S-adenosylmethionine prevents chronic alcohol-induced mitochondrial dysfunction in the rat liver

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CHRONIC ALCOHOL CONSUMPTION causes liver damage by a complex process involving oxidative stress, hypoxia, and energy metabolism defects. Early in this process, endotoxin released from the gut (31) activates Kupffer cells, which in turn leads to an elevated production of cytokines, eicosanoids, and reactive oxygen/nitrogen species (ROS/RNS). This results in a spectrum of responses in the hepatocyte including enhanced hypoxia (2), mitochondrial damage (5), and increased sensitivity to oxidative stress; proteome; blue native gel electrophoresis; cytochrome c oxidase; prohibitin; mitochondrial DNA

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Am J Physiol Gastrointest Liver Physiol 291: G857–G867, 2006. First published July 6, 2006; doi:10.1152/ajpgi.00044.2006.—An early event that occurs in response to alcohol consumption is mitochondrial dysfunction, which is evident in changes to the mitochondrial proteome, respiration defects, and mitochondrial DNA (mtDNA) damage. S-adenosylmethionine (SAM) has emerged as a potential therapeutic for treating alcoholic liver disease through mechanisms that appear to involve decreases in oxidative stress and proinflammatory cytokine production as well as the alleviation of steatosis. Because mitochondria are a source of reactive oxygen/nitrogen species and a target for oxidative damage, we tested the hypothesis that SAM treatment during alcohol exposure preserves organellar function. Mitochondria were isolated from livers of rats fed control and ethanol diets with and without SAM for 5 wk. Alcohol feeding caused a significant decrease in state 3 respiration and the respiratory control ratio, whereas SAM administration prevented these alcohol-mediated defects and preserved hepatic SAM levels. SAM treatment prevented alcohol-associated increases in mitochondrial superoxide production, mtDNA damage, and inducible nitric oxide synthase induction, without a significant lessening of steatosis. Accompanying these indexes of oxidant damage, SAM prevented alcohol-mediated losses in cytochrome c oxidase subunits as shown using blue native PAGE proteomics and immunoblot analysis, which resulted in partial preservation of complex IV activity. SAM treatment attenuated the upregulation of the mitochondrial stress chaperone prohibitin. Although SAM supplementation did not alleviate steatosis by itself, SAM prevented several key alcohol-mediated defects to the mitochondria genome and proteome that contribute to the bioenergetic defect in the liver after alcohol consumption. These findings reveal new molecular targets through which SAM may work to alleviate one critical component of alcohol-induced liver injury: mitochondria dysfunction.

nitric oxide (NO)-dependent inhibition of respiration (77). This increased metabolic activity, which includes increased ROS/RNS production, contributes to hepatocyte death (4).

Disruption in oxidative phosphorylation and the inability to maintain ATP levels are proposed to contribute to the liver damage from chronic alcohol exposure. Specifically, the inability to maintain ATP levels in hepatocytes contributes to necrotic cell death from exposure to toxins including ethanol (32, 36). Chronic alcohol consumption depresses the activities of all the respiratory complexes except complex II (21). Mitochondrial protein synthesis inhibition due to mitochondrial DNA (mtDNA) (14, 75) and mitochondrial ribosome defects (13, 54) are proposed to contribute to the decreased functioning of respiratory complexes and ATP synthesis (67). These disturbances in mitochondria structure and loss of function are also associated with increased mitochondria ROS production and oxidative injury to the organelle after chronic alcohol exposure. Because increased mitochondrial oxidant production has been identified as one of several key factors mediating alcoholic liver injury, agents aimed at attenuating mitochondria ROS production should prevent mitochondrial dysfunction and the progression to permanent liver damage.

One agent that has received considerable attention in recent years as a potential therapeutic against alcohol-induced liver injury is S-adenosylmethionine (SAM). SAM is the primary donor in anabolic metabolism, serves as a precursor for glutathione (GSH), and is synthesized by the enzyme methionine adenosyltransferase (MAT) (47). Chronic alcohol consumption decreases hepatic SAM levels due to depressed activity of the hepatocyte-specific isoform of MAT (42). Depletion of SAM correlates with DNA hypomethylation and strand breaks (42) as well as increased lipid peroxidation and mitochondrial damage (39). These effects may also contribute to changes in the mitochondrial proteome and modification of thiols in critical enzymes involved in ethanol metabolism including aldehyde dehydrogenase (76). Moreover, MATI knockout mice, which are deficient in SAM, spontaneously develop steatohepatitis and hepatocellular carcinoma, thus demonstrating that chronic depletion of SAM predisposes the liver to injury (46). Accordingly, the exogenous administration of SAM has been shown to increase survival in patients with alcoholic cirrhosis (48), presumably through a number of mechanisms including inhibition of steatosis and inflammation as well as repletion of mitochondrial GSH levels (27, 35, 40, 65). Recent studies (1, 25, 59) have indicated that the benefits
of SAM might be mediated through effects on mitochondria; however, the specific mechanisms or mitochondria pathways targeted by SAM are unknown. Thus, we investigated the ability of SAM to prevent chronic alcohol-induced mitochondrial damage. Studies were carried out to evaluate the effect of SAM on parameters of mitochondria function as well as mtDNA damage and alterations to the mitochondrial proteome in response to chronic alcohol exposure. Moreover, the ability of SAM to attenuate the effects of alcohol on mitochondrial function will provide insight into whether defects in mitochondrial function are linked to the development of early stages of alcohol-mediated liver injury.

