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Short Communication

SCAVENGING EFFECT OF SILIPIDE, A NEW SILYBIN-PHOSPHOLIPID COMPLEX, ON ETHANOL-DERIVED FREE RADICALS

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Abstract—Ethanol metabolism by cytochrome P4502E1 (CYP2E1) produces free radical intermediates, identified as hydroxyethyl radicals. We have observed that *in vitro* addition or *in vivo* pretreatment of rats with Silipide, a new 1:1 complex of silybin with phosphatidyl-choline, is able to decrease the spin trapping of hydroxyethyl radicals in microsomes from chronic alcohol-fed rats. This effect is not due to an interference with the metabolism of ethanol by CYP2E1, but is rather related to the capacity of the silybin molecule to scavenge hydroxyethyl radicals. However, such an effect is lost when pure silybin in amounts comparable to those present in Silipide is administered instead, due to the low bioavailability of uncomplexed flavonoid. Further experiments *in vivo* have shown that Silipide administration also decreases hydroxyethyl radical signals detectable in the bile of rats acutely treated with ethanol. The ability of Silipide to scavenge ethanol-derived radicals along with its antioxidant activity suggests that this drug might be potentially useful in counteracting free radical-mediated injuries involved in the development of liver damage caused by alcohol abuse.

Key words: silipide; free radical; ethanol; cytochrome P4502E1; silybin; spin trap

In recent years, increasing attention has been paid to the use of compounds with antioxidant or free radical scavenging properties as possible tools for the treatment of diseases consequent to oxidative injury [1, 2].

A number of studies have demonstrated that alcohol abuse leads to the development of oxidative damage, as indicated by the decrease in liver and blood content of antioxidants such as reduced glutathione or vitamin E, as well as by the detection of lipid peroxidation products in patients with alcoholic cirrhosis [3, 4]. The possible involvement of free radical-mediated oxidative damage in the pathogenesis of alcoholic liver disease is suggested by the observation that in rats fed intragastrically with ethanol, the development of hepatic injury is associated with an increase in lipid peroxidation [5, 6]. Furthermore, the oxidation of ethanol by CYP2E1¶ results in the formation of hydroxyethyl free radicals [7, 8], which are able to bind to liver proteins [9], leading to the development of immunological reactions in alcoholic patients [10].

Silymarin, a standardized extract of the milk thistle Silybum Marianum, is known to have antihepatotoxic effects [11], and *in vitro* studies have suggested that its hepatoprotective effects could be related to its antioxidant action [12]. Silymarin has been shown to prevent ethanol-induced lipid peroxidation [13] and, in a double-blind randomized controlled trial performed in 170 patients, has been shown to significantly reduce mortality by alcoholic cirrhosis [14].

Silybin, the main constituent of silymarin, also exhibits hepatoprotective activities connected with antioxidant properties [15]. However, the possible application of this flavonoid in therapy is hampered by its poor enteral absorption [16]. In the present study we have investigated whether silybin might be able to interact with hydroxyethyl radicals originating during ethanol oxidation either *in vitro* in hepatic microsomes or in the whole liver *in vivo*. For this purpose we have used a new 1:1 silybin complex with phosphatidylcholine (Silipide) that, unlike silybin, undergoes enteral absorbtion following oral administration to either rats or humans [16, 17]. The interaction of silybin with hydroxyethyl free radicals was studied by using ESR spectroscopy coupled with spin trapping technique.

Materials and Methods

Chemicals. $NADP^+$, glucose-6-phosphate dehydrogenase, glucose-6-phosphate, semicarbazide, and aniline sulphate were obtained from Sigma Chemical Co. (St. Louis, MO). The spin trap 4-POBN was purchased from Aldrich Europe (Bersee, Belgium). Silipide, silybin, and phosphatidylcholine were kindly supplied by Indena S.p.A. (Milano, Italy).

