Free Radical Research, Volume 38 Number 3 (March 2004), pp. 315–321

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Evaluation of the Antioxidant Properties of Propofol and its Nitrosoderivative. Comparison with Homologue Substituted Phenols

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Accepted by Professor E. Niki

(Received 24 July 2003; In revised form 18 November 2003)

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Propofol (2,6-diisopropylphenol), some substituted phenols (2,6-dimethylphenol and 2,6-ditertbutylphenol) and their 4-nitrosoderivatives have been compared for their scavenging ability towards 1,1-diphenyl-2-picrylhydrazyl and for their inhibitory action on lipid peroxidation. These products were also compared to the classical antioxidants butylated hydroxytoluene and butylated hydroxyanisole. When measuring the reactivity of the various phenolic derivatives with 1,1-diphenyl-2-picrylhydrazyl the following order of effectiveness was observed: butylated hydroxyanisole > propofol > 2,6-dimethylphenol > 2,6-di-tertbutylphenol > butylated hydroxytoluene. In cumene hydroperoxide-dependent microsomal lipid peroxidation, propofol acts as the most effective antioxidant, while butylated hydroxyanisole, 2,6-di-tertbutylphenol and butylated hydroxytoluene exhibit a rather similar effect, although lower than propofol. In the iron/ascorbate-dependent lipid peroxidation propofol, at concentrations higher than 10 μM, exhibits antioxidant properties comparable to those of butylated hydroxytoluene and butylated hydroxyanisole. 2,6-Dimethylphenol is scarcely effective in both lipoperoxidative systems. The antioxidant properties of the various molecules depend on their hydrophobic characteristics and on the steric and electronic effects of their substituents. However, the introduction of the nitroso group in the 4-position almost completely removes the antioxidant properties of the examined compounds. The nitrosation of the aromatic ring of antioxidant molecules and the consequent loss of antioxidant capacity can be considered a condition potentially occurring *in vivo* since nitric oxide and its derivatives are continuously formed in biological systems.

Keywords: DPPH; Lipid peroxidation; Propofol; Nitric oxide; Nitrosophenols; Phenolic antioxidants

Abbreviations: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; ESR, electron spin resonance; CHP, cumene hydroperoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; MDA, malondialdehyde

INTRODUCTION

General anesthetics are active on mitochondria and their action is mostly referable to a perturbation of membranes.[1] Among other effects, they might behave either as antioxidants or prooxidants,[1] therefore, influencing the oxidation conditions of biological membranes. In particular, propofol acts as a potent antioxidant.[2–4] In a previous paper,[5] it was also shown that nitrosopropofol, formed after interaction between *S*-nitrosoglutathione and propofol, alters mitochondrial respiration to an extent greater than *S*-nitrosoglutathione and propofol alone. Further exploration at physicochemical level indicates that, while 2,6-diisopropylphenol acts on lipid organization

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/03079450310001652031

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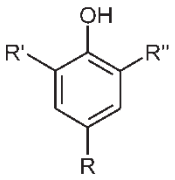

R', R'', R = H	phenol	(Ph)
R', R'' = H ; R = NO	4-nitroso-phenol	(Ph-NO)
R', R'' = CH_3 ; R = H	2,6-di-methyl-phenol	(DMP)
R', R'' = CH_3 ; R = NO	2,6-di-methyl-4-nitroso phenol	(DMP-NO)
R', R'' = $CH(CH_3)_2$; R = H	2,6-di-*iso*-propyl-phenol	(DPP, Propofol)
R', R'' = $CH(CH_3)_2$; R = NO	2,6-di-*iso*-propyl-4-nitroso phenol	(DPP-NO)
R', R'' = $C(CH_3)_3$; R = H	2,6-di-*tert*-butyl-phenol	(DTP)
R', R'' = $C(CH_3)_3$; R = NO	2,6-di-*tert*-butyl-4-nitroso phenol	(DTP-NO)
R', R'' = $C(CH_3)_3$; R = CH_3	2,6-di-*tert*-butyl-4-methyl phenol	(BHT)
R' = H ; R'' = $C(CH_3)_3$; R = OCH_3	2-(3)-*tert*-butyl-4-methoxy phenol	(BHA)

FIGURE 1 Structures of the phenol derivatives and their abbreviations.

