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S-Adenosylmethionine (SAMe) protects against acute alcohol induced hepatotoxicity in mice☆

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Abstract

Although S-Adenosylmethionine (SAMe) has beneficial effects in many hepatic disorders, the effects of SAMe on acute alcohol-induced liver injury are unknown. In the present study, we investigated effects of SAMe on liver injury in mice induced by acute alcohol administration. Male C57BL/6 mice received ethanol (5 g/kg BW) by gavage every 12 hrs for a total of 3 doses. SAMe (5 mg/kg BW) was administrated i.p. once a day for three days before ethanol administration. Subsequent serum ALT level, hepatic lipid peroxidation, enzymatic activity of CYP2E1 and hepatic mitochondrial glutathione levels were measured colorimetrically. Intracellular SAMe concentration was measured by high-performance liquid chromatography (HPLC). Histopathological changes were assessed by H&E staining. Our results showed that acute ethanol administration caused prominent microvesicular steatosis with mild necrosis and an elevation of serum ALT activity. SAMe treatment significantly attenuated the liver injury. In association with the hepatocyte injury, acute alcohol administration induced significant decreases in both hepatic SAMe and mitochondrial GSH levels along with enhanced lipid peroxidation. SAMe treatment attenuated hepatic SAMe and mitochondrial GSH depletion and lipid peroxidation following acute alcohol exposure. These results demonstrate that SAMe protects against the liver injury and attenuates the mitochondrial GSH depletion caused by acute alcohol administration. SAMe may prove to be an effective therapeutic agent in many toxin-induced liver injuries including those induced by alcohol.

Keywords: S-adenosylmethionine; Alcohol-induced liver injury; Mitochondria; Glutathione; CYP2E1; Mitochondrial permeability transition

1. Introduction

Alcoholic liver disease is a major medical complication of alcohol abuse and a common liver disease in the western countries [1]. Although important progress has been made in understanding the pathogenesis of alcoholic liver disease, the mechanisms involved in the development of the disease are not fully understood. Moreover, no FDA (Food and Drug Administration)-approved therapy is available. Thus, novel agents that correct the fundamental cellular disturbances resulting from excessive alcohol consumption represent attractive therapeutic strategies.

Increasing evidence demonstrates that oxidative stress plays an important etiologic role in the development of alcoholic liver disease [2–5]. One of the most prominent antioxidant defense systems in the liver is the presence of reduced glutathione (GSH). In addition to serving as a substrate for glutathione-related enzymes, GSH acts as a free radical scavenger, a regenerator of a-tocopherol, and it plays an important role in the maintenance of protein sulfhydryl groups [6,7]. Short-term ethanol administration has been reported to decrease hepatic GSH content and increase superoxide generation in rat liver and in liver mitochondria [8]. The enhanced superoxide generation increases lipid peroxidation and induces mitochondrial dysfunction in rats that have been subjected to acute ethanol exposure [8,9]. Since liver mitochondria do not contain catalase, the ability

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of the mitochondrial GSH system to reduce hydrogen peroxide is probably the main mechanism for protecting mitochondria against oxidative stress. Chronic ethanol intake induces a selective decrease in the mitochondrial pool of GSH, presumably because of impaired uptake of GSH from the cytosol to mitochondrial matrix [10–13]. The decreased levels of GSH cause greater susceptibility to hepatic injury in chronically alcohol-fed rats via oxidative stress or TNF-α [14,15]. However, there is still no agreement concerning the effects of acute ethanol intoxication on hepatic GSH level, especially mitochondrial GSH.

S-Adenosylmethionine (Adomet, SAM or SAMe – SAMe used here) is a key intermediate in the hepatic transsulfuration pathway and serves as a precursor for GSH as well as the methyl donor in most transmethylation reactions [16,17]. Clinical studies have reported that the therapeutic administration of stable salts of SAMe has beneficial effects on many hepatic disorders ranging from cholestasis to alcoholic liver disease [18–21]. Chronic alcohol consumption has been reported to induce a selective defect in the mitochondrial transport of GSH, and SAMe treatment normalizes the steady state levels of GSH in mitochondria in rats chronically fed ethanol. Moreover, hepatocytes become sensitized to TNF-induced death due to mitochondrial GSH depletion during chronic alcohol consumption, and SAMe protects against the toxicity [22,23].

