Research Paper

The methyl donor S-adenosylmethionine prevents liver hypoxia and dysregulation of mitochondrial bioenergetic function in a rat model of alcohol-induced fatty liver disease

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ABSTRACT

Background: Mitochondrial dysfunction and bioenergetic stress play an important role in the etiology of alcoholic liver disease. Previous studies from our laboratory show that the primary methyl donor S-Adenosylmethionine (SAM) minimizes alcohol-induced disruptions in several mitochondrial functions in the liver. Herein, we expand on these earlier observations to determine whether the beneficial actions of SAM against alcohol toxicity extend to changes in the responsiveness of mitochondrial respiration to inhibition by nitric oxide (NO), induction of the mitochondrial permeability transition (MPT) pore, and the hypoxic state of the liver.
Methods: For this, male Sprague-Dawley rats were pair-fed control and alcohol-containing liquid diets with and without SAM for 5 weeks and liver hypoxia, mitochondrial respiration, MPT pore induction, and NO-dependent control of respiration were examined.
Results: Chronic alcohol feeding significantly enhanced liver hypoxia, whereas SAM supplementation attenuated hypoxia in livers of alcohol-fed rats. SAM supplementation prevented alcohol-mediated decreases in mitochondrial state 3 respiration and cytochrome c oxidase activity. Mitochondria isolated from livers of alcohol-fed rats were more sensitive to calcium-mediated MPT pore induction (i.e., mitochondrial swelling) than mitochondria from pair-fed controls, whereas SAM treatment normalized sensitivity for calcium-induced swelling in mitochondria from alcohol-fed rats. Liver mitochondria from alcohol-fed rats showed increased sensitivity to NO-dependent inhibition of respiration compared with pair-fed controls. In contrast, mitochondria isolated from the livers of SAM treated alcohol-fed rats showed no change in the sensitivity to NO-mediated inhibition of respiration.
Conclusion: Collectively, these findings indicate that the hepato-protective effects of SAM against alcohol toxicity are mediated, in part, through a mitochondrial mechanism involving preservation of key mitochondrial bioenergetic parameters and the attenuation of hypoxic stress.

1. Introduction

Chronic and heavy alcohol (ethanol) consumption remains a significant cause of worldwide morbidity and mortality, remaining a top ten cause of preventable death in the United States [1]. Alcohol is a major causative factor for many diseases, including liver and heart disease, cancer, neurological impairments and other mental health problems, and alcohol dependence. Alcoholic liver disease is the number one cause of death from alcohol consumption [2]. Regrettably, there are still very few effective treatments for patients afflicted with this serious liver disease. As such, intensive basic science research efforts still remain at the forefront to identify the molecular underpinnings of alcohol-induced liver injury.

The etiology of alcoholic liver disease is highly complex involving different disease stages (i.e., steatosis, steatohepatitis, fibrosis, and cirrhosis), as well as disruptions in multiple liver cell types, metabolic and signaling pathways, and sub-cellular organelle function. Notably, no single underlying causative factor has been identified for alcoholic liver disease. One early target of alcohol toxicity in the liver is the mitochondrion. Our laboratory and others have reported that hepatic mitochondrial function, specifically bioenergetic function, is significantly impaired by chronic alcohol drinking in animal models. For example, chronic alcohol consumption depresses hepatic mitochondrial bioenergetics, increases mitochondrial reactive oxygen species (ROS) production, and increases sensitivity of the mitochondrial permeability transition (MPT) pore in rat and mice models of alcohol drinking [3–7]. Hepatocyte death is a direct consequence of impaired bioenergetics, as too little energy is made by mitochondria to fuel metabolism and critical cellular repair mechanisms [8]. Hepatocyte death also is a main trigger for progression from steatosis to alcoholic steatohepatitis [9]. Taken together, these findings reinforce the need to more fully understand the role of mitochondrial damage in alcoholic liver disease.

As the recognition of mitochondrial dysfunction in alcoholic liver disease has grown, there is an expanding list of pharmacological agents being tested in experimental animal models of alcohol toxicity. For example, the aldehyde dehydrogenase 2 activator, Alda-1, reverses alcohol-induced steatosis and attenuates apoptosis [10]. The mitochondrial-targeted antioxidant MitoQ reduces steatosis, mitochondrial ROS production, and ROS-dependent hypoxia inducible factor 1-alpha (HIF1α) stabilization during alcohol consumption [11]. Along these same lines, the methyl donors betaine and S-Adenosylmethionine (SAM) offer protection against alcohol hepatotoxicity, presumably by mitochondrial mechanisms [12–14]. Previously, we reported that SAM supplementation attenuates mitochondrial ROS production, mtDNA damage, and the alcohol-mediated increase in inducible nitric oxide synthase (iNOS) in the liver [13]. Consequently, mitochondrial respiratory function in liver from alcohol-fed rats is maintained by SAM supplementation. Even with this knowledge, we still have an incomplete understanding of how SAM protects against alcohol-mediated mitochondrial dysfunction. Therefore, in the present study we expanded on our previous analyses [13,15] to include a more in-depth assessment of alcohol and SAM treatment on hepatic mitochondrial bioenergetics. Accordingly, studies were undertaken to determine whether the increased sensitivity of mitochondrial respiration to inhibition by nitric oxide (NO) in liver mitochondria from alcohol-fed animals was altered by SAM treatment. Further, we assessed whether SAM supplementation attenuates alcohol-mediated increased sensitivity to undergo the MPT and/or diminishes alcohol-induced liver hypoxia.

2. Methods

2.1. Alcohol feeding protocol

Male Sprague-Dawley rats (200–220 g, Charles River Laboratories, San Diego, CA) were individually housed and acclimated to laboratory rat chow for 1 week before starting the experimental diets. Nutritionally adequate Lieber-DeCarli control and ethanol containing liquid diets [16] were purchased from Bio-Serv (Frenchtown, NJ). The ethanol diet contains 36% of total daily calories as ethanol, 35% as fat, 18% as protein and 11% as carbohydrate. Control rats were fed an identical diet except ethanol calories were substituted with dextrin maltose. Controls were pair-fed so that each pair (control and ethanol) was iso-caloric. A second set of animals were also pair-fed control and ethanol diets supplemented with SAM (0.8 mg active SAM/mL diet) as described previously [15]. Rats were maintained on diets for at least 31 days before experiments to induce fatty liver disease. Serum blood alcohol and alanine aminotransferase (ALT) levels were measured using spectrophotometric-based assays (Pointe Scientific, Inc., Canton, MI). Animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham, 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).

