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Antioxidant properties of ursodeoxycholic acid

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Abstract

We have investigated potential antioxidant properties of the clinically relevant bile acid UDCA, which reaches therapeutic concentrations up to 0.09 and 29 mM, respectively, in human plasma and bile. UDCA was an excellent scavenger of $OH^{\bullet}$ generated by $FeCl_3$–EDTA, H_2O_2 and ascorbate in the deoxyribose oxidation test, showing IC_{min} and IC_{50} values of 0.02 and 0.2 mM, respectively, and a second-order rate constant for reaction with $OH^{\bullet}$ of $2 \pm 0.1 \times 10^{10}\ M^{-1}\ s^{-1}$. Notably, the drug could enhance at 1.5 mM concentration the antioxidant capacity of human bile against $OH^{\bullet}$-induced deoxyribose oxidation. UDCA also showed antioxidant effects in the deoxyribose test performed with nonchelated iron ions, such as Fe^{2+} plus H_2O_2 (IC_{min}: 7 mM, IC_{50}: 20 mM) or Fe^{3+} plus H_2O_2 and ascorbate (IC_{min}: 0.3 mM, IC_{50}: 5 mM), and inhibited ferrozine–Fe^{2+} and desferrioxamine–Fe^{3+} complexes formation with IC_{50} values of, respectively, 12 and 0.3 mM, indicating that the drug interacts more with iron(III) than with iron(II). Moreover, UDCA significantly inhibited phospholipid liposome peroxidation induced by the $OH^{\bullet}$-generating system $FeCl_3$–EDTA, H_2O_2 and ascorbate (IC_{min}: 0.75 mM, IC_{50}: 3 mM), and by peroxyl radicals generated in the aqueous phase by AAPH (IC_{min}: 8 mM, IC_{50}: 14 mM). UDCA, even at 25 mM concentration, was ineffective on the lipoperoxidation mediated by Fe^{2+} alone, but at the same concentration counteracted significantly that by Fe^{3+} plus ascorbate, further pointing to its preferential antioxidant interaction with iron(III).

In conclusion, UDCA has direct antioxidant properties, which are especially relevant against Fe^{3+}- and $OH^{\bullet}$-dependent biomolecular oxidative damage; such properties are evident at therapeutically relevant drug concentrations, suggesting that UDCA could act as an antioxidant *in vivo*.

Keywords: Ursodeoxycholic acid; Antioxidant; Hydroxyl radical; Iron; Lipid peroxidation; Oxidative stress

1. Introduction

UDCA is a therapeutically relevant bile acid capable of dissolving cholesterol gallstones and of preventing their formation in patients with lithogenic bile [1,2]. Notably, radical-induced phospholipid peroxidation has been implicated in the pathogenesis of cholesterol gallstones [3,4]. The efficiency of UDCA in this pathological condition, as well as in cholestatic and alcoholic liver disease, liver transplantation and nonalcoholic steatohepatitis [2], which are characterized by enhanced oxidative stress [5–10], has led us to hypothesize that the drug could also possess some antioxidant properties. Moreover, given the role of oxidative stress in the genesis of cell damage and apoptosis [10,11], it is possible that the cytoprotective and antiapoptotic effects of UDCA [2] may be partly related to antioxidant mechanisms. In such a context, UDCA has been shown to induce endogenous antioxidant defenses in rat hepatocytes [12], and to counteract deoxycholate-related lipid peroxidation in cultured macrophages and liposomes [13,14], indicating an indirect-type drug antioxidant activity; however, in spite of these evidences, direct antioxidant properties of UDCA have not yet been characterized. This issue has been herein addressed in specific model systems,

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Abbreviations: UDCA, ursodeoxycholic acid; $OH^{\bullet}$, hydroxyl radical; EDTA, ethylenediaminetetraacetic acid; PPB, potassium phosphate buffer; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; AAPH, 2,2′-azobis(amidinopropane) dihydrochloride; BHT, butylated hydroxytoluene; IC_{min}, minimal drug concentration inhibiting significantly deoxyribose or phospholipid oxidation, as well as ferrozine–Fe^{2+} or desferrioxamine–Fe^{3+} complexes formation; IC_{50}, drug concentration inhibiting by 50% deoxyribose or phospholipid oxidation, as well as ferrozine–Fe^{2+} or desferrioxamine–Fe^{3+} complexes formation.

