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Inhibition of Membrane Lipid Peroxidation by a Radical Scavenging Mechanism: a Novel Function for Hydroxyl-Containing Ionophores

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In the present study we show that K^+/H^+ hydroxyl-containing ionophores lasalocid-A (LAS) and nigericin (NIG) in the nanomolar concentration range, inhibit Fe^{2+}-citrate and 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP)-induced lipid peroxidation in intact rat liver mitochondria and in egg phosphatidylcholine (PC) liposomes containing negatively charged lipids—dicetyl phosphate (DCP) or cardiolipin (CL)—and KCl as the osmotic support. In addition, monensin (MON), a hydroxyl-containing ionophore with higher affinity for Na^+ than for K^+, promotes a similar effect when NaCl is the osmotic support. The protective effect of the ionophores is not observed when the osmolyte is sucrose. Lipid peroxidation was evidenced by mitochondrial swelling, antimycin A-insensitive O_2 consumption, formation of thiobarbituric acid-reactive substances (TBARS), conjugated dienes, and electron paramagnetic resonance (EPR) spectra of an incorporated lipid spin probe. A time-dependent decay of spin label EPR signal is observed as a consequence of lipid peroxidation induced by both inductor systems in liposomes. Nitroxide destruction is inhibited by butylated hydroxytoluene, a known antioxidant, and by the hydroxyl-containing ionophores. In contrast, valinomycin (VAL), which does not possess alcoholic groups, does not display this protective effect. Effective order parameters (S_{eff}), determined from the spectra of an incorporated spin label are larger in the presence of salt and display a small increase upon addition of the ionophores, as a result of the increase of counter ion concentration at the negatively charged bilayer surface. This condition leads to increased formation of the ion-ionophore complex, the membrane binding (uncharged) species. The membrane-incorporated complex is the active species in the lipid peroxidation inhibiting process. Studies in aqueous solution (in the absence of membranes) showed that NIG and LAS, but not VAL, decrease the Fe^{2+}-citrate-induced production of radicals derived from piperazine-based buffers, demonstrating their property as radical scavengers. Both Fe^{2+}-citrate and ABAP promote a much more pronounced decrease of LAS fluorescence in PC/CL liposomes than in dimyristoyl phosphatidylcholine (DMPC, saturated phospholipid)-DCP liposomes, indicating that the ionophore also scavenges lipid peroxyl radicals. A slow decrease of fluorescence

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is observed in the latter system, for all lipid compositions in sucrose medium, and in the absence of membranes, indicating that the primary radicals stemming from both inductors also attack the ionophore. Altogether, the data lead to the conclusion that the membrane-incorporated cation complexes of NIG, LAS and MON inhibit lipid peroxidation by blocking initiation and propagation reactions in the lipid phase via a free radical scavenging mechanism, very likely due to the presence of alcoholic hydroxyl groups in all three molecules and to the attack of the aromatic moiety of LAS.

Keywords: ABAP, Fe^{2+}-citrate, ionophores, lipid peroxidation, liposomes, mitochondria, EPR spectroscopy, spin label

Abbreviations: ABAP, 2,2′-azobis(2-amidinopropane) dihydrochloride; BHT, butylated hydroxytoluene; DCP, dicetyl phosphate; DMPC, dimyristoyl phosphatidylcholine; EGTA, ethyleneglycolbis (β-aminoethylether)-*N,N,N′,N′*-tetra acetic acid; EPR, electron paramagnetic resonance; FCCP, carbonyl cyanide p-(trifluoromethoxy) phenyl hydrazone; hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; PC, egg phosphatidyl choline, pipes, 1,4-piperazinediethanesulfonic acid; RLM, rat liver mitochondria; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances.

INTRODUCTION

The biochemical mechanisms underlying oxidative damage of mitochondria have attracted increased interest during recent years due to the role that mitochondria play both as a site of reactive oxygen species (ROS) production and as an important target for the action of these radicals when a condition of oxidative stress is generated, for example during pathological situations such as xenobiotic poisoning and ischemia/reperfusion.[1,2] It is proposed that low molecular weight iron complexes are also involved in these mechanisms of cell death, and in liver injuries associated with iron-overload.[3] Since the 60's several papers have shown that iron and iron-complexes induce mitochondrial membrane lipid peroxidation and alterations in membrane proteins.[4–11] Existing data[12–14] demonstrate that the K^+/H^+ ionophores lasalocid A (LAS) and nigericin (NIG), in the nanomolar concentration range, protect mitochondria against oxidative damage induced by Ca^{2+} plus inorganic phosphate,[15] a prooxidant,[16] or phenyl arsine oxide.[17]

LAS, NIG and monensin (MON) belong to a class of linear polyether carboxylic acids, extensively used in experimental biology and technology. Their conformational and dynamical properties, namely, cation-ionophore complexation, incorporation of the complex into the membrane, and the mechanism and kinetics of complex-mediated ion transport are similar, as revealed by a variety of spectroscopic techniques.[18–22] Typically, an acyclic quasi-linear conformer predominates under high polarity conditions and in the absence of adequate ligands (uncomplexed form). On the other hand, a cyclic conformer predominates in apolar environments and in the complexed form.[20,21]

In order to ascertain the mechanisms underlying this protective effect, we have examined the interactions between symport (K^+/H^+, LAS and NIG; Na^+/H^+, monensin (MON)) and uniport (K^+, valinomycin (VAL)) ionophores and intact mitochondria or lipid multibilayers, both from the structural and functional points of view. Lipid peroxidation was induced by two different systems, Fe^{2+}-citrate, a chelate of putative physiological relevance,[23] and 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP).[24] Lipid peroxidation in intact mitochondria or liposomes was evaluated by determination of thiobarbituric acid reactive substances (TBARS) and of conjugated dienes, by O_2 uptake, and by following the loss of electron paramagnetic resonance (EPR) signal intensity in the spectra of a spin label incorporated in liposomes. EPR spectra of an incorporated lipid spin probe were used to monitor the effect of the ionophores on membrane molecular organization and fluorescence spectroscopy was employed to investigate the role of the chemical structural features of the ionophores on lipid peroxidation as well as their alterations during this process.

The ionophore protective effect against lipid peroxidation was examined as a function of membrane surface charge, phospholipid acyl chain degree of unsaturation, and the presence of ionic and nonionic solutes in the medium.

EXPERIMENTAL PROCEDURES

Reagents and Solutions

Ferrous ions ($Fe(NH_4)_2(SO_4)_2$ $6H_2O$) solutions were used immediately after preparation in milli-Q water. Antimycin A, BHT, CL, sodium citrate, DCP, DMPC, 5-SASL, EGTA, hepes, MON, NIG, pipes, rotenone, TBA and VAL were obtained from Sigma Chemical Co (St. Louis, MO). ABAP was obtained from Polysciences, USA. LAS came from Hoffman-La Roche. PC was extracted and purified according to Singleton *et al.*,[25] as modified by Kamp *et al.*[26] All other reagents were commercial products of the highest available grade.

Isolation of Rat Liver Mitochondria and Preparation of Liposomes

Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar rats fasted overnight. The homogenate was prepared in 250 mM sucrose, 1.0 mM sucrose, 1.0 mM EGTA and 5.0 mM hepes buffer, pH 7.2. The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was diluted in 250 mM sucrose to a protein concentration of 80–100 mg/ml. The mitochondria contained 8–10 nmol endogenous calcium/mg protein as determined by atomic absorption spectroscopy. Liposomes were prepared by bath sonication for 30 min at controlled temperature. For PC-CL and PC-DCP liposomes the temperature was 4°C; for DMPC and DMPC-DCP liposomes the temperature was 35°C (above the phospholipid phase transition temperature, T_C, 23°C).

