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Role of elevated S-adenosylhomocysteine in rat hepatocyte apoptosis: Protection by betaine

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Abstract

Previous studies from our laboratory have shown that ethanol consumption results in an increase in hepatocellular S-adenosylhomocysteine levels. Because S-adenosylhomocysteine is a potent inhibitor of methylation reactions, we propose that increased intracellular S-adenosylhomocysteine levels could be a major contributor to ethanol-induced pathologies. To test this hypothesis, hepatocytes isolated from rat livers were grown on collagen-coated plates in Williams' medium E containing 5% FCS and exposed to varying concentrations of adenosine in order to increase intracellular S-adenosylhomocysteine levels. We observed increases in caspase-3 activity following exposure to adenosine. This increase in caspase activity correlated with increases in intracellular S-adenosylhomocysteine levels and DNA hypoploidy. The adenosine-induced changes could be significantly attenuated by betaine administration. The mechanism of betaine action appeared to be via the methylation reaction catalyzed by betaine-homocysteine-methyltransferase. To conclude, our results indicate that the elevation of S-adenosylhomocysteine levels in the liver by ethanol is a major factor in altering methylation reactions and in increasing apoptosis in the liver. We conclude that ethanol-induced alteration in methionine metabolic pathways may play a crucial role in the pathologies associated with alcoholic liver injury and that betaine administration may have beneficial therapeutic effects.
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1. Introduction

It has been demonstrated that ethanol administration is linked to increased hepatocyte apoptosis in both clinical and experimental alcohol-induced liver injury in a variety of species of animals including rats, mice and minipigs [1–13]. Although much progress has been made in understanding the role of critical factors that may be involved, such as cytokines and oxidative stress, there is still no unified hypothesis to explain the pathogenesis of alcohol-induced apoptosis of hepatocytes.

Our laboratory and others have established that multiple steps in the methionine metabolic cycle (Fig. 1) are

affected in the liver by ethanol consumption [14–16]. The earliest studies conducted in our laboratory showed that a major defect elicited by ethanol consumption appears to be the inhibition of methionine synthase (MS) activity, resulting in impaired remethylation of homocysteine to form methionine [3,17,18]. This defect ultimately results in an increased generation of the potentially toxic agent, homocysteine, which is released from the liver [12–14,19–21]. Ethanol consumption also results in increased hepatocellular levels of S-adenosylhomocysteine (SAH) [3,21,22]—a product of methyl transfer reactions involving S-adenosylmethionine (SAM) and the metabolic precursor of homocysteine and adenosine. Since the reaction that converts SAH to homocysteine and adenosine (catalyzed by SAH hydrolase) is reversible and proceeds toward hydrolysis only if the products are removed [15], the accumulation of homocysteine follow-

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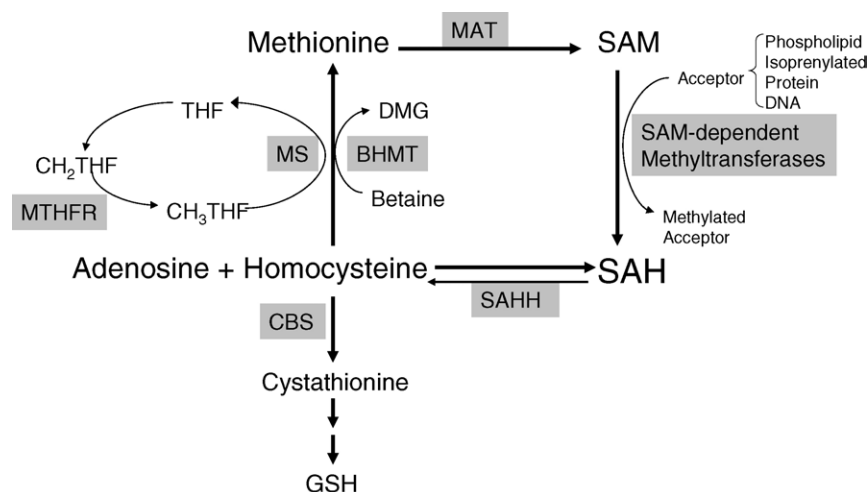 *E-mail address:* kkharbanda@unmc.edu (K.K. Kharbanda).