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focusing attention on pharmacological inhibition of oxidative damage by iron and $OH^{\bullet}$, which is the most toxic oxygen radical [10] and may foster phospholipid peroxidation and cell injury in various pathological conditions, including cholestatic and alcoholic liver disease [4,5,8,10].

2. Materials and methods

2.1. Materials

Reagents were from Sigma Aldrich, except for UDCA sodium salt, which was a generous gift of Aldo Roda, Universita' di Bologna, Italy, and AAPH, which was from Polisciences. Human bile was kindly provided by Roberto Cotellese, Patologia Chirurgica, Universita' G. d'Annunzio, Chieti, Italy.

2.2. $OH^{\bullet}$ scavenging and drug–iron interaction

We used the deoxyribose test, which gives results very similar to those obtained using pulse radiolysis techniques [15], to detect possible $OH^{\bullet}$ scavenging properties of UDCA [10,15,16]. When the test is performed without EDTA, namely with nonchelated iron ions, drug metal-binding capacity may also be evaluated [10,15,16].

Reaction mixtures contained, in a final volume of 1.0 mL, 10 mM PPB, pH 7.4, 2.8 mM deoxyribose, 20 μM $FeCl_3$ (premixed or not with 100 μM EDTA before addition to the reaction system) and 1.42 mM H_2O_2, in the absence or presence of UDCA sodium salt; 100 μM ascorbic acid started the reaction, allowing 60 min incubation at 37°. In other experiments, drug effects were tested on deoxyribose oxidation induced by 60 min incubation at 37° with nonchelated Fe^{2+}, namely 20 μM $FeSO_4$, with or without 1.42 mM H_2O_2, in PPB, pH 7.4. The TBA-test, which detects aldehydic products, such as malondialdehyde (MDA), resulting from deoxyribose (or lipid) oxidation, was the carried out adding to 0.5 mL of sample, 1.0 mL of 2.8% trichloroacetic acid and 1.0 mL of 0.6% aqueous solution of TBA; tubes were heated at 95° for 30 min to develop the color, and successively cooled in ice. The red chromogen, expression of the TBA:MDA adduct formation, was extracted with *n*-butanol kept at 4°; after a brief centrifugation to favor organic phase separation, the upper *n*-butanol layer was removed and read spectrophotometrically at 532 nm against an appropriate blank. Extraction with *n*-butanol was performed because UDCA, especially at high concentrations, resulted in some turbidity of reaction mixtures. It is of note that in control experiments of iron-dependent deoxyribose oxidation carried out without UDCA, the absorbance values at 532 nm detected in the organic layer after red chromogen extraction with cold *n*-butanol were lower by only about 2% than those recorded directly in the TBA-reacted cold aqueous mixture before extraction; thus, TBARS were almost totally extracted under our experimental conditions, with a negligible experimental error related to the extractive procedure. Results were calculated as nmol TBARS per mL, using a molar extinction coefficient of 154,000 at 532 nm [10]. The drug gave no interference in the TBA assay of deoxyribose (or phospholipid) oxidation.

From the slope of competition plot for inhibition of deoxyribose oxidation by various UDCA concentrations (from 0.02 to 3 mM) in the iron–EDTA system, the second-order rate constant (k_2) for reaction of the drug with $OH^{\bullet}$ was calculated according to the equation:

$$k_2 = \text{slope} \times k_{DR} \times [DR] \times A^0$$

where k_{DR} is the k value of deoxyribose (3.1×10^9 M^{-1} s^{-1}), [DR] is the deoxyribose concentration and A^0 is the absorbance detected at 532 nm without the drug in the TBA-test [15,16].

To further evaluate UDCA–iron interaction, we also performed the ferrozine test [17], which assesses drug capability to interact with iron(II) preventing the formation of the complex between the reduced metal form and the specific Fe^{2+} colorimetric detector ferrozine. A similar experimental approach was used for iron(III) using desferrioxamine, which forms a 430 nm absorbing complex with Fe^{3+}. In order to avoid possible interference of buffers with iron, such as phosphate-mediated iron(II) autoxidation, reactions were carried out in 0.15 M NaCl. For the ferrozine test, experimental tubes contained 5 μM $FeSO_4$, with and without various UDCA sodium salt concentrations, and 30 μM ferrozine to start the reaction [17], in 0.15 M NaCl; after 10 min incubation at 25°, absorbance values at 562 nm related to the ferrozine–iron(II) complex were recorded spectrophotometrically against appropriate blanks. For the desferrioxamine test, we used the same chelator:iron molar ratio (i.e. 6:1) of the ferrozine test; reaction mixtures contained therefore 100 μM $FeCl_3$, UDCA sodium salt and 600 μM desferrioxamine, in 0.15 M NaCl; after 10 min incubation at 25° and centrifugation at 12,000 *g* for 10 min, absorbance values at 430 nm related to the desferrioxamine–iron(III) complex were recorded spectrophotometrically against appropriate blanks.

