S-Adenosylmethionine Protects Against Cyclosporin A-Induced Alterations in Rat Liver Plasma Membrane Fluidity and Functions

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ABSTRACT

We studied the effect of cyclosporin A (CyA) on liver plasma membrane (LPM) composition, fluidity, and functions and on hepatic glutathione (GS) and oxidative status. We also evaluated the ability of S-adenosylmethionine (SAMe) to antagonize the CyA-induced disturbances in rats. The animals were randomly divided into four groups and treated daily with saline, CyA vehicle, CyA, and SAMe plus CyA, respectively, for 1 week. Bile, blood, and liver samples and LPM vesicles were obtained at the end of the treatments. CyA-induced cholestasis was associated with alterations in LPM composition and fluidity. The contents of total phospholipids, phosphatidylcholine, and proteins were decreased and cholesterol and the cholesterol/phospholipid molar ratio increased. Na⁺,K⁺-ATPase activity was decreased, whereas those of 5'-nucleotidase, Mg²⁺-ATPase, and γ-glutamyltransferase increased. The hepatic contents of proteins and GS and the reduced/oxidized glutathione molar ratio were decreased and hepatic malondialdehyde increased. SAMe cotreatment 1) significantly improved or abolished the CyA-induced changes in LPM fluidity and composition and the changes in the activity of the carrier and enzymes tested, 2) counteracted the hepatic depletion of GS and proteins caused by CyA and normalized the reduced/oxidized glutathione ratio, and, as expected, 3) prevented cholestasis and the inhibitory effect of CyA on hepatobiliobiliary transport of the major bile components. We conclude that CyA-induced cholestasis and hepatotoxicity in the rat is associated with changes in LPM composition and fluidity, liver GS depletion, and oxidative stress. SAMe cotreatment significantly improves or totally protects against these hepatotoxic effects.

Bile formation and the hepatobiliary transport of the biliary components and xenobiotics eliminated into bile require the coordinated action of many sinusoidal and canalicular hepatocyte plasma membrane-embedded proteins responsible for transport and enzymatic processes or serving as channels or receptors (Oude Elferink et al., 1995). Alterations in liver plasma membrane (LPM) composition and fluidity can influence carrier-mediated transport processes and membrane-bound enzyme activities, thus causing cholestasis (Smith and Gordon, 1987; Muriel and Mourelle, 1992; Bossard et al., 1993; Simon, 1993).

Cyclosporin A (CyA), a widely used immunosuppressor drug, induces intrahepatic cholestasis and hepatotoxicity in humans and rats and reduces the capacity of the liver to excrete endo- and xenobiotics into bile (Galán et al., 1992; Faulds et al., 1993; Chan et al., 1998; Morán et al., 1998).

Inhibition of the sinusoidal bile acid (BA) transporters (Moseley et al., 1990; Böhme et al., 1994) and of the canalicular multispecific organic anion transporter (Böhme et al., 1994), the ATP-dependent multidrug export carrier, and of the canalicular transporters for BA (Moseley et al., 1990; Böhme et al., 1994) and glutathione (GS) (Böhme et al., 1994; Morán et al., 1998) has been shown to be the main causative mechanism of CyA-associated cholestasis. The underlying mechanisms of interaction between CyA and these carriers have not been definitively established. Competitive and noncompetitive inhibition of the sinusoidal uptake of BA on other compounds has been observed (Zimmerli et al., 1989; Moseley et al., 1990), and specific (Ziegler and Frimmer, 1986; Moseley et al., 1990) and nonspecific (Zimmerli et al., 1989; Schramm et al., 1993) interactions have been suggested to account for this. A direct interaction with the BA uptake system is supported by photoaffinity labeling studies (Ziegler and Frimmer, 1986), although more recent studies have demonstrated indirect interactions between CyA and LPM (Schramm et al., 1993). Additionally, the activation energy for transport in the
presence of CyA is unchanged for taurocholate but decreases for ouabain, indicating that CyA does not cause changes in membrane fluidity when taurocholate is used as substrate, whereas the data for ouabain are consistent with increased membrane fluidity (Kukongviriyapan and Stacey, 1991).