Fig. 1. Methionine metabolic pathway in the liver. Using ATP as a substrate, methionine adenosyltransferase (MAT) converts methionine to S-adenosyl-methionine (SAM), which then serves as a methyl-group donor substrate for methyltransferases. The other product of methyltransferase reaction S-adenosylhomocysteine (SAH) is hydrolyzed to adenosine and homocysteine by S-adenosylhomocysteine hydrolase (SAHH). The thermodynamic of this reaction favors the formation of SAH if the products are not removed. The methionine cycle is completed when homocysteine is remethylated back to methionine by B_{12}-dependent methionine synthase (MS) using 5-methyltetrahydrofolate (CH_3THF) as substrate. The latter is generated by methylene tetrahydrofolate reductase (MTHFR). Homocysteine can also be remethylated to methionine by betaine homocysteine methyltransferase (BHMT) with the formation of dimethylglycine (DMG). Homocysteine can also be catabolized through the transulfuration pathway initiated by B_6-dependent cystathionine β-synthase (CBS) to generate glutathione (GSH).

ing ethanol exposure drives SAH hydrolase to catalyze the energetically favorable reverse reaction resulting in increased SAH generation [22].

In recent years there have been reports of increased intracellular SAH levels inducing apoptosis in many different cell types [23–29]. Therefore, we put forth the hypothesis that increased intracellular SAH generation may be responsible for hepatocyte apoptosis seen after ethanol administration. To test our hypothesis, we needed to achieve elevated intracellular SAH levels in hepatocytes without ethanol exposure. We accomplished this undertaking by exploiting the unique characteristic of the enzyme SAH hydrolase—the only enzyme involved in SAH catabolism. Since SAH hydrolase catalyzes the reversible hydrolysis to adenosine and homocysteine with equilibrium of the reaction favoring the synthesis of SAH [30,31], altering the levels of its products (adenosine and/or homocysteine) has been shown to play a critical role in the control of tissue levels of SAH [24–26,32]. Therefore in this study, hepatocytes were exposed to varying concentrations of adenosine in order to promote the generation of SAH in order to increase intracellular SAH levels. Following exposure to adenosine, indices of apoptosis were determined.

In addition, our laboratory has also been interested in the therapeutic properties of betaine, a naturally occurring trimethylglycine that principally functions as an osmolyte and methyl donor [33]. As an osmolyte, betaine protects cells, proteins and enzymes from environmental stress, including extreme temperature, high salinity and low water content. As a methyl donor, betaine participates in the methionine cycle, and by remethylating homocysteine via betaine-homocysteine-methyltransferase (BHMT) has

been shown to alleviate ethanol-induced changes in intracellular SAH levels [22].

Therefore, the objective of this study was: (1) to induce elevated intracellular SAH levels in hepatocytes without ethanol intervention and observe its effect on hepatocyte apoptosis and (2) to evaluate the therapeutic affect of betaine on hepatocyte apoptosis under these conditions.

2. Materials and methods

2.1. Animals

Male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 150–175 g were used. The animals were fed the Purina 5001 diet (Ralston Purina, St. Louis, MO) and housed at the Omaha Veterans Affairs Medical Center Animal Research Facility. The care and the use of the rats was approved by the Institutional Animal Care and Use Committee in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals [34].

2.2. Hepatocyte isolation and experimental design

Hepatocytes were obtained from the livers of the rats by a modified collagenase-perfusion technique as previously described [22]. Viabilities of the different cell populations were determined by trypan blue exclusion, and only cell preparations attaining a viability of >90% were used. Isolated hepatocytes were plated (4.5 million viable

cells/dish) on 10 μg collagen IV-coated 100 mm culture dishes in Williams' medium E supplemented with antibiotics and 5% FCS. After 2 h of plating at 37 °C in a humidified atmosphere of 95% O_2/5% CO_2, the medium and unattached cells were removed and replaced with 10 ml of serum-supplemented Williams' medium E containing 0–1 mmol/L adenosine. In some dishes, 2 mmol/L betaine and/or 10 mmol/L dimethylglycine (DMG) were added just before addition of adenosine. DMG is the demethylated product of betaine formed by the BHMT catalyzed reaction and is shown to be a potent feedback inhibitor of porcine, rat and human BHMT [35]. Betaine was obtained from Danisco USA Incorporated, Ardsley, NY. All other chemicals were obtained from Sigma–Aldrich Corp., St. Louis, MO.

After overnight treatment, the following analyses were done.