**MATERIALS AND METHODS**

**Feeding protocols.** Male Sprague-Dawley rats (3 mo of age, Charles River Laboratories) were individually housed and maintained under a 12:12-h light-dark cycle for the entire duration of the feeding period. Animals had free access to standard laboratory rat chow and water for at least 1 wk and weighed at least 200 g before being provided the liquid diets. Nutritional adequacy control and ethanol-containing liquid diets were formulated by Bio-Serv (Frenchtown, NJ). The ethanol-containing diet provided 36% of the total daily caloric intake as ethanol, 35% as fat, 18% as protein, and 11% as carbohydrate. Control animals were pair fed identical diets except that ethanol calories were replaced isocalorically with carbohydrate (dextin maltose). A second set of animals were also pair fed the same control and ethanol diets supplemented with SAM (0.8 mg active SAM/ml diet). SAM was provided as the stable 1,4-butanedisulfonate salt form (Denosyl SD4, Nutramax Laboratories, Edgewood, MD). SAM was dissolved in the diets 1 h before animals were fed. Animals were maintained on these feeding protocols for at least 31 days before being used in experiments. These studies were approved by the institutional animal care and use committee, and animals received humane care in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23).

**Mitochondria isolation.** Mitochondria were isolated by differential centrifugation of the liver homogenate (6). Mitochondria function was assessed by measuring state 3 and 4 respiration and the respiratory control ratio (RCR) using glutamate/malate or succinate as substrates. Cytochrome c oxidase and citrate synthase activities were measured using standard spectrophotometric measures (63, 80). Mitochondrial GSH concentrations were measured using an enzymatic recycling assay (72). Ethanol and SAM treatments had no effect on mitochondrial protein yield (control, 23 ± 2.0 mg protein/g liver; ethanol, 24 ± 1.8 mg protein/g liver; control + SAM, 20 ± 2.0 mg protein/g liver; and ethanol + SAM, 21 ± 0.9 mg protein/g liver, P = 0.9).

**Liver histology and biochemical measurements.** Liver sections were evaluated by two pathologists blinded to the experimental groups. Steatosis was assessed by determining the percentages of hepatocytes containing fat droplets and were graded as 0 (<5%), 1+ (6–25%), 2+ (26–50%), and 3+ (51–75%). Intra-acinar inflammation was scored as the presence of inflammatory cell aggregates: absence of inflammation was scored as 0, one focus was scored as 1, and two to four foci were scored 2 per ×10 objective (78). Liver samples were homogenized in 0.5 M perchloric acid, and the filtered extracts were directly subjected to HPLC analysis for the determination of hepatic SAM levels following the procedures of Fu et al. (26) as detailed in Ref. 8. Triglycerides were measured in the whole liver homogenate as described in Ref. 35.

**Mitochondrial superoxide production.** Rates of O$_2^-$ production in mitochondria were determined using a chemiluminescent coelenterazine assay (70). The production of O$_2^-$ was stimulated by the addition of succinate (1 mM) to mitochondria (60 μg) suspended in the same buffer used to measure rates of respiration. This allowed for the measurement of O$_2^-$ production to be done under the same conditions used to measure oxygen consumption. Coelenterazine (prepared in ethanol) was added to the buffer immediately before the measurements to achieve a concentration of 5 μM for assays. Increases in chemiluminescence were monitored for 5 min at 37°C using an AutoLumat plus model 953 luminometer (Berthold, Germany), and rates of O$_2^-$ production were corrected for background rates, i.e., signals generated solely with reactants. The signal (relative light units) was integrated over a 30-s period (30–60 s) and used in the final calculation.