The current study was undertaken to determine the effect(s) of SAMe on acute alcohol-induced hepatotoxicity and to explore a possible mechanistic link between mitochondrial oxidative stress and acute alcohol-induced liver injury. Specifically, we plan to evaluate [1] whether acute alcohol intake induces lipid peroxidation, mitochondrial dysfunction, or GSH depletion and [2] whether SAMe supplementation can attenuate the alcohol-induced abnormalities.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in the animal quarters at the University of Louisville Research Resources Center. They were maintained at 22°C with a 12:12-hr light-dark cycle, and they had free access to rodent chow and tap water. The experimental procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care.

2.2. SAMe treatment and ethanol administration

The binge drinking mouse model developed by Carson and Pruett [24] was utilized for ethanol challenge. This model was designed to achieve blood alcohol levels, behavioral effects, and physiological changes comparable with human binge drinking. Nine week old mice were divided into 3 groups: control, ethanol treatment, and SAMe/ethanol treatment, with 10 mice in each group. Mice received ethanol (5 g/kg BW) by gavage every 12 hrs for a total of 3 doses. Control mice received an isocaloric maltose solution. In the SAMe/ethanol group, SAMe was injected i.p. (50 mg/kg BW) every 12 hrs x 3 before the start of ethanol administration. At 4 hr after the final ethanol dose, the mice were anesthetized with urethane (1.25 g/kg BW). Blood was drawn from the vena cava, and serum was obtained by centrifugation using a serum separator tube.

2.3. Sub-cellular fractionation of liver tissue

Liver tissue was homogenized with cold HMS (220 mM D-Mannitol, 70 mM sucrose, 2 mM K-HEPES, pH 7.4) and centrifuged at 4°C for 5 min at 600*g*. The supernatants were further centrifuged at 7000*g* for 10 min. The heavy membrane fraction (mitochondria) was washed once again in HMS by centrifugation at 600*g* for 5 min to remove any contaminating particles and then recovered by centrifugation at 7000*g* for 10 min. The isolated mitochondria were resuspended in incubation buffer (250 mM sucrose, 10 mM K-HEPES, pH 7.2, 2 mM KH_2PO_4, 5 mM sodium succinate, 2.5 mM EGTA). For mitochondrial permeability transition (MPT) measurement, mitochondria were resuspended in the same incubation buffer without EGTA. MPT was monitored within 2 hrs. The remaining samples of mitochondria were frozen immediately for GSH assay.

2.4. Intracellular SAMe assay by HPLC

Deproteinized cellular extracts (4% metaphosphoric acid) were prepared and SAMe was determined by an HPLC method [25], using a 5-mm Hypersil C-18 column (250 $\times$ 4.6 mm). The mobile phase consisted of 40 mM ammonium phosphate, 8 mM heptanesulfonic acid (ion-pairing reagent, pH 5.0), and 6% acetonitrile and was delivered at a flow rate of 1.0 mL/min. SAMe was detected using a Waters 740 UV detector at 254 nm. An internal standard, S-adenosylethionine (SAE), was added to all samples and standard solutions to a concentration of 100 nmol/mL. Protein concentrations were measured by protein assay kit from Bio-Rad (Catalog No. 500 to 0006, Bio-Rad Laboratories, Hercules, CA) in accordance with the manufacturer's instructions.

2.5. Serum enzymes assay

Serum alanine aminotransferase (ALT, EC 2.6.1.2.) activity was measured colorimetrically using a Diagnostic kit (Procedure No.505, Sigma Chemical Co., St. Louis, MO) according to the instructions provided.

