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Effects of *S*-adenosylmethionine on intrabiliary glutathione degradation induced by long-term administration of cyclosporin A in the rat

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Abstract

We investigate the ability of *S*-adenosylmethionine (SAMe) to antagonize the cyclosporine A (CyA)-induced inhibition of biliary glutathione efflux induced by long-term administration of CyA (10 mg/kg per day-CyA_{10} or 20 mg/kg per day-CyA_{20} for 4 weeks) in rats. CyA treatment reduced the liver content of total glutathione and caused a significant increase in the oxidized-to-reduced glutathione ratio and the thiobarbituric acid-reactive substances (TBARS) concentration. When the rats were concurrently treated with SAMe (10 mg/kg twice daily) and CyA, all these parameters did not significantly differ from control values. Treatment with CyA induced a significant increase in liver GGT activity that was attenuated by coadministration of SAMe. Biliary efflux of total glutathione was significantly reduced in animals treated with CyA. These changes were abolished by SAMe administration. Following inhibition of the intrabiliary catabolism of the tripeptide by acivicin, glutathione efflux rates increased to a lesser extent in animals cotreated with SAMe when compared to those receiving only CyA. The significant decrease in biliary efflux of oxidized glutathione induced by CyA was totally ($S + CyA_{10}$) or partially ($S + CyA_{20}$) prevented by coadministration of SAMe. Our observations confirm that SAMe cotreatment in rats antagonizes CyA-induced inhibition in the biliary efflux of glutathione and suggest that protection against intrabiliary glutathione degradation plays a major role in this protective effect.

Keywords: Bile; Cyclosporin; Glutathione; *S*-adenosylmethionine

1. Introduction

Cyclosporin A (CyA) is an immunosuppressive drug widely used to prevent rejection of solid organ transplantation and in the treatment of several autoimmune diseases. However, therapy is often associated

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with serious side effects, which include hepatic and renal dysfunction (Borel et al., 1996; Deters et al., 1997; Galán et al., 1999). The hepatotoxic effect of the drug manifests as a cholestatic syndrome characterized by alterations in bile formation and in the capacity of the liver to excrete different xenobiotics (Schade and Van Thiel, 1983; Galán et al., 1995; Chan et al., 1998; Palomero et al., 2001). It has been reported that CyA decreases Na, K-ATPase expression in rat liver (Chanussot and Benkoel, 2003), impairs synthesis and hepatobiliary transport of bile salts (Hulzebos et al., 2003) and inhibits the multidrug resistance (MDR) protein P-glycoprotein (Pgp) (Litman et al., 2003). Alterations of glutathione homeostasis appear to play an important role in the pathogenesis of CyA-induced side effects and depletion in the hepatic pool of glutathione (Deters et al., 1997), inhibition of biliary glutathione secretion (Morán et al., 1998; Palomero et al., 2003) and reduced expression of its canalicular transporter Mrp2 (Bramon et al., 2001) have been reported.

The liver is the major site of glutathione synthesis and also the major supplier of plasma and bile glutathione (Ookhtens and Kaplowitz, 1998). Transport of the tripeptide across the canalicular membrane not only contributes to bile formation but also serves to deliver glutathione and its constituent amino acids to the biliary tree and intestinal epithelium and plays an important role in the maintenance of the balance between the formation and removal of reactive oxygen species (Lauteburg, 1991). Alterations in glutathione hepatic content and export into bile caused by CyA could compromise glutathione-based detoxification processes and protection against oxidative stress.

S-adenosylmethionine (SAMe) is a thiolic compound that plays an important role in transmethylation and transsulphuration reactions. SAMe has been reported to prevent and reverse the cholestasis and hepatotoxicity associated with several drugs and chemical compounds, either by normalizing membrane fluidity through methylation of membrane phospholipids or by maintaining the hepatic pool of glutathione (Corrales et al., 1992; Jover et al., 1992). We have previously shown that SAMe cotreatment protects against glutathione depletion, changes in hepatocyte membrane fluidity and composition, and abnormalities in biliary bile acid, lipid and protein secretion induced by CyA (Fernández et al., 1995; Galán et al., 1995).

