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STUDIES ON THE ANTIOXIDANT AND FREE RADICAL SCAVENGING PROPERTIES OF IdB 1016 A NEW FLAVANOLIGNAN COMPLEX

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Silybin has been complexed in 1:1 ratio with phosphatidyl choline to give IdB 1016 in order to increase its bioavailability. The antioxidant and free radical scavenger action of this new form of silybin has beenn evaluated.

One hour after the intragastric administration to rats of IdB 1016 (1.5 g/kg b.wt.) the concentration of silybin in the liver microsomes was estimated to be around 2.5 μg/mg protein corresponding to a final concentration in the microsomal suspension used of about 10 μM. At these levels IdB decreased by about 40% the lipid peroxidation induced in microsomes by NADPH, CCl_4 and cumene hydroperoxide, probably by acting on lipid derived radicals. Spin trapping experiments showed, in fact, that the complexed form of silybin was able to scavenge lipid dienyl radicals generated in the microsomal membranes. In addition, IdB 1016 was also found to interact with free radical intermediates produced during the metabolic activation of carbon tetrachloride and methylhydrazine.

These effects indicate IdB 1016 as a potentially protective agent against free radical-mediated toxic damage.

KEY WORDS: Silybin, lipid peroxidation, free radicals, antioxidants, IdB 1016, flavonoids.

INTRODUCTION

Flavonoids are a wide group of plant-derived compounds which have been demonstrated to have antihepatotoxic effects.[1] Among the flavonoids the purified extract of *Silybum Marianum* (silymarin) and its main active constituent silybin have received substantial attention because of their antioxidant properties. Both silymarin and silybin, in fact, have been shown to be very active in preventing iron-stimulated lipid peroxidation in microsomes and mitochondria.[2,3] Moreover, silybin when administered parenterally to experimental animals was found to protect the liver against oxidative damage induced by acute ethanol intoxication, as well as the erythrocytes against lipid peroxidation and hemolysis consequent to the exposure to phenylhydrazine.[4,5] Nonetheless, the possible application of these flavonoids in therapy is

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hampered by their low solubility in water and by the poor enteral absorption.[6,7] In order to increase its bioavailability silybin has been complexed in a molar ratio of 1:1 with phosphatidylcholine to give IdB 1016. After oral administration of IdB 1016 to rats the plasma levels of silybin were found to be significantly higher than those present after the administration of either silybin to rats[3] or silymarin to humans.[8] In the present study we have investigated the antioxidant properties of IdB 1016 by evaluating the susceptibility to oxidative stress of microsomes isolated from the liver of rats treated orally with IdB 1016.

MATERIALS AND METHODS

IdB 1016 and pure silybin were suspended in water and administered intragastrically to male Sprague-Dawley rats (200 g body w.t.) at the doses of 1.5 and 0.6 g/kg body w.t. one hour before sacrifice. Liver microsomes were prepared according to Slater and Sawyer[9] and resuspended in 0.15 M KCl 10 mM Tris-HCl buffer pH 7.4 to a concentration of 20 mg protein/ml. The actual concentration of silybin in plasma and liver microsomes was estimated by a specific HPLC method.[7] The content of cytochrome P-450, and the activity of cytochrome P-450 reductase, aminopyrine demethylase and 7-ethoxycoumarin de-ethylase was determined as previously described.[10] Oxidative stress was induced by incubating the microsomes (2 mg protein/ml) for 15 min at 37°C in the presence of: (i) an NADPH regenerating system consisting of 5 mM glucose-6-phosphate, 0.5 mM $NADP^+$ and 10 I.U. of glucose-6-phosphate dehydrogenase plus or minus 8.6 mM CCl_4; (ii) 0.1 mM cumene hydroperoxide; (iii) 0.1 mM ascorbate and 2 μM $FeSO_4$. The stimulation of lipid peroxidation was measured in terms of accumulation of malondialdehyde (MDA) according to Poli *et al.*[11]

Spin trapping experiments were performed in microsomes incubated 30 min with a NADPH regenerating system, 25 mM 4-pyridyl-1-oxide-t-butyl nitrone (4-POBN) and either 2 mM methyl-hydrazine (MHD) or 2.5 mM ADP and 0.1 mM $FeCl_3$ as previously reported.[12,13] For the spin trapping of trichloromethyl radicals the microsomes were incubated instead with 4 mM CCl_4 and 25 mM phenyl-t-butyl nitrone (PBN).[14]

Glucose-6-phosphate, $NADP^+$, glucose-6 -phosphate dehydrogenase, aminopyrine, 7-ethoxycoumarin and adenosine diphosphate sodium salt were obtained from Sigma Chemical Co. (St Louis, USA). The spin traps 4-POBN and PBN and methylhydrazine were purchased from Aldrich Europe (Beerse, Belgium). IdB 1016, silybin and silybin dihemisuccinate were from Inverni della Beffa (Milano, Italy).