CyA, a highly lipophilic molecule that is extensively cleared and metabolized and is eliminated by the biliary into the bile, binds to membrane lipids (Galán et al., 1992; Faulds et al., 1993), inhibits the hepatic synthesis of proteins (Bäckman et al., 1988) and phospholipids (PHOs) (Bäckman et al., 1986), and blocks their vesicle-mediated transhepatocytic transport (Román et al., 1990). It also induces liperoxidation in rat and human liver (Barth et al., 1991; Wolf et al., 1997) and depletes hepatic GS (Morín et al., 1998). Accordingly, it is possible that CyA might alter the recycling and repair of LPMs, thus affecting LPM fluidity, membrane transport processes, and hepatobiological functions. To our knowledge, no studies examining the potential effect of CyA on both LPM composition and fluidity and membrane carrier/enzyme activity have been performed, with the exception of a preliminary report by Whittington et al. (1988) indicating that CyA alters LPM fluidity.

Our study, carried out in short-term CyA-treated rats, aimed at investigating the possibility that CyA-induced changes in LPM composition, fluidity, and the activities of several key membrane carriers and enzymes, might be involved in the cholestatic and hepatotoxic effects of the drug. We also evaluated the possibility of antagonizing these adverse effects by simultaneous administration of CyA and S-adenosyl-L-methionine (SAME). Cotreatment with SAME was chosen because we (Fernández et al., 1995; Jiménez et al., 1996) have recently observed that exogenous SAME is able to antagonize CyA-induced cholestasis in rats. This is in agreement with many studies (for reviews, see Friedel et al., 1989; Mato et al., 1994, 1997; Lu, 1998) reporting the efficacy of SAME in preventing and reversing the cholestasis and hepatotoxicity associated with several drugs and chemical compounds, either by modulating LPM fluidity or by maintaining the hepatic pool of GS, and hence improving the detoxifying capacity of the liver cells and protecting them against oxidative stress.

Materials and Methods

Chemicals. CyA, in powder form, was a gift from Sandoz A.G. (Basel, Switzerland). SAME was kindly provided by Europharma S.A. (Madrid, Spain). 1,6-Diphenyl-1,3,5-hexatriene (DPH), olive oil, bilirubin, 3α-hydroxy steroid dehydrogenase, BA, reduced glutathione (GSH), glutathione reductase, NADPH, 5,5'-dithio-bis(2-nitrobenzoic acid), and the different kits and reagents for determination of the enzymatic activities and substrate concentrations evaluated in plasma, bile, liver homogenates, and plasma membrane fractions were purchased from Sigma-Aldrich-Quimica (Madrid, Spain) and Boehringer Mannheim GmbH (Mannheim, Germany). All other reagents were of the highest quality available commercially.

Animals and Experimental Procedures. Forty-eight male Wistar rats weighing 240 to 260 g were used. The animals were treated humanely, and the study protocols were in compliance with our institution’s guidelines for the use of laboratory animals. Before the experiments, the animals were adapted to a 12:12 h light/dark cycle and kept in a temperature- and humidity-controlled room. Animals were provided with food (rodent cubes, Purina A03; Panlab, Barcelona, Spain) and water ad libitum. Rats were randomly divided into four groups of 12 rats each kept in separate cages and were treated for 1 week as follows: One group was treated with physiological saline, another group was given the CyA vehicle (olive oil), and the experimental groups were treated with either CyA or CyA plus SAME. CyA (10 mg/kg b.wt., once daily) and its vehicle were administered intraperitoneally, and SAME (10 mg/kg b.wt., twice per day) was administered s.c. The rats were weighed daily and were not starved before experiments. The volumes of solutions administered daily to the rats ranged between 0.20 and 0.27 ml, according to the animals’ body weight.

On the day of experiments, six animals from each group were anesthetized with pentobarbital sodium (50 mg/kg b.wt. i.p.) 12 h after the last dose. A median laparotomy and a routine tracheotomy were performed. The bile duct and left femoral artery were catheterized for bile and blood sampling. Losses in body temperature were prevented by a thermostatically controlled warming plate, and rectal temperature was maintained at 37°C. After an equilibration period of 25 to 30 min to allow bile flow to stabilize, a blood sample was taken for plasma biochemical assays. Then, bile was collected into preweighed tubes on melting ice over two 15-min periods. Mean bile flow was estimated gravimetrically, assuming a bile density of 1.0. At the end of the experiments, the rats were sacrificed by exsanguination. Livers were quickly removed, weighed, and washed with an ice-cold isotonic saline solution; small pieces weighing 0.5 g were harvested from the liver for biochemical determinations. Plasma, bile, and liver samples were stored at −80°C until required for analysis. To avoid variations due to circadian rhythms, all experiments were started at the same time of day.