Extent of apoptosis was evaluated by the measurements of the following markers of apoptosis:

Caspase-3 activation assay: Treated hepatocytes were lysed and then assayed for specific caspase activity following manufacturers' instructions using commercially available caspase-3 Ac-DEVD-AMC fluorogenic substrate (BD Biosciences, San Diego, CA). Caspase activity was evaluated by measuring the release of AMC (7-Amino-4-methylcoumarin) obtained by the cleavage of the defined synthetic peptide sequence by caspase-3 using a Perkin-Elmer Luminescence Spectrophotometer LS 50B. Free AMC obtained from Sigma–Aldrich, St. Louis, MO was used as the standard. The caspase-3 activity was expressed as pmoles of AMC produced per mg hepatocyte lysate protein.

DNA-fragmentation analysis: The treated hepatocytes were harvested, fixed in ethanol and incubated with DNA staining solution (RNAase A/propidium iodide mixture) [36]. After incubation, the nuclear DNA content in hepatocytes was evaluated using a FACSCalibur (excitation, 488; emission 575 nm; Becton Dickinson, San Jose, CA) flow cytometer. DNA fluorescence pulse processing was used to discriminate between single cells and aggregates of cells by evaluating the FL2-width versus FL2-area scatter plot. Light scatter gating was used to eliminate smaller debris from analysis. DNA content was displayed on a linear scale. Apoptosis was determined by DNA hypoploidy quantification by identifying the percentage of cells in the sub-G1 hypodiploid zone of the DNA fluorescent cytogram using Modfit analysis program obtained from Varity software house (VSH, Topsham, ME). Data was collected from 10,000 nuclei/assay.

Measurement of cell necrosis: Cytotoxicity measurements were done by determining the release of lactate dehydrogenase (LDH) in the culture medium. The leakage of LDH was measured by incubating a volume of the medium with NADH (0.2 mmol/L) and pyruvic acid (0.4 mmol/L) diluted in phosphate buffer as described [37] and expressed as % LDH release over controls.

SAH levels: Hepatocyte pellets were homogenized in 0.5 mol/L $HClO_4$, and the filtered acid extracts directly subjected to HPLC analysis for the determination of hepatocellular SAH levels following the procedure of Fu et al. [38] as detailed in our publication [22].

Homocysteine levels: Hepatocyte pellets and the conditioned media were subjected to HPLC analysis for the determination of total (hepatocellular + secreted) homocysteine [39] as detailed in our publication [22].

Statistical analysis: Data were analyzed by ANOVA, followed by Student's Newman–Keuls post hoc test. A P value <0.05 was regarded as statistically significant.

3. Results

Hepatocytes cultured in vitro overnight on collagen IV matrix in serum-supplemented Williams' medium E maintained their normal phenotype with flattened morphology. In dishes with adenosine supplementation, the hepatocytes showed changes in morphology to a more rounded appearance.

The caspases are a family of cysteine-aspartic acid-specific proteases involved in the cleavage of cellular substrates that ultimately leads to apoptosis. This family contains some members that act as signal transduction molecules, while other members carry out the executioner function in the process of apoptosis. The measurement of executioner caspase activation (caspase-3) has become a widely accepted marker of the process of apoptosis. We observed that incubation with increasing concentrations of adenosine induced a dose-dependent increase in caspase-3 activity in hepatocytes (Fig. 2). An 8–10-fold increase in its activity was seen with the highest dose of adenosine tested
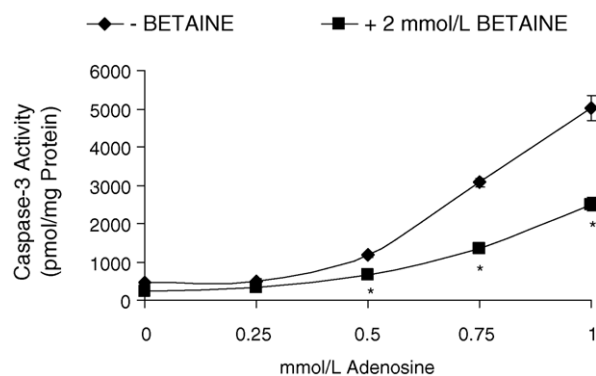
Fig. 2. Effect of adenosine and betaine on caspase-3 activity in rat hepatocytes. Isolated hepatocytes from chow-fed rats were seeded on collagen IV-coated 100 mm petri plates at 4.5 million cells/plate in Williams' medium E supplemented with 5% FCS. After 2 h of plating, the unattached cells were removed and replaced with serum-supplemented Williams' medium E containing varying amounts of adenosine (0–1 mmol/L) in the presence and absence of 2 mmol/L betaine. After overnight incubation, caspase-3 activity was determined in the cellular lysate. Data represent mean of five determinations. *P < 0.05 as compared to betaine-untreated samples.