2.3. $OH^{\bullet}$ scavenging properties of human bile with and without UDCA

In specific experiments, we investigated whether human bile had scavenging antioxidant properties against $OH^{\bullet}$, and whether such properties were enhanced *in vitro* by UDCA added to bile itself. Aliquots of 0.1 mL of bile with or without 1.5 mM UDCA sodium salt (present at final concentration of 0.15 mM in reaction mixtures) were added to experimental tubes containing, in a final volume of 1.0 mL, 10 mM PPB, pH 7.4, 2.8 mM deoxyribose, 20 μM $FeCl_3$ premixed with 100 μM EDTA, 1.42 mM H_2O_2 and 100 μM ascorbic acid, followed by 60 min

incubation at 37°. The TBA-test was then carried out as reported above, including however 0.8 mM BHT just before heating to prevent possible artifactual peroxidation of phospholipids present in bile. The red chromogen was extracted with cold *n*-butanol and read at 532 nm against appropriate bile- and drug-containing blanks.

2.4. Lipid peroxidation

Effects of UDCA on lipid peroxidation were investigated using phospholipid liposomes, which adequately represent the biological lipid bilayer. Soybean asolectin (a source of naturally occurring phospholipids) was previously dissolved in chloroform, and then dried under a stream of argon; PPB (10 mM, pH 7.4) was added to the lipid residue, followed by vortex mixing and sonication. The resulting liposomes, containing a final phospholipid concentration of 1.5 mg mL^{-1}, were induced to peroxidize with or without UDCA sodium salt by different oxidant systems: (1) 20 μM $FeCl_3$, precomplexed with 100 μM EDTA, plus 2.84 mM H_2O_2 and 200 μM ascorbic acid; (2) 100 μM $FeSO_4$ alone; (3) 300 μM $FeCl_3$ plus 200 μM ascorbic acid; (4) 4 mM AAPH, in the presence of 100 μM diethylenetriaminepentaacetic acid. In this latter system, where the oxidizing species are peroxyl radical generated thermally by the water-soluble azo-initiator AAPH, incubation was at 37° for 60 min; in the iron-related systems, incubation was instead for 20 min at 37°. Lipid peroxidation was evaluated through the aforementioned TBA-test performed in the presence of 0.1 mL of 8.1% sodium dodecyl sulfate and 0.8 mM BHT. When turbidity occurred, as in the oxidant systems based on nonchelated iron and AAPH owing to high UDCA concentrations and/or AAPH, TBARS were extracted with cold *n*-butanol before spectrophotometric reading at 532 nm.

2.5. Statistics

Results were calculated as means ± SD and analyzed by ANOVA plus Student–Newman–Keuls test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Effect of UDCA on $OH^{\bullet}$- and iron-dependent deoxyribose oxidation, and drug–iron interaction

UDCA was a powerful inhibitor of deoxyribose oxidation in the presence of EDTA, indicating its excellent $OH^{\bullet}$ scavenging ability. It should be noted that the drug, at concentrations inhibiting biomolecular oxidant damage induced by the iron–EDTA system, was apparently incapable of removing the metal from EDTA. Indeed, after 60 min incubation at 37° in PPB, pH 7.4, of the complex between 20 μM $FeCl_3$ and 100 μM EDTA with a drug concentration up to 3 mM, the absorbance values at 258 and 328 nm of the iron–EDTA complex [18] were not significantly different from the control ones detected without the drug (0.130 ± 0.009 and 0.030 ± 0.003, respectively, at 258 and 328 nm with 3 mM UDCA vs. 0.122 ± 0.006 and 0.028 ± 0.002 of controls, P = NS; $N = 5$), with similar spectral characteristics of the iron–EDTA complex in the absence or presence of UDCA (not shown). Thus, the antioxidant effects of UDCA in the iron–EDTA system are not due iron chelation but rather due to $OH^{\bullet}$ scavenging. From the slope of competition plot for inhibition of $OH^{\bullet}$-induced deoxyribose oxidation by UDCA (Fig. 1), a second-order rate constant for the reaction of the drug with $OH^{\bullet}$ of $2 \pm 0.1 \times 10^{10}$ M^{-1} s^{-1} was calculated, essentially a diffusion-controlled rate. The IC_{min} and IC_{50} of UDCA towards $OH^{\bullet}$-induced deoxyribose oxidation in the iron–EDTA system were 20 and 200 μM, respectively. The drug has therefore a significant $OH^{\bullet}$ scavenging activity also at therapeutically achievable mean plasma concentrations, which are about 60–90 μM in patients with primary biliary cirrhosis [13,19].