RESULTS AND DISCUSSION

One hour after the intragastric administration to rats of IdB 1016 (1.5 g/kg body w.t.) the plasma levels of silybin were about 290 μg/ml while the content in the microsomes was approximately 2.5 μg/mg protein corresponding to a final concentration of silybin in the microsomal suspensions used for the experiments of about 10μM.

At this concentration the complexed silybin was found not to interfere with the function of the microsomal monoxygenase system. The levels of cytochrome P-450 and the activities of cytochrome P-450 reductase, in fact, were 0.48 $\pm$ 0.04 nmol/mg

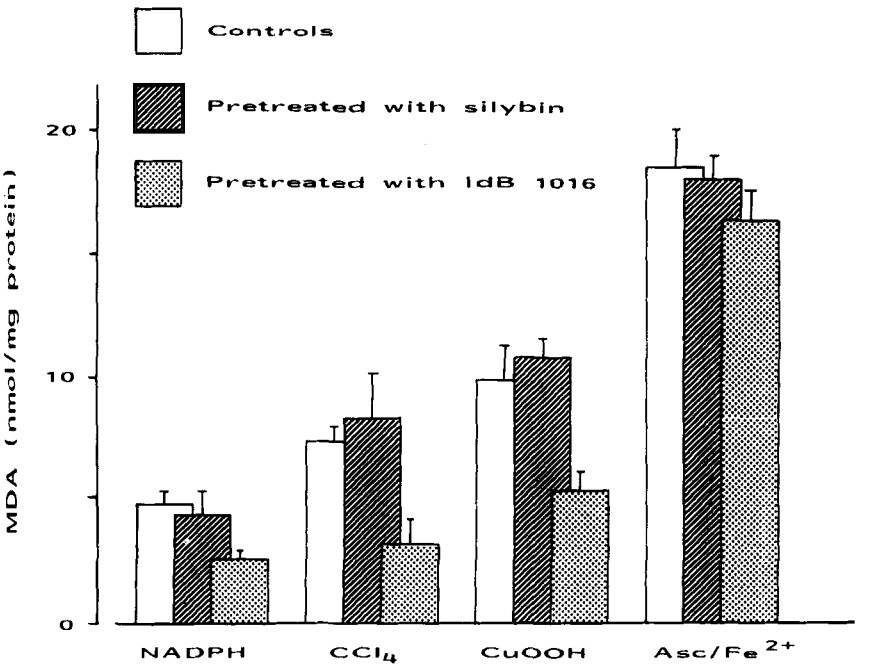

FIGURE 1 Effect of the intragastric administration of IdB 1016 (1.5 g/kg b.wt.) or of pure silybin (0.6 g/kg b.wt.) on malondialdehyde (MDA) production by rat liver microsomes treated *in vitro* with: a NADPH regenerating system (NADPH) plus or minus 8.6 mM carbon tetrachloride (CCl_4); 0.1 mM cumene hydroperoxide (CuOOH); 0.1 mM ascorbate and 2μM $FeSO_4$. The results are means ± S.E. of 6-8 different microsomal preparations obtained from single animals.

protein and 50 ± 2.8 nmol/min/mg protein, respectively, not significantly different from the values of 0.46 ± 0.04 nmol cytochrome P-450/mg protein and 54 ± 4.6 nmol cytochrome c reduced/min/mg protein found in the controls. Similarly, also the activities of aminopyrine demethylase and 7-ethoxycoumarin de-ethylase were not affected by the administration of IdB 1016 (not shown).