2.6. Histopathological examination

Liver tissues were cut into ~3-mm-thick slices and fixed with 10% neutral formalin. The tissue slices were embedded in paraplast. Tissue sections of 5 μm were stained by hematoxylin and eosin (H&E), and were observed with a Nikon Eclipse E400 light microscope.

2.7. Determination of reduced glutathione (GSH) concentrations in mitochondria

GSH concentrations in mitochondria were determined using a Glutathione Assay Kit (Cat. No. 354102, Calbiochem, San Diego, CA) according to the instructions provided and the result was confirmed by HPLC. The GSH content was expressed as nanomoles per milligram protein.

2.8. Lipid peroxidation assay

Liver tissue was homogenized in 9 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 180 mM KCl, 10 mM EDTA, and 0.02% butylated hydroxytoluene. To 0.2 mL of the tissue homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid, 1.5 mL of 0.9% thiobarbituric acid, and 0.6 mL of distilled water were added and vortexed. The reaction mixture was placed in a water bath at 95°C for 1 hr. After cooling on ice, 1.0 mL of distilled water and 5.0 mL of butanol/pyridine mixture (15:1,v/v) were added and vortexed. After centrifugation at 10,000*g* for 10 min, the resulting lower phase was determined at 532 nm. The Thiobarbituric Acid Reactive Substances (TBARS) concentration was calculated using 1,1,5,3-tetraethoxypropane as a standard.

2.9. Determination of cytochrome P450 2E1 (CYP2E1) activity

Hydroxylation of *p*-nitrophenol to 4-nitrocatechol, a reaction catalyzed specifically by cytochrome P450 2E1 (CYP2E1), was determined colorimetrically as described by Koop and Laethem [26]. Liver tissue was homogenized in 0.15 KCl and was spun at 10,000*g* for 30 min. Microsomes were isolated by further centrifugation at 105,000*g* for 60 min. For the assay, 300 μl of microsomal protein was incubated for 5 min at 37°C, and absorbance at 535 nm was measured with 4-nitrocatechol as a standard. The CYP2E1-catalized *p*-nitrophenol hydroxylation was expressed as nanomoles of product formed per minute per milligram of microsomal protein.

2.10. Assessment of mitochondrial permeability transition (MPT)

Mitochondrial permeability transition was estimated from the decrease in the absorbance at 540 nm. Briefly, mitochondria (0.5 mg protein/mL) were equilibrated in a total volume of 1 mL incubation buffer consisting of 250 mM sucrose, 10 mM K-HEPES, pH 7.2, 2 mM KH_2PO_4, 5 mM sodium succinate at 25°C for 10 min. The suspension was then preincubated with 10 mM succinate for 5 min. Mitochondrial swelling was initiated by the addition of 300 μM calcium chloride. The decrease of the absorbance at 540 nm was determined with a Beckman model DU-650 spectrophotometer (Beckman Instruments, Inc, Fullerton, CA, USA). Results presented are representative of duplicate determinations.

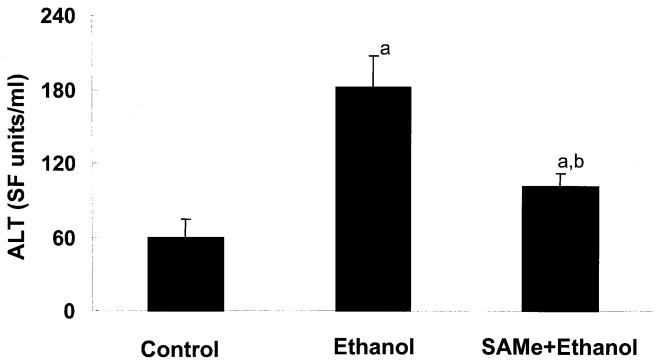

Fig. 1. Serum ALT activity in different groups. [a]($P < 0.05$ vs. Control). [b]($P < 0.05$ vs. Ethanol). Values are means ± SD (n = 5 to 7).