Standard Incubation Procedure

The experiments were carried out at 35°C in the reaction media described in the figure legends. The results are representative or averages of at least three experiments reproducible within 10%.

The inductor systems of lipid peroxidation were Fe^{2+}-citrate at concentrations 25 to 50 μM−2 to 4 mM,[23] respectively, and ABAP, 20 to 50 mM.[24] Typically, Fe^{2+}-citrate at 25 μM−2 mM or ABAP 20 mM were used for 1 mM phospholipid or 1 mg protein/ml mitochondria.

Determination of Mitochondrial Swelling

Mitochondrial swelling was monitored by the decrease in absorbance at 520 nm measured in an SLM Aminco DW2000 spectrophotometer.[9–15]

Oxygen Uptake Measurements

Oxygen uptake was measured using a Clark-type electrode (Yellow Springs Instruments Co.) in a glass chamber equipped with magnetic stirring.

Thiobarbituric Acid Reactive Substances (TBARS) Determination

TBARS (expressed as malondialdehyde) production by mitochondria were evaluated according to Buege and Aust.[27] Briefly, 1 ml samples were taken after 10 min incubation under the conditions described in Figure 1A and mixed with 0.4 ml of 1% TBA in 0.05 M NaOH, 0.2 ml of 20% H_3PO_4 and 40 μl of 10 M NaOH. The mixture was heated at 90–100°C for 15 min in the presence of 1 mM BHT. After cooling, 1.5 ml butanol was added to the solution. The mixture was shaken and centrifuged at 3000 rpm during 5 min. The malondialdehyde concentration was determined in the supernatant at 532 nm using a molar extinction coefficient of $1.56\times10^5 M^{-1}cm^{-1}$.

Conjugated Diene

Total lipids from liposome samples were extracted with chloroform : methanol (1 : 1, v/v), dried under N_2, dissolved in heptane and read at 233 nm. The amount of conjugated diene formed was calculated using a molar absorption coefficient of $2.52\times10^4 M^{-1}cm^{-1}$.

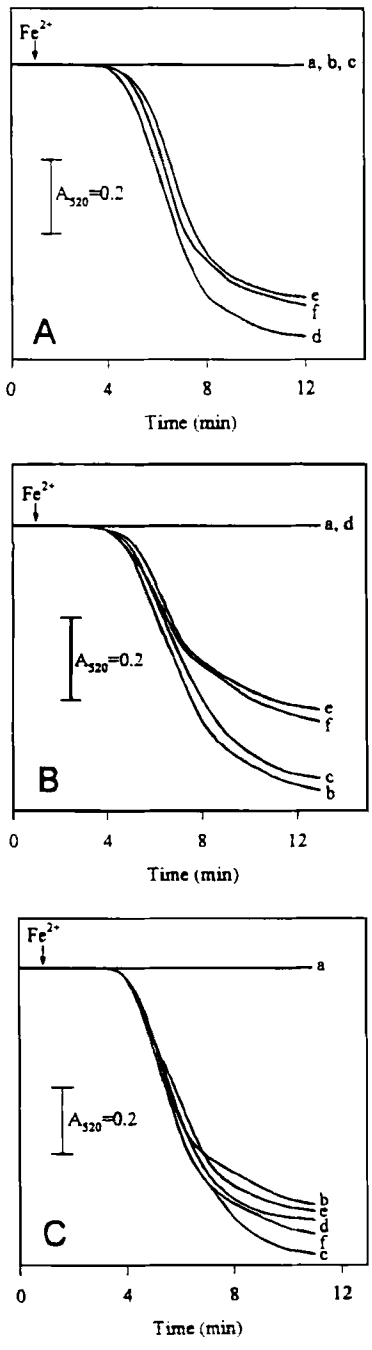


FIGURE 1 Effect of ionophores on Fe^{2+}-citrate-induced mitochondrial swelling. RLM (0.4 mg protein/ml) were incubated in reaction medium containing 2 mM citrate, 4 μM rotenone, 0.5 μM antimycin A, 0.5 μM FCCP, 10 mM hepes (pH 7.2) and 100 mM KCl (panel **A**), 100 mM NaCl (panel **B**), or 250 mM sucrose (panel **C**) as osmotic support. Fe^{2+} (25 μM) was added in the presence of: (a) 5 μM BHT; (b) 100 nM NIG; (c) 100 nM LAS; (d) 6 μM MON; (e) 60 nM VAL; (f) no other addition.

Spin Labeling of Samples and EPR Measurements

Stock solutions of the spin label 5-doxylstearic acid (5-SASL) in chloroform were stored at −20°C. To prepare labeled liposomes, lipids and spin label (1 mol%) were dried under N_2 and submitted to vacuum for no less than 2 h. Buffer was added to the film and the sample was sonicated in a bath sonicator for 30 min at adequate temperature to obtain liposome suspensions.

Effective order parameters (S_{eff}) were calculated from EPR spectra of membrane-bound 5-SASL according to Hubbell and McConnell.[28] This parameter reflects the amplitude of motion of the long molecular axis plus intra-molecular *trans-gauche* isomerization. For 5-SASL, whose long molecular axis is approximately parallel to the bilayer normal, S_{eff} values were calculated from Eq. (1):

$$S_{eff} = \frac{A_{||} - A_{\perp}}{A_{zz} - (A_{yy} + A_{xx})/2} \times \frac{a'_0}{a_0} \qquad (1)$$

where $A_{||}$ ($A_{\perp}$) is the hyperfine splitting corresponding to the spin label long molecular axis oriented preferentially parallel (perpendicular) to the external magnetic field. $A_{||}$ ($A_{\perp}$) is measured as half the separation between the spectra outer (inner) extrema. A_{xx}, A_{yy} and A_{zz} are the principal components of the hyperfine tensor and were taken as 6.0, 6.0 and 32.0 gauss,[29] respectively. Polarity effects are taken into account by the a'_0/a_0 ratio, where a'_0 and a_0 correspond to the isotropic hyperfine splitting in the single crystal and in the membrane, respectively, and are given by:

$$a'_0 = (1/3)(A_{zz} + A_{yy} + A_{xx}) \qquad (2)$$

and

$$a_0 = (1/3)(A_{||} + 2A_{\perp}) \qquad (3)$$

The kinetics of spin label signal decay during Fe^{2+}-citrate and ABAP-mediated lipid peroxidation in liposomes were followed by monitoring the intensity of the center field line (h_0) in the EPR

spectra of 5-SASL. The values are the average of four different experiments, in which the initial spin label concentrations were not strictly the same. EPR spectra were obtained in a Bruker ER-200D-SRC spectrometer at room temperature.

Fluorescence Measurements

Fluorescence spectra of LAS[18] were obtained in a Hitachi 4500 steady state spectrofluorometer equipped with a thermostatized cell, with a built-in computer, and using 1 cm path-length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used in all experiments. The excitation wavelength used was 310 nm in all cases.

Statistics

Data in the Tables were compared by unpaired Student's *t* test.

