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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.

Abbreviations: ALT, alanine aminotransferase; CN-PAGE, clear native polyacrylamide gel electrophoresis; CsA, cyclosporin A; GSH, glutathione; HIF1α, hypoxia-inducible factor 1 alpha; iNOS, inducible nitric oxide synthase; MPT, mitochondrial permeability transition; NO, nitric oxide; $ONOO^-$, peroxynitrite; RCR, respiratory control ratio; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAM, *S*-adenosylmethionine; $O_2^{\bullet}$-, superoxide anion

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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 dephariffinized 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].