2.11. Statistics

All data are expressed as mean ± SD (n = 5 to 7). Statistical analysis was performed using Student's *t*-test and ANOVA where appropriate. Differences between groups were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Effects of SAMe treatment on serum enzyme levels and histopathological changes

Ethanol administration caused an elevation of serum ALT activity. However, SAMe pretreatment attenuated the ethanol-induced increases in ALT activity (Fig. 1). Liver sections from mice treated with ethanol showed prominent microvesicular steatosis (arrows) along with necrosis (arrowheads) (Fig. 2B). The necrotic hepatocytes were characterized by cell enlargement and nuclear dissolution. In the SAMe pretreated group, only microvesicular steatosis, which was less extensive than in livers from mice receiving ethanol alone, was observed (Fig. 2C).

3.2. Attenuation of ethanol-induced lipid peroxidation by SAMe

Ethanol-induced lipid peroxidation was assessed by measuring the extent of TBARS. In comparison with controls,

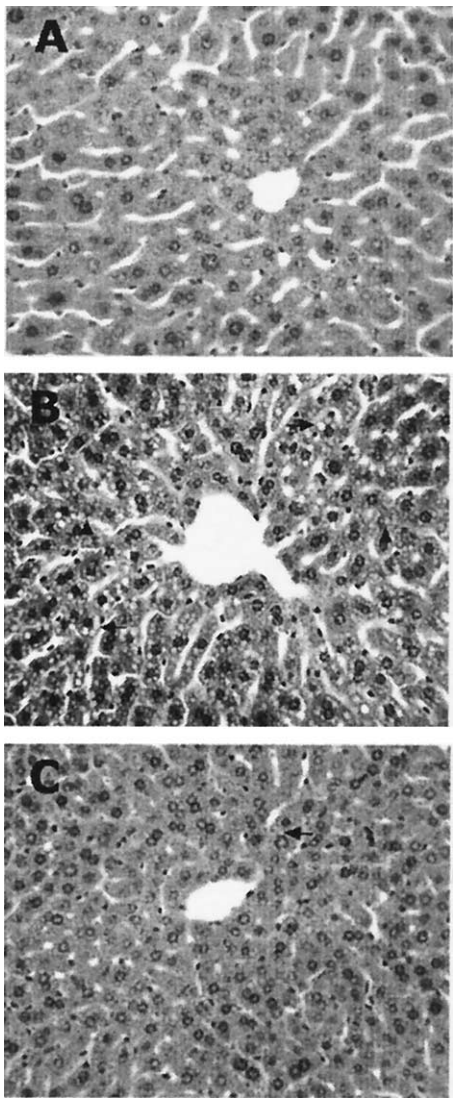

Fig. 2. Histopathological changes in the liver of different groups. (A) Control mice. (B) Ethanol treatment induced prominent microvesicular steatosis (arrows) along with necrosis (arrowheads) in the liver. The necrotic hepatocytes are characterized by cell enlargement and nuclear dissolution. (C) SAMe pretreated livers showed only microvesicular steatosis, which was less extensive than in livers from mice receiving ethanol alone. CV, central vein. x40.

ethanol administration caused an almost 9-fold increase in the TBARS content of the liver. In the SAMe-pretreated mice, the ethanol-induced elevation of TBARS was significantly attenuated, being less than twice that of control mice (Fig. 3).

3.3. Intracellular SAMe concentration

In comparison to control, acute ethanol administration induced a significant decrease in SAMe concentration in the liver (Table 1). In contrast, mice pretreated with exogenous SAMe had a significant elevation in hepatic SAMe level in comparison with control.