RESULTS

Inhibition by Ionophores of Fe^{2+}-Citrate-Induced Mitochondrial Lipid Peroxidation as Monitored by Swelling, O_2 Uptake, and TBARS Production

Inner mitochondrial membrane permeabilization can be studied using the classical swelling technique (decrease in light scattering) to follow the net influx of the osmotic support associated with nonspecific increase in permeability.[30] Experiments with deenergized mitochondria (in the presence of antimycin A and FCCP) have the advantage of eliminating the involvement of respiration, membrane potential, changes in membrane ΔpH, and Ca^{2+} cycling in the swelling process.[16] Figure 1 (panels A–C) illustrates the Fe^{2+}-citrate-induced swelling of deenergized mitochondria (line f). When KCl was the osmotic support (panel A), the swelling was completely inhibited by 100 nM NIG (line b) or LAS (line c), ionophores that promote the electroneutral K^+/H^+ exchange. In contrast, 6 μM MON (line d) or 60 nM VAL (line e) had no effect on mitochondrial swelling. MON promotes electroneutral Na^+/H^+ exchange and VAL is an uniporter for K^+. On the other hand, when NaCl was the osmotic support (panel B) only MON (line d) inhibited mitochondrial swelling. When sucrose was used as osmotic support (panel C), none of the ionophores inhibited swelling. Finally, when the experiments were conducted in the presence of BHT, a known inhibitor of lipid peroxidation, no mitochondrial swelling was observed either (panels A–C, lines a), confirming that swelling was secondary to permeabilization caused by lipid peroxidation.

Figure 2 (panels A–C) shows that addition of Fe^{2+}-citrate to mitochondria caused a transitory burst of antimycin A-insensitive oxygen uptake (lines f). Previous data[9–11] showed that this BHT-sensitive oxygen burst (line a) is due to lipid peroxidation. The pattern of oxygen uptake in the presence of ionophores was similar to that observed for swelling, that is, uptake is inhibited by NIG (panel A, line b) and LAS (panel A, line c) only in the presence of KCl and by MON (panel B, line d) only in the presence of NaCl.

Lipid peroxidation was also assayed by measurement of TBARS production.[9,10,15,16] Table I shows that NIG, LAS, and BHT had a significant inhibitory effect on the Fe^{2+}-citrate-induced TBARS production when KCl was the osmotic support, while VAL had no inhibitory effect.

Inhibition by Ionophores of Fe^{2+}-Citrate-Induced Liposome Lipid Peroxidation as Monitored by O_2 Uptake and Spin Label EPR Signal Loss

Liposomes are a well known model for lipid peroxidation studies, allowing the analysis of important parameters, such as lipid composition, charge effects, acyl chain degree of unsaturation, presence of intrinsic or extrinsic proteins, composition of the external and/or internal medium.

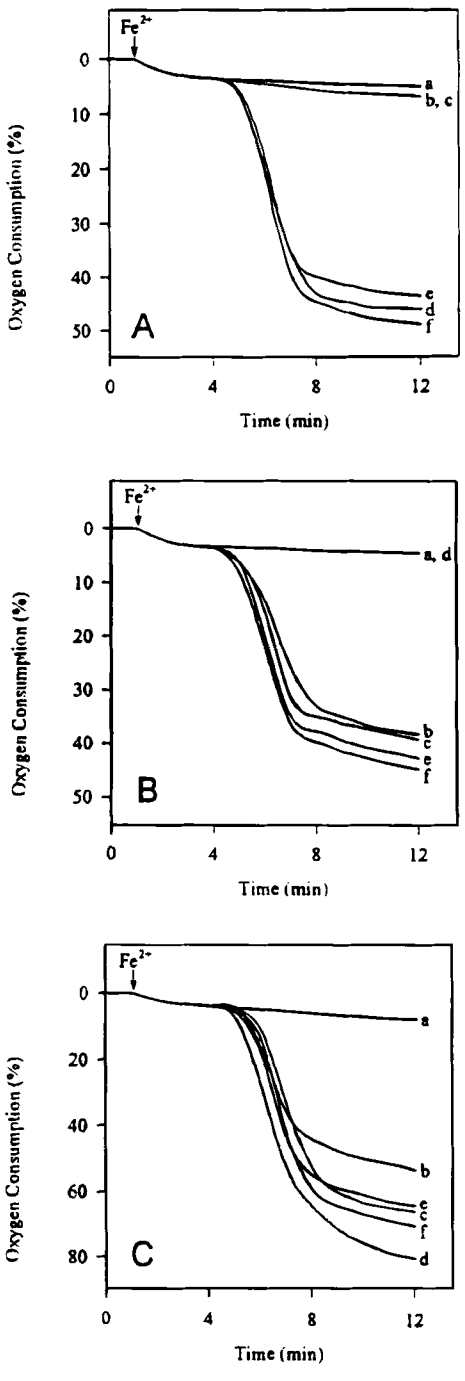


FIGURE 2 Effect of ionophores on oxygen uptake due to Fe^{2+}-citrate-induced RLM lipid peroxidation. Same experimental conditions as in Fig. 1; 100 mM KCl (panel **A**), 100 mM NaCl (panel **B**), and 250 mM sucrose (panel **C**) were used as osmotic support. Fe^{2+} (50 μM) was added in the presence of: (a) 10 μM BHT; (b) 100 nM NIG; (c) 100 nM LAS; (d) 6 μM MON; (e) 60 nM VAL; (f) no other addition.

TABLE I Effect of ionophores on Fe^{2+}-citrate-induced lipid peroxidation in RLM[a]

Additions	TBARS (nmol/ml)[b]
control	3.95 ± 1.30
BHT (10 μM)	0.14 ± 0.05
NIG (100 nM)	0.19 ± 0.10
LAS (100 nM)	0.60 ± 0.47
VAL (60 nM)	4.42 ± 0.87
control without Fe^{2+}	0.10 ± 0.04

[a] Experimental conditions as in Figure 1A. Experiments were initiated by addition of 25 μM Fe^{2+}; the TBARS assay was performed after 20 min incubation.
[b] Values are averages of three experiments ± SD.

These parameters are known to modulate a large number of membrane processes.[31,32] In Figure 3 a comparison is made of the ionophore effects on O_2 uptake due to Fe^{2+}-citrate-induced lipid peroxidation in PC-20 mol% CL liposomes. No effect was observed in the presence of 250 mM sucrose (panel B), in contrast with a complete inhibition of the process by NIG (panel A, line b) and LAS (panel A, line c) in 100 mM KCl (panel A), and partial inhibition by MON in NaCl (not shown). BHT inhibited oxygen uptake under all experimental conditions (lines a).

The ionophores protective ability against Fe^{2+}-citrate-induced lipid peroxidation was tested in spin labeled PC-DCP liposomes. Nitroxides are destroyed by free radical peroxidation products such as lipid alkyl and peroxyl radicals, causing loss of the EPR signal, rendering these reactions useful for probing the early steps of free radical processes.[33–37] Figure 4 illustrates the effect of NIG on the EPR spectra of 5-SASL in PC-DCP liposomes after 15 min incubation with Fe^{2+}-citrate in either sucrose (Column A) or KCl (Column B) containing media. In the absence of NIG, the addition of Fe^{2+}-citrate leads to a large decrease in the EPR signal due to lipid peroxidation in both media (Column A and Column B, spectra c), whereas, in the presence of the ionophore, signal loss is prevented in KCl (Column B, spectrum d), but not in sucrose-containing medium (Column A, spectrum d). Signal loss was also prevented by NIG in the