The six remaining animals from each group were stunned and decapitated, and their livers were immediately removed and weighed. Liver homogenates and LPM vesicles were prepared immediately; thereafter, following the method of Van Ameloot et al. (1978), LPM vesicles were rapidly frozen in liquid nitrogen and stored until use within 4 weeks.

Plasma, Hepatic, and Biliary Parameters Studied. The effects of CyA and SAME on several selected indicators of cholestasis and hepatotoxicity were estimated by the determination in plasma of total bilirubin and BA levels and γ-glutamyltransferase (γ-GT), alkaline phosphatase (ALP), aspartate aminotransferase, and alanine aminotransferase activity. Bile flow, biliary concentrations, and the secretion of BA, cholesterol (CHO), and total PHOs, together with the biliary activity and excretion rates of γ-GT, were also assessed. In addition, total proteins in plasma and liver and the hepatic content of GSH, oxidized glutathione (GSSG), total GS (GSH + GSSG), and malondialdehyde (MDA) were determined.

Total bilirubin concentrations and γ-GT, ALP, aspartate aminotransferase, and alanine aminotransferase activities in plasma were measured by optimized methods routinely used at our laboratory on an automated analyzer (Hitachi model 717; Noka Works, Tokyo, Japan). γ-GT activity in bile was determined as reported previously (Galán et al., 1992). Total BA concentrations in plasma and bile and total CHO and PHO concentrations in bile were determined enzymatically via commercial kits (Galán et al., 1992). Total protein concentrations in plasma and liver homogenates were assayed by the method of Lowry et al. (1951). GSH and GSSG in liver homogenates were determined enzymatically according to Griffith (1980), and total GS and the GSH/GSSG molar ratio were calculated. MDA was determined in liver homogenates according to Ohkawa et al. (1979).

Measurement of LPM Fluidity and Composition. The fluidity of LPM preparations was determined by measuring steady-state fluorescence polarization of the lipid probe DPH incorporated into the hydrophobic core of LPM, following the method of Shinitzky and Barenholz (1978), with an SLM-Amino spectrofluorimeter (model LH-700; SLM Instruments, Urbana, IL) equipped with polarizers in both the excitation and emission beams. The fluorescence intensity of DPH-labeled LPM were measured perpendicularly (I_p) and parallel (I_p) to the polarization phase of the exciting light. Steady-state fluorescence polarization (P) was calculated with Perrin’s equation: 

\[ P = \frac{I_p - G \cdot I_p}{I_p + G \cdot I_p}, \]

where G is the correction factor I/I_p.
The composition of LPM aliquots and enzyme activities were also evaluated. Protein determinations were performed with the method of Lowry et al. (1951). The activities of three LPM marker enzymes (i.e., Na$^+$,K$^+$-ATPase, 5'-nucleotidase, and γ-GT) and the degree of purity of LPM were evaluated with previously reported methods (Torres et al., 1994; Fernández et al., 1995). Total lipids were extracted from membrane vesicles by the method of Folch et al. (1957), and total CHO and PHO contents were evaluated in each extract with previously reported methods (Galán et al., 1992). The two main PHO species, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were analyzed by thin-layer chromatography with silica gel chromatoplates and the following developing solvent mixture: chloroform/ethanol/acetic acid/water (170:40:18:10, v/v/v/v). Plates were dried under nitrogen, sprayed with 50% sulfuric acid, and charred. Separated PHO bands were then scraped off and relative phosphorous contents determined (Muriel and Mourelle, 1992). Each PHO species was identified by comparison with the R$_s$ of pure standards chromatographed in parallel.

Statistics. Results are expressed as means ± S.E. for all data. Data were compared by the Kruskal-Wallis test; when the analysis indicated significant differences among groups, means were compared via the nonparametric Mann-Whitney U test. A P value of .05 or less was considered significant.

Results

Studies of Hepatobiliary Function in Anesthetized Rats. The mean values of the different parameters evaluated in rats treated with physiological saline over 1 week (data not shown) and those obtained in rats treated with CyA vehicle were similar, and no significant differences were observed in any case. Thus, the rats treated with CyA vehicle were considered controls for the rest of the groups.