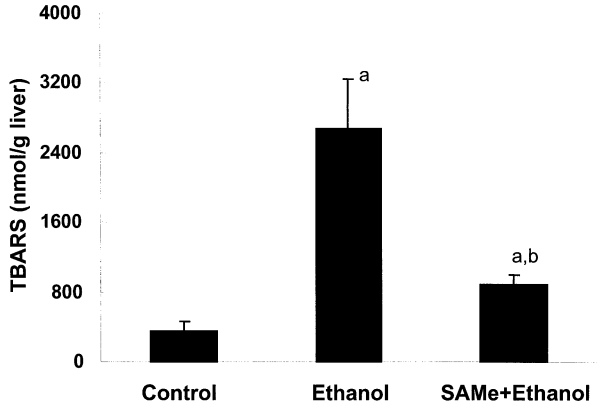

Fig. 3. Hepatic lipid peroxidation (TBARS content) in different groups. [a]($P < 0.05$ vs. Control). [b] ($P < 0.05$ vs. Ethanol). Values are means ± SD ($n = 5$ to 7).

3.4. Effects of SAMe on GSH concentration in mitochondria

Ethanol-treated mice had significant decreases in mitochondrial GSH concentrations compared to control mice. However, SAMe pretreatment significantly attenuated the mitochondrial GSH decrease, although values were still lower than controls (Fig. 4).

3.5. Changes in CYP2E1 activity

To determine whether SAMe attenuated oxidative stress by altering activity of the ethanol metabolizing enzyme CYP2E1, activity of this enzyme was determined (Fig. 5). There were about a 1.5-fold increase in hepatic microsomal CYP2E1 activities in both ethanol alone and SAMe plus ethanol groups in comparison to controls.

3.6. The effect of SAMe on Ca^{2+}-induced nitochondrial permeability transition (MPT)

MPT is characterized by a progressive permeabilization of the inner mitochondrial membrane dependent on the excessive amount of intra-mitochondrial Ca^{2+} and results in mitochondrial swelling, release of accumulated Ca^{2+}, and decrease in mitochondrial $\Delta\psi$. In order to determine whether acute ethanol accelerated Ca^{2+}-induced MPT (and

Table 1
Hepatic SAMe levels in different groups

	Control	Ethanol	SAMe+Ethanol
SAMe (nmol/mg protein)	0.621 ± 0.051	0.180 ± 0.016[a]	13.606 ± 8.768[a,b]

Values are means ± SD (n = 5–7).
[a] ($P < 0.05$ vs. Control).
[b] ($P < 0.05$ vs. Ethanol).

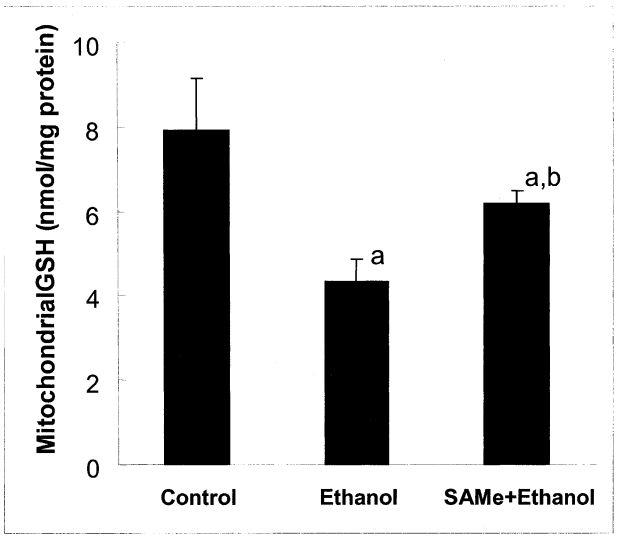

Fig. 4. Mitochondrial GSH concentrations in different groups. [a]($P < 0.05$ vs. Control). [b] ($P < 0.05$ vs. Ethanol). Values are means ± SD ($n = 5$ to 7).