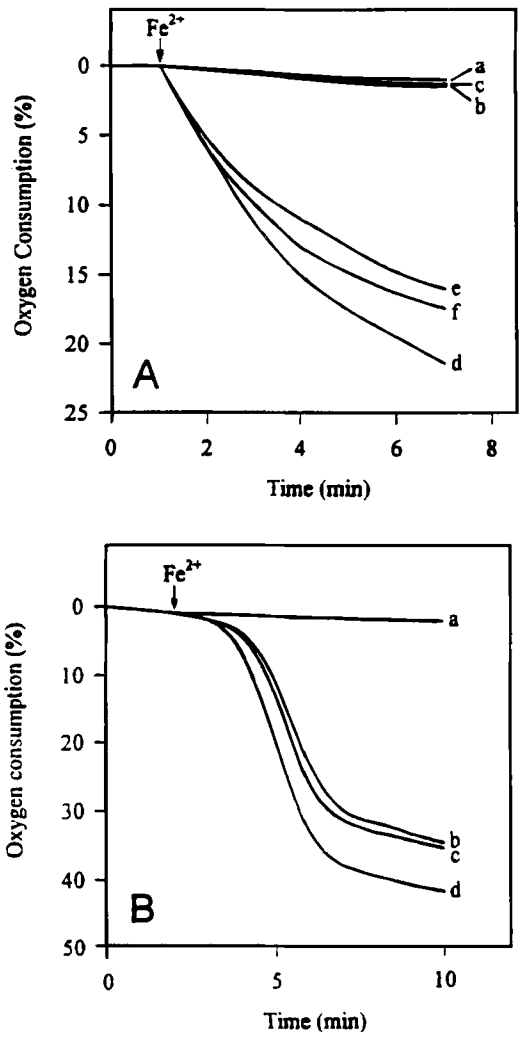


FIGURE 3 Effect of ionophores on oxygen uptake due to Fe^{2+}-citrate-induced liposome lipid peroxidation. Liposomes consisting of PC-20 mol% CL were incubated in reaction medium containing 2 mM citrate, 10 mM hepes (pH 7.2) and 100 mM KCl (panel **A**) or 250 mM sucrose (panel **B**). Fe^{2+} (50 μM) was added in the presence of: Panel A, (a) 10 μM BHT; (b) 100 nM NIG; (c) 100 nM LAS; (d) 6 μM MON; (e) 60 nM VAL; (f) no other addition. Panel B, (a) 10 μM BHT; (b) 100 nM NIG; (c) 60 nM VAL; (d) 6 μM MON.

presence of hepes alone (not shown). It is known that LAS can complex and transport hepes across lipid membranes.[38] This is likely to occur with NIG, too.

Figure 5 summarizes the effect of NIG on Fe^{2+}-citrate-induced loss of 5-SASL EPR signal. In the absence of the ionophores, the rate of lipid peroxidation is slower in hepes alone than in the presence of 130 mM KCl. This could be due to the increased lipid packing in the latter system as seen by the larger degree of order (see below, and Table II) induced by the electrostatic interaction between K^+ and DCP. It is known that increased lipid packing favors the propagation of lipid peroxidation.[39] Protection by NIG (Figure 5, Panel A) is observed in KCl medium, and also in hepes alone, but not in the presence of sucrose. Although the antibiotic displays a large selectivity for K^+, hepes complexation probably also occurs, explaining the protection observed in this medium. In contrast, sucrose tends to stabilize the open, uncomplexed form of the ionophore, rendering cation-ionophore complex formation and subsequent membrane incorporation less favorable processes. As found in the previous experiments, VAL has no protective effect in KCl medium (Figure 5, Panel A), in hepes alone or in hepes-sucrose (data not shown). In NaCl medium, an effective protection was obtained with MON, which, at much higher concentration displayed a protective effect even in the presence of KCl. Figure 5 (Panels A and B) also shows that inhibition of lipid peroxidation by the ionophores does not take place in the presence of sucrose.

Inhibition by NIG of ABAP-Induced Liposome Lipid Peroxidation as Monitored by Spin Label EPR Signal Loss, and Production of TBARS and Conjugated Dienes

CL contains a considerable amount of polyunsaturated fatty acids, mainly linoleic acid (80–90%);[40] it is, therefore, a very convenient system to study the formation of conjugated dienes and to examine the ability of hydroxyl containing-ionophores to react with lipid alkoxyl and peroxyl radicals formed during ABAP-induced oxidation. Exposure of PC-20 mole% CL liposomes to free radicals generated by ABAP thermal decomposition showed a significant increase in the formation of MDA and conjugated dienes and a concomitant

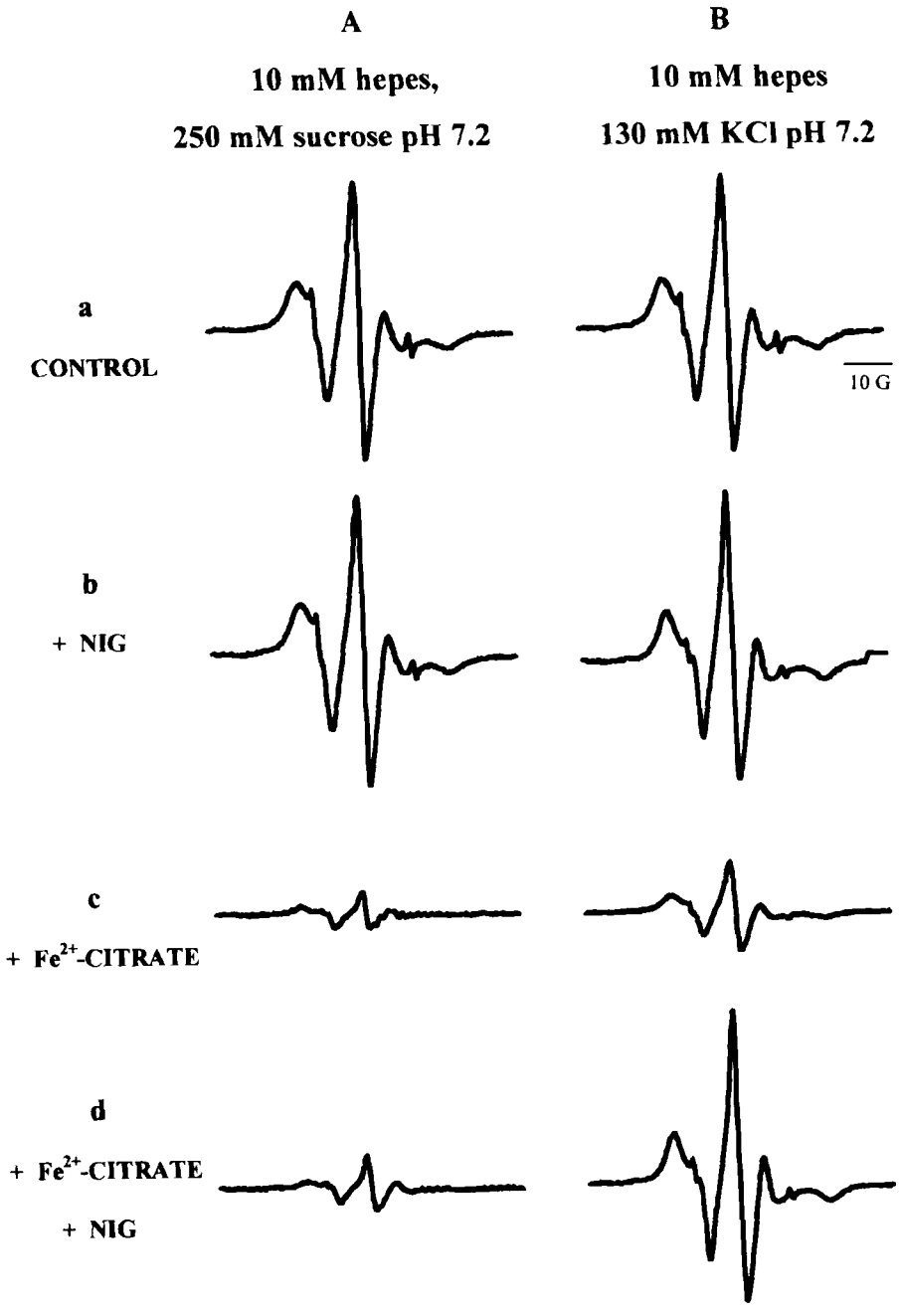


FIGURE 4 Effect of NIG on the EPR spectra of 5-SASL incorporated in liposomes. Spectra of 5-SASL (1 mol%) in PC-20 mol% DCP (1.2 mM total lipid) were obtained after 15 min incubation with NIG (150 nM) and/or Fe^{2+} (50 μM)-citrate (4 mM) under the conditions described in the Figure. Spectrometer receiver gain was the same for all samples.