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quite similarly to phenol, 2,6-diisopropyl-4-nitrosophenol behaves as a stronger perturbing agent, since, as suggested by ESR spectra, this molecule accumulates into the interfacial region of the bilayer.[6] This behavior has been generalized and confirmed by comparing 2,6-dialkyl-phenols to the corresponding 2,6-dialkyl-4-nitrosophenols.[7] In fact, it was observed that 2,6-dialkyl-phenols place themselves in the phospholipid bilayer with their plane parallel to the fatty acid chains, therefore minimizing distortion of the chain alignment. On the contrary, the presence of the nitroso group in the 4-position of 2,6-dialkyl-phenols favors their anchoring at the interface of the phospholipid bilayer probably by hydrogen bond interactions with the glycerol groups or dipole-ion interactions with the negative phosphate group.[7]

In the present paper, various substituted phenols and nitrosophenol derivatives, including the widely employed anesthetic propofol and its product of interaction with nitric oxide, were tested and compared for their reactivity towards the stable free radical 1,1-diphenyl-2-picrylhydrazyl and for their effect on membrane lipid peroxidation (Fig. 1).

MATERIALS AND METHODS

Synthesis of Nitrosophenols

2,6-Dialkyl-4-nitrosophenols were synthesized according to Momo *et al.*[7] essentially following the procedure described for preparing nitrosophenol.[8] Briefly, 2 ml of concentrated HCl was slowly added to 25 ml of an ethanolic solution of 3 g of 2,6-dialkylphenol kept at −5°C. Therefore, sodium nitrite (1.1 g in 5 ml of water) was added in about 15 min, under vigorous agitation and maintaining the temperature below 0°C. The resulting product was left under agitation for another 30 min, poured into cold water and the yellow precipitate was crystallized from toluene. The obtained product was characterized by GC/MS analysis and a purity grade higher than 95% was confirmed by thin layer chromatography.

Antioxidant Activity Measured with the DPPH Method

Antioxidant activity was estimated by the DPPH method where antioxidants are able to reduce the nitrogen-centered free radical 2,2′-diphenyl-1-picryl-hydrazyl.[9] The reduction of the latter was followed spectrophotometrically at 517 nm. The reaction was performed at 25°C in a medium formed by mixing equal volumes of ethanol (or methanol) and 0.2 M phosphate buffer (pH 6.0).[10] DPPH was added at the final concentration of 150 μM. Reactions were started by the addition of the various phenolic compounds dissolved in ethanol. The rate constants of the reaction of the various substituted phenols with DPPH were determined after performing the reaction under pseudo-first order conditions and using the equation: $kt = \ln[(A_0 - A_\infty)/(A_t - A_\infty)]$, where A_t is the absorbance of DPPH at various times, A_0 is the initial absorbance and A_∞ is the final estimated absorbance of the reaction.[11]

Microsomal Lipid Peroxidation

Liver microsomes were prepared according to Ernster and Nordenbrand[12] and proteins were measured with the biuret test. Microsomal lipid peroxidation was measured as malondialdehyde formation using the 2-thiobarbituric acid assay[13] or as oxygen uptake estimated polarographically using a Clark-type oxygen electrode connected to a computerized system.

Statistics

The data obtained from the various experiments and generated via the oxygraph or the spectrophotometer software were stored and utilized for averaging the different curves. All values are the mean ± SD of not less than five measurements.