(1 mmol/L). When betaine at 2 mmol/L was added along with adenosine, a 40–50% decrease in adenosine-induced caspase-3 activity increase was seen.

Apoptosis was also quantified by determining the extent of internucleosomal DNA cleavage or DNA-fragmentation by subjecting the treated hepatocytes to flow cytometric analysis following PI staining. By this methodology, the apoptotic nuclei stained with PI appeared as a broad hypodiploid peak that was easily distinguishable from viable nuclei with diploid DNA. DNA-fragmentation or percent apoptosis determined from the cytograms obtained from one representative experiment is shown in Fig. 3. The average of the percent apoptosis obtained from five such independent experiments are shown as Table 1. We observed low percent apoptosis to the extent of $2.6 \pm 0.5\%$ in freshly isolated hepatocytes. There was a slight enhancement of percent apoptosis in hepatocytes cultured overnight under basal conditions. Treatment with 0.75 mmol/L adenosine led to a $\sim$3-fold increase in apoptosis as compared to controls. Co-incubation with betaine led to a significant decrease in DNA-fragmentation in adenosine-exposed hepatocytes to near control levels (Fig. 3). These flow cytometric results correlated with the results obtained for caspase activities ($r^2 = 0.91$, $P < 0.05$).

Since one pathway of intracellular adenosine metabolism relates to increased intracellular SAH levels, these levels were determined in hepatocyte pellets after treatments. We observed that when rat hepatocytes were incu-

Table 1
Effects of adenosine and betaine on hepatocyte apoptosis

Treatment	Percent apoptosis[a]
Before incubation	$2.6 \pm 1.9a$
After overnight incubation with	
Media alone	$7.4 \pm 2.2a$
+Betaine (2 mmol/L)	$4.2 \pm 1.2a$
+Adenosine (0.75 mmol/L)	$19.2 \pm 4.2b$
+Adenosine (0.75 mmol/L) + betaine (2 mmol/L)	$8.6 \pm 3.1a$

Rat hepatocytes, either freshly isolated or following overnight culture in the presence and absence of 0.75 mmol/L adenosine and 2 mmol/L betaine, were fixed in ethanol, incubated with DNA staining solution and the nuclear DNA content in hepatocytes evaluated using a FACSCalibur (excitation, 488; emission 575 nm) flow cytometer. Apoptosis was determined by DNA hypoploidy quantification by identifying the percentage of cells in the sub-G1 hypodiploid zone of the DNA fluorescent cytogram using Modfit analysis program obtained from Varity software house (VSH, Topsham, ME).
[a] Values listed are means $\pm$ S.E.M. for five independent experiments. Values not sharing a common letters (a and b) are significantly different by ANOVA; $P < 0.01$.

bated with varying amounts of adenosine, a corresponding increase in intracellular SAH levels was seen (Fig. 4). A 10-fold increased level was observed with 1 mmol/L adenosine treatment. While adenosine treatment increased SAH levels, the presence of adenosine decreased the total homocysteine levels. The homocysteine values (mean $\pm$ S.E.M.) for controls were 675 ± 70 pmol/μg DNA compared to values for 0.25, 0.5, 0.75 and 1 mmol/L adenosine
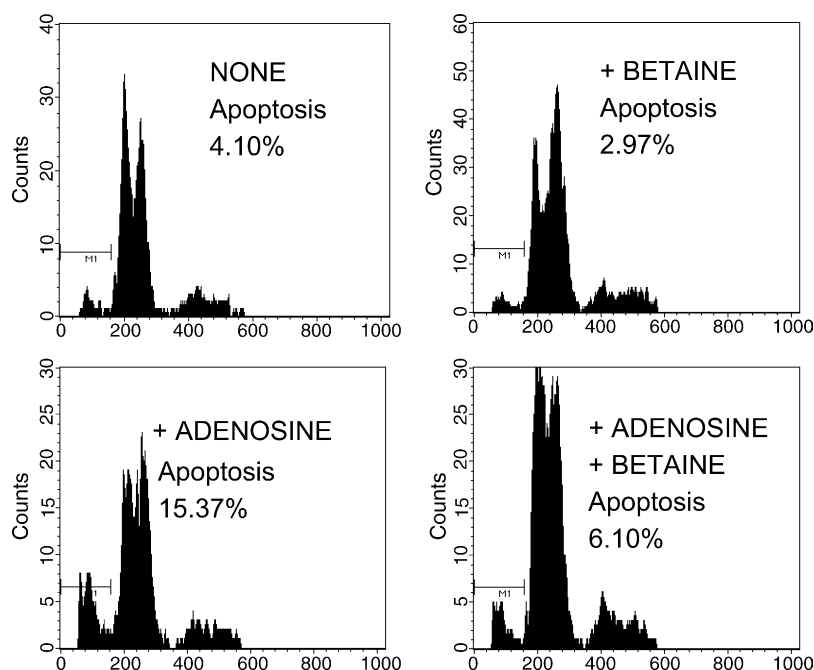