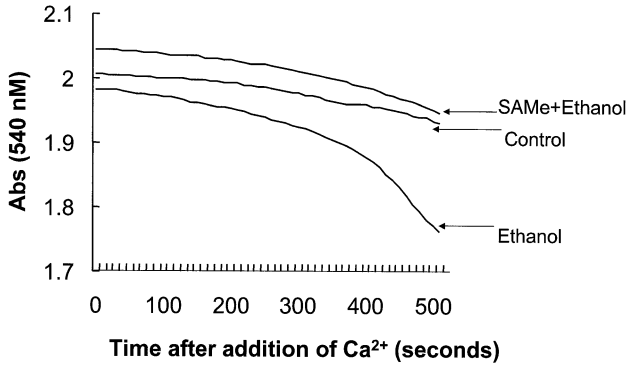

Fig. 6. Ca^{2+}-induced mitochondrial permeability transition in different groups. Mitochondrial swelling was initiated by the addition of calcium chloride (300 μM) and succinate (10 mM) to a suspension of mitochondria (0.5 mg protein/mL) in incubation buffer at 25°C. Swelling was monitored by a decrease in absorbance at 540 nm. Results presented are representative of duplicate determinations.

whether SAMe could attenuate this), we examined the effects by adding 300 μM Ca^{2+} into mitochondria suspension in the incubation buffer and assayed MPT. Fig. 6 illustrates that mitochondria isolated from ethanol-administrated animals had a rapid onset of MPT when compared with both control and SAMe-pretreated animals. SAMe attenuated the Ca^{2+} induced MPT, characterized by a slower onset of the absorbance decrease which mimicked that of control mice.

4. Discussion

S-adenosylmethionine is an important methyl donor and precursor of glutathione. SAMe can be produced in all cells, but the liver is the major organ responsible for the conversion of dietary methionine to SAMe. The hepatic form of methionine adenosyltransferase (MAT), the enzyme responsible for this conversion, is highly susceptible to oxidative stress [16,17]. Decreased SAMe levels are observed in multiple forms of the experimental liver injury, and exogenous SAMe has been demonstrated to be an effective hepatoprotective agent in many experimental liver injuries including alcoholic liver disease [18–21,27]. Although a number of the studies have reported that exogenous SAMe might be taken up by liver or isolated hepatocytes [27,28], whether or not SAMe can be taken up intact is still controversial. Some investigators reported that exogenous SAMe is not taken up by hepatocytes and that only its methyl group is incorporated into the phospholipid moiety of cellular membranes [29–31]. In current experiments, we used SAMe in an in vivo animal model of acute binge drinking and acute ethanol toxicity. Our results showed that acute alcohol administration significantly decreased hepatic SAMe level, however, exogenous addition prevented ethanol-induced hepatic SAMe depletion. This result is consistent with the findings by Feo et al. [32]. Although we did not examine the mechanism(s) for this increase, it is possible that such an increase could have resulted from either a change in its endogenous metabolism or from cellular uptake of the exogenous SAMe.

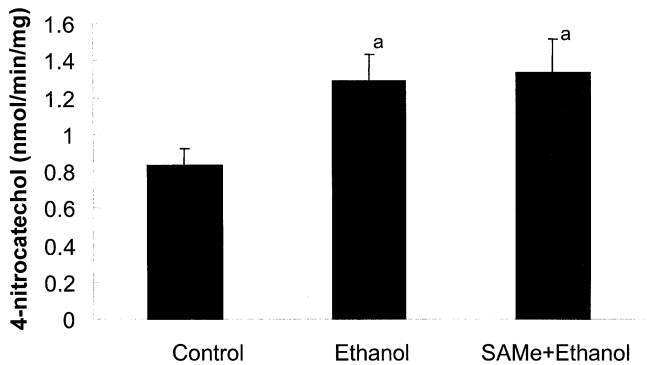

Fig. 5. CYP2E1 enzymatic activity in different groups. [a]($P < 0.05$ vs. Control). Values are means ± SD ($n = 5$ to 7).

Oxidative stress plays an important role in the development of alcohol-induced liver injury. Oxidative stress is generally considered the result of an imbalance between pro-oxidants and antioxidants [2]. Hepatic lipid peroxidation associated with acute ethanol administration has often been assessed in both animal models and in human clinical trials as an indicator of oxidative stress [33,34]. Excess lipid peroxidation as measured by the formation of TBARS or diene conjugates has been observed in many studies [2]. A marked increase in lipid peroxidation was noted after acute alcohol administration in our study, and this increase was significantly attenuated by SAMe pretreatment.