The present study was aimed to investigate the capacity of SAMe to antagonize the inhibition of biliary glutathione efflux in rats with long-term administration of different CyA doses and to identify mechanisms responsible for the protective effect of the thiolic compound.

2. Methods

2.1. Animals

Male Wistar rats weighing 240–260 g were obtained from Charles River, Barcelona, Spain. They were kept on standard rat chow (Panlab, Barcelona, Spain) with free access to tap water, in a temperature- and humidity-controlled animal quarter under a 12 h light–dark cycle. All experiments were performed in compliance with the indications of the Guide to the Care and Use of Experimental Animals, routinely used at our laboratory.

2.2. Experimental procedures

Rats were randomly divided into three groups and were treated for 4 week as follows: one group (control) was given the CyA vehicle and the other groups were treated with either CyA or CyA plus SAMe. CyA (10 mg/kg: group CyA_{10} or 20 mg/kg b.wt.: group CyA_{20}, once daily) and its vehicle were administered i.p., and SAMe (10 mg/kg b.wt.: groups $S + CyA_{10}$ or $S + CyA_{20}$, twice per day) was administered s.c. The rats were weighed daily and the volumes of solutions administered were adjusted between 0.20 and 0.30 ml, depending on their body weights.

Experiments were carried out 12 h after the last treatment. They were planned so that they could be initiated for all animals at the same time daily (between 9 a.m. and 10 a.m.) to avoid variation due to the circadian rhythm. Rats were anaesthetized with sodium pentobarbital (50 mg/kg b.wt., i.p.) and kept at a constant temperature of $37.0 \pm 0.5\,^{\circ}C$. Losses in body temperature were prevented using a rectal probe connected to a thermostatically controlled heating lamp. Routine laparatomy was performed and the common bile duct cannulated with PE-10 polyethylene

tubing for collecting bile samples. Bile collection was started 30 min after finishing the surgical procedure, a time estimated as sufficient to allow bile flow stabilization. In some experiments, with the aim of inhibiting γ-glutamyl transpeptidase (γ-GT) activity, an irreversible inhibitor of this enzyme (acivicin) was administered after collecting a 15 min baseline bile sample; acivicin, at a dose of 40 μmol/kg b.wt. (in 0.4 ml/kg b.wt. of Ringer solution), was administered by retrograde intrabiliary infusion in accordance with previous reports (Morán et al., 1998). After this, three additional bile samples were collected at 15 min intervals. Bile was collected in pre-weighed tubes on melting ice containing 15 μl of 5% metaphosphoric acid to prevent oxidation of GSH. At the end of the assays rats were killed by exsanguination; livers were quickly washed in situ with ice-cold 0.154 M NaCl, removed and weighed. Small pieces weighing 0.5 g were harvested from the liver for biochemical determinations and immediately stored in liquid nitrogen until analysis.

2.3. Tissue preparation and biochemical analysis

Bile flow was determined gravimetrically, assuming a bile density of 1.0 g/ml. Total glutathione (GSH + GSSG) and GSSG concentrations in bile and liver homogenates were evaluated by the enzymatic recycling procedure using glutathione reductase and 5,5′-dithio-bis (2-nitrobenzoic acid), according to Griffith (1980). Gamma glutamyltranspeptidase (γ-GT) (EC 2.3.2.2) activity was determined according to Meister et al. (1981). The amount of aldehydic products generated by lipid peroxidation was quantified in liver homogenates by measurement the thiobarbituric acid-reactive substances (TBARS) (Ohkawa et al., 1979). Protein concentration was measured according to Lowry et al. (1951) using serum bovine albumin as standard.

2.4. Statistical analysis

Results are expressed as means ± standard error of means (S.E.M.) for all data. The effects of CyA and SAMe treatments on all parameters studied were tested for significance by a two-way analysis of variance (ANOVA) followed by the Fisher PLSD test. *P*-values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Effects of CyA and SAMe on liver glutathione and TBARS

CyA treatment reduced the liver content of total glutathione (CyA_{10}: −36% and CyA_{20}: −35%) and caused a significant increase in the oxidized-to-reduced glutathione ratio (CyA_{10}: +66% and CyA_{20}: +70%). Liver TBARS concentration was also significantly elevated (CyA_{10}: +51% and CyA_{20}: +52%). When the rats were concurrently treated with SAMe and CyA all these non dose-dependent changes improved significantly and did not significantly differ from control values (Table 1).