2.2. Liver hypoxia assessment - immunohistochemistry for pimonidazole adducts

Liver hypoxia was assessed using the hypoxia-sensitive marker pimonidazole (Chemicon International, Billerica, MA) as described in [17]. Rats were injected with pimonidazole (60 mg/kg in saline, i.p.) and after 1 h livers were harvested and processed for immunohistochemistry. Formalin-fixed sections were deparaffinized in xylene and rehydrated through incubations in graded ethanol concentrations. Liver sections were incubated with 5% (w/v) BSA in Tris Buffered Saline-Tween 20 for 10 min followed by 1:50 dilution of pimonidazole-1MAb1 conjugated with FITC for 1 h. Slides were washed, incubated for 1 h with anti-FITC conjugated with HRP, and bound antibody was visualized with DAB chromagen followed by hematoxylin nuclear counterstain. Positive pimonidazole protein adduct staining was visualized by brown staining, and the area of staining was quantified using ImageJ (National Institutes of Health, Bethesda, MD).

2.3. Liver mitochondria isolation and measurement of respiratory function

Mitochondria were prepared by differential centrifugation of liver homogenates using ice-cold mitochondria isolation buffer containing 250 mM sucrose, 1 mM EDTA, and 5 mM Tris–HCl, pH 7.5 [18]. Protease inhibitors were added to the isolation buffer to prevent protein degradation. Respiration rates were measured using a Clark-type O_2 electrode (Oxygraph, Hansatech Instruments Limited, Norfolk, UK). Mitochondria were incubated in respiration buffer containing 130 mM KCl, 3 mM HEPES, 1 mM EGTA, 2 mM $MgCl_2$, and 2 mM KH_2PO_4, pH 7.2. Respiratory function was assessed by measuring state 3 and 4 respiration rates using 15 mM succinate/5 μM rotenone and 0.5 mM ADP to stimulate state 3 respiration. Coupling was determined by calculating the respiratory control ratio, which is defined as state 3 (ADP-dependent) divided by state 4 (ADP-independent) respiration. As reported previously, mitochondrial protein yield per liver and citrate synthase activities were unaltered by ethanol, SAM, or both treatments [13,15].

2.4. In-gel activity assays for Complex I and IV

Activities of respiratory Complex I (NADH dehydrogenase) and Complex IV (cytochrome c oxidase) were measured using clear native polyacrylamide gel electrophoresis (CN-PAGE) for in-gel activity assays [19,20]. Preparation of mitochondrial samples and methods used for the separation of complexes by CN-PAGE were similar to those described previously [15, 21], except protein extracts were mixed with Ponceau S (50% w/v glycerol, 0.1% w/v) before electrophoresis. Gels were run overnight (4 °C) at constant 30 V to separate complexes. After electrophoresis, gels were incubated with substrates at room temperature and reactions were stopped by fixing gels in a 50% v/v methanol and 10% v/v acetic acid solution. Loading different amounts of protein and varying incubation times achieved optimization for visualization of in-gel activities. After optimization, samples (0.2 mg protein) from all experimental groups were run on each gel. For Complex I activity staining, gels were incubated for 5 min in 100 mL of 5 mM Tris–HCl, pH 7.4, containing 0.1 mg/mL NADH and 1.25 mg/mL nitroblue tetrazolium. For Complex IV activity staining, gels were incubated for 45 min in 100 mL of 50 mM sodium phosphate, pH 7.2, containing 0.05% w/v DAB chromagen and 50 µM horse heart cytochrome c. Following reactions and fixations, gels were stored in 10% v/v acetic acid. Gels were imaged and analyzed using a Bio-Rad Chemi-Doc Imager with Quantity One software (Bio-Rad Laboratories, Hercules, CA).

2.5. Mitochondrial permeability transition pore formation – swelling assay

MPT pore formation was assessed as the degree of calcium (Ca^{2+})-induced swelling and was measured spectrophotometrically (540 nm) as described previously [6,22,23]. The change in absorbance after Ca^{2+} addition was used to quantify degree of swelling. Isolated liver mitochondria were depleted of Ca^{2+} by 15 min incubation in Ca^{2+} depletion buffer (1 mM EGTA, 10 mM NaCl, 5 mM succinate). Mitochondria (1 mg/mL) were suspended in 1.0 mL of swelling buffer (195 mM mannitol, 25 mM sucrose, 40 mM HEPES) plus 5 mM succinate and 1 µM rotenone, pH 7.2. After 2 min incubation in the spectrophotometer, 100 µM Ca^{2+} was added to initiate MTP opening and swelling (decrease in absorbance) was monitored for 20 min.

2.6. Evaluation of nitric oxide (NO)-dependent control of mitochondrial respiration

The effect of exogenous NO on mitochondrial respiration was studied using a closed chambered oxygraph respirometer (Oroboros, Innsbruck, Austria) essentially as described in [17,24,25]. The respirometer chambers were modified to accommodate a NO sensor (ISO-NOP, World Precision Instruments, Sarasota, FL) for simultaneous real-time measurements of O_2 and NO concentrations. The O_2 sensor signal, calibrated in 100% air equilibrated respiration buffer, was converted to µM O_2 by the respirometer-specific software (DatLab) using the O_2 solubility of the buffer and daily barometric pressure. The NO sensor was calibrated before each experiment using a fast releasing NO donor, PROLI NONOate (t½=1.3 s), over a concentration range of 1.25–5.0 µM at low O_2 levels. The NO sensor signal in nA was recorded with a free radical analyzer (Apollo 4000, WPI, Sarasota FL) and converted to µM NO using the generated NO calibration curve.

For experiments, mitochondria (0.5 mg/mL) were injected into the chambers and mixed with air-equilibrated respiration buffer (∼200 µM O_2), succinate/rotenone (15 mM/5 µM), and ADP (0.5 mM) to start state 3 respiration. After a baseline rate of respiration was attained, the NO donor (PAPA NONOate, 5 µM,

t½=15 min) was added at 160 µM O_2 (∼80% O_2 saturation). This resulted in NO release and gradual inhibition of respiration. The acquired NO concentration traces were corrected for baseline and aligned to corresponding O_2 traces in order to plot the O_2 consumption rates, calculated as derivatives of the O_2 concentration versus time traces, and normalized to the maximum respiration rate, as a function of NO concentration [17].