When the deoxyribose test was performed with iron alone, namely not precomplexed with EDTA, UDCA also showed significant antioxidant effects, which were however considerably lower than those observed with iron–EDTA. It is of note that the yield of TBARS was nearly 2.5-fold higher in the $FeCl_3$–H_2O_2–ascorbate than in the $FeSO_4$–H_2O_2 system (14.7 ± 1 vs. 6.2 ± 0.4 nmol TBARS per mL, $P < 0.0001$; $N = 6$), pointing out the relevant role of iron redox cycling in biomolecular oxidant damage. The specific antioxidant activity of UDCA was higher in the former than in the latter system; indeed, drug IC_{min} and IC_{50}

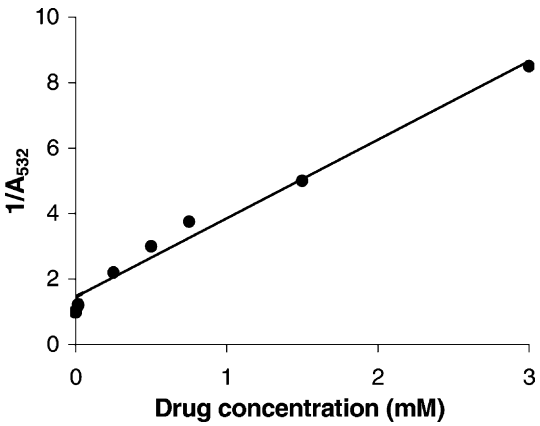

Fig. 1. Scavenging of $OH^{\bullet}$ by UDCA assessed in the deoxyribose oxidation assay performed with $FeCl_3$–EDTA, H_2O_2 and ascorbate. On the *y*-axis are reported the absorbance values at 532 nm (expressed as $1/A_{532}$) detected in the TBA-test in the absence or presence of various drug concentrations, i.e. from 0.02 to 3 mM, which inhibited significantly $OH^{\bullet}$-induced deoxyribose oxidation in a dose-related manner. The results are representative of six independent experiments with average variations <6%. From the slope of competition plot reported in the figure, the second-order rate constant for reaction of UDCA with $OH^{\bullet}$ was calculated to be 2×10^{10} M^{-1} s^{-1}. See Sections 2 and 3 for further explanations.

Table 1
Interaction of UDCA with iron(II) and iron(III) evaluated by drug inhibition of the ferrozine–Fe^{2+} and desferrioxamine–Fe^{3+} complexes formation

	IC_{min} (mM)	IC_{50} (mM)
Ferrozine–Fe^{2+} complex	8	12
Desferrioxamine–Fe^{3+} complex	0.1	0.3

Experimental mixtures contained 5 μM $FeSO_4$ and 30 μM ferrozine, or 100 μM $FeCl_3$ and 600 μM desferrioxamine, with or without UDCA, in 0.15 M NaCl. The absorbance values at 562 nm of the ferrozine–Fe^{2+} complex and those at 430 nm of the desferrioxamine–Fe^{3+} complex were, respectively, 0.128 ± 0.004 and 0.170 ± 0.009 in six independent control experiments. See Sections 2 and 3 for further explanations.

values were, respectively, 0.3 and 5 mM in the deoxyribose test performed with $FeCl_3$, H_2O_2 and ascorbate, and 7 and 20 mM in that performed with $FeSO_4$ plus H_2O_2. Similar values of IC_{min} and IC_{50} (6 and 18 mM, respectively) were observed when deoxyribose oxidation was mediated by $FeSO_4$ alone, which resulted in a TBARS yield about 20% lower than that observed with $FeSO_4$ plus H_2O_2 (not shown).