**Blue native gel electrophoresis.** Ethanol and/or SAM-mediated alterations in the levels of proteins that comprise the oxidative phosphorylation system were assessed using blue native (BN)-PAGE proteomics (11, 75). Oxidative phosphorylation complexes were extracted intact from 1 mg of mitochondrial protein using 1% n-dodecyl-β-d-maltoside in 0.75 M ammonium acid and 50 mM BisTris (pH 7.0). Protein extracts were mixed with 2.5 µl of a 5% (wt/vol) suspension of Coomassie brilliant blue G-250 in 0.5 M ammonium acid before being applied to non-denaturing 5–16.5% gradient gels to separate individual intact oxidative complexes. After native one-dimensional gel electrophoresis was performed, the entire vertical gel lane containing all the complexes was cut from the gel and laid on top of a denaturing Tris-tricine-SDS-PAGE gradient gel to resolve the individual polypeptides of the complexes based on molecular weight. Samples from each pair of untreated or SAM-treated control and ethanol-fed animals were resolved on one gel to minimize for intergel differences. Gels were stained with Coomassie blue using identical staining and destaining conditions. Image analysis on two-dimensional BN-PAGE gels was performed using Quantity One software (Bio-Rad).

**Immunoblot analysis.** For inducible NO synthase (iNOS) and cytochrome P-450 2E1 (CYP2E1) detection, whole liver homogenate was used, whereas isolated mitochondria were used to detect mitochondria proteins. Levels of iNOS protein were detected using a 1:5,000 dilution of antibody (Chemicon). Cytochrome c oxidase subunits I, IV, and Vb were detected using 1:10,000, 1:5,000, and 1:500 dilutions, respectively (Molecular Probes). Cytochrome c was detected using a 1:1,000 dilution (BD Pharmingen). CYP2E1 protein was detected using a 1:1,000 dilution (Chemicon). Prohibitin protein was detected using a 1:1,000 dilution (Oncogene), whereas BAP37 protein was detected using a 1:20,000 dilution of antibody (provided by Dr. Phillip Coates). Mitochondrial heat shock protein (Hsp)70 and Hsp60 proteins were detected using 1:1,000 and 1:2,500 dilutions, respectively (StressGen Biotechnologies). After membranes had been incubated with the appropriate secondary antibodies, proteins were visualized using ECL, and the intensities of immunoreactive protein bands were quantified using NIH Image (Bethesda, MD). The protein concentration loaded onto gels was within the linear range of the ECL system used for all proteins.

**Quantitative PCR.** Genomic DNA was extracted from livers and quantified using PicoGreen (Molecular Probes), and 15 ng of DNA were used for quantitative PCR. The sensitivity of the assay is increased via amplification of large targets, thereby increasing the probability of encountering a DNA lesion. A 16,059-bp quantitative PCR product that included all but 236 bp of the NADH5/6 genes in the mtDNA was amplified using a specified primer set (38). Copy number differences in mtDNA were normalized using a short quantitative PCR, which yielded products directly related to gene copy numbers. mtDNA damage was quantified by comparing the relative efficiency of amplification of large (>15 kb) fragments of DNA and normalizing this with gene copy numbers by the amplification of smaller (<250 bp) fragments, which have a statistically negligible likelihood of containing damaged bases (38, 75).

**Statistical analyses.** Data represent means ± SE for 6 pairs of animals/group. Significant differences between treatments were obtained using paired t-tests and two-factor ANOVA. P < 0.05 was
METHODS, the mitochondria protein yield was equivalent among all groups. As mentioned in MATERIALS AND METHODS, ethanol consumption and is associated with depressed mitochondrial respiration. Slightly, but not statistically, decreased in ethanol-fed animals compared with the ethanol-only group. Ethanol consumption induced a mild inflammation that was treated ethanol group compared with the ethanol-only group.