Ethanol metabolism-associated increases in NADH and CYP2E1 activity are the major contributors to the generation of ROS in hepatocytes. Ethanol oxidization leads to NADH elevation and thereby increased intramitochondrial

reductive potential, which then results in intramitochondrial oxidative stress [35]. CYP2E1 catalyzes the conversion of ethanol to acetaldehyde, and at the same time reduces dioxygen to a variety of reactive oxygen species, including superoxide [36]. In order to investigate the possible mechanisms by which SAMe attenuated acute alcohol-induced liver injury, we first evaluated the effect of SAMe on CYP2E1 enzymatic activity induced by ethanol administration. Our study indicated that ethanol increased slightly hepatic CYP2E1 activity, and this increase was not diminished by SAMe treatment.

Mitochondria represent another major source of ROS during ethanol metabolism, since elevated generation of NADH by alcohol dehydrogenase alters the intramitochondrial redox potential and results in enhanced ROS production. Mitochondria do not contain catalase, thus GSH in the mitochondrial matrix appears to be a major defense available to cope with the potential toxic effects ROS produced endogenously in the electron transport chain [37,38]. GSH is a tripeptide that exists in high concentration (mM range) in all cells in nature. Despite its universal presence in cells, GSH is not homogeneously distributed within cellular organelles. Eighty to eighty-five percent of total cellular GSH is found in the cytosol and a small proportion of the total cellular GSH pool, 10 to 15%, is found in mitochondria [14]. Considering that mitochondria are a major source for ROS production, mitochondrial GSH plays a critical role in maintaining normal mitochondrial function and resisting oxidative stress. Decreased intracellular GSH content associated with acute ethanol liver injury has been reported [39, 40], however, the effect of acute alcohol administration in vivo on mitochondrial GSH level has not been examined. Previous studies established that hepatocytes from rats chronically fed ethanol are selectively depleted of GSH in mitochondria due to defective function of the carrier responsible for transport of GSH from the cytosol into the mitochondrial matrix. Mitochondrial GSH depletion represents a critical contributory factor that sensitizes alcohol-exposed hepatocytes to the pro-oxidant effects of cytokines and the pro-oxidants generated by oxidative metabolism of ethanol [23]. SAMe prevents development of the ethanol-induced defect of the GSH transporter on mitochondrial membrane, and thereby attenuates chronic alcohol-induced liver injury [23]. Although a recent in vitro study has shown that the mitochondrial permeability transition contributes to acute ethanol-induced apoptosis in rat hepatocytes [41], it is unclear whether acute alcohol administration also causes mitochondrial dysfunction in the in vivo animal model and whether SAMe can protect hepatocytes from acute alcohol-induced injury. Results obtained from the present study showed that acute ethanol administration significantly decreased the mitochondrial GSH level, and exogenous SAMe administration significantly attenuated the decrease. In addition, by measuring the sensitivity of mitochondria to Ca^{2+} - induced mitochondrial permeability transition, we noted that SAMe treatment protected mitochondria from acute alcohol-induced dysfunction. All the above results suggest that SAMe attenuated acute alcohol-induced liver injury, at least in part, by preventing mitochondrial GSH depletion and dysfunction following ethanol administration.

In conclusion, we have demonstrated that acute ethanol administration caused prominent microvesicular steatosis with mild necrosis and an elevation of serum ALT activity. SAMe treatment significantly attenuated the liver injury. In association with the hepatocyte injury, acute alcohol administration induced marked decreases in both hepatic SAMe and mitochondrial GSH levels along with enhanced lipid peroxidation. SAMe treatment prevented hepatic SAMe and mitochondrial GSH depletion following acute alcohol exposure and thereby inhibited ethanol-associated intramitochondrial oxidative stress. SAMe may prove to be an effective therapeutic agent in many toxin-induced liver injuries, including those induced by alcohol.

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