As shown in Table 1, UDCA inhibited ferrozine–Fe^{2+} and desferrioxamine–Fe^{3+} complex formation, with IC_{50} values of, respectively, 12 and 0.3 mM, indicating that it may act as a better iron(III)- than iron(II)-binding agent. As for the EDTA–iron complex, UDCA did not influence instead the specific absorbance of the ferrozine–Fe^{2+} and desferrioxamine–Fe^{3+} complexes when added after their formation (not shown), suggesting that the drug does not alter the iron-binding properties of these iron chelators.

In some experiments, we also used the sodium salt of tauroursodeoxycholic acid, which resulted in antioxidant effects similar to those of UDCA (not shown).

3.2. Effect of human bile with and without UDCA on $OH^{\bullet}$-dependent deoxyribose oxidation

Human bile markedly counteracted $OH^{\bullet}$-dependent deoxyribose oxidation (Table 2), indicating that it is physiologically endowed with antioxidant compounds, such as bile acids, mucin and proteins, capable of scavenging $OH^{\bullet}$ (it was however out of the scope of the present paper to characterize the specific antioxidant properties of human bile). As also shown in Table 2, bile with addition of 1.5 mM UDCA was significantly more effective than bile alone on $OH^{\bullet}$-induced deoxyribose oxidation. In this regard, it is possible that the drug, also acting as an emollient, could have decreased the dimensions of the bile hydrophobic clusters, enhancing their reactivity towards $OH^{\bullet}$; such a mechanism might have been therefore operative together with a direct $OH^{\bullet}$ scavenging effect of UDCA. In any event, limitedly to our experimental setting, UDCA can augment efficiently the antioxidant capacity of human bile against $OH^{\bullet}$-dependent oxidative injury.

Table 2
Effect of human bile with and without 1.5 mM UDCA on $OH^{\bullet}$-induced deoxyribose oxidation

	TBARS (nmol mL^{-1})
Control (deoxyribose and iron/EDTA–H_2O_2–ascorbate)	52 ± 3
Control plus	
Human bile (0.1 mL) alone	11 ± 0.9*
Human bile (0.1 mL) with UDCA	5.7 ± 0.6*,**

Means ± SD of five independent experiments.
* $P < 0.05$ vs. control.
** $P < 0.05$ vs. control plus bile alone (ANOVA plus Student–Newman–Keuls or Bonferroni test). See Sections 2 and 3 for further explanations.

3.3. Effect of UDCA on lipid peroxidation

As depicted in Fig. 2, phospholipid peroxidation induced by the $OH^{\bullet}$-generating system iron/EDTA–H_2O_2–ascorbate was significantly counteracted by UDCA in a dose-dependent fashion, with IC_{min} and IC_{50} values of, respectively, 0.75 and 3 mM. The drug was instead ineffective on phospholipid peroxidation mediated by 100 μM $FeSO_4$ alone, which resulted in 1.9 ± 0.13 and 2 ± 0.15 nmol TBARS per mL with and without 25 mM UDCA, respectively (P = NS, N = 5); on the other hand, lipid peroxidation was totally inhibited by 5 mM α-tocopherol or 2 mM BHT (not shown). UDCA (25 mM) counteracted significantly phospholipid peroxidation mediated by 300 μM $FeCl_3$ plus 200 μM ascorbate (4 ± 0.22 vs. 4.9 ± 0.27 nmol TBARS per mL, $P < 0.05$, N = 5). We deemed not adequate to use in these experiments higher drug concentrations because they gave some interference with phospholipid liposomes, such as formation of a flocculent material; thus, IC_{50} could not be determined.