SAM prevents alcohol-mediated decreases in mitochondria respiration. Mitochondrial dysfunction occurs after chronic alcohol consumption and is associated with depressed mitochondrial respiration and ATP synthesis. As mentioned in MATERIALS AND METHODS, the mitochondria protein yield was equivalent among all groups. Similarly, there were no differences in citrate synthase activity, a commonly used mitochondrial marker enzyme, among all groups (control, 0.244 ± 0.01 μmol·min⁻¹·mg⁻¹; ethanol, 0.273 ± 0.02 μmol·min⁻¹·mg⁻¹; control + SAM, 0.267 ± 0.1 μmol·min⁻¹·mg⁻¹; and ethanol + SAM, 0.253 ± 0.1 μmol·min⁻¹·mg⁻¹, p = 0.1). To determine whether SAM supplementation prevented chronic alcohol-mediated defects in mitochondria function, oxygen consumption in the presence of substrates (glutamate/malate or succinate) and ADP was measured in coupled mitochondria isolated from livers of animals fed control and ethanol diets with and without added SAM. State 3 respiration (ADP dependent) and the RCR were significantly decreased in animals fed ethanol compared with controls (Fig. 1, A–D). Chronic alcohol exposure also resulted in a significant decrease in cytochrome c oxidase activity (Fig. 1E). In contrast, state 4 respiration (ADP independent) was unaffected by ethanol consumption, suggesting that uncoupling does not contribute to mitochondrial dysfunction (data not shown). State 3 respiration and the RCR were largely preserved in ethanol-fed animals coadministered SAM (Fig. 1, A–D). Moreover, SAM partially prevented the alcohol-dependent loss in cytochrome c oxidase activity (Fig. 1E). This alcohol-associated defect in mitochondrial function was not accompanied by significant alterations in mitochondrial OSH levels (control, 6.9 ± 1.1 nmol/mg protein; ethanol, 8.6 ± 1.1 nmol/mg protein, control + SAM, 7.1 ± 0.7 nmol/mg protein; and ethanol + SAM, 8.5 ± 0.5 nmol/mg protein, p = 0.84). However, the coadministration of SAM prevented the ethanol-associated decrease in hepatic levels of SAM (control, 2.47 ± 0.26 mmol·liver⁻¹·g body wt⁻¹; ethanol, 1.67 ± 0.029 mmol·liver⁻¹·g body wt⁻¹; control + SAM, 2.81 ± 0.042 mmol·liver⁻¹·g body wt⁻¹; and ethanol + SAM, 2.11 ± 0.039 mmol·liver⁻¹·g body wt⁻¹, p = 0.03). Taken together, these data demonstrate that SAM supplementation prevents alcohol-dependent mitochondrial dysfunction because SAM preserved respiratory rates at control levels.

SAM prevents alcohol-dependent losses in oxidative phosphorylation proteins. It is possible that SAM maintains mitochondrial respiration through preservation against the alcohol-associated loss in oxidative phosphorylation proteins. Indeed, two-dimension BN-PAGE proteomics supported the hypothesis that SAM protects against the loss in both mitochondria- and nuclear-encoded polypeptides that comprise the oxidative phosphorylation system (75). Representative BN-PAGE proteomic maps of the oxidative phosphorylation proteins are shown in Fig. 2. From these maps, it was apparent that chronic alcohol consumption caused a loss of respiratory chain proteins (Fig. 2, A and B). This was especially true for the proteins that comprise cytochrome c oxidase (circled proteins, Fig. 2, A and B) and, to a lesser extent, those subunits that comprise complex I. Quantification of the band density within each complex revealed a significant decrease in the overall content of cytochrome c oxidase and NADH dehydrogenase (complex I) in response to chronic alcohol consumption (Fig. 2E) with only minor effects on ubiquinol cytochrome bc1 reductase (complex III) and ATP synthase (complex V). It is postulated that decreases in respiratory complex proteins contribute to the depressed state 3 respiration and cytochrome c oxidase activity in ethanol-fed animals. Whereas SAM supplementation only modestly prevented the alcohol-associated loss in complex I subunits, SAM prevented a loss in cytochrome c oxidase subunits in response to chronic alcohol (Fig. 2, C–E). Thus, changes in some of the cytochrome c oxidase subunits were verified by Western blot analysis. Immunoblot analysis indicated a 70% decrease in the level of mitochondria-encoded cytochrome c oxidase subunit I protein in mitochondria from ethanol-fed rats vs. controls, which was partially attenuated by the coadministration of SAM (Fig. 3A). Similarly, SAM also prevented the alcohol-mediated loss in two of the nuclear-encoded subunits, subunits IV and Vb (Fig. 3, C and D). SAM alone had no effect on the expression of these subunits in control animals, and ethanol and/or SAM treatment had no effect on the levels of cytochrome c (Fig. 3B). These data indicate that SAM protects at the molecular level through

Table 1. Effect of EtOH and SAM on liver parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Con</th>
<th>EtOH</th>
<th>Con + SAM</th>
<th>EtOH + SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain, g</td>
<td>118±12</td>
<td>111±13</td>
<td>115±7.3</td>
<td>99±13</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>12.7±0.6</td>
<td>13.9±0.7*</td>
<td>12.2±0.5</td>
<td>13.1±1.0</td>
</tr>
<tr>
<td>Liver-to-body weight ratio, %</td>
<td>3.3±0.08</td>
<td>3.7±0.12*</td>
<td>3.6±0.12</td>
<td>3.9±0.14</td>
</tr>
<tr>
<td>Steatosis score</td>
<td>0.0±0.0</td>
<td>1.7±0.43*</td>
<td>0.0±0.0</td>
<td>1.5±0.5</td>
</tr>
<tr>
<td>Triglycerides, μmol/liver</td>
<td>183±31</td>
<td>947±304*</td>
<td>242±25</td>
<td>804±167*</td>
</tr>
<tr>
<td>Inflammation score</td>
<td>0.3±0.2</td>
<td>1.2±0.17*</td>
<td>0.16±.16</td>
<td>0.66±0.3</td>
</tr>
</tbody>
</table>