When lipid peroxidation was stimulated in liver microsomes by the incubation with a NADPH regenerating system and CCl_4 or by the addition 0.1 mM cumene hydroperoxide, the formation of MDA was decreased by about 40% in the microsomal fractions prepared from rats pretreated with IdB 1016 (Figure 1). A lower degree of protection against lipid peroxidation was observed in microsomes following addition of ascorbate-iron or the incubation in the presence of NADPH (Figure 1). On the other hand, no inhibition of lipid peroxidation was evident in microsomes prepared from rats receiving intragastrically 0.6 mg/kg b.wt. of pure silybin which represents the amount of flavonoid present in IdB 1016 (Figure 1). This discrepancy can be explained considering the poor enteral absorption of uncomplexed silybin, which limits its bioavailability to the liver.[7] Similarly, no interference with the formation of MDA was observed in microsomes isolated from rats pretreated with the phospholipid fraction present in IdB 1016 (not shown). It is interesting to note that the antioxidant action of IdB 1016 was more evident when peroxidative reactions were initiated within the microsomal membranes by CCl_4 and cumene hydroperoxide,

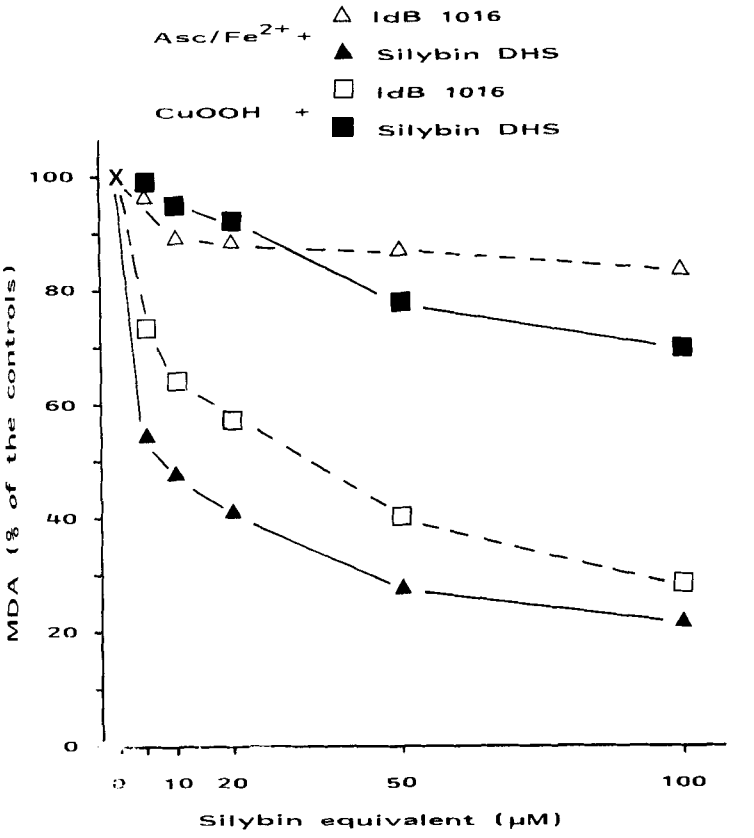

FIGURE 2 Differential inhibition of lipid peroxidation induced by 0.1 mM ascorbate and 2 μM $FeSO_4$ (Asc-Fe^{2+}) or 0.1 mM cumene hydroperoxide (CUOOH) by the addition *in vitro* to liver microsomes of equivalent concentrations of silybin as the water-soluble dihemisuccinate (Silybin DHS) form or as the liposoluble complex IdB 1016. The results are expressed as percent of the respective controls and were calculated from two experiments in duplicate.

rather than by free radicals generated in the aqueous environment, such as in the cases of NADPH or ascorbate-iron stimulated lipid peroxidation. Such a different effect could be ascribed to the preferential partitioning of the flavonoid-phospholipid complex in the lipid phase of the membranes.

Consistent with this interpretation, we observed that the direct addition to microsomal suspensions of increasing amounts of IdB 1016 and of similar concentrations of silybin in the water-soluble form of dihemisuccinate displayed different protection against the peroxidative effects of ascorbate-iron or cumene hydroperoxide. Silybin in the water-soluble form was very effective in inhibiting lipid peroxidation stimulated by ascorbate-iron, while it exerted a modest action (25% inhibition) on that induced by cumene hydroperoxide (Fig. 2). In contrast, the addition of equivalent concentrations of silybin as the lipid-soluble complex IdB 1016 had the reverse effect since at about 30 μM it inhibited MDA formation stimulated by cumene hydroperoxide by 50%, but required concentrations up to 100 μM to decrease that produced by the addition of ascorbate-iron by only 15% (Figure 2).