Fig. 3. Flow cytograms of DNA-fragmentation in hepatocytes: effects of adenosine and betaine. Rat hepatocytes seeded on collagen IV-coated 100 mm petri plates in Williams' medium E supplemented with 5% FCS were treated overnight in the presence and absence of 0.75 mmol/L adenosine and 2 mmol/L betaine. Following treatment, the hepatocytes were harvested, fixed in ethanol, incubated with DNA staining solution and the nuclear DNA content evaluated using a FACSCalibur flow cytometer. Apoptosis was determined by DNA hypoploidy quantification by identifying the percentage of cells in the sub-G1 hypodiploid zone (recognized at the left of the major peak, labeled M1 in the cytogram) using Modfit analysis program obtained from Varity software house (VSH, Topsham, ME). The cytograms and the percent apoptosis are from one of five independent experiments, all of which had similar results.
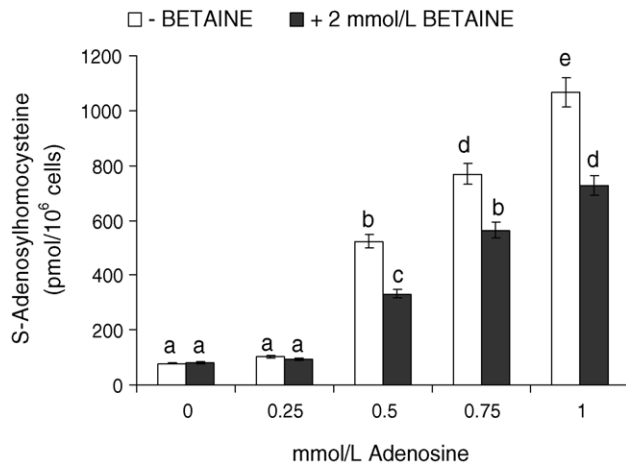
Fig. 4. Effect of adenosine and betaine on intracellular S-adenosylhomo-cysteine (SAH) levels. Isolated hepatocytes from chow-fed rats were seeded on collagen IV-coated 100 mm petri plates at 4.5 million cells/plate in Williams' medium E supplemented with 5% FCS. After 2 h of plating, the unattached cells were removed and replaced with serum-supplemented Williams' medium E containing varying amounts of adenosine (0–1 mmol/L) in the presence and absence of 2 mmol/L betaine. Following overnight incubations, the intracellular SAH levels were determined. Data represent mean of three determinations. Values not sharing a common subscript letter are significantly different by ANOVA; $P < 0.05$.

of 430 ± 122, 351 ± 174, 303 ± 177 and 292 ± 112 pmol/ μg DNA, respectively ($P < 0.05$).

We further observed that adenosine-induced increases in SAH levels were significantly decreased by 40–50% following co-incubation with betaine, indicating that betaine may be protecting against adenosine-induced apoptosis presumably via BHMT catalyzed methylation reactions to decrease SAH levels.

Therefore, to evaluate whether the protective mechanism of betaine incubation is via methylation reactions, hepatocytes were simultaneously also exposed to DMG, a potent inhibitor of BHMT activity. Addition of DMG alone to control hepatocytes or adenosine-treated hepatocytes resulted in a small increase in caspase-3 activity (Fig. 5). More importantly, it attenuated the protective effect of betaine under basal as well as under adenosine-supplemented condition.

No significant changes in LDH release under any experimental conditions were observed (data not shown).