Data represent means ± SE; n = 6 animals/group. Con, control; EtOH, ethanol; SAM, S-adenosylmethionine *P < 0.05 compared with Con; †P < 0.05 compared with Con + SAM.
preservation of the mitochondrial proteome in response to chronic alcohol consumption.

**Effect of ethanol and SAM on the mitochondrial chaperone prohibitin.** To date, the impact of chronic alcohol consumption on the mitochondrial chaperone system is undefined. The proteins prohibitin and BAP37 together form a complex within the mitochondrial inner membrane that functions in the assembly of respiratory complexes (50). Studies have shown that metabolic stress associated with mitochondrial protein synthesis inhibition causes an upregulation of prohibitin protein. It is suggested that this occurs as an adaptive response to help protect against a continued loss of mitochondrial gene products that might occur in response to the imbalance between the content of mitochondria- and nuclear-encoded subunits in the inner membrane (19, 51). Because chronic alcohol consumption induces a similar imbalance (75), we chose to examine the effect of chronic alcohol and SAM on prohibitin. Both prohibitin and BAP37 protein were increased by 50% and 40%, respectively, in mitochondria from ethanol-fed rats compared with controls (Fig. 4, A and B). In contrast, there were no differences in prohibitin and BAP37 levels in mitochondria isolated from control and ethanol-fed animals in the SAM group (Fig. 4, A and B). Chronic alcohol also had no effect on the levels of two additional mitochondria-associated chaperones, Hsp70 and Hsp60 (Fig. 4, C and D).

**Effect of SAM on mitochondria O$_2^-$ production and iNOS expression.** It is hypothesized that the effect of SAM to prevent the alcohol-associated losses in oxidative phosphorylation proteins (Figs. 2 and 3) will also attenuate chronic alcohol-associated increases in mitochondrial O$_2^-$ production. As shown in Fig. A, O$_2^-$ production was significantly increased in mitochondria isolated from ethanol-fed animals compared with controls. Mitochondria from ethanol-fed animals also generated 40% more O$_2^-$ compared with control mitochondria in

![Graph](http://ajpgi.physiology.org/)}
the presence of antimycin (Fig. 5B, inset), which is consistent with increased $O_2^-$ production related to ethanol-elicited damage to the cytochrome $bc_1$ region of complex III (4, 20). Importantly, SAM treatment attenuated the alcohol-dependent increase in mitochondrial $O_2^-$ production in both the presence and absence of antimycin (Fig. 5). Another potential source of $O_2^-$ in the chronic alcohol-exposed liver is CYP2E1 (34). Although CYP2E1 protein was induced by chronic alcohol consumption, SAM did not prevent this increase (Fig. 6A). These results indicate that the ability of SAM to attenuate oxidant production is largely mediated through its ability to preserve the functioning of the electron transport chain via preventing losses in proteins that comprise respiratory complexes (Fig. 2).

Accompanying the attenuation of mitochondrial $O_2^-$ production was the concomitant suppression of iNOS protein induction in ethanol-fed animals coadministered SAM. Immunoblot analysis indicated a 70% increase in the level of iNOS protein in livers of ethanol-fed animals vs. controls, which was largely attenuated by the coadministration of SAM (Fig. 6B). It is postulated that SAM contributes to the preservation of mitochondrial function through attenuation of iNOS, thus preventing oxidative damage to mitochondrial proteins, as well as mtDNA, from alcohol-induced increases in RNS.

**SAM attenuates chronic alcohol-mediated mtDNA damage.** We (75) have previously shown that chronic alcohol consumption induces mtDNA damage in young animals (75), which in turn is postulated to negatively impact mitochondrial protein...
synthesis and functioning of the oxidative phosphorylation system, and thus leads to increased mitochondrial O$_2$ production. The ability of SAM to prevent chronic alcohol-mediated mtDNA damage was assessed using quantitative PCR. The principle of this assay is that DNA lesions block the polymerase leading to decreased amplification of the target of interest, i.e., the mitochondrial genome. Thus, a decreased PCR product compared with control indicates mtDNA damage. There was a significant loss in the PCR product from ethanol-fed animals compared with controls, which was prevented by the coadministration of SAM (Fig. 7). There were no differences in mtDNA copy numbers among all groups (data not shown). These results are consistent with the hypothesis that chronic alcohol consumption induces damage to the mitochondrial genome and that this effect can be prevented by the coadministration of SAM.