Animals treatment and microsome preparation. Male Sprague-Dawley rats (200–250 g) were obtained by Nossan (Corezzana, Italy) and fed a liquid diet containing ethanol as 36% of the total calory intake for two weeks, according to DeCarli and Lieber [18]. Silipide was suspended in water and administered intragastrically to the rats at a dose of 1.5 g/kg b wt one hour before killing. Silibin or phosphatidylcholine in amounts equivalent to those present in Silipide were administered by the same route. Liver microsomes were prepared from alcohol-fed rats as previously described and resuspended in 10 mM Na/K phosphate buffer pH 7.4 [19]. In some experiments, silipide was

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¶Abbreviations: CYP2E1, cytochrome P4502E1; DMSO, dimethylsulphoxide; 4-POBN, 4-pyridyl-1-oxide-t-butyl nitrone; and ESR, electron spin resonance spectroscopy.

added directly to microsomal suspensions as a solution in dimethylsulfoxide. For spin trapping experiments, microsomal suspensions (5 mg protein/mL) were incubated for 30 min at 37°C with an NADPH-regenerating system, 25 mM 4-POBN, and 20 mM ethanol as previously reported [7].

Biochemical analysis. Aniline hydroxylase activity was measured in the microsomes of rats pretreated or not with Silipide, as previously reported [19]. Acetaldehyde formation was determined spectrophotometrically at 224 nm as semicarbazide complex [18] using the same microsomal incubation employed for spin trapping experiments, except that 4-POBN was omitted.

Spin trapping experiments in vivo. Spin trapping of ethanol radicals *in vivo* was performed using ethanol-fed rats. Animals pretreated or not with Silipide were injected with a single dose of ethanol (2 g/kg b wt) i.p., and after 10 minutes received i.p. 4-POBN (100 mg/kg). Thirty minutes later, rats were anesthetized with thiopental, and the bile duct cannulated. The bile was collected for 1.5 hours (20) and extracted with chloroform and methanol for ESR analysis.

ESR analysis. ESR spectra were recorded at room temperature using a Brucker D 200SRC spectrometer with the following instrument setting: microwave power 10 mW; modulation frequency 100 KHz; modulation amplitude 1G; and field scan 100 G.

Results and Discussion

In a previous study we showed that one hour after the intragastric administration of Silipide to rats, the content of silybin in the microsomes, as measured by a specific HPLC assay, was approximately 2.5 μg/mg protein, corresponding to a final concentration of approximately 10 μM silybin in the microsomal suspensions used for the experiments [19]. Spin trapping experiments performed with liver microsomes from alcohol-fed rats showed that the intensity of the ESR signals due to the trapping of hydroxyethyl free radicals was decreased by approximately 50% in microsomes prepared from rats receiving Silipide (Fig. 1). No effect on hydroxyethyl free radical trapping was observed in liver microsomes prepared from rats receiving phosphatidylcholine alone (not shown). A dose-dependent decrease in the ESR signals due to hydroxyethyl free radicals adducts of 4-POBN was also evident when increasing concentrations of silipide were added *in vitro* to liver microsomes incubated with ethanol (Fig. 2). However, liver microsomes prepared from rats treated *in vivo* with uncomplexed silybin in amounts comparable to that present in Silipide did not show any effect on the trapping of hydroxyethyl free radicals (Fig. 1) because of the low bioavailability of the uncomplexed flavonoid [16, 17]. Indeed, in those microsomal preparations, negligible amounts of silybin were detected (not shown).

The effect of Silipide on the spin trapping of hydroxyethyl radicals cannot be ascribed to interferences with the functions of the microsomal monoxygenase system, since in microsomes from rats pretreated with Silipide, aniline hydroxylase activity was not significantly different from that of untreated animals (2.52 ± 0.23 vs 2.31 ± 0.13 nmol/min/mg protein). Furthermore, the measurement of acetaldehyde production by liver microsomes incubated with ethanol and NADPH showed that Silipide did not affect ethanol metabolism by CYP2E1 (Fig. 1).