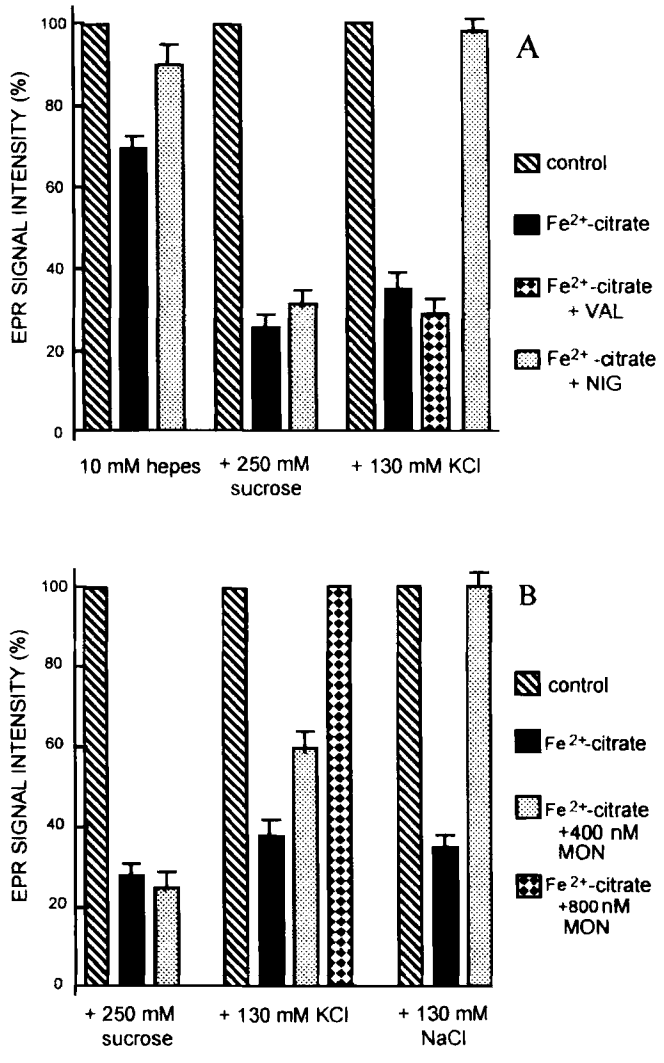


FIGURE 5 Effect of ionophores on lipid peroxidation as monitored by EPR signal loss. Effect of 300 nM NIG and 300 nM VAL (**A**) and 20 μM MON (**B**) on the EPR signal loss (height of the center field line) of 5-SASL (1 mol%) in PC- 20 mol% DCP (3.25 mM total lipid) submitted to Fe^{2+} (150 μM)-citrate (10 mM) in the presence of electrolytes (KCl or NaCl) or sucrose. Spectra were taken after 15 min incubation.

TABLE II Stoichiometry of free radical formation and of conjugated diene and TBARS accumulation in PC-20 mole % CL liposomes[a]

Addition	Free radical Formation $\times 10^{-5}$ (M)	TBARS $\times 10^{-6}$ (M)	Conjugated diene $\times 10^{-4}$ (M)
None	1.632	4.7	4.0
100 nM NIG	–	1.6	2.0

[a] Calculated from the data in Figure 6 in the presence of 130 mM KCl, according to Niki[24]

decrease in the 5-SASL EPR signal (Fig. 6 A,B,C). NIG greatly prevents damage of the liposome lipids when KCl is the osmotic support (Fig. 6).

The stoichiometry of radical formation and of conjugated diene and TBARS accumulation was calculated from the data in Figure 6 according to Niki.[24] Table II shows that the concentrations of free radicals, conjugated diene, and TBARS formed after ten minutes are in the range found for similar systems by other authors.[41–44] Moreover, it is seen that 100 nM NIG inhibits formation of conjugated diene and TBARS by 66 and 50%, respectively.

Effect of NIG on Liposome Lipid Organization

Ions can influence membrane structure, especially in the presence of charged lipids.[31,39,45,46] These effects can be monitored by changes in EPR spectral parameters such as S_{eff} (see Methods). Table III presents the effect of NIG and VAL on this parameter obtained from the spectra of 5-SASL incorporated in PC-20 mole% DCP liposomes in different media. The EPR spectra of 5-SASL in liposomes in the absence of nigericin display a large increase of S_{eff} when going from buffer alone (S_{eff}=0.588) to buffer plus 130 mM KCl (S_{eff}=0.632). This effect is a consequence of the counter ion-promoted increased lipid packing due to screening of negative charges on the lipid surface.[45] In contrast, in the presence of sucrose, the S_{eff} value is very close to that obtained in buffer alone (0.584). The buffer cation (hepes) can also interact with charged surfaces, but its concentration is very low to cause significant alterations in the order parameter. Addition of NIG leads to an increase in S_{eff} in PC-DCP liposomes both in buffer and in buffer/KCl media. S_{eff} changes from 0.588 to 0.635 and from 0.632 to 0.658, respectively (Table III). Similarly, VAL leads to an increase in S_{eff} in PC-DCP liposomes in buffer/KCl media (S_{eff} changes from 0.632 to 0.654), but not in buffer alone (S_{eff}=0.588 and 0.590, in the absence and in the presence of VAL, respectively). No changes were found in the

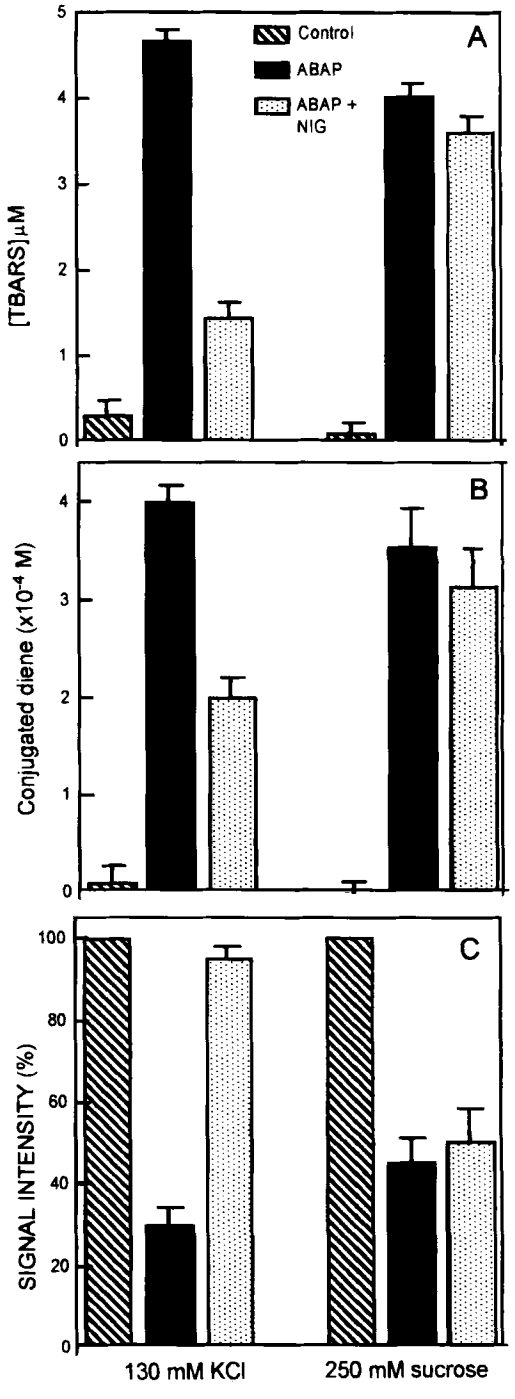


FIGURE 6 Protective effect of NIG on ABAP-induced lipid peroxidation. Effect of 100 nM NIG upon formation of TBARS (**A**) and conjugated dienes (**B**), and EPR signal loss (**C**). 1 mM PC-20 mole % CL liposomes were incubated for 10 min with 20 mM ABAP in standard medium (see Material and Methods) containing 130 mM KCl (left) or 250 mM sucrose (right).