DISCUSSION

As a source of ROS/RNS and a locus of resulting oxidative/nitrative modifications, mitochondria are recognized as critical components in the cellular stress response to toxic agents. Ethanol consumption causes increased mitochondrial O$_2$ production and iNOS induction, which in turn results in increased NO generation, an enhanced sensitivity of the respiratory chain to inhibition by NO, and the subsequent production of more toxic RNS like peroxynitrite (ONOO$^-$). This oxidative/nitrative stress contributes to mitochondrial injury, which manifests as an overall defect in oxidative phosphorylation. This causes further oxidant production and escalating levels of damage at multiple sites. The consequence of this mechanism with various feedback loops is an overall decrease in mitochondrial function that places the hepatocyte under a bioenergetic stress. This is important because a loss in the capacity for maintenance of hepatic ATP levels will ultimately predispose the tissue to permanent damage due to a depression in anabolic processes responsible for replacing damaged and/or lost cellular components. Thus, agents aimed at “short circuiting” these responses are predicted to prevent mitochondrial dysfunction and potentially delay the onset or severity of disease. In this study, we evaluated the ability of SAM to prevent several indexes of alcohol-induced mitochondrial damage.

Whereas SAM supplementation partially preserved hepatic SAM levels in ethanol-fed animals, it had only a minimal effect on preventing alcohol-induced steatosis (7, 33, 40). Because SAM did not significantly impact steatosis, it is possible that the concentration of SAM used in the present study was insufficient to alleviate the inhibitory effect of S-adenosylhomocysteine (SAH) on methyltransferase activity. SAH is formed as a by-product of methyltransferase reactions involving SAM and is increased after alcohol consumption due to the impaired activity of methionine synthase and subsequent removal of homocysteine (8, 28). It is proposed that SAH inhibits the methyltransferase that converts phosphatidylethanolamine to phosphatidylcholine. Duce and colleagues (24) demonstrated a reduction in phospholipid methyltransferase activity in human cirrhotic patients, which was also observed.
in baboons fed ethanol chronically (41). Because phosphatidylcholine is required for the synthesis and secretion of very-low-density lipoprotein, a defect in its synthesis could be expected to lead to the accumulation of triglyceride in the liver (52). It is predicted that an increased dose of SAM might have been more effective in reducing triglycerides levels via normalization of the SAM-to-SAH ratio in the liver. This is likely because betaine, a choline metabolite that remethylates homocysteine to methionine, prevents alcohol-induced steatosis in rodents when administered in much higher concentrations than were used in the present study (33).

In contrast, SAM had a profound effect to attenuate ethanol-elicited defects in the mitochondrial respiratory chain. Thus, studies were focused on elucidating the potential mitochondrial targets and pathways through which SAM works to prevent alcohol-induced mitochondrial dysfunction. An early study (27) has demonstrated that the coadministration of SAM during chronic alcohol feeding maintained mitochondrial GSH levels and preserved mitochondria functions. These results suggested that SAM protects mitochondria from ethanol-associated oxidative damage by maintaining GSH. However, several recent studies (6, 22, 45, 53, 57, 60) have demonstrated that mitochondria GSH depletion may not be a consistent feature of chronic alcohol exposure. We observed a small increase in mitochondrial GSH levels in response to alcohol that may represent an adaptive response to counteract the mild to moderate oxidative stress induced by chronic alcohol consumption (43). Similar adaptive increases in GSH have been shown in other systems of mild oxidant exposure (23). Thus, our results suggest that the protective effects of SAM on mitochondria might be mediated through effects on other mitochondrial targets, such as preservation of the proteins that comprise the oxidative phosphorylation system.

Indeed, recent evidence suggests the involvement of SAM in the regulation of mitochondrial proteins. It is possible that...
SAM maintains mitochondrial respiration by preventing alcohol-associated losses in the polypeptides that comprise the respiratory complexes (75). BN-PAGE proteomics clearly demonstrates the preservation in respiratory chain proteins in SAM-treated ethanol animals compared with the low levels detected in animals exposed to ethanol alone. This is particularly true for cytochrome c oxidase, whose activity and subunit composition were partially preserved in ethanol-fed animals coadministered SAM. Whereas the mechanism responsible for the ethanol-related decrease in mitochondria-encoded subunits has been attributed to lesions in the mitochondrial ribosome (12, 54), the decrease in nuclear-encoded subunits has only recently been recognized, and the mechanism is undefined (75). Recent work has demonstrated a coordinated downregulation of both mitochondria- and nuclear-encoded subunits of cytochrome c oxidase, specifically subunits IV and Vb, in response to hypoxia due to reduced transcription (79). Similarly, Chandel et al. (17) proposed that alterations in cytochrome c oxidase activity occur in response to hypoxia and play a role in oxygen sensing. Our findings demonstrating the preservation of these hypoxia-sensitive subunits in ethanol-fed animals coadministered SAM suggests that SAM prevents alcohol-associated mitochondria dysfunction in part through interactions with pathways involved in the cellular response to hypoxic stress.