2.7. Statistical Analysis

Two-factor ANOVA and Student's t-tests were performed using KaleidaGraph software version 4.0 (Synergy Software, Reading PA) with statistical significance set at $p < 0.05$. For ANOVA, the main effect factors are defined as diet (control vs. ethanol), SAM effect (absent vs. present), and interaction (ethanol X SAM). The sample size ranged from three to eight rats per treatment group.

3. Results

3.1. Animals and markers of liver injury

Addition of SAM to the control and ethanol-containing diets had no effect on the total amount of the liquid diets consumed by rats (data not shown), and weight gain during the feeding protocol (Weight gain: control, 105 ± 9; ethanol, 96 ± 11; control+SAM, 103 ± 9; and ethanol+SAM, 103 ± 7 g). While rats fed the ethanol diet alone gained slightly less weight than the other groups this difference was not statistically different (2-factor ANOVA: diet effect, p=0.6; SAM effect, p=0.8; interaction, p=0.60). Moreover, there was no difference in blood alcohol concentration between the ethanol and ethanol+SAM groups (Blood alcohol concentration: ethanol, 153 ± 20 and ethanol±SAM, 142 ± 18 mg/dL, p=0.70). Previously, we reported that SAM co-administration modestly decreases ethanol-mediated fat accumulation and inflammation in liver [15]. In the current study we observed similar results on histopathology (data not shown) and serum ALT levels. For example, ethanol feeding increased serum levels of ALT compared to control groups with lower ALT levels measured in the ethanol+SAM group (Serum ALT: control, 37 ± 3; ethanol 102 ± 35; control+SAM, 40 ± 2; ethanol+SAM, 67 ± 5 IU/L). Two-factor ANOVA showed a statistically significant effect of ethanol with no effect of SAM on serum ALT levels (diet effect, p=0.02; SAM effect, p=0.4, interaction, p=0.3).

3.2. SAM attenuates ethanol-mediated hypoxia

Early studies indicate that hypoxia is increased by both acute and chronic ethanol consumption and contributes to hepatotoxicity [26,27]. However, what is not known is whether the hepatoprotective effects of SAM can be linked to attenuation of ethanol-mediated hypoxia. To address this question, the hypoxia marker chemical pimonidazole was used to assess the degree of liver hypoxia. Pimonidazole is reductively activated and covalently binds to macromolecules, most likely proteins, at low pO₂ to form adducts that can be detected via an antibody directed against the pimonidazole adduct [28]. Using an immunohistochemistry approach, we observed a significant increase in pimonidazole staining (i.e., brown staining) in the liver of ethanol fed rats that extended out from the centrilobular (i.e., zone 3) region into the midzonal and periportal regions of liver as compared to control (Fig. 1A). In contrast, the extent of pimonidazole staining was significantly reduced in the ethanol+SAM group and approached control levels (Fig. 1A). Quantification of the percent area for pimonidazole staining showed approximately 25% coverage of the lobule in liver from ethanol-fed animals versus only 10% in the
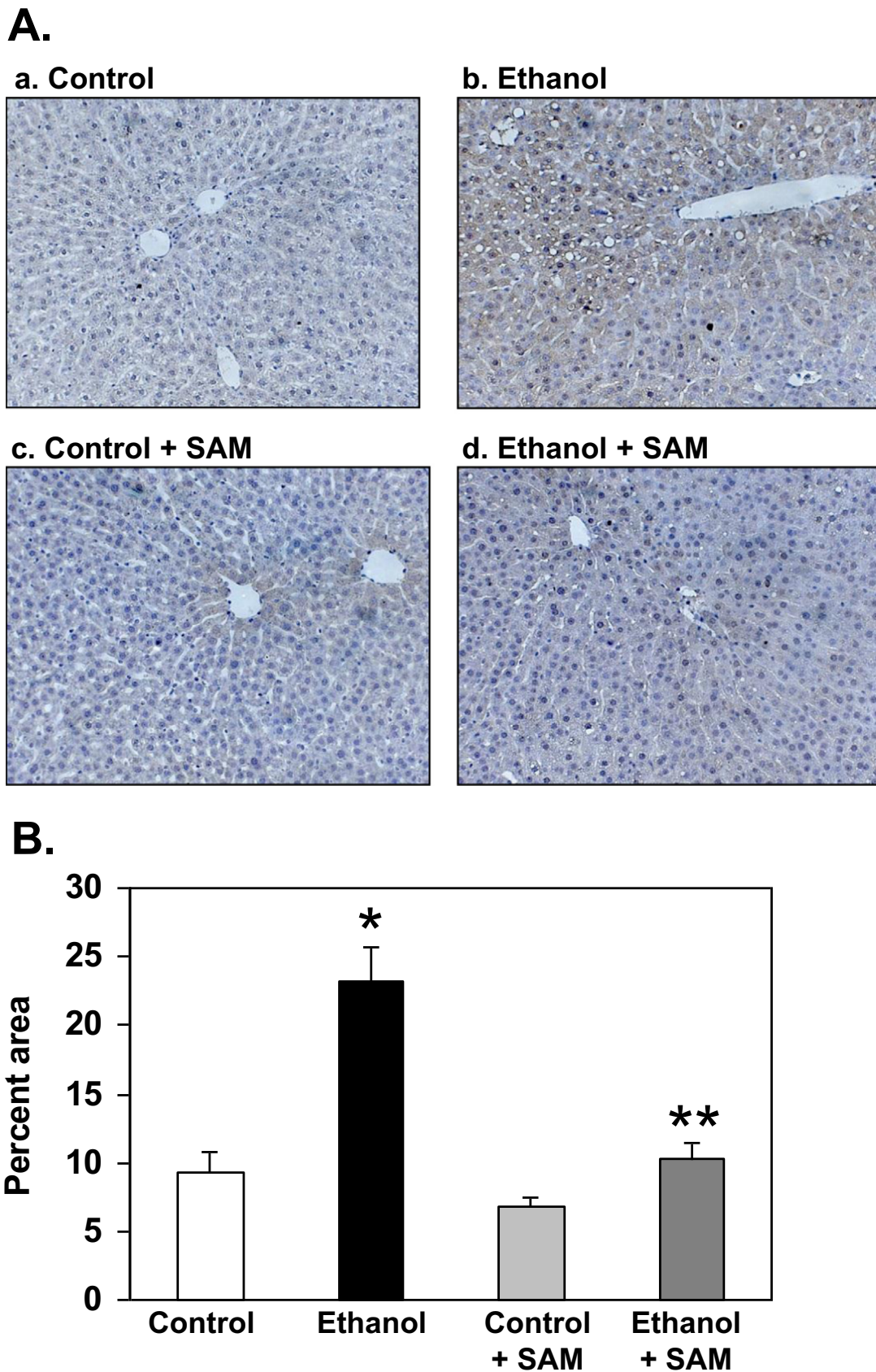
A.

a. Control

b. Ethanol

c. Control + SAM

d. Ethanol + SAM

B.