TABLE III Effect of ionophores on S_{eff} measured from the EPR spectra of 5-SASL in 3.3 mM PC-20 mole% DCP multilamellar liposomes, pH 7.2

	S_{eff}		
MEDIUM	no addition	+300 nM NIG	+300 nM VAL
10 mM hepes	0.588	0.635	0.590
10 mM hepes, 25 mM sucrose	0.584	0.587	0.591
10 mM hepes, 130 mM KCl	0.632	0.658	0.654

presence of sucrose (S_{eff}=0.584, 0.587, and 0.591, in the absence and in the presence of NIG and VAL, respectively). The increase in S_{eff} observed in buffer alone corroborates the hypothesis that NIG complexes hepes and that this species is transported across the membrane.

Effect of Ionophores on Fe^{2+}-Citrate-Induced Radical Formation by Piperazine-Based Buffers

To further investigate the scavenging properties of the ionophores we examined their effect on radical formation by piperazine-based buffers.[47] Pipes was used as a target for the Fe^{2+}-citrate-catalyzed process. It is well established that radicals can be formed from piperazine-derived buffers, at high concentration (0.4 M), when they are submitted to several Fe^{2+} complexes.[47] The alcoholic hydroxyl groups present in these compounds are oxidized giving rise to relatively stable free radicals, detectable by EPR. Figure 7 summarizes the results obtained when pipes is exposed to Fe^{2+}-citrate in the absence (spectra A), and presence of 1, 3, and 10 μM NIG (spectra B, C and D, respectively). A significant decrease in the signal intensity of the pipes radical in a NIG dose-dependent manner strongly suggests that the ionophore can act as a scavenger, protecting pipes from oxidation. The control with 0.237 M ethanol (spectrum E), the solvent used for ionophore solubilization, indicates that the protection is much lower than that provided by NIG in the presence of the same concentration of ethanol (spectrum D). Similar results were obtained employing LAS (data not shown). In contrast, VAL did not have an effect on pipes oxidation. Since these experiments were done in aqueous solution, much higher ionophore concentrations were required than in the presence of membranes, where the ionophore-cation complexes are concentrated.

Effect of Fe^{2+}-Citrate and ABAP on LAS Fluorescence

Another aspect of this work concerns the molecular mechanism of the protection process. The scavenger properties are most likely due to the presence of hydroxyl groups in LAS, NIG and MON. In contrast, VAL, which does not carry hydroxyl groups, does not exert this effect. The salicylic acid moiety present in LAS is also a potential target for free radical attack. Since LAS is an intrinsically fluorescent molecule,[18] this possibility was investigated by monitoring its fluorescence spectrum. Addition of Fe^{2+}-citrate (Fig. 8A) or ABAP (Fig. 8B) causes a decrease in the fluorescence, the rate of the decay depending on the presence of liposomes and on the degree of acyl chain unsaturation. The decay of LAS fluorescence in homogeneous medium containing KCl or sucrose indicates that the primary radicals formed by both inductor systems are able to react with the ionophore (Figure 8). A comparison of the kinetic profiles in the presence of PC-CL in KCl, PC-CL in sucrose and in buffer with KCl or sucrose indicates that LAS destruction is more intense in the presence of liposomes, particularly when its incorporation is maximal. When liposomes were made with fully saturated lipids (DMPC-DCP), the destruction of ionophore was slower in KCl than in sucrose medium (Figure 8A

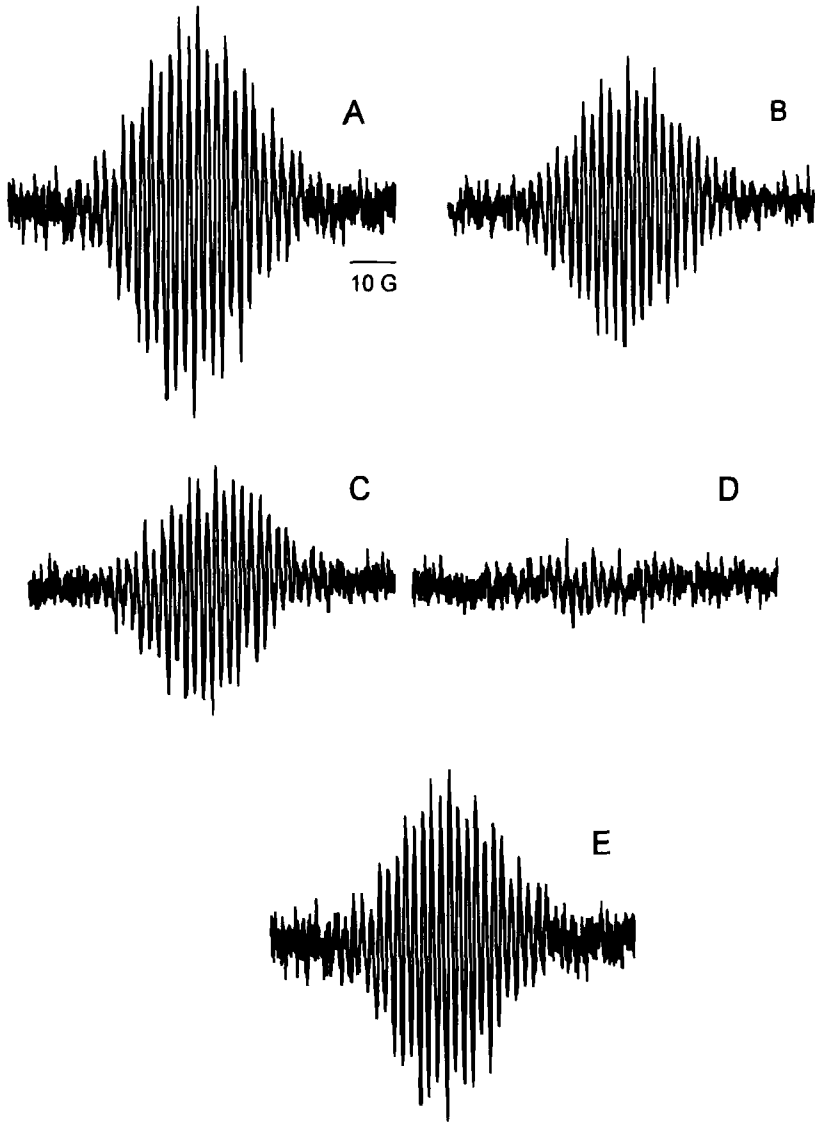


FIGURE 7 **NIG prevention of pipes radical formation.** Effect of NIG on the EPR spectra of the radical produced by oxidation of 0.2 M pipes, pH 7.2, by 150 μM Fe^{2+}-10 mM citrate. [NIG] (μM): (**A**) 0; (**B**) 1, (**C**) 3, (**D**) 10 and (**E**) ethanol at the same concentration (0.237 M) as in (**D**). Spectrometer receiver gain was the same for all samples.

and B, solid and open squares, respectively). In the presence of KCl, the rate of LAS destruction was slower than in homogenous media, probably because LAS incorporation is maximal under this condition. However, since lipid peroxidation does not occur due to the absence of double bonds, the ionophore is protected from the free radicals produced in the aqueous medium.