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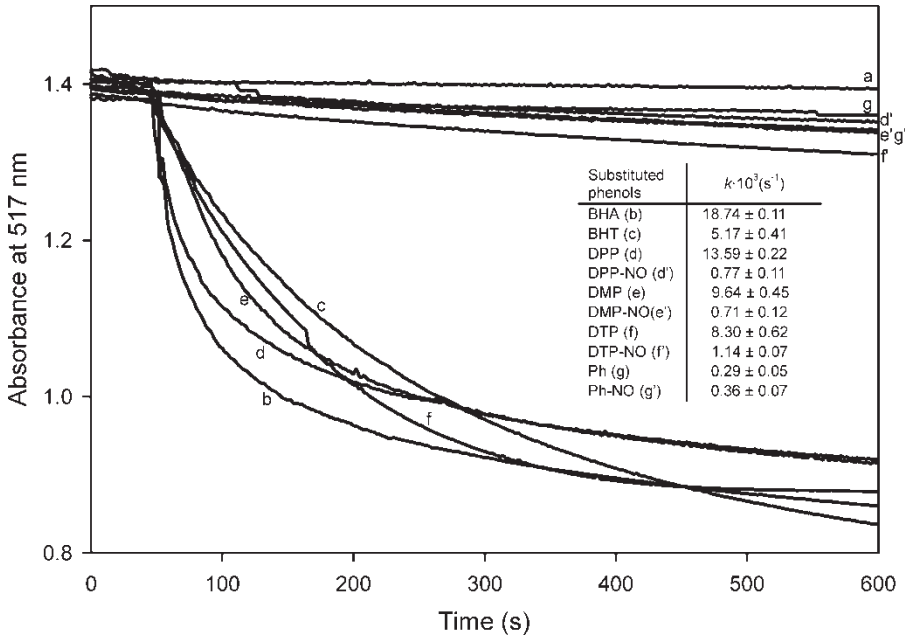

FIGURE 2 DPPH scavenging activity of the phenol derivatives. Reactions were performed in a medium formed by mixing equal volumes of 0.2 M Na^+, K^+ phosphate (pH 6.0) and ethanol and containing 0.150 mM DPPH (final concentration) at 25°C. The absorbance of DPPH was recorded spectrophotometrically at 517 nm in a continuously stirred cuvette. The various phenol derivatives were added at 20 μM concentration. Optical density was followed for 30 min. (a) none; (b) BHA; (c) BHT; (d) DPP; (f) DTP; (g) Ph; (d′) DPP-NO; (e′) DMP-NO; (g′) DTP-NO; (g′) Ph-NO. The pseudo-first order rate constants (k, s^{-1}) for the various compounds were determined as indicated under the "Materials and Methods" section.

Multiple comparisons were done by one-way analysis of variance followed by the Tukey post-test.

RESULTS

As shown in Fig. 2, the rate of decolorization of DPPH in the presence of the various substituted phenols strongly depends on the ring substituents. In fact, while phenol is completely ineffective, the presence of methyl, isopropyl and tertbutyl groups is of fundamental importance in conferring antioxidant properties to the molecule. Isopropyl and tertbutyl groups appear more efficient than methyl groups in conveying the antioxidant characteristics probably because of their greater electron-donating effects. However, the presence of excessively bulky groups around the hydroxyl group decreases the radical scavenging capacity. In fact, the following sequence of free radical scavenging ability was obtained: BHA > DPP > DMP > DTP > BHT. As apparent, BHT is the least effective in interacting with DPPH in accordance with previous results,[14] while the anesthetic propofol seems to excellently balance the steric and electronic effects on the molecule in order to impart the antioxidant qualities. The behavior observed was further confirmed by following the ESR signal decrease of DPPH in the presence of the various derivatives (not shown). Interestingly, the presence of the nitroso group in the 4-position of the phenolic ring almost completely eliminates the antioxidant capacity of all the molecules tested. This effect can be attributed to the electron-withdrawing properties of this substituent. The DPPH bleaching experiments were also performed using methanol, instead of ethanol, since, in the former solvent, DPPH shows an absorbance higher than in ethanol (not shown); however, since in these conditions a fine microprecipitate was observed, the experiments have been usually run in ethanol. Interestingly, with relatively long incubation times, an extremely small reactivity with DPPH is also elicited by the nitrosoderivatives.

The different substituted phenols were then examined in a biological membrane environment in order to observe, in addition to their free radical scavenging properties in solution also their antioxidant effectiveness in a natural context. Figure 3 reports the effects of the various substituted phenols and the corresponding nitrosoderivatives on lipid peroxidation elicited by cumene hydroperoxide in rat liver microsomes. This lipid peroxidation is due to the interaction between cytochrome P450 and cumene hydroperoxide with the formation of peroxyl and alkoxyl radicals.[15,16] The propagation of lipid peroxidation induced by cytochrome P450 occurs even in the presence of chelating agents indicating that free metal catalysis is not required. As apparent (Fig. 3A), propofol acts as the most efficient inhibitor of CHP-induced lipid peroxidation, DMP is scarcely active, and DTP exhibits an intermediate behavior again indicating that DPP has