As expected, in comparison to the controls, the CyA-treated rats developed cholestasis and bile flow, and the biliary concentrations (data not shown) and excretion rates of BA, CHO, and PHO were significantly decreased. The biliary excretion rates of γ-GT was also significantly reduced (Table 1). In contrast, CyA treatment markedly increased plasma bilirubin (0.22 ± 0.03 mg/dl) and BA concentrations (9.11 ± 1.38 mg/dl) with respect to the control values (0.07 ± 0.01 and 0.76 ± 0.12 mg/dl, respectively). CyA treatment reduced total protein concentrations in plasma (3.93 ± 0.19 versus 5.81 ± 0.26 g/dl), but the activities of transaminases and the other hepatic indicator enzymes evaluated remained unchanged with respect to the controls, indicating that the cholestatic effect developed with a moderate degree of hepatotoxicity in this species, in agreement with previous studies (data not shown) (Fernández et al., 1995; Galán et al., 1995). When CyA and SAMe were administered simultaneously, the adverse effects of the immunosuppressor agent on biliary function were significantly attenuated or totally abolished; the biliary concentration and excretion rates of BA, CHO, and PHO fully returning to control values; and bile flow and the biliary excretion of γ-GT being significantly improved (Table 1). In addition, CyA-induced changes in the plasma levels of bilirubin, BA, and proteins were totally or partially abolished after cotreatment with SAMe (0.11 ± 0.01, 2.05 ± 0.71, and 5.39 ± 0.33 mg/dl, respectively).

Regarding the changes in liver GS and MDA, we observed that treatment with CyA significantly depleted the hepatic pool of total GS and GSH and increased the relative amount of GSSG with respect to the values found in the controls. These effects led to significant decreases in the GSH/GSSG ratio and were accompanied by increases in MDA concentrations (Table 2), which are usually considered indicators of increased production of reactive oxygen species and accelerated lipid peroxidation (Aruoma, 1998). Also, the amount of total proteins in liver was decreased (data not shown). Co-treatment with SAMe counteracted all the above effects, and the livers were found to have significantly higher protein, total GS, and GSH concentrations than livers of the rats treated with CyA alone; despite this, total GS and GSH values still remained slightly lower than those observed in the controls. SAMe cotreatment also inhibited excess free radical formation, because the hepatic levels of MDA and the calculated GSH/GSSG ratio were totally restored to control values (Table 2).

Studies on LPM Composition and Fluidity. As previously stated, many studies have shown that changes in hepatocyte plasma membrane composition alter membrane fluidity and can influence several important hepatobiliary transport and enzymatic processes. Therefore, these assays in LPM were restricted to measuring their contents in total CHO and PHOs and proteins, the relative amounts of PC and PE, and fluorescence polarization (which is inversely related to membrane fluidity). The functional significance of the changes in LPM fluidity was explored by comparing the changes in the activity of four selected membrane-bound enzymatic proteins: a basolateral membrane-transport enzyme (Na$^+$,K$^+$-ATPase) and three canalicular membrane enzymes (i.e., γ-GT, Mg$^{2+}$-ATPase, and 5'-nucleotidase), which have no known transport functions. Enrichment of the enzymes tested in LPM fractions was within the previously reported range (Torres et al., 1994), indicating that, under the conditions used, treatment with olive oil, CyA, or SAMe plus CyA did not significantly alter the purity of isolated LPMs (data not shown).

After CyA treatment, the CHO content and the CHO/PHO molar ratio in LPM increased, and these effects were accompanied by a significant decrease in membrane fluidity be-

### Table 1

<table>
<thead>
<tr>
<th>Effect</th>
<th>Controls</th>
<th>CyA</th>
<th>SAMe + CyA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow, µl·min$^{-1}$·g$^{-1}$ liver</td>
<td>1.82 ± 0.09</td>
<td>1.19 ± 0.07$^a$</td>
<td>1.62 ± 0.08$^{a,b}$</td>
</tr>
<tr>
<td>Bile acids, nmol·min$^{-1}$·g$^{-1}$ liver</td>
<td>45.5 ± 2.6</td>
<td>24.6 ± 2.5$^b$</td>
<td>43.1 ± 3.0$^b$</td>
</tr>
<tr>
<td>CHO, nmol·min$^{-1}$·g$^{-1}$ liver</td>
<td>0.82 ± 0.09</td>
<td>0.48 ± 0.11$^c$</td>
<td>0.79 ± 0.04$^c$</td>
</tr>
<tr>
<td>Phospholipids, nmol·min$^{-1}$·g$^{-1}$ liver</td>
<td>5.77 ± 0.37</td>
<td>3.26 ± 0.18$^c$</td>
<td>5.82 ± 0.41$^d$</td>
</tr>
<tr>
<td>γ-GT, µIU·min$^{-1}$·g$^{-1}$ liver</td>
<td>346 ± 44</td>
<td>32 ± 10$^c$</td>
<td>207 ± 26$^{c,d}$</td>
</tr>
</tbody>
</table>

Data are means ± S.E. for six rats in each group.

