidizing systems. DMPO and POBN concentrations in the incubates were 500 and 300 mM, respectively. SUVs dispersions used with DMPO or POBN contained 2 mg lipid ml^{-1}. Incubates were diluted to obtain a DMPO and POBN concentration of 100 and 60 mM, respectively, prior to ESR measures. (A) DMPO/$^{\bullet}$OH adduct with Fe^{2+}/H_2O_2 0.1/1 mM. (B) DMPO/NAC$^{\bullet}$ adduct with HRP 1.15 mg ml^{-1}, H_2O_2 1 mM and NAC 12.5 mM. (C) DMPO/GS$^{\bullet}$ adduct with HRP 1.15 mg ml^{-1}, H_2O_2 1 mM and GSH 12.5 mM. (D) POBN/A$^{\bullet}$ adduct with AAPH 10 mM. (E) POBN/L$^{\bullet}$ adduct with Fe^{2+}/H_2O_2/ascorbic acid 0.1/1/2 mM.

and DMPO/GS$^{\bullet}$ were in agreement with those reported for the NAC[30,45] and glutathionyl[30,46] radical adducts of DMPO.

The ESR spectrum obtained with AAPH (Fig. 5, D) gave hfc values ($A_N = 14.95$ G and $A^{\beta}_H = 2.6$ G) different from those found in the literature ($A_N =$ 15.5–15.8 G and $A^{\beta}_H = 2.5$–2.7 G) for a POBN adduct with carbon centred lipidyl radicals derived from lipid peroxidation.[42,43,47]

To assess the nature of the radicals trapped with POBN when AAPH was used as oxidant, it was assayed the influence of the liposomal concentration (2, 4 and 8 mg lipid ml^{-1}) on the ESR signal intensity (results not shown). The results showed that as the concentration of lipid was increased, the intensity of the signal in the ESR spectra, with the hfc indicated in D, decreased. Moreover, the spectrum acquired in the same conditions but in the absence of lipid had a higher intensity. The results are in contradiction with those of Freyaldenhoven *et al.*,[43] who reported the absence of any POBN ESR signal due to the thermal decomposition of 3 mM AAPH, in the absence of lipid, and the DMPO/L$^{\bullet}$ ESR signal due to the oxidation of 18:2 micelles with AAPH. This fact suggests that the radical trapped by POBN, observed in D spectrum (Fig. 5), is not a lipidyl radical obtained from the peroxidation of SUVs, but a radical generated directly from AAPH. As POBN traps carbon centred radicals,[42] the radical trapped in D (Fig. 6) could be a carbon centred radical (A$^{\bullet}$) derived from the thermal decomposition of AAPH by the sequence of reactions indicted below.[43],



AAPH: 2,2′-azobis-(2-amidinopropane) dihydrochloride (A − N = N − A).

$$A\text{–}N = N\text{–}A \rightarrow [A^{\bullet}N_2A^{\bullet}] \rightarrow 2A^{\bullet} + N_2 \quad (4)$$

$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet} \quad (5)$$

$$AOO^{\bullet} + LH \rightarrow AOOH + L^{\bullet} \quad (6)$$

To confirm this hypothesis, the ternary oxidant system Fe^{2+}/H_2O_2/ascorbic acid was used (Fig. 5, E) to produce the L$^{\bullet}$. The ESR spectrum obtained 12 h after the addition of the oxidant in the SUVs gave hfc values of $A_N = 15.55$ G and $A^{\beta}_H = 2.8$ G, which were very similar to those found in the literature[42,43,47] for the POBN/L$^{\bullet}$ spin adduct.

The ESR signal intensities of POBN/A$^{\bullet}$ were dependent both on the reaction time after mixing and on the presence and the concentration of the thiols NAC and GSH (Fig. 6). The addition of both thiols produced a decrease in the signal intensity, suggesting that NAC and GSH serve as scavengers for the reactive radical species A$^{\bullet}$ generated by thermal decomposition of AAPH. After 2 and 4 h, the effects of the two concentrations of the thiols assayed on the signal intensity were very similar and the scavenging of A$^{\bullet}$ was almost total. Nevertheless, after 6 h the inhibition was dependent on the thiol nature and concentration. Again, 3.75 mM of both thiols inhibited the formation of the POBN/A$^{\bullet}$ adduct almost completely, whereas at 1.25 mM the scavenging of A$^{\bullet}$ occurred in a lower extent. Moreover, NAC was a bit more efficient in the reduction of the amount of the A$^{\bullet}$ radical.