Knecht and coworkers [20] have recently reported that the formation of hydroxyethyl free radicals can be detected in the bile of deer mice receiving *in vivo* ethanol along with the spin-trapping agent 4-POBN. These findings prompted us to investigate whether the scavenging effect of Silipide toward hydroxyethyl free radical might be detectable *in vivo* in the whole animal. Figure 3 shows that ESR signals with spectral features compatible with those of hydroxyethyl-4-POBN adduct were detectable in the bile of ethanol-fed rats 1 hour after the intraperitoneal administration of ethanol and 4-POBN. Conversely, no ESR signal was evident in the bile of animals not receiving acute alcohol intoxication. The pretreatment of rats with Silipide one hour before ethanol administration effectively reduced detection in the bile of ESR signals due to hydroxyethyl radicals (Fig. 2).

In previous studies we reported that a single oral administration of Silipide to rats prevented lipid peroxidation in liver microsomes [19] and protected isolated hepatocytes from the toxic effects of several compounds acting through oxidative mechanisms [21]. The present results demonstrate that the supplementation of liver microsomes from alcohol-fed rats with silybin by pretreating the animals with Silipide decreased the intensity of hydroxyethyl free radical signals detectable by ESR

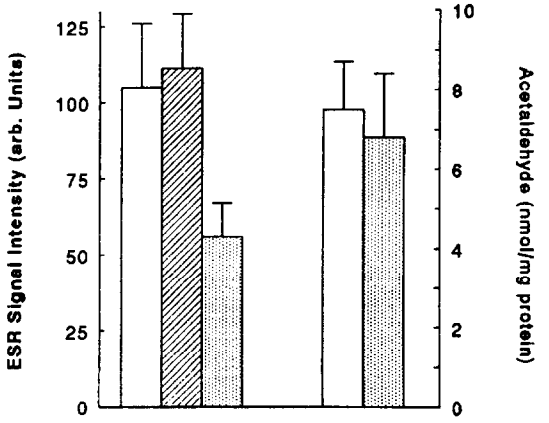

Fig. 1. Effects of silybin on the trapping of hydroxyethyl free radicals and acetaldehyde formation by liver microsomes from alcohol-fed rats incubated *in vitro* with ethanol. Liver microsomes were prepared from untreated rats (open bars) or animals pretreated with Silipide (1.5 g/kg b wt) (dotted bars) or silybin (0.6 g/kg b wt) (hatched bar) one hour before killing. The microsomes were incubated for 30 min at 37°C with an NADPH regenerating system in the presence of 20 mM ethanol. For spin trapping experiments, 20 mM 4-POBN was added to the incubation mixture. The results are means of 3 different experiments ± SD.

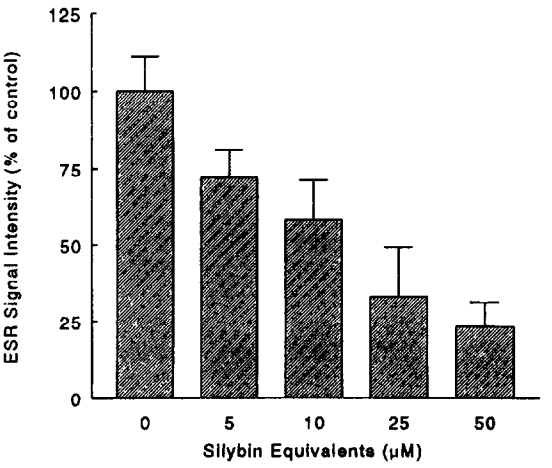

Fig. 2. Effect of the *in vitro* addition of increasing concentrations of Silipide on the intensity of the ESR spectra due to 4-POBN hydroxyethyl radical adducts in rat liver microsomes. Liver microsomes from ethanol-fed rats were preincubated for 5 min at 37° with different amounts of Silipide dissolved in 2 μL/mL of DMSO to obtain the concentrations of silybin reported. Controls received DMSO alone, Microsomal suspensions were then incubated for 30 min at 37°C with an NADPH regenerating system, 20 mM ethanol and 20 mM 4-POBN. The results are expressed as percent of the control values and means of 3 different experiments ± SD.