$^{a,b}$ Means different from controls and CyA groups, respectively (P < .05).
cause, with DPH as sensor, fluorescence polarization was significantly enhanced (Fig. 1). Total protein and PHO contents together with the relative concentrations of PHO subclasses were also modified by CyA (Fig. 2). Thus, decreases in protein, PHO, and PC contents and increases in PE content were observed at the end of CyA treatment. These elicited significant decreases in the PC/PE (Fig. 2) and PHO/protein (data not shown) ratios. When the rats were concurrently treated with CyA and SAMe, all the CyA-induced changes in LPM composition and fluidity were significantly palliated (which was the case in LPM fluidity, CHO content, and CHO/PHO ratio) or totally prevented (total protein, PHO, PC, and PE contents and the PHO/protein and PC/PE ratios) (Figs. 1 and 2).

Regarding the activity of the enzymes tested in LPM, we found that, whereas Na\(^{+},K^{+}\)-ATPase activity underwent a 2.3-fold decrease after CyA treatment (4.18 ± 0.55 versus 9.66 ± 0.82 μmol P\(_{1}\)·mg\(^{-1}\) protein·h\(^{-1}\) in the CyA-vehicle treated rats), Mg\(^{2+}\)-ATPase and 5′-nucleotidase activities were increased. The changes in Na\(^{+},K^{+}\)-ATPase and 5′-nucleotidase activity were totally prevented when the rats were cotreated with SAMe plus CyA, and those observed in Mg\(^{2+}\)-ATPase activity were partially abolished (Fig. 3, top). A CyA-induced increase and a SAMe-dependent normalization of γ-GT activity were also found in LPM and liver homogenates (Fig. 3, bottom). Moreover, when the activity of the latter enzyme was evaluated in bile, the CyA-induced decreases were partially antagonized after SAMe cotreatment (Fig. 3, bottom).

In summary, our data show that CyA hepatotoxicity is
associated with changes in LPM composition and fluidity. In addition, the drug alters the activity of the four enzymes tested in LPM, induces oxidative stress, and depletes hepatic GS and proteins. In contrast, cotreatment with SAMe 1) prevents or totally abolishes the changes in LPM composition, fluidity, and activity of the enzymes and carrier evaluated; 2) counteracts the oxidative stress and depletion of GS and proteins; and 3) in accordance with our previous findings (Fernández et al., 1995; Jiménez et al., 1996), prevents or partially antagonizes the inhibitory effect of CyA on bile flow and the hepatobiliary transport of BA, CHO, PHO, proteins, and bilirubin.

Discussion

Among the many factors involved in bile formation and hepatobiliary transport processes, LPM fluidity has been assigned a major role. CHO and PHO contents are the major determinants of plasma membrane fluidity, and high CHO levels and/or low PHO contents lead to increased CHO/PHO ratio and decreased fluidity (Boelsterli et al., 1983; Rosario et al., 1988; Simon, 1993), thus altering membrane functions (Smith and Gordon, 1987; Rosario et al., 1988; Thalhammer et al., 1993). Although our study did not allow us to unequivocally identify the cellular mechanisms through which CyA treatment alters LPM composition, fluidity, and functions (not those through that SAMe cotreatment antagonizes these disturbances), several hypotheses could be proposed.