To establish the mechanism of the pro-oxidant effect of NAC and GSH observed by the TBARS assay and by fluidity and DPH-PA fluorescence measures, ESR spectra using the spin trap DMPO

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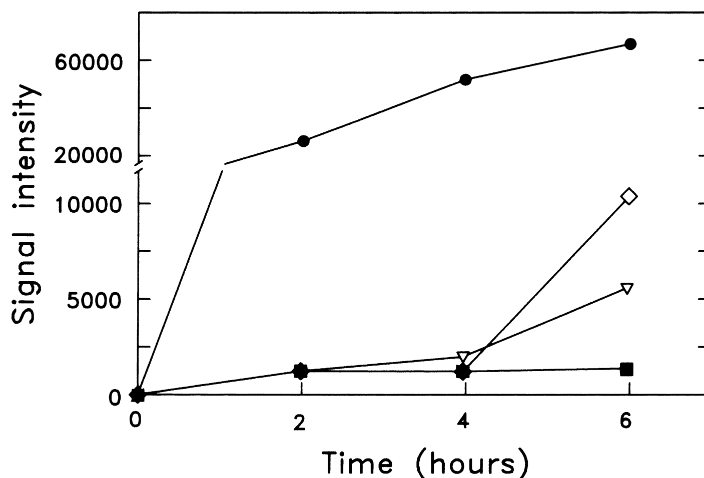

FIGURE 6 Effect of NAC and GSH on the time course of the signal intensities of POBN/A˙ adducts in AAPH-induced lipid peroxidation. Peroxidation was initiated by the addition of 10 mM AAPH in SUVs containing 2 mg lipid ml^{-1} (2.84 mM lipid), 300 mM POBN and NAC ((●), none; (▽), 1.25 mM; (▲), 3.75 mM) or GSH ((●), none; (◇), 1.25 mM; (■), 3.75 mM) suspended in 20 mM Tris-HCl buffer (pH 7.4). Incubates were diluted to obtain a POBN concentration of 60 mM prior to obtain ESR measurements at 37°C.

and the oxidative system Fe^{2+}/H_2O_2 were acquired. When SUVs were oxidised with 0.1/1 mM Fe^{2+}/H_2O_2, which produces hydroxyl radicals by the Fenton reaction, in the presence of DMPO, the typical spectra of the DMPO/$^{\bullet}$OH adducts were obtained (Fig. 7, B). The hfc $A_N = A_H^{\beta} = 14.9$ G are in agreement with those found in the literature.[47,48] When liposomes were incubated with thiols, prior to the addition of the oxidant, the intensity of ESR signals was modified in a thiol concentration dependent manner. At 1.25 and 3.75 mM of thiols (Fig. 7, C–D), the intensity of the signal was equivalent to that obtained in the absence of thiols (Fig. 7, B). However, the spectrum obtained at

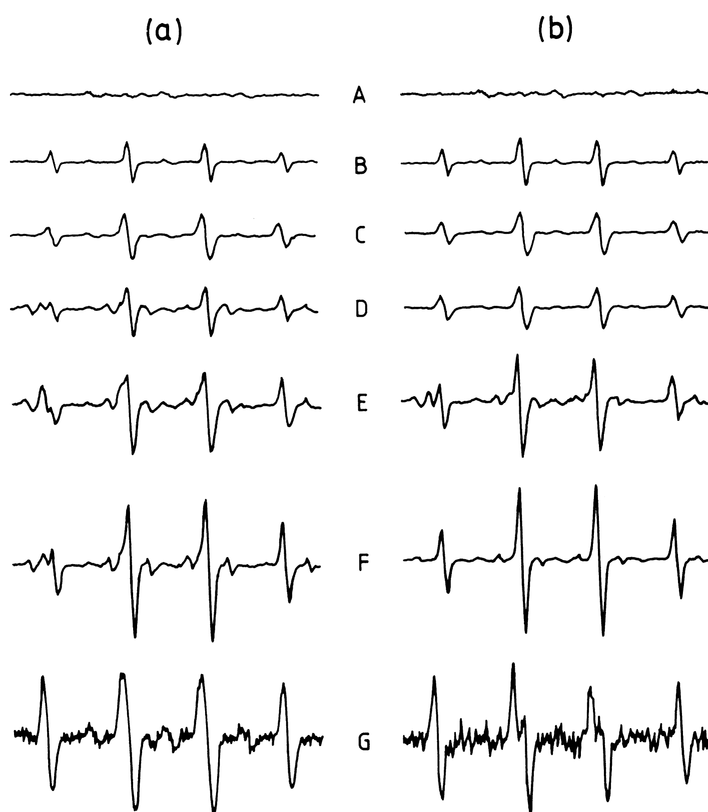

FIGURE 7 Effect of GSH (a) and NAC (b) on ESR spectra of DMPO/˙OH adducts obtained at room temperature. For the spectra A to F, peroxidation was initiated by the addition of Fe^{2+}/H_2O_2 0.1/1 mM in SUVs containing 2 mg lipid ml^{-1} (2.84 mM lipid), 500 mM DMPO and the amounts of thiols indicated, suspended in 20 mM Tris-HCl buffer (pH 7.4). Spectra A, B, C, D, E and F correspond to control, oxidised sample without thiols, and oxidised sample in the presence of 1.25, 3.75, 12.5 and 37.5 mM thiols, respectively. Spectra G were obtained using 1.15 mg HRP/ml and 1 mM H_2O_2 in the presence of the thiolic compounds at a concentration of 12.5 mM. Incubates were diluted to obtain a DMPO concentration of 100 mM prior to ESR measures.