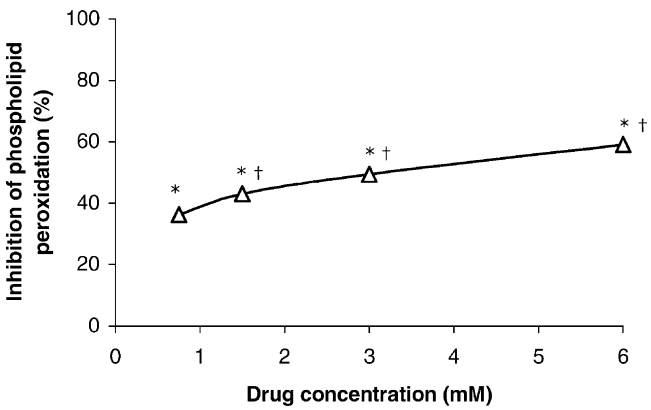

Fig. 2. Concentration-dependent inhibition by 0.75, 1.5, 3 and 6 mM UDCA of phospholipid liposome peroxidation induced by the $OH^{\bullet}$-generating system $FeCl_3$–EDTA, H_2O_2 and ascorbate. Lipid peroxidation was evaluated by the TBA-test, which resulted in 1.1 ± 0.07 nmol TBARS per mL in control experiments. The results represent the means of percentage inhibition of phospholipid peroxidation calculated from seven independent experiments (standard deviations are less than 6% and were omitted as they do not exceed the size of the symbols). *$P < 0.05$ vs. control; †$P < 0.05$ vs. the lower drug concentration (ANOVA plus Student–Newman–Keuls test). See Sections 2 and 3 for further explanations.

Finally, UDCA inhibited AAPH-mediated, peroxyl radical-induced phospholipid peroxidation, which gave 2.8 ± 0.16 nmol TBARS per mL in control experiments ($N = 6$); in such oxidant system the drug showed an IC_{min} of 8 mM and an IC_{50} of 14 mM.

4. Discussion

The present study shows that UDCA has specific antioxidant properties. The $OH^{\bullet}$ scavenging efficiency of UDCA appears remarkable, considering that its rate constant for reaction with this radical species is about 10-fold higher than that of the well known pharmacological scavenger mannitol and of the physiological scavengers glucose or histidine [10,15]. Thus, given also the high therapeutic concentrations of UDCA reached in human bile, namely up to 29 mM [20], the drug could readily act as an effective $OH^{\bullet}$ scavenger especially in the biliary milieu; in this regard, it is noticeable that in our experimental setting the scavenging antioxidant capacity of human bile against $OH^{\bullet}$ is significantly enhanced by an UDCA concentration of only 1.5 mM. UDCA also counteracts deoxyribose oxidation mediated by nonchelated iron, indicating that it has some iron-binding effects [10,16]. This is basically in line with previous reports showing an iron-complexating activity of bile salts, although only their interaction with iron(II) has been specifically assessed [21,22]. UDCA is however considerably more effective on the oxidation of deoxyribose by $FeCl_3$–H_2O_2–ascorbate than on that by $FeSO_4$–H_2O_2, suggesting that the drug interacts more with iron(III) than with iron(II). Accordingly, the drug is also far more effective as inhibitor of the desferrioxamine–Fe^{3+} than of the ferrozine–Fe^{2+} complex formation, pointing to its preferential interaction with iron(III). On the other hand, it has been proposed that deoxyribose oxidation mediated by nonchelated iron(II) and H_2O_2 is due to an hypervalent iron species, such as iron(IV) [23]; it is therefore possible that UDCA may act not only as a site-specific $OH^{\bullet}$ scavenger, but also, at high therapeutic concentrations, as an antioxidant against iron(IV)-induced oxidative damage.

Our data also show that UDCA has antilipoperoxidative effects. Lipid peroxidation is a very complex phenomenon, which is initiated by an oxidizing species, such as $OH^{\bullet}$, capable of abstracting hydrogen from the allylic bond of polyunsaturated fatty acids, and then propagated by peroxyl radicals [10]. When iron alone, such as Fe^{2+}, is used as oxidative catalyst, lipid peroxidation may occur *via* initiation by alkoxyl radicals and, especially, propagation by peroxyl radicals [10]; these oxidizing species are generated from metal decomposition of trace amounts of preformed lipid hydroperoxides, which are very difficult to remove totally from biological lipids [10]. We have used different prooxidant systems to induce phospholipid peroxidation, including iron/EDTA–H_2O_2–ascorbate, nonchelated Fe^{2+} alone and Fe^{3+} plus ascorbate. UDCA shows the highest antioxidant efficiency in the first system with an IC_{50} of 3 mM, whilst it is ineffective in the second and inhibits at 25 mM Fe^{3+}/ascorbate-mediated lipid peroxidation by only about 18%. Such findings, together with the evidence of drug incapability to remove iron from EDTA, indicate that the antilipoperoxidative activity exerted by UDCA in the iron/EDTA–H_2O_2–ascorbate system, which generates $OH^{\bullet}$ [10,15,16], is not due to iron chelation but rather to $OH^{\bullet}$ scavenging. In this regard, it is of note that lipid peroxidation catalyzed by some iron-dependent systems, such as chelated Fe^{3+} plus H_2O_2 and the iron reductant superoxide anion, is inhibited by $OH^{\bullet}$ scavengers [24], which are instead poorly effective when simple iron ions are used to foster lipoperoxidative processes [10]. UDCA is an excellent $OH^{\bullet}$ scavenger and can so counteract efficiently $OH^{\bullet}$-induced lipid peroxidation. The drug has also some interaction with iron(III), reducing Fe^{3+}/ascorbate-mediated lipid peroxidation possibly through decreased binding to phospholipids and/or oxidant activity of the metal, but it does not interact with ferrous ions in a way that they become less active as catalysts of lipid peroxidation. Consistently, Sreejayan and von Ritter [14] have reported that tauroursodeoxycholic acid is ineffective on iron(II)-mediated phospholipid peroxidation; these authors have however shown that the drug counteracts significantly the enhancement of Fe^{2+}-dependent phospholipid peroxidation induced by taurodeoxycholate, probably as a result of competitive displacement of this hydrophobic bile acid from the lipid surface [14]. A similar indirect-type antioxidant mechanism, conceivably associated with induction of endogenous antioxidant defenses [12], could enhance drug antioxidant potential *in vivo*.