The data indicate that lipid-centered radicals generated in PC-CL liposomes are responsible for the faster decay of LAS fluorescence in this system. Fe^{3+} does not cause a significant quenching of LAS fluorescence, as indicated by the spectrum obtained upon addition of the ionophore 30 min after Fe^{2+}/citrate cycling was started (data not shown).

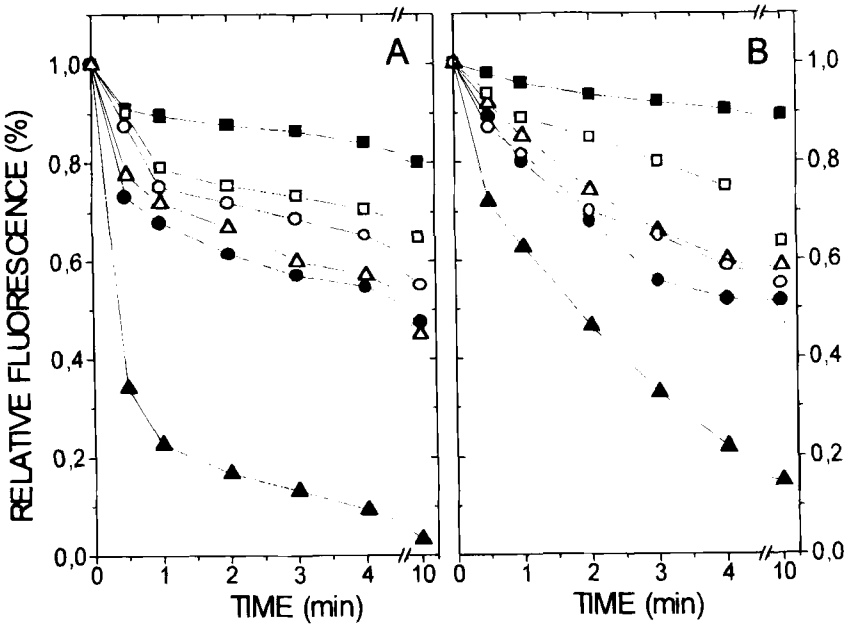


FIGURE 8 Effect of Fe^{2+}-citrate and ABAP upon the fluorescence spectra of LAS. Kinetics of fluorescence decay at 412 nm of 250 nM LAS induced by 50 μM Fe^{2+}-4 mM citrate (**A**) or by 40 mM ABAP (**B**) in 10 mM hepes, pH 7.4: (●,○) aqueous medium; (■,□) 2 mM DMPC-20 mole% DCP; (▲,△) 2 mM PC-20 mole% CL; solid symbols, 130 mM KCl, open symbols, 250 mM sucrose. Incubation at 35°C, λ_{EXC}=310 nm, slits: 5 nm/5 nm.

DISCUSSION

The present study has provided an insight into the mechanistic aspects of the protection conferred by hydroxyl-containing ionophores against lipid peroxidation in mitochondria and in model systems. Peroxidation of liposomes consisting of egg PC and the negatively charged lipids CL and DCP was induced by the addition of either Fe^{2+}-citrate or ABAP, and the protective effect was independent of the inductor system employed.

Evaluation of lipid peroxidation by monitoring mitochondrial swelling (Figure 1), oxygen uptake (Figures 2 and 3), TBARS (Figure 6, Table I) and conjugated diene (Figure 6), and loss of the EPR signal of a lipid spin probe (Figures 4, 5 and 6), clearly demonstrated that this process does not occur in the presence of hydroxyl-containing ionophores and that protection, of either intact mitochondria or liposomes, requires the presence of the complexing ions (K^+ or Na^+) as osmotic support. That the protection against lipid peroxidation is not related to changes in pH is evidenced by the experiments in the presence of FCCP (Figures 1–6).

Table II shows that the stoichiometry of radical formation and of TBARS and conjugated diene accumulation lies in the same range as found by other authors.[41–44] It is seen that the production of TBARS and conjugated diene decreased by 66 and 50%, respectively, in the presence of considerably low ionophore concentration. In the present study, for 1 mM lipid, the antioxidant concentration was 0.1 μM. A rough analysis of these data suggests that the ionophores could be more effective antioxidants than vitamin E. A synergistic effect has been observed when water soluble (vitamin C) and lipid soluble (vitamin E) antioxidants were used together.[41–44] Moreover, it has been suggested that the lack of mobility of vitamin E in the membrane is responsible for a decrease in antioxidant activity,[43,44] when a water soluble radical generating system was used.

Although the intrinsic (chemical) ability of the ionophores to react with lipid radicals has not been evaluated, it is tempting to speculate that, since these molecules possess the ability to partition between the water and membrane phases, and since they can traverse the lipid bilayer, these properties could confer them the ability to act as very effective antioxidants. In this context, it is worthwhile noticing that a small antioxidant activity is observed when the ionophores remain in the aqueous phase (in sucrose medium, Figure 6).

Increased lipid packing is known to favor propagation of the peroxidative reaction chain.[39] In the present work, inhibition of peroxidation occured (Table I) when the lipid packing increased (Table III). In this case, however, increased lipid packing is coupled to ionophore-cation complex formation and intercalation in the lipid bilayer, leading to optimization of the radical scavenging effect.

Order parameter measurements (Table III) are in agreement with a less organized lipid bilayer in PC/DCP liposomes in buffer alone and in the presence of sucrose. At high ionic strength, the molecular degree of order increases as a result of DCP's head groups charge screening by the positively charged cations. Table III shows that S_{eff} increases upon addition of the ionophores, suggesting that these molecules further increase the interfacial cation concentration. The data clearly show that the protective effect of the ionophores is not due to their membrane ordering ability, since VAL, which exerts this effect to the same extent as the hydroxyl-containing ionophores, is incapable of preventing lipid peroxidation.

The role of membrane negative charge associated with the presence of salt was fully demonstrated (Figures 3–6, 8), leading to the conclusion that the charge at the membrane-water interface in PC/DCP, PC/CL and DMPC/DCP liposomes, as well as on the inner mitochondrial membrane causes an increased cation concentration at the interface, facilitating formation of the cation-ionophore complex, the membrane-incorporated species.[19–22] On the other hand, in sucrose medium, inhibition of lipid peroxidation by the ionophores is not observed, probably because the sugar stabilizes ionophore structures in the linear backbone conformation through hydrogen bonding, thus preventing cation complexation and the consequent complex incorporation into the membrane. The inhibition by NIG of lipid peroxidation in hepes buffer in the absence of KCl (Fig. 5A) is in agreement with the fact that the ionophore probably also transports this cation, albeit less efficiently.[38]