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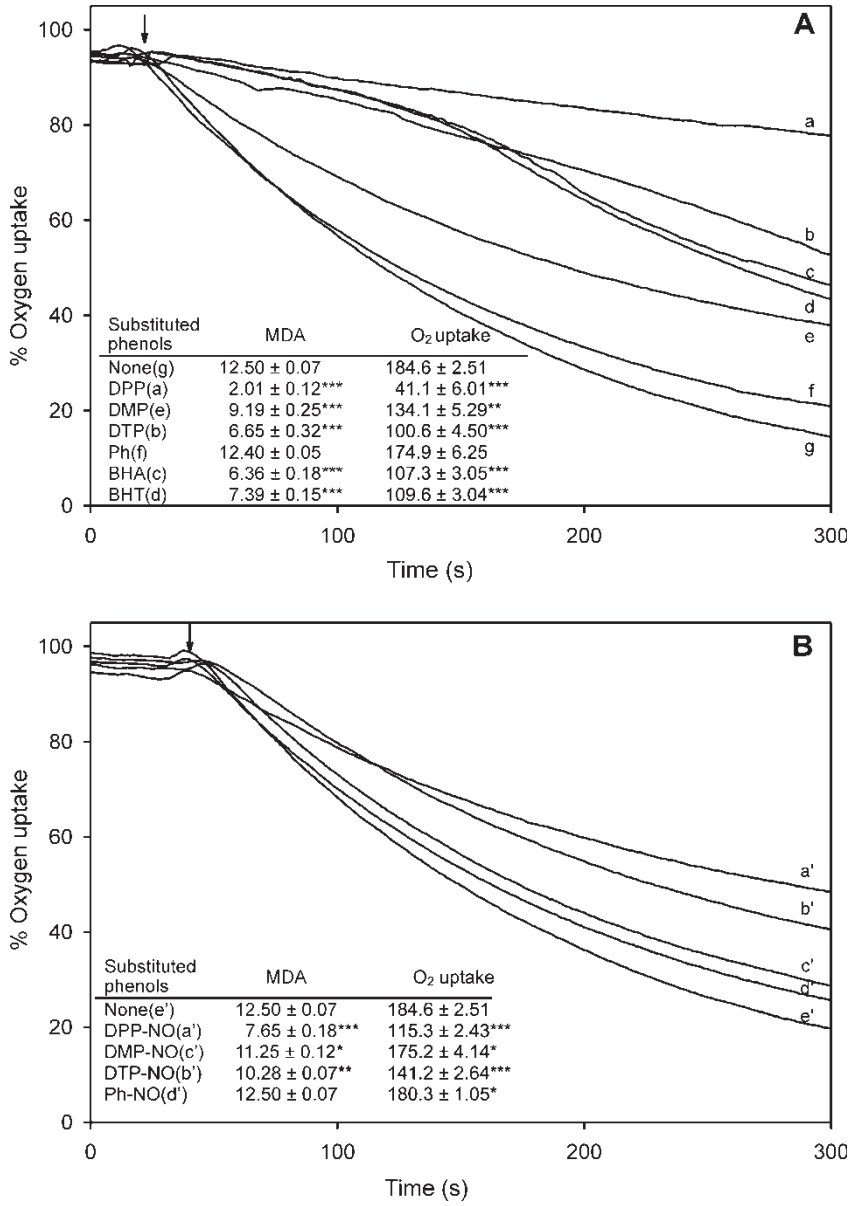

FIGURE 3 Effect of the phenol derivatives on cumeme hydroperoxide-dependent microsomal lipid peroxidation. Rat liver microsomes (1 mg ml^{-1}) were incubated at 25°C in 0.125 M KCl, 20 mM Hepes/Tris (pH 7.4) and in the presence of the indicated phenols derivatives at 20 μM concentration. After 1 min of equilibration, the reaction was initiated by the addition of 0.75 mM CHP (arrow). The inset reports MDA formation and oxygen uptake both indicated as nmol mg^{-1} protein and estimated at the end of the reaction (5 min). 100% oxygen, indicated in the ordinate axis, corresponds to 278 nmol ml^{-1} of oxygen dissolved in the vessel. A: (a) DPP, (b) DTP, (c) BHA, (d) BHT, (e) DMP, (f) Ph, (g) none. B: (a′) DPP-NO, (b′) DTP-NO, (c′) DMP-NO, (d′) Ph-NO, (e′) none. In each column of the inset, values are compared versus the control (no additions) *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