Table 1
Effects of treatment with cyclosporin A and SAMe on liver glutathione concentration, GSSG/GSH molar ratio and thiobarbituric acid-reactive substances concentration

	GS (μg/g liver)	GSSG/GSH.100	TBARS (nmol/g liver)
Control	3.84 ± 0.14	1.38 ± 0.07	21.3 ± 1.8
CyA_{10}	2.44 ± 0.22[a]	2.29 ± 0.18[a]	32.1 ± 3.4[a]
CyA_{20}	2.48 ± 0.14[a]	2.34 ± 0.13[a]	32.3 ± 0.7[a]
$S + CyA_{10}$	3.49 ± 0.33[b]	1.40 ± 0.15[b]	23.0 ± 1.2[b]
$S + CyA_{20}$	3.69 ± 0.69[b]	1.35 ± 0.19[b]	24.6 ± 1.2[b]

Values are means ± S.E.M. from 6 to 8 animals. GS: total glutathione; GSH: reduced glutathione; GSSG: oxidised glutathione; TBARS: thiobarbituric acid-reactive substances; Cy1: CyA 10 mg/kg per day; Cy2: CyA 20 mg/kg per day; *S*+Cy1 or *S*+Cy2: Cy1 or Cy2 plus SAMe at 10 mg/kg twice per day.

[a] $P < 0.05$ significantly different from control rats.

[b] $P < 0.05$ significantly different from the corresponding CyA group.

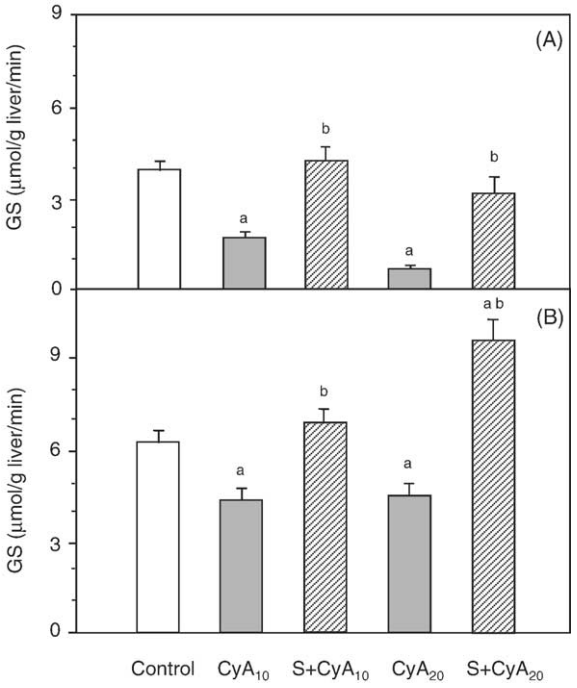

Fig. 1. Effects of treatment with cyclosporin A and *S*-adenosylmethionine on biliary secretion of glutathione (GS) before (A) and after (B) acivicin administration. Values are means ± S.E.M. from 6 to 8 animals. CyA_{10}: CyA 10 mg/kg per day; CyA_{20}: CyA 20 mg/kg per day; $S + CyA_{10}$ or $S + CyA_{20}$: CyA_{10} or CyA_{20} plus SAMe at 10 mg/kg twice per day. [a] $P < 0.05$ significantly different from control rats; [b] $P < 0.05$ significantly different from the corresponding CyA group.

3.2. Effects of CyA and SAMe on biliary glutathione

Biliary efflux of total glutathione before and after acivicin administration is shown in Fig. 1. Biliary efflux was significantly reduced in animals treated with CyA (CyA_{10}: −58%; CyA_{20}: −82%). These changes were abolished when SAMe was administered to the animals. Following inhibition of the intrabiliary catabolism of the tripeptide by acivicin, glutathione efflux rates increased by 225 and 547% (CyA_{10} and CyA_{20}, respectively) in animals treated with CyA and only by 60 and 184% ($S + CyA_{10}$ and $S + CyA_{20}$, respectively) in those cotreated with SAMe (Fig. 1).