Fig. 1. *S*-Adenosylmethionine (SAM) prevents alcohol-mediated increase in liver hypoxia. (A) Representative photomicrographs showing pimonidazole adduct formation (brown staining) against hematoxylin counterstain (blue stained nuclei) in liver from rats fed control (a), ethanol (b), control+SAM (c), and ethanol+SAM (d) diets. The increased pimonidazole (brown) staining in the ethanol group (b) demonstrates increased tissue hypoxia compared to all other groups. (B) Image analysis shows increased area of pimonidazole adduct formation (*i.e.,* hypoxia) in liver from ethanol fed rats (b) compared to control (a) whereas addition of SAM to the ethanol diet prevented ethanol-mediated hypoxia (compare panels b and d). Data represent the mean $\pm$ SEM for three pairs per group. *p < 0.05, compared to corresponding control; **p < 0.05, compared to ethanol. Two-factor ANOVA; Diet, p=0.0007; SAM, p=0.0015; and Interaction, p=0.013. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
S-Adenosylmethionine (SAM) preserves liver mitochondrial respiration during chronic alcohol.

Treatment	State 3[1]	State 4	RCR[2]
Control	0.160 ± 0.009	0.046 ± 0.002	3.47 ± 0.15
Ethanol	$0.117 \pm 0.007^{*}$	0.048 ± 0.003	$2.54 \pm 0.11^{*}$
Control+SAM	$0.252 \pm 0.009^{*,**}$	$0.065 \pm 0.003^{*}$	$4.02 \pm 0.07^{*}$
Ethanol+SAM	$0.193 \pm 0.011^{**,***}$	0.052 ± 0.002	$3.61 \pm 0.05^{**,***}$
ANOVA (p values)[3]			
Diet effect	< 0.0001	0.0479	< 0.0001
SAM effect	< 0.0001	< 0.0001	< 0.0001
Interaction	0.376	0.0129	0.0126

Mitochondrial respiration was measured using the complex II-linked substrate succinate.

Data represented as mean $\pm$ SEM for n=8 animals per treatment group.

* $p < 0.05$, compared to Control.
** $p < 0.05$, compared to Ethanol.
*** $p < 0.05$, compared to Control+SAM.
[1] Oxygen utilization for state 3 and 4 respiration expressed as μg atom oxygen consumed/min/mg protein.
[2] RCR – respiratory control ratio (state 3 respiration/state 4 respiration).
[3] Results from 2-Factor ANOVA with Diet effect (control vs. ethanol), SAM effect (absent vs. present), and Interaction (Ethanol X SAM).

ethanol+SAM group (p=0.005), which was not different from controls. These results demonstrate that SAM attenuates ethanol-induced hypoxia in the liver.

3.3. SAM maintains hepatic mitochondrial respiratory capacity in rats fed ethanol

To ascertain whether SAM prevents ethanol-mediated loss in mitochondrial respiratory capacity, oxygen consumption was measured using standard respirometry techniques [18]. Using succinate, a complex II-linked oxidizable substrate, we observed a significant decrease in state 3 respiration and the respiratory control ratio (RCR) in mitochondria isolated from livers of ethanol-fed animals compared to controls (Table 1). The RCR is an indicator of the tightness of coupling between respiration (i.e., electron transport) and oxidative phosphorylation (i.e., ATP synthesis). Therefore, a decrease in the RCR indicates less coupled mitochondria and decreased ATP synthesis in liver mitochondria from ethanol-fed rats. State 3 respiration and the RCR were maintained at control levels in mitochondria from the ethanol+SAM group (Table 1).