also the hydrophobic characteristics able to allow its correct positioning in the biological membranes. In general, the nitrosoderivatives are poorly efficient in eliciting a consistent antioxidant effect (Fig. 3B). However, the nitrosoderivative of propofol (DPP-NO) is, in the nitrosoderivatives family, the most effective also in preventing the lipid peroxidation induced by CHP. Inhibition of lipid peroxidation by the various phenol derivatives was also tested employing the well-known iron/ascorbate system (Fig. 4). In the latter, iron, active in the initiation of lipid peroxidation and in the decomposition of hydroperoxides, is maintained reduced by ascorbate. Again, DPP completely inhibits lipid peroxidation together with BHA and BHT (Fig. 4A). In the same system, DTP is also very active (Fig. 4A), while DMP (Fig. 4A) and the nitrosoderivatives (Fig. 4B) are poorly effective. Table I shows the extent of lipid peroxidation induced by either CHP or Fe^{2+}/ascorbate in the presence of increasing concentrations of phenol derivatives. It can be further observed that DPP, together with BHT and BHA, act as the most effective in both the systems, while the NO-derivatives lose almost completely their antioxidant capacity with the exception of dimethyl nitrosophenol that, solely in the iron/ascorbate

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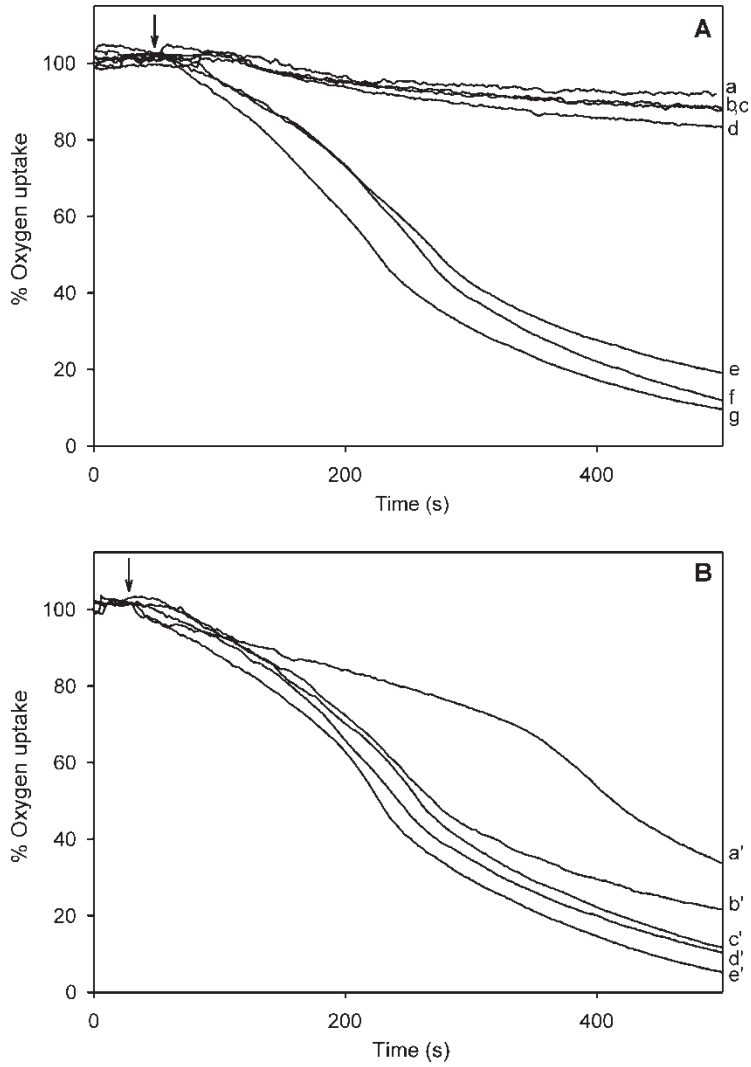