First, we observed that CyA treatment reduces PHO content, the PC/PE ratio, and fluidity and that SAMe cotreatment antagonizes these effects. Accordingly, it is possible that changes in PHO synthesis or methylation flux of PC from PE could be involved, because membrane PHO content influences membrane fluidity and protein functions (Boelsterli et al., 1983; Mato, 1986; Rosario et al., 1988; Simon, 1993). It has been reported that CyA inhibits PHO synthesis in rat liver (Bäckman et al., 1986) and that inhibition of PHO synthesis and methylation reactions is associated with PHO depletion in LPM (Chagoya De Sánchez et al., 1991), increased CHO/PHO molar ratio, and decreased rat LPM fluidity (Mato, 1986; Simon, 1993). Also, SAMe is a limiting factor in the methylation of membrane PHO, mainly in regard to the conversion of PE to PC (Mato, 1986; Chagoya De Sánchez et al., 1991; Mato et al., 1994; Bontemps and Van Den Berghe, 1998), and hepatic SAMe depletion impairs transmethylation reactions, alters LPM fluidity, reduces Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, and exacerbates the liver membrane injury associated with ethanol (Feo et al., 1986; Pascale et al., 1989), CCl\textsubscript{4} (Corrales et al., 1992), or ethynylestradiol (Boelsterli et al., 1983; Fricker et al., 1988). In contrast, when exogenous SAMe is administered, it is efficiently cleared and metabolized by the liver, and its methyl groups are incorporated into membrane PHO (Hirata and Axerold, 1980; Bontemps and Van Den Berghe, 1998). A SAMe-dependent normalization of the PC content of LPM and transmethylation reactions in the liver has been proposed as one of the major mechanisms through which SAMe exerts its anticholestatic and hepatoprotective effects against several drugs and chemical compounds. It has been reported that SAMe cotreatment with ethanol (Pascale et al., 1989; Alvaro et al., 1995), ethynylestradiol (Boelsterli et al., 1983; Fricker et al., 1988), or chlorpromazine (Friedel et al., 1989) normalizes PC content, the PC/PE ratio, and LPM fluidity in rat liver, which in turn normalizes the Na\textsuperscript{+} pump and Na\textsuperscript{+}/H\textsuperscript{+} antiport activities in rats intoxicated with these drugs (Hirata and Axerold, 1980; Smith and Gordon, 1987; Fricker et al., 1988).

Second, our data on liver GSH, GSSG, and MDA suggest that changes in GS homeostasis, free radical formation, and lipid peroxidation might also be involved in the CyA- and SAMe-induced effects on LPM, because GS protects against free radicals, and reductions in the liver GSH/GSSG ratio and/or increases in the formation of GSSG and MDA are indicators of free radical formation and accelerated lipid peroxidation (Kaplowitz and Tsukamoto, 1996; Aruoma, 1998). This hypothesis is supported by the facts that 1) CyA, in addition to reducing hepatic GS in the rat (Morán et al., 1998), increases H\textsubscript{2}O\textsubscript{2} formation in cultured rat hepatocytes (Wolf et al., 1997) and induces excess free radical formation and lipid peroxidation in rat liver in both in vitro (Barth et al., 1991; Deters et al., 1997; Wolf et al., 1997) and in vivo (Morán et al., 1996) models; 2) excess free radical formation leads to oxidation of the fatty acyl chains of membrane PHO, which are especially susceptible, and leads to oxidation of sulphydryl protein groups (Simon, 1993; Sundari and Ramakrishna, 1997; Aruoma, 1998; Vendemiale et al., 1998), and both effects are known to alter LPM composition and fluidity, and the PC/PE ratio, and decreased rat LPM fluidity (Mato, 1986; Simon, 1993). Also, SAMe is a limiting factor in the methylation of membrane PHO, mainly in regard to the conversion of PE to PC (Mato, 1986; Chagoya De Sánchez et al., 1991; Mato et al., 1994; Bontemps and Van Den Berghe, 1998), and hepatic SAMe depletion impairs transmethylation reactions, alters LPM fluidity, reduces Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity, and exacerbates the liver membrane injury associated with ethanol (Feo et al., 1986; Pascale et al., 1989), CCl\textsubscript{4} (Corrales et al., 1992), or ethynylestradiol (Boelsterli et al., 1983; Fricker et al., 1988), or chlorpromazine (Friedel et al., 1989) normalizes PC content, the PC/PE ratio, and LPM fluidity in rat liver, which in turn normalizes the Na\textsuperscript{+} pump and Na\textsuperscript{+}/H\textsuperscript{+} antiport activities in rats intoxicated with these drugs (Hirata and Axerold, 1980; Smith and Gordon, 1987; Fricker et al., 1988). Accordingly, the effects we observed on LPM fluidity and hepatobiliary functions after treatment with CyA or SAMe plus CyA could probably be related to a reduction in the liver pool of SAMe during CyA treatment and its normalization during SAMe cotreatment, although this remains to be verified.