3.4. SAM preserves respiratory Complex I and IV activities in liver of ethanol-fed rats

To determine whether maintenance of respiration by SAM in the ethanol-fed rats was linked to the respiratory complex activity, we measured Complex I and IV activities. We were able to visualize and quantify the activities of Complex I and IV of our liver
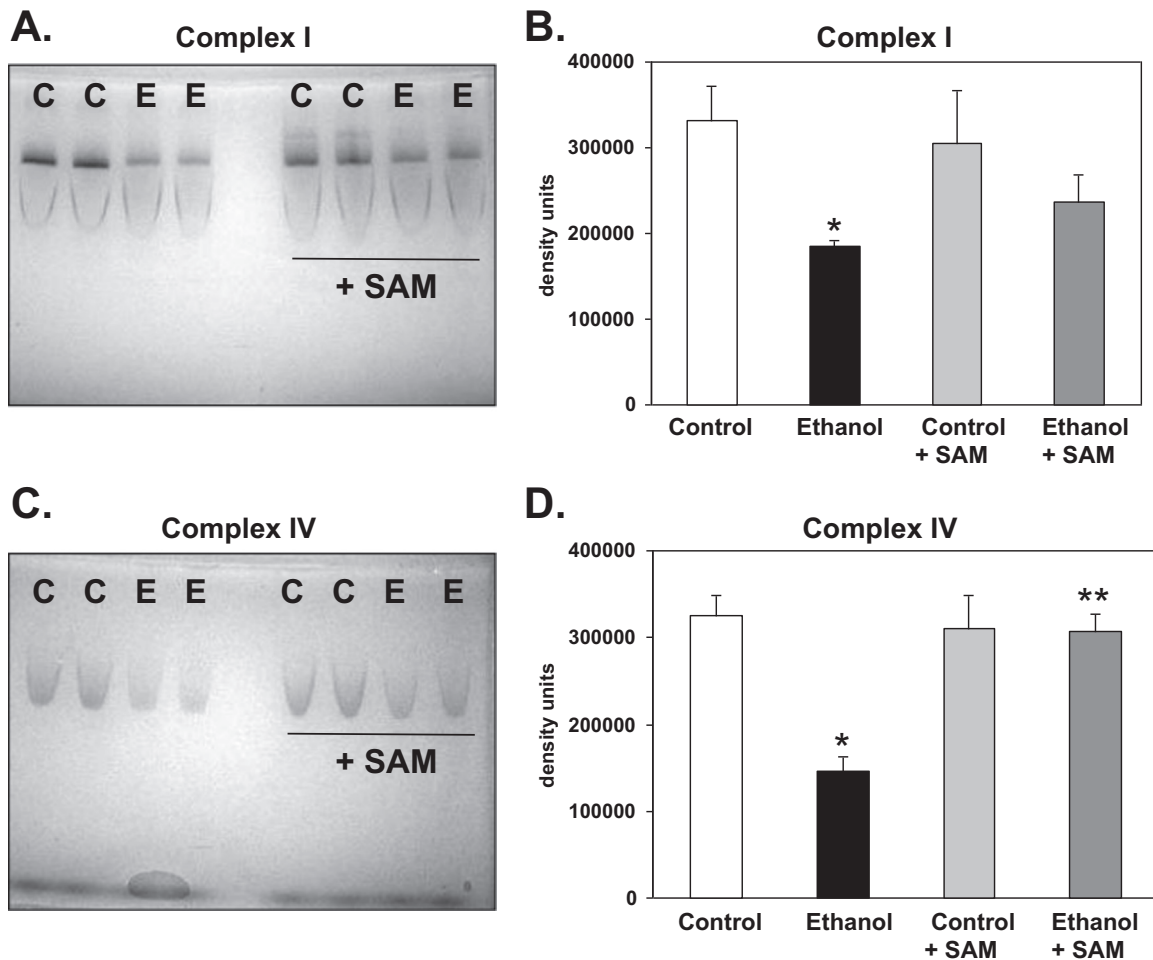

Fig. 2. S-Adenosylmethionine (SAM) maintains respiratory complex activities during chronic alcohol consumption. Liver mitochondria (0.2 mg) was separated using clear native gel electrophoresis and activities of Complex I and IV were visualized by in-gel activity assays. Representative gels showing activities of Complex I (A) and Complex IV (C) in liver mitochondria from control, ethanol, control+SAM, and ethanol+SAM treated rats. (B, D) Quantification of Complex I and IV activities within the gel. Data represent the mean $\pm$ SEM for three to four pairs per group. *$p < 0.05$, compared to corresponding control, **$p < 0.01$, compared to ethanol. Two-factor ANOVA for Complex I; Diet, p=0.020, SAM, p=0.753 and Interaction, p=0.349. Two-factor ANOVA for Complex IV; Diet, p=0.004; SAM, p=0.013; and Interaction, p=0.005.

mitochondrial samples within a native gel format using a well-established in-gel activity assay format (Fig. 2). As expected, chronic alcohol consumption alone significantly decreased the activities of Complex I and IV compared to samples from control-fed rats (Fig. 2A and C). Complex IV activity was maintained in the ethanol+SAM group and did not differ from activities measured in the control groups (Fig. 2C and D). While Complex I activity was higher in the ethanol+SAM group compared to the ethanol alone group this did not reach statistical significance (Fig. 2A and B). Together, these results support the idea that SAM supplementation prevents a loss in respiratory complex activity during chronic ethanol feeding, which preserves hepatic respiratory function of mitochondria.

3.5. SAM prevents the ethanol-mediated increase in sensitivity to undergo the mitochondrial permeability transition

To extend investigations into the effect of ethanol and SAM on mitochondrial function, experiments were performed to determine responses to Ca^{2+}-mediated induction of the MPT pore. For this, we monitored mitochondrial swelling spectrophotometrically in response to Ca^{2+}. Mitochondria from all treatment groups showed swelling (i.e., decrease in absorbance) in response to Ca^{2+} (dashed lines) with little change in absorbance observed in mitochondria incubated in the absence of Ca^{2+} (solid lines) (Fig. 3A and B). Mitochondria from ethanol-fed animals showed a greater degree of swelling compared to corresponding controls (Fig. 3A and C). Notably, this increased sensitivity to Ca^{2+}-induced swelling was absent in mitochondria from the ethanol+SAM group (Fig. 3B and C). Inclusion of cyclosporin A (CsA, 1 μM), which inhibits MPT pore opening by binding to the proposed regulatory protein cyclophilin D blocked Ca^{2+}-mediated swelling in all groups (results not shown). These results provide further evidence that the hepato-protective effects of SAM against alcohol toxicity can be attributed, in part, to preservation in various mitochondrial functions.

3.6. SAM prevents ethanol-mediated increased sensitivity to nitric oxide-mediated inhibition of respiration

To determine whether SAM preserves NO-control of respiration during chronic alcohol consumption, we assessed NO-dependent inhibition of mitochondrial respiration using high-resolution respirometry. Oxygen traces from a representative experiment using mitochondria from a control animal are shown in Fig. 4A. For these measurements, mitochondria are incubated in the respirometer chamber with succinate and state 3 respiration is initiated by the addition of ADP. When the O_2 concentration in the chamber reaches approximately 80% of the initial O_2 concentration, approximately 160 μM O_2, the NO donor PAPA NONOate was added (see arrow, panel A), which causes an inhibition of mitochondrial respiration (solid line, panel A). In the absence of PAPA NONOate, a constant rate of O_2 consumption is observed until O_2 is depleted (dashed line, panel A). In these experiments there was no difference in the amount or rate of NO produced from PAPA NONOate in mitochondria from all treatment groups indicating equal rates of NO consumption and that addition of oxyhemoglobin, a NO scavenger, restores respiration to pre-NO rates of O_2 consumption (results not shown).