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3.75 mM GSH (Fig. 7a, D) corresponds to a composite signal, which includes two different radical adducts, DMPO/$^{\bullet}$OH and DMPO/GS$^{\bullet}$. The hfc for the additional signal ($A_N = 15.3$ G and $A_H^{\beta} = 16.2$ G) correlated well with those obtained from the spectrum G (Fig. 7) with GSH and the oxidant system HRP/H_2O_2, in which case only thiyl radicals were generated.[30,31,41] In the same way, in the composite spectrum E (Fig. 7b) the signals of DMPO/$^{\bullet}$OH (hfc, $A_N = A_H^{\beta} = 14.9$ G) and DMPO/-NAC$^{\bullet}$ (hfc, $A_N = 15.25$ G and $A_H^{\beta} = 16.75$ G)[30,45] were shown. The NAC$^{\bullet}$ and GS$^{\bullet}$ radicals may be derived from NAC and GSH scavenging of initial $^{\bullet}$OH radicals produced by the Fenton reaction,

$$NAC + {}^{\bullet}OH \rightarrow NAC^{\bullet} + H_2O \quad (7)$$

$$GSH + {}^{\bullet}OH \rightarrow GS^{\bullet} + H_2O \quad (8)$$

The ESR signal intensities for the adducts DMPO/GS$^{\bullet}$ and DMPO/NAC$^{\bullet}$ were dependent on the concentration of both thiols in the media. Moreover, the decrease in the ESR signal intensities of these thiyl radicals, with the higher concentration of the thiol (37.5 mM), and the increase in the signal intensities of the DMPO/$^{\bullet}$OH adducts suggest the enhanced production of hydroxyl radicals in the presence of NAC and GSH. This behaviour can be explained by means the following reactions,

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-} \quad (9)$$

$$GSH + Fe^{3+} \rightarrow GS^{\bullet} + Fe^{2+} \quad (10)$$

Thus, the iron-mediated oxidative metabolism of thiols would account for their pro-oxidant action.

SUMMARY

The pivotal role of GSH in protecting cells against free radical damage has been widely demonstrated. However, the oxidative metabolism of GSH can generate thiyl free radicals that have been increasingly considered as intermediates in processes that may be involved in the development of biological damage resulting from oxidative or reductive stress. As in the case of oxygen free radicals, the formation and reaction of thiyl radicals can occur in many different ways and is likely to depend on factors such as the nature of the sulphur compound and the presence of oxygen or trace metals. The results obtained show the different behaviour of the assayed thiolic compounds in relation to lipid peroxidation. The antioxidant and pro-oxidant effects of both NAC and GSH have been demonstrated, as well as their dependence on the nature of the radicals generated by the oxidative systems used in this study. The evaluation of the TBARS produced as the result of the oxidative action shows the pro-oxidant effect of the thiols in the presence of hydroxyl radicals, whereas an antioxidant behaviour was observed against AAPH-derived radicals. The results obtained with the fluorescent probes DPH and DPH-PA also showed that the antioxidant and pro-oxidant effects of both NAC and GSH were dependent on the way that radical species were generated. The changes in the bilayer fluidity and the measurements of the decay in the fluorescence of DPH-PA, associated to lipid peroxidation, show the ability of the thiolic compounds to remove the effect of the AAPH radicals, but not the hydroxyl radicals produced by the iron-mediated Fenton reaction. Moreover, a decrease in the bilayer fluidity was observed as the result of the oxidative attack. The ESR spectra have allowed the identification of the hydroxyl and AAPH-derived radicals and the effect of the thiols on the scavenging of these radicals. The abilities of NAC and GSH to suppress the AAPH-dependent, but not the Fe^{2+}/H_2O_2-dependent, oxidative actions are in agreement with the results of the fluorescent studies and can be explained on the basis of the ESR spectra. The use of DMPO and POBN spin traps has allowed the identification of hydroxyl, AAPH-derived, lipidyl and thiyl radicals, the formation of thiyl radicals in the iron-mediated oxidation and the interaction of thiols with the different radicals species. Then, the whole of the results obtained gives actual information on the antioxidant and pro-oxidant capabilities of the thiols NAC and GSH against the oxidative action of different oxidants and provides information to propose a mechanism for such a dual behaviour in an *in vitro* situation.

Acknowledgements

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