When rats were treated with CyA, the drug significantly reduced the biliary efflux of oxidized glutathione (CyA_{10}: −56%; CyA_{20}: −80%). The decrease was totally ($S + CyA_{10}$) or partially ($S + CyA_{20}$) prevented by coadministration of SAMe (Fig. 2).

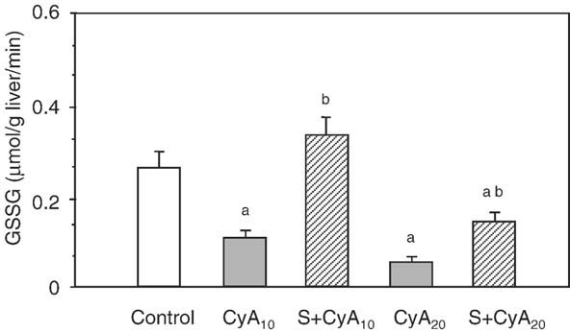

Fig. 2. Effects of treatment with cyclosporin A and *S*-adenosylmethionine on biliary secretion of oxidized glutathione (GSSG). Values are means ± S.E.M. from 6 to 8 animals. CyA_{10}: CyA 10 mg/kg per day; CyA_{20}: CyA 20 mg/kg per day; $S + CyA_{10}$ or $S + CyA_{20}$: CyA_{10} or CyA_{20} plus SAMe at 10 mg/kg twice per day. [a] $P < 0.05$ significantly different from control rats; [b] $P < 0.05$ significantly different from the corresponding CyA group.

3.3. Effects of CyA and SAMe on gamma glutamyl transpeptidase activity

Treatment with CyA induced a significant dose-dependent increase in liver GGT activity (CyA_{10}: +129%; CyA_{20}: +396%, versus control). These changes were attenuated by coadministration of SAMe ($S + CyA_{10}$: +35%; $S + CyA_{20}$: +200%, versus control) (Fig. 3).

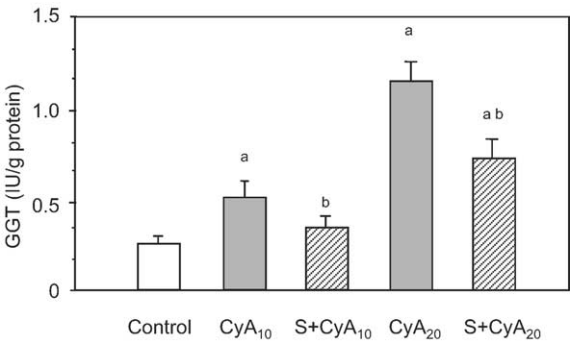

Fig. 3. Effects of treatment with cyclosporin A and *S*-adenosylmethionine on liver activity of gamma glutamyltranspeptidase (GGT). Values are means ± S.E.M. from 6 to 8 animals. CyA_{10}: CyA 10 mg/kg per day; CyA_{20}: CyA 20 mg/kg per day; $S + CyA_{10}$ or $S + CyA_{20}$: CyA_{10} or CyA_{20} plus SAMe at 10 mg/kg twice per day. [a] $P < 0.05$ significantly different from control rats; [b] $P < 0.05$ significantly different from the corresponding CyA group.

4. Discussion

Previous studies have indicated that CyA inhibits biliary glutathione secretion (Morán et al., 1998; Palomero et al., 2003) and SAMe treatment protects against reduced bile formation induced by CyA (Román et al., 1990; Fernández et al., 1992, 1995; Galán et al., 1999). Results obtained in the present study demonstrate that SAMe cotreatment antagonizes alterations induced by CyA on the biliary efflux of glutathione. Different mechanisms could contribute to this effect. First, SAMe prevented the CyA-induced increase in GGT activity. Glutathione is degraded in the biliary tree by a process initiated by this enzyme (Ballatori et al., 1988) and we have previously reported that CyA treatment increases GGT activity in both hepatocyte canalicular plasma membrane vesicles and liver homogenates from 2 month old rats (Galán et al., 1999). This effect could, as has been previously proposed in different experimental situations (Ballatori et al., 1986; Lauteburg, 1991), intensify the intrabiliary hydrolysis of glutathione and reduce biliary glutathione content. When we blocked hepatic GGT activity with acivicin a dose-dependent increase in biliary glutathione secretion was observed in animals treated with CyA, but the magnitude of this effect was lower in rats cotreated with SAMe. This indicates that reduced intrabiliary degradation of glutathione would be involved in the normalization of the biliary efflux of the tripeptide induced by SAMe. The mechanism responsible could be related to the SAMe-induced normalization in the hepatobiliary transport of bile acids (Fernández et al., 1995), which are known to solubilize lipids and proteins from the canalicular membrane during their excretion into bile. A higher bile acid-dependent solubilization and extraction of GGT is consistent with the increase in the biliary excretion of the enzyme that we have previously reported in animals treated with CyA plus SAMe (Galán et al., 1999).