Our results are also consistent with those demonstrating disrupted mitochondrial function in the MAT1A knockout mouse, which is deficient in SAM (59). Depletion of hepatic SAM in these mice results in decreased levels of mitochondria-encoded subunits of cytochrome c oxidase, reduced membrane potential, and alterations in prohibitin levels (59). As discussed earlier, the best-described function of the prohibitin complex is as a chaperone within the inner membrane (50). This complex stabilizes mitochondrial translation products by binding to them before the subunits are transferred to the newly assembled complex (51). According to this concept, an alteration in the level or functioning of the prohibitin complex may impact the assembly and activity of the oxidative phosphorylation system. While prohibitin levels are depressed in the MAT1A knockout mouse, we observed an upregulation in prohibitin and its binding partner BAP37 in response to chronic alcohol consumption, which was attenuated by SAM. This response appears to be specific for inner membrane chaperones because chronic alcohol consumption had no effect on the classic “heat shock” chaperones Hsp70 and Hsp60, which are associated with mitochondria (29). The upregulation of prohibitin and BAP37 suggests an attempt of the hepatocyte to “adapt” to the metabolic stress induced by chronic alcohol consumption by trying to minimize losses in mitochondria-encoded polypeptides and thus preserve mitochondrial function. Similar adaptive increases in prohibitin have been found using other models of metabolic stress (19, 51) and apoptosis (71). It is possible that the decrease in prohibitin levels observed in the MAT1A knockout mouse (59) reflects a failure of this adaptive response in more severe models of liver disease (46). Clearly, the effect of chronic alcohol and SAM on the function of prohibitin warrants further investigation.

It is well established that there are chronic alcohol-induced defects in complexes I, III, and IV due to losses in several of the polypeptides and redox centers that comprise these enzyme complexes. These defects are the result of alcohol-induced damage to the mitochondrial genome and ribosomes (12, 13, 54, 75). Decreases in the content of proteins downstream from the redox active centers of complex I and III, as well as a loss in complex IV subunits, impedes electron flow within the respiratory chain. As a consequence, the respiratory complexes
are in a more reduced state, which facilitates electron transfer to molecular oxygen to generate $O_2^-$ . This occurs at complexes I and III because they contain stable semiquinone intermediates as redox components. Thus, disruption of the electron transport chain in response to chronic alcohol consumption results in increased mitochondrial $O_2^-$ production. Recent data by Cahill and colleagues have demonstrated that the coadministration of SAM during ethanol exposure prevents the ethanol-related dissociation of mitochondrial ribosomes (69), presumably through preserving the methylation status of both tRNA and rRNA (30, 61, 62). This would have the effect of preserving mitochondrial protein synthesis and thus maintaining the integrity of oxidative phosphorylation system complexes, which was verified in our study using BN-PAGE proteomics (Fig. 2). Moreover, we observed that SAM prevented the hepatic mtDNA damage caused by chronic alcohol consumption. These results are significant because they indicate that the effect of SAM to prevent oxidative damage to mtDNA will also contribute to the preservation of the electron transport chain, which will have the ultimate effect of inhibiting further increases in mitochondrial $O_2^-$ generation. Thus, a mechanism linking the preservation of the electron transport chain system to attenuation of mitochondrial oxidant production by SAM can be proposed. In contrast, SAM had no effect on CYP2E1 levels, another source of oxidants after chronic alcohol feeding. Although a study (15) has demonstrated that SAM can inhibit CYP2E1 activity at high concentrations in vitro, it is highly unlikely that these concentrations were achieved in our study, where animals consumed $\sim 100 – 150 \mu$ mol SAM/day. CYP2E1 activity was also unaffected by SAM administration in an acute model of alcohol hepatotoxicity (66). These data support the hypothesis that alcohol consumption initiates molecular alterations to liver mitochondria, specifically the electron transport chain, that lead to the increased production of $O_2^-$, which is attenuated by SAM.