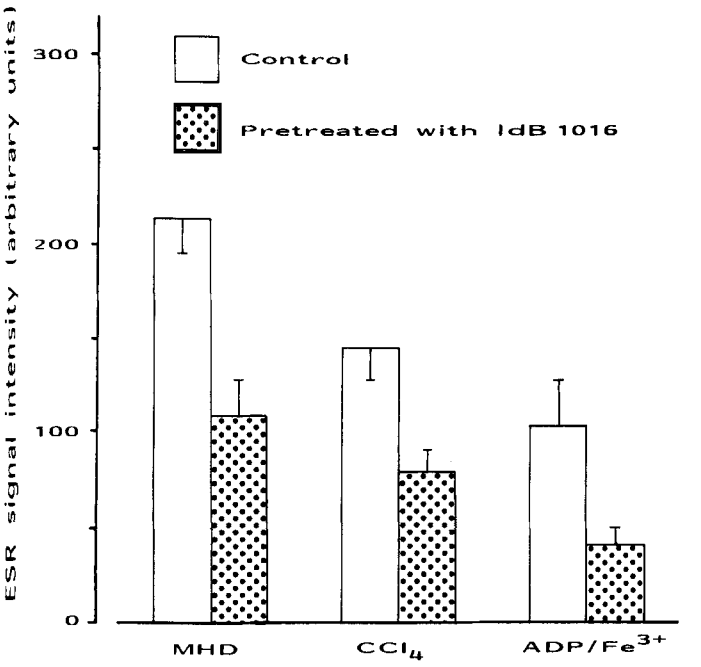

FIGURE 3 Effect of the *in vivo* administration of IdB 1016 on the spin trapping of lipodienyl radicals produced in liver microsomes by the addition of ADP/Fe^{3+} complex or on the trapping of free radical species formed during the metabolic activation of carbon tetrachloride (CCl_4) or methyl-hydrazine (MHD). The results are means ± S.E. of 6–8 determinations in microsomes obtained from different animals.

These findings are consistent with the observation of Valenzuela and Guerra[3] that silybin dihemisuccinate at 5 μM lowered ADP-Fe^{3+}-induced lipid peroxidation by 75%, while at 100μM it decreased the prooxidant effect of t-butylhydroperoxide by only 20–25%. Thus, we suggest that silybin might act mainly as a scavenger of reactive oxygen species when is preferentially located in the polar environment, whereas it displays more specific antioxidant effect when it is present in the lipid phase of the membranes.

The capacity of the silybin-phospholipid complex to interact with the radical species involved in the peroxidative process is shown by spin trapping experiments which demonstrated that IdB 1016 when incorporated in the cellular membranes acted as an effective free radical scavenger towards lipid dienyl radicals produced during the incubation of liver microsomes with ADP-$FeCl_3$ in the presence of the spin trap 4-POBN (Figure 3).

Besides the scavenging of lipid-derived radicals the oral treatment of rats with IdB 1016 was found to nearly halve the trapping of trichloromethyl ($CCl_3^{\cdot}$)[14] and methyl ($CH_3^{\cdot}$)[13] free radicals by liver microsomes exposed to, respectively, CCl_4 or methyl-hydrazine (Figure 3), indicating that the complexed form of silybin is also capable of scavenging different types of radical species generated within the microsomal membranes.

This latter effect is consistent with the observations that in chemical systems many flavonoids displayed high rate constants for the reaction with both azide radical and

superoxide anion.[15,16] Valenzuela and Guerra[17] have postulated that the protective action of silybin against the oxidative damage induced in the liver by phenylhydrazine might be due to the scavenging of phenyl radicals. Our results confirm that, when incorporated in the microsomal membranes, silybin has the capability to interact with radical species generated during the cytochrome P-450-mediated metabolism of xenobiotics, suggesting that such an effect might be of relevance in the hepatoprotective action of this compound.

In conclusion, these results demonstrate that silybin, orally given as IdB 1016, is capable of reaching intracellular sites at concentrations which makes it effective as an antioxidant without interfering with the functions of the microsomal monoxygenase system. Furthermore, the preferential location of silybin-phospholipid-complex in the membrane environment makes this flavanolignan capable of scavenging free radicals generated by the action of the cytochrome P-450-dependent monooxygenase system and this effect, along with the inhibition of lipid peroxidation, suggests that IdB 1016 may be a potentially useful protective agent against free radical- mediated toxic damage.

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