4. Discussion

This study was conducted with the goal to causally link elevated intracellular SAH levels seen in hepatocytes following ethanol consumption to alcoholic apoptosis observed in a variety of animal species. In this study, we conducted our experiments on isolated hepatocytes obtained from chow-fed rats rather than ethanol-fed rats because we wanted hepatocytes without any previous alterations in pathways of methionine metabolism. These naïve hepatocytes were then exposed in vitro to agents that
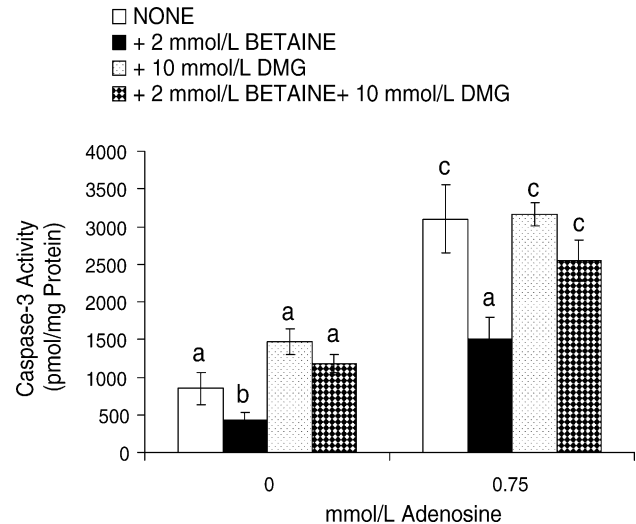

Fig. 5. Effect of adenosine, betaine and dimethylglycine (DMG) on caspase-3 activity. Isolated hepatocytes from chow-fed rats were seeded on collagen IV-coated 100 mm petri plates at 4.5 million cells/plate in Williams' medium E supplemented with 5% FCS. After 2 h of plating, the unattached cells were removed and replaced with serum-supplemented Williams' medium E in the presence and absence of 0.75 mmol/L adenosine, 2 mmol/L betaine and 10 mmol/L DMG. After overnight incubation, caspase-3 activity was determined in the cellular lysate. Data represent mean of five determinations. Values not sharing a common subscript letter are significantly different by ANOVA; $P < 0.05$.

are known to increase intracellular SAH levels by exploiting the unique characteristic of the enzyme, SAH hydrolase, which catalyzes the reverse hydrolysis of SAH to adenosine and homocysteine. Incubations with SAH-hydrolase inhibitor, or under conditions of excess adenosine and homocysteine either added alone or in combination have all been shown to generate increased intracellular SAH [24–26,32]. But in our study, we did not use homocysteine as an agent to achieve increased intracellular SAH levels but instead focused on adenosine. This agent elevated intracellular SAH without increasing either hepatocellular or secreted homocysteine levels, thus facilitating the effects of elevated intracellular SAH to be distinguished from those implicated via elevated homocysteine-induced ER stress [12,13].

The data in this study indicate that increased intracellular SAH functions as a physiological modulator of hepatocyte apoptosis. Our study also corroborates a recent publication that implicated increased intracellular SAH levels with hepatotoxicity [40] and supports of the concept that removal of intracellular SAH is a potential therapeutic option. Although the message is similar, this recent published study differs from ours in that no toxicity was observed in HepG2 on exposure to agents, such as adenosine, homocysteine and 3-deaza-adenosine (a SAH hydrolase inhibitor) that are known to increase intracellular SAH levels [40]. These HepG2 cells with elevated SAH levels, however, showed increased sensitivity to TNFα toxicity [40]. The disparity between their study and our study may be due to the fact that Song et al. conducted their

experiments in a hepatoma-derived cell line and not with hepatocytes in primary culture that were employed in the current study. Recent reports from our laboratory have also shown that exposure to 7-deaza-adenosine (another potent SAH hydrolase inhibitor) alone also induces apoptosis in rat hepatocytes in primary cultures [41].

We further show that betaine confers protection against apoptosis. This evidence is based on the following observations. First, addition of adenosine that increase intracellular SAH levels, induce increases in activity of effector caspase (caspase-3) as well as cause increases in DNA-fragmentation. A linear correlation existed between SAH levels and caspase-3 activity ($r^2 = 0.91$; $P < 0.01$). Second, betaine by decreasing intracellular SAH by virtue of remethylating homocysteine, results in significant attenuation in adenosine-induced increased caspase activities and DNA-fragmentation.