FIGURE 4 Effect of the phenol derivatives on Fe^{2+}/ascorbate-induced microsomal lipid peroxidation estimated as oxygen uptake. Rat liver microsomes (1 mg ml^{-1}) were incubated at 25°C in 0.125 M KCl, 20 mM Hepes/Tris (pH 7.4) and in the presence of the indicated phenol derivatives at 10 μM concentration. After about 1 min of equilibration, the reaction was initiated by the addition of 0.24 mM ascorbate and 24 μM $FeSO_4$ (arrow). 100% oxygen, indicated in the ordinate axis, corresponds to 278 nmol ml^{-1} of oxygen dissolved in the vessel. A: (a) BHA, (b) DPP, (c) BHT, (d) DTP, (e) DMP, (f) none, (g) Ph. B: (a′) DMP-NO, (b′) DPP-NO, (c′) Ph-NO, (d′) none, (e′) DTP-NO.

TABLE 1 Effect of the various phenol derivatives at increasing concentrations on cumene hydroperoxide- and Fe^{2+}/ascorbate-induced lipid peroxidation

	Cumene hydroperoxide				Iron /ascorbate			
Substituted phenols	3 μM	5 μM	10 μM	20 μM	1 μM	3 μM	5 μM	10 μM
DPP	89.5 ± 0.6	64.1 ± 2.5	29.4 ± 3.8	16.4 ± 2.5	91.4 ± 0.2	65.2 ± 0.8	36.1 ± 2.1	13.1 ± 2.7
DPP-NO	96.2 ± 0.4	93.8 ± 1.4	82.2 ± 2.1	64.1 ± 3.4	103.2 ± 0.2	92.5 ± 0.5	81.7 ± 1.8	68.4 ± 3.2
DMP	100.1 ± 0.5	98.7 ± 1.8	74.2 ± 4.3	65.7 ± 2.6	104.1 ± 0.5	90.5 ± 0.7	87.1 ± 2.9	68.2 ± 2.8
DMP-NO	99.8 ± 0.8	98.7 ± 1.5	83.5 ± 2.5	71.7 ± 3.8	106.1 ± 1.2	92.2 ± 0.5	59.0 ± 3.2	31.0 ± 3.9
DTP	99.5 ± 0.8	90.7 ± 2.2	75.2 ± 3.2	49.6 ± 3.8	98.5 ± 0.4	92.4 ± 1.2	45.7 ± 3.5	29.9 ± 4.2
DTP-NO	99.6 ± 0.9	86.3 ± 2.9	77.3 ± 3.4	76.9 ± 1.6	98.7 ± 0.3	97.2 ± 1.3	94.2 ± 0.9	94.1 ± 0.5
Ph	99.5 ± 1.2	100.9 ± 1.1	98.9 ± 1.2	92.7 ± 1.1	106.0 ± 1.2	99.1 ± 1.2	100.1 ± 0.1	98.9 ± 0.4
Ph-NO	103.1 ± 0.9	102.4 ± 1.1	97.5 ± 1.1	93.1 ± 0.8	110.2 ± 2.1	104.1 ± 1.5	93.9 ± 0.7	86.7 ± 0.6
BHA	92.2 ± 0.2	81.4 ± 1.5	37.1 ± 0.8	16.4 ± 1.2	100.2 ± 2.1	44.5 ± 0.5	23.4 ± 1.9	4.4 ± 1.1
BHT	94.4 ± 0.3	92.2 ± 1.2	61.9 ± 0.8	29.6 ± 1.4	100.1 ± 1.9	88.5 ± 0.4	39.5 ± 1.9	22.7 ± 1.1

Rat liver microsomes (1 mg ml^{-1}) were incubated at 25°C in 0.125 M KCl, 20 mM Hepes/Tris (pH 7.4) and in the presence of the indicated concentrations of the phenol derivatives. Reaction was initiated by the addition of 0.75 mM CHP or 0.24 mM ascorbate/24 μM $FeSO_4$ and carried out for 5 min (CHP) or 15 min (Fe^{2+}/ascorbate). Lipid peroxidation was measured as MDA formation and expressed as percentage decrease with respect to the control (100%) ± SD. MDA formed in the absence of antioxidants was 12.7 ± 0.5 and 66.4 ± 1.2 for CHP and Fe^{2+}/ascorbate, respectively.