UDCA has also inhibitory effects on phospholipid peroxidation mediated by AAPH, which generates peroxyl radicals essentially in the aqueous phase. Given the hydrophilic properties of UDCA sodium salt, AAPH-generated peroxyl radicals are expected to be scavenged by the drug in the aqueous phase preventing their oxidizing effects on liposomal phospholipids. The ineffectiveness of UDCA on Fe^{2+}-catalyzed lipid peroxidation suggests that the drug, although able to scavenge peroxyl radicals, is not soluble enough in the lipid phase to act, like the lipophilic chain-breaking antioxidants Vitamin E and BHT, as an efficient scavenger of alkoxyl/peroxyl radicals generated metal-dependently within the hydrophobic liposomal compartment. It may be therefore inferred that the antilipoperoxidative activity exerted by UDCA in the iron/EDTA–H_2O_2–ascorbate system is due to scavenging of $OH^{\bullet}$ more than of peroxyl radicals. However, it is possible that UDCA could counteract *in vivo* oxidant damage by alkoxyl/peroxyl radicals formed from protein hydroperoxides in the biological aqueous compartment [25].

As a further extrapolation to the *in vivo* setting of our experimental data, it should be noted that catalytic iron exists essentially in a chelated form with low molecular

weight ligands, such as nucleotides [10], which are present together with iron in human liver and bile [26,27]; the complex of these ligands with iron, similarly to that of EDTA with the metal, can react with H_2O_2 inducing $OH^{\bullet}$ generation [10,28]. Moreover, a pool of ''free'' iron, capable of fostering oxidant injury even under physiological conditions, has been shown to be present as Fe^{3+} in hepatic cells [29]. Thus, the antioxidant properties of UDCA against Fe^{3+}- and $OH^{\bullet}$-dependent oxidative damage appear of particular relevance. It is also of note that $OH^{\bullet}$ acts as a mediator of mucin hypersecretion [30] and induces phospholipid peroxidation [4,10], which are involved in cholesterol gallstone formation [1,3,4]. Indeed, aldehydic end-products of lipid peroxidation have been found in lithogenic human bile [31]. Since in patients with lithogenic bile the excess of cholesterol is solubilized also in lamellar, liposome-like phospholipid vesicles containing only traces of bile acids [1], the biliary and hepatic UDCA enrichment could result in antilithogenic and cytoprotective effects partly related to drug antioxidant activity. $OH^{\bullet}$ also causes DNA deoxysugar oxidation eventually resulting in mutagenic and apoptotic processes [10]; such oxidation may be prevented by the nuclear bile acid pool of the liver, where UDCA concentrations markedly increase after oral administration to humans [32].

In conclusion, UDCA has direct and significant antioxidant properties, which are especially relevant against Fe^{3+}- and $OH^{\bullet}$-dependent biomolecular oxidative damage; these properties are evident at therapeutically relevant drug concentrations, suggesting that UDCA could act as an antioxidant *in vivo*. The drug has in fact been shown to suppress extent of hepatic lipid peroxidation in experimental cholestatic liver disease [33], which is characterized by enhanced $OH^{\bullet}$ generation [5]. However, whether the antioxidant activity of UDCA is an essential component of its therapeutic efficiency in humans warrants further clinical investigation.

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