In our hands, the minimum concentration of adenosine required to observe increases in intracellular SAH levels in cultured hepatocytes was 0.5 mmol/L, which was also the minimum concentration required to observe increases in caspases activation. Earlier studies had employed much lower amounts of adenosine ($\sim$50 μmol/L) in order to achieve elevated intracellular SAH and induce toxicity in cultured mouse lymphoblasts [23]. It is possible that the use of different cell-types or animal sources may be responsible for the inconsistency observed between the two studies. This speculation is based on the fate of adenosine metabolism that relates not only to homocysteine metabolism and the ability of adenosine to form a stable complex with SAH hydrolase to inhibit SAH hydrolase activity, but also to the metabolism of adenosine within seconds by adenosine deaminase (ADA) to inosine or its phosphorylation by adenosine kinase to form 5′AMP. It is likely that lower adenosine concentration could induce toxicity in lymphoblasts in previous studies because these cells were isolated from ADA-deficient mice and therefore were probably more susceptible to lower adenosine concentrations because of its inhibited metabolism to inosine.

More recent studies using various cell types that implicate increased adenosine toxicity via increased SAH levels have employed comparable adenosine concentrations as used by us [24,26–29,42]. The mechanism of action of adenosine appears to be via its ability to act as a substrate for as well as an inhibitor of SAH hydrolase thereby elevating intracellular SAH.

Further implicating the role of elevated SAH as one of the principle factors responsible for apoptosis were the experiments conducted with betaine supplementation. Betaine by virtue of decreasing elevated SAH levels as shown in this study as well as in our previous publication [22], also significantly attenuated-induced apoptosis. Additional experiments conducted with the inclusion of excess DMG—a potent feedback inhibitor of porcine, rat and human BHMT [35]—demonstrated the mechanism of action of betaine. We observed that DMG added in 5-fold excess of the concentration of betaine partially blocked the protective effect of betaine on adenosine-induced apoptosis. This reaffirmed that the protective effect of betaine was primarily via methyl-group transfer reaction catalyzed by BHMT that alleviates the increased intracellular SAH levels by remethylating homocysteine.

The protective effect of betaine in preventing hepatocyte apoptosis has also been corroborated by reports from different laboratories in which apoptosis was induced by various conditions including hypertonicity [43], bile salts [44] and perfusion–reperfusion liver injury [45]. It is not known whether these experimental manipulations of hypertonicity, bile salts exposure and perfusion–reperfusion injury also result in alterations in methionine metabolic pathways, especially elevations of intracellular SAH levels.

Betaine has also been shown to protect against alcoholic liver injury [12,13,20,22,46–49], prevent carbon tetrachloride-induced liver injury [50] as well as to considerably decrease indices of steatosis in NASH patients [51,52]. In addition, betaine has been effectively used as a treatment regimen for patients with inherited genetic disorders of hyperhomocysteinemia such as cystathionine β-synthase deficiency and methylene tetrahydrofolate reductase deficiency [53]. Although the untreated patients with these deficiencies always present with hepatic abnormalities, of particular interest to us is the generation of animal models with these genetic deficiencies where a more detailed biochemical analysis has been done. The livers of these animals have high SAH levels [54–56], show liver injury [57] and betaine administration appears to reverse these biochemical alterations [57].

Our future studies are aimed at elucidating the mechanism by which elevated intracellular SAH promotes apoptosis. Since the homeostatic levels of SAH are critically important on account of SAH being a potent product-inhibitor of many SAM-dependent methyltransferases [58], we postulate a critical role of one or more liver methyltransferases in preventing hepatocyte apoptosis. Recent studies have shown that the carboxyl methylation reaction of small GTPases is a crucial activation step that facilitates these proteins to participate in anti-apoptotic signaling pathways [59]. We speculate that inhibition of the critical methyltransferase (such as isoprenyl cysteine methyltransferase) by elevated intracellular SAH levels may be responsible for adenosine-induced apoptosis in hepatocytes.

To conclude, we have data that provides a definite link between elevated intracellular SAH and increased hepatocyte apoptosis and presents a therapeutic approach of betaine as a modality to prevent alcoholic apoptosis. These results indicate that the elevation of SAH levels in the liver by ethanol appears to be a major factor in altering methylation reactions and in increasing apoptosis in the liver. Considering the link between hepatocyte apoptosis and liver fibrosis [60], inhibition of hepatocyte apoptosis by betaine may also be an anti-fibrotic therapeutic strategy.

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