NO and its metabolites have also been linked to mechanisms of mitochondria damage (58), including that observed after chronic alcohol exposure (49, 77, 78). In the present study, chronic alcohol consumption induced iNOS protein, which is in agreement with previous work (49, 78). Furthermore, a study (44) has indicated that the protective effects of SAM may be due in part through inhibition of NO and RNS formation in the liver via the suppression of TNF-$\alpha$-mediated iNOS induction. Similarly, we observed an attenuation of iNOS induction in ethanol-fed animals coadministered SAM. Although the inhibition of NO production has been shown to exert both detrimental and beneficial effects against liver toxicity, it is generally accepted that the generation of ONOO$^-$ during conditions of excessive NO and $O_2^-$ production, as in the case of chronic alcohol exposure, would cause mitochondrial damage through the posttranslational modification of proteins (10). The interaction of NO and ONOO$^-$ with the respiratory chain may also play a role in predisposing hepatocytes to hypoxic injury during ethanol metabolism (64, 77, 78). These concepts provide a mechanism for the RNS-dependent amplification of mitochondrial dysfunction at multiple levels that contribute to chronic alcohol-associated losses in hepatocyte viability. Thus, the ability of SAM to downregulate iNOS in the alcohol-exposed liver would be expected to prevent organelle damage by modulating the interaction of NO and other RNS at the level of the mitochondrion. Specifically, it is postulated that SAM treatment may prevent the alcohol-associated increased sensitivity of mitochondrial respiration to inhibition NO through the attenuation of iNOS induction.

Although experiments were not conducted in the present study to determine the mechanism by which SAM attenuates iNOS induction by ethanol, it is predicted that SAM modulates iNOS expression via downregulation of TNF-$\alpha$ expression (74) and effects on the NF-$\kappa$B system (44). Moreover, it is now recognized that iNOS expression is induced by hypoxia (37, 73), which occurs from both acute and chronic alcohol exposure (2, 3). It is possible that in our model SAM inhibited the alcohol-mediated increase in iNOS by modulation of hypoxia in the liver. This is in part supported by the observation that SAM treatment prevented the alcohol-associated decrease in cytochrome c oxidase subunits IV and Vb, whose expression is known to be regulated by oxygen tension (17, 79). Further studies are necessary to determine the mechanism through which SAM may modulate hypoxia or hypoxia-sensitive pathways in the liver, particularly in the context of alcohol exposure.

Interestingly, these findings indicate that SAM may be functioning to preserve mitochondrial function through mechanisms independent of oxidant production. As mentioned above, it is likely that SAM supplementation is preserving transmethylation reactions of nucleic acids, proteins, and lipids. This is important because methylation of tRNA and rRNA is known to be crucial for synthesis of mitochondrial proteins. Moreover, Walker and colleagues (16, 18) have identified several respiratory chain subunits that are methylated posttranslationally. Whether SAM deficiency in response to chronic alcohol alters the methylation status of these proteins and impacts mitochondrial functioning is not known at present. Another study (9) has also indicated arginine methylation as a crucial posttranslational modification of proteins. SAM is also required for the stabilization of cystathionine $\beta$-synthase (55), thus identifying novel impacts on the transsulfuration pathway and the formation of hydrogen sulfide, which is now recognized as an important signaling molecule in mammalian systems (56, 68).

In summary, we have demonstrated that SAM prevents alcohol-dependent mitochondrial dysfunction via the preservation of mitochondrial respiration, attenuation of mitochondrial $O_2^-$ production, and maintenance of the integrity of the mtDNA. Even though the coadministration of SAM did not prevent steatosis per se, SAM had a profound effect to attenuate ethanol-elicted defects in the mitochondrial respiratory chain. These seemingly disparate results highlight the important concept that alcohol-elicted mechanisms that impair the function of the mitochondrial electron transport chain and those that initiate steatosis may not be the same, overlap, or interact. Similarly, our results underscore that SAM almost certainly works through ROS/RNS-dependent and -independent mechanisms at multiple levels and sites of action to improve mitochondria functionality. Specifically, this work reveals that SAM prevents alterations to both the mitochondrial genome and proteome induced by chronic alcohol consumption and lays the groundwork to identify novel mechanisms that link SAM-mediated protection to effects on mitochondrial function. These results predict that SAM will preserve the capacity of mitochondria to maintain ATP levels and sustain the energy-requiring processes needed to replace nucleic acids.
structural lipids, and proteins damaged in response to continued alcohol exposure. As long as ATP levels remain normal, much of the damage elicited by ethanol can be repaired, and hepatocyte necrosis will not occur. Thus, the ability of SAM to preserve mitochondrial function after early exposure to alcohol may prevent the progression to more permanent liver injury and cell death in the chronic alcohol consumer when used in combination with other therapeutic agents or strategies.

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