To quantify the difference in sensitivity of mitochondrial respiration to NO among treatment groups, the respiration rate was calculated as the instantaneous derivative of the O_2 concentration trace (Fig. 4A) and plotted as a function of NO concentration, and recorded simultaneously by the NO sensor following the bolus addition of PAPA NONOate. As shown in Fig. 4B, mitochondria
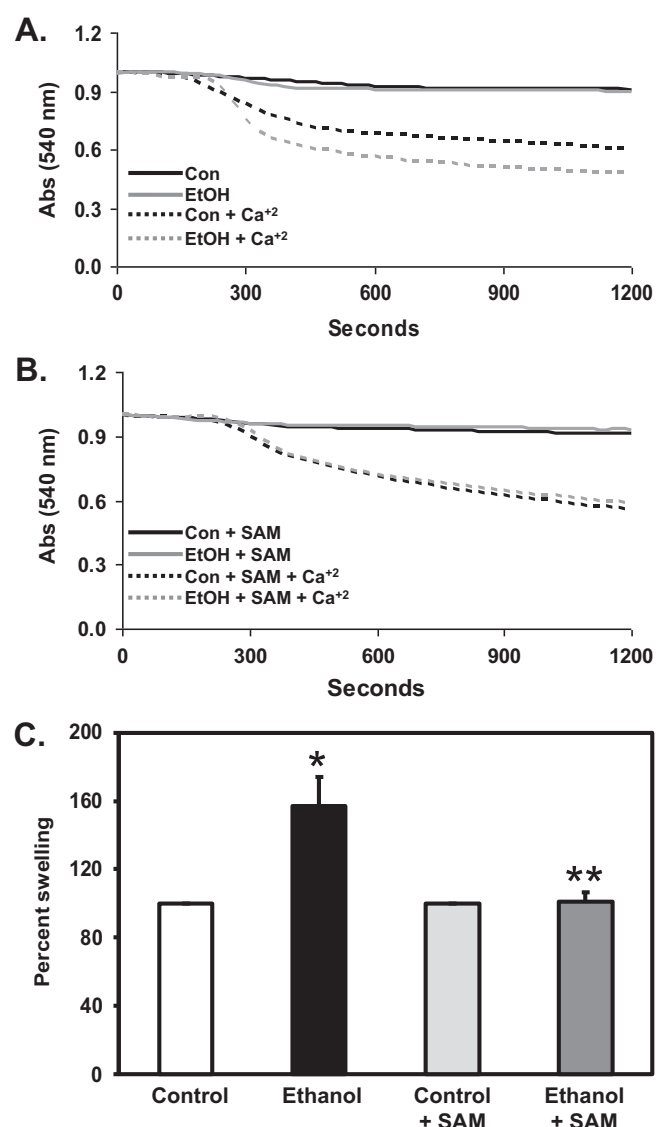

Fig. 3. S-Adenosylmethionine (SAM) prevents ethanol-associated increase in sensitivity to calcium-induced mitochondrial swelling. Liver mitochondria (1.0 mg/mL) from control and ethanol (A) and control+SAM and ethanol+SAM (B) treated animals were incubated in the spectrophotometer and calcium (Ca^{2+}) was added (dashed lines) at 2 min to induce mitochondrial swelling indicated by a decrease in absorbance at 540 nm. Solid lines are in the absence of Ca^{2+}. (C) Quantification of mitochondrial swelling. The immediate change in absorbance (slope) after the addition of Ca^{2+} was calculated and used to determine the extent of swelling. Results are presented as percent change from control set at 100%. Data represent the mean ± SEM for five pairs per group. *p < 0.05, compared to control, **p < 0.05, compared to ethanol. Two-factor ANOVA; Diet, p=0.048; SAM, p=0.023; and Interaction, p=0.043.

isolated from the livers of ethanol-fed rats were more sensitive to the inhibitory effects of NO as evident by the leftward-shift in the NO concentration versus respiration rate dose-response curve. In contrast, the NO-respiration curve for the ethanol+SAM group was not different from control. The NO concentration required to elicit a 50% inhibition in respiration (i.e., IC_{50}) decreased by 46% from 0.30 ± 0.02 μM in control to 0.16 ± 0.03 μM in ethanol. In comparison, the IC_{50} only decreased by 15% from 0.32 ± 0.02 μM in control+SAM to 0.27 ± 0.04 μM in ethanol+SAM (Fig. 4C). This result shows that SAM largely prevents the ethanol-mediated increase in sensitivity to NO-mediated inhibition of mitochondrial respiration.
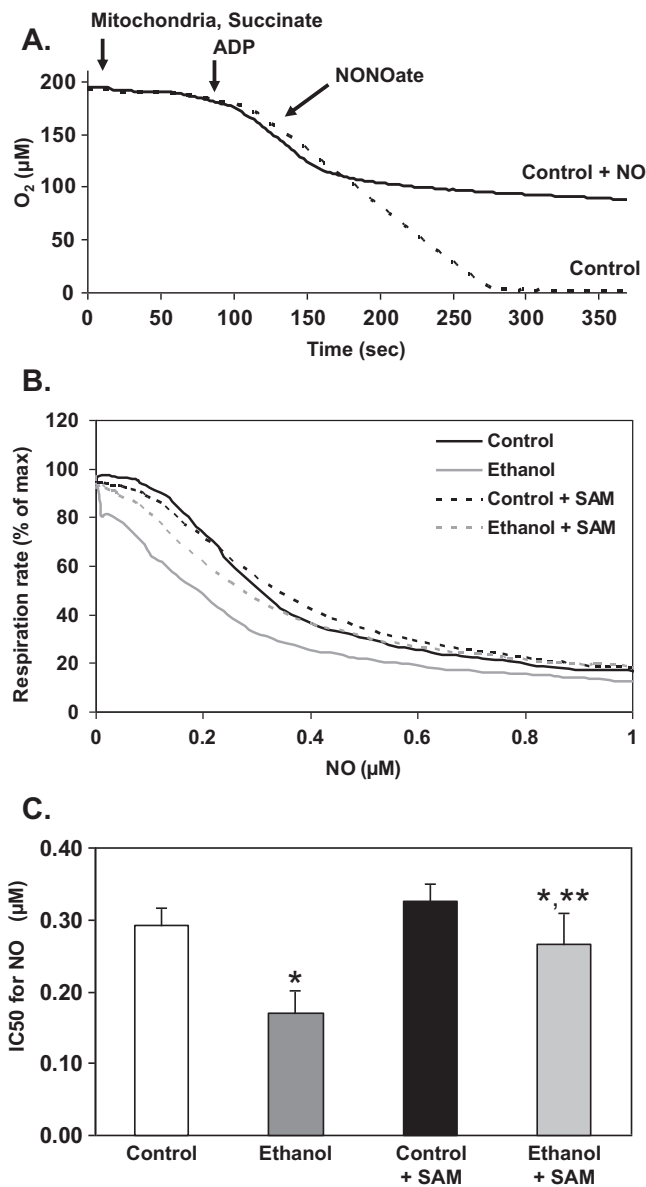
Fig. 4. *S*-Adenosylmethionine (SAM) prevents alcohol-mediated increased sensitivity to nitric oxide-mediated inhibition of mitochondrial respiration. (A) Representative respiration experiment with liver mitochondria (0.5 mg/mL) from a control fed animal incubated with succinate/rotenone (15 mM/5 μM) and ADP (0.5 mM). Oxygen concentration traces are shown in the absence (black dashed line) and presence (black solid line) of PAPA NONOate (5 μM), which was added to the respirometer chamber at approximately 80% O_2 saturation (160 μM O_2). There was no difference in the rate or amount of NO produced among the four treatment groups (*results not shown*). (B). Trace of respiration rate, % of maximum, plotted as a function of NO concentration with mitochondria isolated from liver of control (black solid line), ethanol (gray solid line), control+SAM (black dashed line), and ethanol+SAM (gray dashed line) fed rats. In these experiments, both O_2 and NO concentrations are measured simultaneously for the duration of each respiration measurement. (C) Presented is the concentration of NO that results in a 50% decrease in the mitochondrial respiration rate (inhibitory concentration 50, IC_{50}) for the four treatment groups. Note that there is a large decrease in the IC_{50} for ethanol group compared to control, which is attenuated in the ethanol+SAM group. Data represent the mean ± SEM for six pairs per group. *$p < 0.05$, compared to corresponding control, **$p < 0.05$, compared to ethanol. Two-factor ANOVA; Diet, $p=0.009$; SAM, $p=0.054$; and Interaction, $p=0.31$.