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system, shows a significant protection (see also Fig. 4B). In the CHP system, DPP, even at low concentrations, is the most effective antioxidant, while, in the iron/ascorbate system, BHA is prevailing over DPP at concentrations ranging from 1 to 10 μM. Moreover, at concentrations higher than 10 μM, the antioxidant effect of DPP, BHA and BHT is leveled off.

DISCUSSION

The various substituted phenols utilized in the present study exhibit a differential sensitivity either in interacting with the stable free radical DPPH or in inhibiting microsomal lipid peroxidation. Substituent characteristics are important and involve polar, electronic and steric factors. For instance, the steric hindrance for hydrogen abstraction together with the hydrophobicity of the compounds and hence their correct interaction with membrane sites undergoing lipid peroxidation, is relevant. Propofol appears to act both as a very good scavenger of free radicals and also as a potent inhibitor of lipid peroxidation elicited by different peroxidizing systems, indicating that it is endowed with all the characteristics to act as an optimal antioxidant. It should also be noted that, in the clinical practice, the plasmatic concentration levels of propofol required for deep sedation are higher than 10 μM, for deep hypnosis are between 20 and 30 μM while, for the induction of anesthesia are about 35–40 μM,[17,18] therefore, in a concentration range higher than that needed *in vitro* for obtaining a maximal antioxidant effect.

The observed behavior of the substituted phenolics is in line with previous results by Burton and Ingold[19] obtained by measuring the rate constants for the reaction of peroxyl radicals with a variety of synthetic phenols where electron-donating groups reinforce the antioxidant power, while electron withdrawing groups act in the opposite way. Therefore, all the factors stabilizing the phenoxyl radical determine a weakening of the oxygen–hydrogen bond of the phenol group that can be readily cleaved by the peroxyl radical, making the compound an effective antioxidant. Similar conclusions can be drawn for the interaction of different substituted phenols with DPPH.[20]

A major observation resulting from this work indicates that the introduction of the nitroso group in the ring of a phenolic compound, almost completely removes its antioxidant capacity. This is observable either with the DPPH test or by comparatively measuring microsomal lipid peroxidation. Nitric oxide is a relevant signaling molecule involved in many physiological processes but also able to contribute to several pathological conditions.[21] Nitric oxide, in the presence of oxygen, generates an oxidizing environment formed of nitrogen oxides mostly consisting of NO_2 and N_2O_3 which are better oxidizing species as compared to NO.[22,23] In addition, after interaction with the superoxide radical anion, nitric oxide gives rise to peroxynitrite that is another powerful oxidizing agent.[23,24] Consequently, these derivatives can be potentially formed in biological systems with toxic effects for the cell[24] and also appear to be associated with the chemistry of air pollution and cigarette smoke.[25,26] However, since nitric oxide, *per se*, is a potent antioxidant,[27,28] it might appear rather surprising that the combination of two effective antioxidants such as substituted phenolics and nitric oxide gives rise to species devoid of antioxidant power such as the nitrosoderivatives. This action of nitric oxide and its derivatives can be of general interest. For instance, different phenolic antioxidants in their phenoxyl form (after scavenging a free radical) can interact with nitric oxide and therefore, instead of being regenerated to their native form, lose their ability to scavenge other free radicals similarly to a chain termination reaction. This property can be included among the prooxidant effects of nitric oxide. It was recently shown[29] that some flavonoids, a large class of phenolic antioxidants, are able to prevent nitration/nitrosation induced by peroxynitrite although it remains to be defined if the products formed after this interaction can still retain their antioxidant properties.[30] According to our results, at least for simple phenols, the interaction with nitric oxide eventually brings to compounds inactive as antioxidants.

Another important factor endowing phenolic compounds with the ability to restrain lipid peroxidation is their extent of interaction with biological membranes. As already discussed, nitrosation at the 4-position of the phenolic ring completely removes its antioxidant properties essentially for electronic reasons. However, also the positioning of the molecule in the bilayer is important in imparting the antioxidant effect. In the case of the nitrosoderivatives of phenolic compounds, their arrangement at the interface of the bilayer,[7] at variance with their parent compounds, is characterized by a lack of interaction with the potentially peroxidizable hydrophobic tails of phospholipids. In conclusion, the nitrosation of propofol and the other derivatives, in addition to a stronger perturbing action on the membranes[7] also eliminates, for different reasons, the antioxidant capacity of the molecule.

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