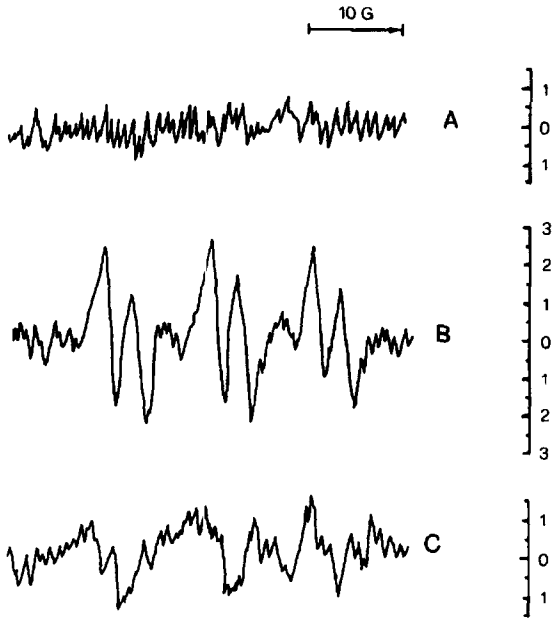

Fig. 3. Effect of Silipide administration on the ESR spectra of 4-POBN hydroxyethyl radical adducts detected in the bile of rats acutely treated with ethanol. Ethanol-fed rats were injected i.p. with 4-POBN (100 mg/kg) (Trace A) or with 4-POBN and ethanol (2 g/Kg b wt) (Trace B). Trace C refers to animals pretreated with Silipide (1.5 g/kg b wt) one hour before the administration of ethanol. Amplification gain for all traces was 10^6. The spectral features of the nitroxide adduct detected in the bile of alcohol-treated rats were: a_N = 14.90 G; a_H = 3.45 G.

spetroscopy both *in vitro* in liver microsomes and *in vivo* in the bile of rats exposed to an acute dose of ethanol. Valenzuela *et al.* [22] reported that silybin dihemisuccinate interferes with ethanol oxidation in liver microsomes, and that concentrations as low as 20 μM reduced acetaldehyde formation by 30% [22]. In our hand, silybin administered as phospholipid complex did not show any effect on ethanol oxidation. It has been suggested that superoxide anion and hydroxyl radicals might contribute to the formation of hydroxyethyl free radicals by liver microsomes through a non-enzymatic reaction involving iron [23]. However, a recent report demonstrates that silybin is a poor scavenger of superoxide anion (IC_{50} = 2.8 mM) and a weak iron chelator [24]. The same authors have also shown that although silybin can react rapidly with hydroxyl radicals (rate constant = 1.0–1.2 × 10^{10}/M/sec), concentrations in millimolar range are required to effectively compete with other targets of hydroxy radicals [24], such as alcohols. Thus, it is likely that the lowering by Silipide of the ESR signals due to hydroxyethyl radical-4-POBN adducts can be ascribed to the direct scavenging by the silybin molecule of hydroxyethyl radicals produced at the level of CYP2E1. We consistently observed that in liver microsomes from Silipide-pretreated rats, silybin reduced the spin trapping of trichloromethyl and methyl radicals from carbon tetracloride and methyl hydrazine, respectively [19], two compounds also metabolized by CYP2E1 [25]. In this respect, it is possible that the distribution of silybin-phospholipid complex in the lipid environment of the membranes might favor the scavenging activity of silybin toward free radicals generated by cytochrome P450.

We recently reported that hydroxyethyl free radicals covalently bind to microsomal proteins [9], and that these adducts are immunogenic [26]. Furthermore, both IgGs and IgAs directed specifically against proteins modified by hydroxyethyl radicals are detectable in the serum of patients with alcoholic cirrhosis [10]. Thus, the scavenging action of silipide toward hydroxyethyl radicals might prevent the formation of immunogenic epitopes, which could be involved in the development of autoimmune reactions [27].

In conclusion, the free radical scavenging properties of Silipide along with its antioxidant action [19, 21] makes this drug potentially useful in counteracting alcohol damage mediated by free radical mechanisms.

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