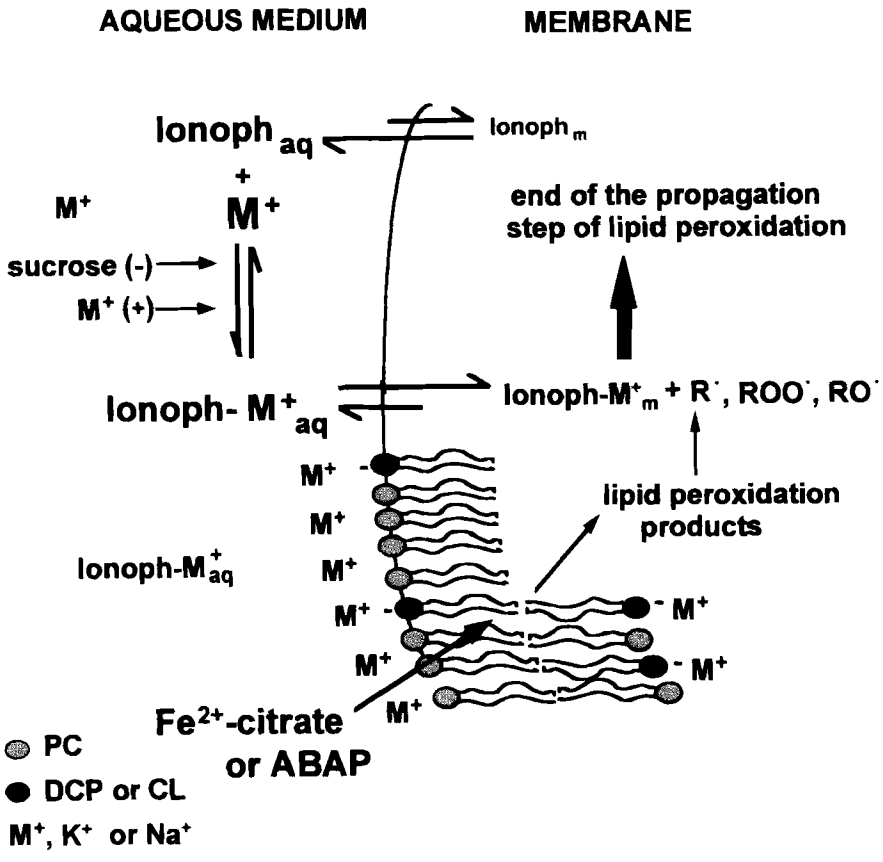
With regard to the chemical nature of the primary radicals, the autoxidation product of the Fe^{2+}-citrate complex is not known. It has been suggested to consist of a Fe^{2+}-O_2-Fe^{3+}-complex.[23] The thermal decomposition of ABAP gives rise to a peroxyl radical.[24] Moreover, no evidence was found for the participation of the hydroxyl radical in the initiation of lipid peroxidation by Fe^{2+}-citrate. The inhibitory effect can be explained by a radical scavenging property of the OH-containing ionophores, that are able to react with both primary radicals from Fe^{2+}-citrate and ABAP, and secondary radicals, such as lipid peroxyl radicals, produced by the attack of the primary radical to the unsaturated phospholipid acyl chains (Figure 8A and B). That primary radicals can react with the ionophores is supported by the observed protection against pipes radical formation (Fig. 7), and by the decrease in LAS fluorescence in the absence of liposomes (Fig. 8A and B). The slower rate of fluorescence decay in DMPC-DCP liposomes in KCl medium submitted to both radical generating systems (Figure 8A and B), as compared to PC-CL liposomes (Figure 8A and B) under the same conditions, strongly supports the conclusion that lipid peroxyl radicals are able to react with the OH containing ionophores. In sucrose medium, when the ionophores remain preferably in the aqueous phase, the rate of radical attack, as seen by the fluorescence decay, is very similar in aqueous phase and in the presence of PC-CL, and DMPC-DCP liposomes. The absence of protection in sucrose medium when the Fe^{2+}-citrate

system was used is in agreement with other reports[23] and reinforces the lack of hydroxyl radical participation in the initiation step of lipid peroxidation. Again, these results indicate that when the ionophores are in the aqueous phase, the reactions occur because they are exposed to the primary radicals. Only when the ionophores are compartmentalized, a condition favored by the presence of counter ions in the medium and by negatively charged lipids, they will be in contact with lipid radicals generated during the peroxidation process and, therefore, will be destroyed at a faster rate. In this condition, the effective ionophore concentration in the membrane is much higher than that in solution. In addition, despite ionophore compartmentalization, if lipid peroxidation does not occur due to the lack of double bonds (in DMPC/DCP), the destruction of ionophores should be a consequence of the attack only by primary radicals formed in the aqueous phase, and the fluorescence decay would be expected to be slower (Figure 8A and B). Clearly, the ionophores alcoholic hydroxyl groups are very good targets for radicals generated during lipid peroxidation. In contrast, VAL, although capable of ion transport, does not bear hydroxyl groups and, accordingly, does not inhibit lipid peroxidation.

It was possible to rule out Fe^{2+}-ionophore complex formation as being responsible for the protective effect in view of the following observations and information: (i) the kinetics of autoxidation of the citrate complex, as monitored by the optical absorption of the Fe^{2+}-citrate-1,10-phenanthroline adduct[23] were similar in the absence and presence of NIG (data not shown); (ii) the protection is minimal in sucrose medium where Fe^{2+}-ionophore complex formation would be favored, due to the absence of competing K^+ ions; (iii) in contrast, the protection is maximal in the presence of competing K^+. These results are probably due to the high association constant of the Fe^{2+}-citrate complex.[48]

The above results are in contrast with *in vivo* studies of lipid peroxidation showing that increased MON concentrations lead to increased peroxidation in liver[49] and heart,[50] but have no effect on the lipid peroxide status of the blood plasma and breast muscle.[49] However, much higher ionophore concentrations were used in these reports. It is not clear that increased ionophore concentrations should provide a stronger effect against lipid peroxidation, since: (i) the primary ionophore function is cation transport; an increase in transport rates can have other spurious effects intrinsic to the system under study; (ii) increased ionophore concentrations could alter the physico-chemical properties of the membrane, such as fluidity and viscosity, and, as a consequence, its susceptibility to oxidative processes and potential protection by antioxidants. In this context, is important to emphasize that BHT causes tissue damage when used at higher concentrations than the usual 1–10 μM range.[51] Finally, we propose that although our *in vitro* results cannot be applied directly to *in vivo* systems, data from simple model systems can provide a more detailed picture at a molecular level, establishing a basis for further studies, focusing more complex living systems.

Scheme I summarizes the fundamental events, leading to the protection of model and biological membranes against lipid peroxidation by low ionophore concentrations. The rate of ionophore diffusion in the acyclic conformation into and across the bilayer is very slow due to the relatively high hydrophilicity of this form (compared with the cyclic form), and to the electrostatic repulsion between the uncomplexed species and the bilayer negative surface charge. In contrast, the cation-bound ionophores are neutral and their three-dimensional conformation renders them lipophilic, facilitating incorporation into the less polar membrane environment.[19–22] Clearly, the protective effect requires ionophore incorporation into the membrane, a process dependent on cation complexation.[19–22] On the other hand, sucrose does not seem to be an innocuous additive, as suggested by the EPR data, preventing ionophore complexation probably by stabilizing



SCHEME 1 General scheme summarizing the molecular mechanism by which complexed ionophores can protect membranes from lipid peroxidation. The scheme shows the main steps of ionophore cation-complexation and the consequent membrane incorporation. Ionophores in solution ($Ionoph_{aq}$) complex cations (M^+), and the negatively charged surface can catalyze this process by concentrating M^+ at the interface, rendering its local concentration higher than in the bulk solution. This condition strongly favors the ionophore complexation step, leading to an increased concentration of membrane-incorporated complexed ionophore ($Ionoph\text{-}M^+_m$) which, in turn, can act as a radical scavenger, inhibiting lipid peroxidation.

the acyclic, hydrophilic form. In addition, if, as proposed,[43,44] antioxidant mobility in membranes is important for the radical scavenging ability, this could explain the high efficiency of the hydroxyl groups in these molecules in inhibiting lipid peroxidation processes at concentrations lower than those usually employed for other membrane soluble antioxidants.

Altogether, the data indicate that the action of the hydroxyl-containing ionophores in biological systems must be analyzed with care. The present studies show, for the first time, that, in addition to their transport properties, their chemical structure confers them the ability to act as free radical scavengers.

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