4. Discussion

Mitochondria provide the majority of energy required to fuel cellular metabolism and repair reactions in most organs. They mediate the controlled production of ROS for redox signaling,

regulate intracellular Ca^{2+} homeostasis, and serve as gatekeepers for apoptotic and necrotic cell death pathways. Further highlighting the importance of the mitochondrion in maintaining cellular physiology and health is the fact that dysregulated mitochondrial function underlies numerous diseases, including alcoholic liver disease. Therefore, improved understanding of the role of mitochondrial dysfunction in the etiology of alcoholic liver disease remains an intense area of investigation.

Confirming previous studies from our laboratory [6,7], we observed that chronic alcohol consumption disrupts several key mitochondrial bioenergetic parameters in rat liver. Chronic alcohol intake decreases mitochondrial oxygen consumption and increases the susceptibility of mitochondria to induce formation of the MPT pore, a key component of cell death programs. We also validated that supplementation of the alcohol-containing diet with SAM preserves hepatic mitochondrial respiratory function (Table 1) and cytochrome *c* oxidase activities at levels similar to those measured in livers of control rats (Fig. 2). While the molecular mechanism(s) underlying these protective effects on respiratory function is unknown, we and others have shown that SAM supplementation has a significant impact on the mitochondrial proteome [12,15], prevents alcohol-mediated mtDNA damage [13] and mitoribosome dissociation [29]. SAM prevents the alcohol-mediated decrease in select Complex I and IV subunits [13,15]. We propose this as a component of the mechanism by which SAM maintains mitochondrial respiratory during chronic alcohol consumption. We have also shown that the methyl donor betaine prevents alcohol-mediated losses in respiratory chain proteins [14]. Herein, we observed that SAM also decreases alcohol-induced liver injury as serum ALT levels were lower in alcohol-fed rats supplemented with SAM compared to ALT levels measured in rats fed alcohol alone. These findings support previous studies demonstrating that SAM and other methionine metabolism intermediates decrease alcohol-induced steatosis and liver injury [30–32].

Several investigators have described the importance of NO in alcohol hepatotoxicity. It is widely accepted that generation of the reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$) during conditions of elevated superoxide anion ($O_2^{\bullet-}$) and NO production (as in the case of chronic alcohol consumption) causes mitochondrial dysfunction through post-translational modification (*e.g.,* cysteine oxidation and tyrosine nitration) and subsequent inactivation of mitochondrial proteins [33,34]. Indeed, studies from our lab and others show oxidative modification of mitochondrial protein cysteinyl groups in the livers of rats chronically fed alcohol [35,36]. Increased mitochondrial ROS production occurs as a consequence of alcohol-mediated alterations to the respiratory chain [3,7,37] and increased iNOS occurs in response to upregulation of pro-inflammatory mediators during chronic alcohol consumption [25,38,39]. Additionally, NO is a key regulator of mitochondrial respiration through its ability to inhibit cytochrome *c* oxidase activity and O_2 consumption [40,41]. While the physiologic significance of this NO-cytochrome *c* oxidase interaction remains poorly defined, it is speculated that controlling respiratory function at the level of Complex IV can affect tissue O_2 gradients and ROS production from mitochondria [42,43]. Within this context, we hypothesize that dysregulation of the NO-mitochondria signaling axis contributes, in part, to alcohol-induced hepatotoxicity.

Previously, we reported that chronic consumption of an alcohol diet [24,39] or a high fat diet [17] increases the sensitivity of hepatic mitochondrial respiration to inhibition by NO. Importantly, this alcohol-dependent alteration in the function of Complex IV is iNOS-dependent. We found that the alcohol-dependent increase in the sensitivity of respiration to inhibition by NO is not observed in liver mitochondria isolated from iNOS knockout mice chronically fed the alcohol diet [25]. This result implicates a major role of NO

derived from iNOS in mediating this critical alteration in bioenergetic responsiveness, which we propose is due to NO-mediated posttranslational modifications to Complex IV, other regulatory components of the respiratory chain, and/or other mitochondrial proteins.

Interestingly, we have also revealed that alcohol-mediated hypoxia is attenuated in livers of alcohol-fed iNOS knockout mice [39], which again supports an important role for NO-derived iNOS in alcohol-induced hypoxic stress and injury. Herein, we show for the first time similar responses with the pharmacological intervention of SAM. Hypoxia was significantly blunted in the livers of alcohol-fed rats treated with SAM (Fig. 1). Additionally, the alcohol-dependent increase in NO sensitivity was not detected in liver mitochondria isolated from alcohol-fed rats co-administered SAM (Fig. 4). Of importance, our group and others have reported that SAM blocks hepatic iNOS induction in alcohol feeding models [13,44,45]. Similar findings (decreased iNOS) have been reported for betaine in alcohol feeding models [14] and for SAM in an LPS model of inflammation [46]. The latter study showed that SAM normalized the methylation status of the iNOS promoter, which prevented iNOS induction [46]. Whether this SAM-mediated action occurs in response to chronic alcohol feeding requires further investigation. Collectively, these data strongly support a role for iNOS and the ensuing chronic exposure to NO in alcohol-induced mitochondrial dysfunction.