Fig. 3. Effects of treatment with CyA alone ( ) and cotreatment with SAMe plus CyA ( ) on the specific activities of three canalicular plasma membrane marker enzymes from livers of treated rats. Mg\textsuperscript{2+}-ATPase and 5'-nucleotidase activities (top) were measured in LPM vesicles. γ-GT activity (bottom) was evaluated in LPM, liver homogenates (Liver), and bile. Rats treated with the CyA vehicle were used as controls ( ). Treatments lasted 1 week; CyA (i.p.) and SAMe (s.c.) were administered at doses of 10 mg/kg per day and 10 mg/kg every 12 h, respectively. Data are given as means ± S.E. for six rats in each group. *, #, means different from control and CyA groups, respectively (P < .05).
lipid-protein interactions, thus altering fluidity and protein functions. The protective role of exogenous SAMe against these adverse effects of CyA could be mediated by its participation in trans-sulfuration reactions (Friedel et al., 1989; Pisi and Marchesini, 1990; Mato et al., 1994). In this context, it has been reported (Barth et al., 1991; Deters et al., 1997) that addition of GS or other antioxidants to a recirculating system of isolated rat liver preparations minimizes CyA-induced injury and lipid peroxidation. It has also been shown (Pascale et al., 1989) that administration of [35S]SAMe to rats leads to the appearance of labeled GS in the liver, because SAMe acts as a precursor for GS biosynthesis. A SAMe-dependent normalization of trans-sulfuration reactions and of the liver GS pool has been proposed as yet another mechanism that antagonizes the hepatotoxicity induced by agents known to deplete GS or produce peroxidation, such as acetaminophen (Bray et al., 1992), bromobenzene and D-galactosamine (Wu et al., 1996), CCl4 (Corrales et al., 1992; Muriel and Mourelle, 1992), ethanol (Pascale et al., 1989; Alvaro et al., 1995; Lieber, 1997), and other GS-depleting drugs (Pisi and Marchesini, 1990; Lu, 1998). In addition, inhibition of SAMe synthetase probably occurred in our CyA-treated rats, because it has been shown, on the one hand, that some drugs and conditions that reduce liver GS and/or induce oxidative stress are associated with inactivation of this enzyme (Feo et al., 1986; Corrales et al., 1992; Lieber, 1997; Mato et al., 1997) and with SAMe and GS depletion (Corrales et al., 1991; Mato et al., 1997; Lu, 1998) and, on the other hand, that exogenous SAMe administration antagonizes these effects (Feo et al., 1986; Pascale et al., 1989; Corrales et al., 1992). Were this the case, SAMe cotreatment might bypass the blockade of SAMe synthetase, thus enabling reconstitution of the hepatic pool of SAMe and GS and in turn helping to protect the liver against CyA-induced liperoxidation and GS depletion. However, further studies are needed to determine how SAMe antagonizes these CyA-induced alterations.

Third, the inhibitory effect of CyA on protein synthesis in rat liver might also be involved (Bäckman et al., 1988), because we observed that CyA reduces protein levels in plasma, liver, and LPM, whereas SAMe prevented these reductions. In this regard, it has been shown (Mato, 1986; Simon, 1993) that lipid-protein interactions in plasma membrane may also modify fluidity, which tends to decrease with the PHO/protein ratio, similar to our observations. In addition, oxidative sulfhydryl protein groups damage resulting from excess free radical formation, an effect observed in the livers of CyA-treated rats (Wolf et al., 1997), might lead to damage of secondary structures, loss of catalytic functions, and increased proteolytic digestion and degradation of proteins (Sundari and Ramakrishna, 1997; Wolf et al., 1997; Aruoma, 1998; Vendemiale et al., 1998). Regarding the SAMe-induced normalization of protein levels, it has been demonstrated that SAMe, by normalizing transmethylation reactions in the liver (Mato, 1986) or perhaps by maintaining a high methionine pool that might be partially used for protein synthesis, is able to increase protein synthesis in patients with alcoholic liver diseases (Avogaro et al., 1979), to restore the protein balance in CCl4-intoxicated rats (Stramentinoli, 1987), and to stimulate methylation of membrane proteins (Mato, 1986; Mato et al., 1994). In this sense, Rosario et al. (1988), working with ethynylestradiol, which also alters LPM fluidity and functions (Boelsterli et al., 1983; Fricker et al., 1988; Rosario et al., 1988; Bossard et al., 1993), have suggested that SAMe cotreatment affords protection against the effects of this drug and normalizes membrane fluidity by preventing alterations in membrane protein contents.