Biliary glutathione secretion following acivicin administration tended to increase in animals treated with CyA, confirming its reduced intrabiliary hydrolysis (Ballatori et al., 1986; Morán et al., 1998). However, values still remained lowered when compared to control rats, while a marked increase was observed in animals cotreated with SAMe. This allows to suggest the presence of additional factors responsible for the maintainance of biliary glutathione efflux. The protective role of SAMe could be mediated by its participation in trans-sulfuration reactions. It is known that depletion of liver glutathione and/or oxidative stress are associated with inactivation of methionine adenosyltransferase (Corrales et al., 1992) which in turn produces a further decrease in glutathione levels. The addition of SAMe might break this vicious circle, enabling reconstitution of the hepatic pool of SAMe and helping to protect against CyA induced glutathione depletion and oxidative stress. However, changes in glutathione efflux in animals treated with CyA or CyA plus SAMe were dose-dependent but no significant difference between both CyA doses was observed in the alterations of liver glutathione concentration or markers of oxidative stress. In fact, the direct contribution of glutathione depletion to the inhibition of its biliary efflux in CyA-treated rats remains unclear, because a tendency to increased hepatic glutathione has been found in different models of cholestasis in association to the biliary defect (Bouchard et al., 1994, 2000) and biliary glutathione secretion is rapidly reduced following a single i.v. dose of CyA, even when the hepatic content of the tripeptide remains unaltered (Morán et al., 1998).

Changes of GSH and GSSG canalicular transport might also contribute to the normalization of biliary glutathione efflux induced by SAMe. The canalicular multispecific organic anion transporter (cMOAT or Mrp2) is responsible for the biliary excretion of GSSG (Muller et al., 1996; Keppler and Arias, 1997) and might also be involved in that of GSH (Ballatori and Rebbeor, 1997; Paulusma et al., 1999). It has been previously reported that CyA inhibits the activity of Mrp2 in canalicular membrane vesicles (Bohme et al., 1994) and reduces the biliary excretion of substrates from Mrp2 (Román et al., 1990; Galán et al., 1991). CyA treatment diminishes phospholipid content, the phosphatidylcholine/phosphatidylethanolamine ratio and fluidity of hepatocyte membranes (Galán et al., 1999). In addition, oxidative damage to sulphydril protein groups resulting from the free radical formation in CyA-treated rats could give place to loss of catalytic functions and increased degradation of proteins (Wolf et al., 1997), and both factors may lead to alteration in membrane-bound enzyme activities such as transporter proteins (Vendemiale et al., 1999). When SAMe is administered, its methyl groups are

incorporated into the membrane phospholipids (Bontemps and Van Den Berghe, 1998) and this normalizes membrane fluidity and the Na^+ pump (Fricker et al., 1988). Additionally, it has been shown that SAMe, by normalizing methylation reactions or maintaining a high methionine pool, is able to increase protein synthesis and to stimulate methylation of membrane proteins (Mato et al., 1997). Finally, the SAMe-induced increased in glutathione content helps to protect against effects of oxidative stress. The fact that GSSG biliary secretion was reduced by CyA and preserved by SAMe in a dose-dependent manner suggests that the thiolic compound could maintain the functionality of transporters through indirect interactions related to changes in membrane fluidity and composition.

In summary, our observations confirm that SAMe cotreatment in rats antagonizes CyA-induced inhibition in the biliary efflux of glutathione and suggest that, although other factors may be involved, protection against intrabiliary glutathione degradation plays a major role in this effect.

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