Multiple studies have shown that inhibiting the ability of mitochondria to undergo MPT prevents or lessens the extent of mitochondrial and tissue damage in a large number of organs systems [47,48]. As such, we investigated whether SAM supplementation alters MPT sensitivity in liver mitochondria from alcohol-fed rats. For these studies, we used the classic Ca^{2+}-induced mitochondrial swelling assay [49]. We observed that SAM had no effect on Ca^{2+}-induced swelling in liver mitochondria from control-fed rats (Fig. 3). As expected, mitochondria isolated from the livers of alcohol-fed rats are the most sensitive group to Ca^{2+}-induced swelling. In contrast, mitochondria isolated from alcohol-fed rats treated with SAM have similar responsiveness to Ca^{2+}-induced swelling compared to mitochondria isolated from livers of both control-fed groups (Fig. 3). In addition to Ca^{2+}, oxidants can trigger and/or facilitate induction of the MPT pore. It has been proposed that, in addition to the classic regulated MPT pore model (*i.e.*, a protein pore consisting of multiple mitochondrial proteins and Cyclophilin D that is inhibited by CsA), there is a less-regulated mechanism of MPT pore formation involving aggregation of oxidized and misfolded mitochondrial membranes proteins and/or lipids [50]. Previous studies from our laboratory suggest that both MPT pore mechanisms are functioning in liver mitochondria following chronic alcohol exposure; the classic Cyclophilin D/CsA-sensitive MPT pore and the less-regulated pore consisting of oxidatively-damaged proteins and lipids [23]. Taken together, we hypothesize that SAM decreases sensitivity of the MPT pore, in part, by attenuating oxidant damage to the mitochondrion. This concept is supported by our earlier work showing that SAM normalizes mitochondrial $O_2^{\bullet-}$ production and iNOS levels in livers of alcohol-fed animals [13].

In this current study, we have provided several exciting and novel pieces of data supporting the hypothesis that SAM hepato-protection is mediated, in part, through preservation of key mitochondrial functions. Early studies on this topic examined the possibility that SAM protection is mediated by the antioxidant glutathione (GSH). Work by Fernandez-Checa and colleagues showed that chronic alcohol consumption depletes hepatic mitochondrial GSH in rodents [51–53]. Moreover, they showed that adding SAM, a GSH precursor, to the ethanol diet helped to maintain mitochondrial GSH in the liver [54]. These findings therefore supported the hypothesis that SAM protects mitochondria from

ethanol-induced oxidative stress by preserving GSH. With this said, however, the picture is not so clear, as several groups have reported that mitochondrial GSH depletion is not a consistent characteristic of chronic alcohol-induced liver injury [55–57]. For example, we found that chronic alcohol feeding in rats causes a small increase in mitochondrial GSH levels [13,58] with no effect of SAM [13]. Similar results were found with betaine [14]. In these studies, we proposed that increased mitochondrial GSH represents an adaptive physiological response to the mild oxidant stress induced by chronic alcohol consumption. Taken together, these findings suggest SAM protection is multi-faceted and mediated by both GSH dependent and independent pathways.

As discussed, SAM is the main cellular methyl donor and disruption in hepatic SAM metabolism occurs during chronic alcohol consumption [59]. It is believed that one mechanism through which SAM repletion functions to protect against toxicity and disease is by sustaining methylation of lipids, proteins, and nucleic acids. For example, Halsted and colleagues showed that decreases in hepatic SAM content, hypo-methylation of genomic DNA, and injury are absent in livers of alcohol-fed mice treated with betaine [60]. In line with the importance of nuclear DNA methylation status in determining health or disease, mounting evidence suggests that methylation of mtDNA occurs *in vivo* and is altered by disease [61]. Abnormal methylation patterns are observed in livers of nonalcoholic steatohepatitis patients and correlated with decreased mitochondrial transcripts [62]. In addition to this potential epigenetic mechanism, methylation of tRNA and rRNA is essential for mitochondrial protein synthesis and maintenance of mitochondrial function [63]. Protein methylation is a low abundance post-translational modification; however, Walker and colleagues recently identified methylated proteins within the respiratory chain [64,65]. Although no such research regarding methylation of mtDNA and/or mitochondrial proteins has been reported in experimental or clinical alcoholic liver disease, it is conceivable that alcohol-mediated SAM depletion could alter the mitochondrial 'methylome' in the liver. Therefore, future studies should be aimed at characterizing the liver mitochondrial methylome under control and alcohol-exposed conditions, and determining whether alcohol-mediated changes, if present, impact the mitochondrial bioenergetic parameters examined in this study.

5. Conclusion

In summary, these new data support the hypothesis that the mitochondrion is a central player in SAM hepato-protection. We show that SAM prevents alcohol-induced hypoxia, normalizes responsiveness for MPT pore induction, and prevents increased sensitivity to NO-mediated inhibition of respiration. As these SAM-mediated responses mirror those seen using the iNOS knockout mouse model [25,39], it is likely that NO and/or other RNS derived from iNOS mediate the damaging effects of alcohol on the mitochondrion. And, while we cannot determine from the current data whether the protective effect of SAM derives directly from blunting NO (or RNS)-mediated toxic reactions, we do know from previous work that SAM prevents iNOS induction [13]. Thus, we speculate that SAM prevents alcohol-induced mitochondrial damage by blocking iNOS induction in the liver. Although pre-clinical data like those included herein show hepato-protective actions of SAM early in the disease process, the effectiveness of SAM in treating end-stage liver disease patients with alcoholic cirrhosis is mixed [66,67]. However, as reviewed by Anstee and Day [68], only a handful of small clinical trials have been performed to assess the efficacy of SAM in treating a variety of liver diseases. Therefore, targeting methyl metabolism with SAM or related agents still remains a viable therapy for treating alcoholic liver disease.

Acknowledgments

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