Finally, the CyA-induced increases in CHO content and the CHO/PHO ratio must surely also affect LPM fluidity. These changes could be related to alterations in CHO metabolism and to its elimination from the liver in these animals. CyA enhances CHO plasma levels in humans (Loss et al., 1995), drastically reduces the hepatic conversion of CHO to BA (Chan et al., 1998), and, in agreement with our observations, markedly depresses the biliary excretion of CHO and BA in the rat (Galán et al., 1992, 1995; Fernández et al., 1995; Chan et al., 1998) which is the major pathway for elimination of CHO in mammals. Despite this, we observed that SAMe cotreatment does normalize the biliary excretion of BA and CHO and improves BA plasma levels, suggesting that besides the canalicular transport of BA (Fernández et al., 1995), SAMe cotreatment restores the hepatic synthesis of BA and the biliary elimination rates of CHO. This could account for the observed improvement in the CHO/PHO ratio and CHO content in LPM. A SAMe-dependent normalization of fat deposition in the liver and in CHO content, the CHO/PHO ratio, membrane fluidity, and Ca2+- and Na+-ATPase activities have been reported to occur in LPM from rats intoxicated with CCl4, ethanol, or ethynylestradiol (Boelsterli et al., 1983; Feo et al., 1986; Pascale et al., 1989; Muriel and Mourelle, 1992).

Regarding the mechanisms involved in the differential response of the basolateral and canalicular marker enzymes on treatment with CyA or with SAMe plus CyA, these cannot be easily judged with the results obtained here. In principle, they might be related to the changes observed in membrane fluidity or to the different topographical location of the enzymes in LPM. Na+-K+-ATPase is a basolateral PHO-dependent transport enzyme (Kimelberg, 1975; Boelsterli et al., 1983) whose activity is directly correlated with LPM fluidity (Kimelberg, 1975; Boelsterli et al., 1983; Rosario et al., 1988; Simon, 1993), suggesting that the CyA- and SAMe-induced changes in its activity might be causally related to those observed in LPM fluidity, as has been observed (Boelsterli et al., 1983; Friedel et al., 1989) in rats treated with SAMe plus ethanol, CCl4, ethynylestradiol, and other chemical compounds. Regarding Mg2+-ATPase, 5′-nucleotidase, and γ-GT, it is likely that the changes we observed in their activities could be related to their topographical location in the membrane or to the CyA-induced reductions and SAMe-induced normalization in the hepatobiliary transport of BA, which are known to solubilize and remove lipids and proteins from the canalicular membrane during their excretion into the bile (Verkade et al., 1995). A lower BA-dependent solubilization and extraction of γ-GT (and Mg2+-ATPase and 5′-nucleotidase?) from the canalicular membrane after CyA treatment is consistent with the lower biliary excretion rate of γ-GT and the observed increase in its activity in liver homogenates and LPM from these rats. It is also consistent with the parallel normalization of biliary excretion rates of BA and γ-GT in the SAMe plus CyA-treated rats, probably because of the improvement in the solubilizing capability of the circulating BA pool and of the release of membrane proteins into bile. This hypothesis is in keeping with previous reports (Boelsterli et al., 1983; Rosario et al., 1988; Arrese et al., 1995) that ethy-
nylestradiol, which also reduces fluidity, Na\(^+\)K\(^+\)ATPase activity, and bile flow, and the biliary excretion of BA and lipids, increases ALP, Mg\(^2+\)ATPase, and \(\gamma\)-GT activities in liver homogenates (Bossard et al., 1993; Arrese et al., 1995) and LPM (Boelsterli et al., 1983; Fricker et al., 1988; Rosario et al., 1988). However, a different interaction between enzymes and lipids may occur, because not all membrane proteins respond uniformly to similar modifications in fluidity (Mato, 1986; Simon, 1993; Thalhammer et al., 1993). In this sense, ethanol and ethyleneestradiol alter LPM fluidity and Na\(^+\)K\(^+\)ATPase activity (Fricker et al., 1988; Rosario et al., 1988; Pascale et al., 1989; Kukongviriyapan and Stacey, 1991) without affecting Mg\(^2+\)ATPase activity (Boelsterli et al., 1983; Pascale et al., 1989). However, the former decreases \(5\)‘-nucleotidase activity (Pascale et al., 1989), whereas the latter increases ALP (Rosario et al., 1988; Bossard et al., 1993) and \(\gamma\)-GT (Boelsterli et al., 1983) activity.

In sum, our data show for the first time, as far as we know, that SAMe co-treatment in the rat antagonizes CyA-induced alterations in hepatic GS levels, oxidative status, and LPM composition and fluidity and the functionality of several membrane marker proteins, and support the notion that CyA would interfere in the functionality of LPM transporters not only through direct interactions but also through indirect interactions probably related to changes in LPM composition, hepatic GS depletion, and oxidative stress. Three hepatocellular processes seem to be involved in the beneficial effects of SAMe: 1) the action of the drug as a methyl donor and restorer of physicochemical LPM properties, 2) its ability to preserve the hepatic pool of GS and oxidative status, and 3) its capacity to maintain the hepatic pool of proteins and their recycling in LPMs.

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