Effect of betaine supplementation on changes in hepatic metabolism of sulfur-containing amino acids and experimental cholestasis induced by α-naphthylisothiocyanate

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Abstract

Alterations in the hepatic metabolism of sulfur amino acids in experimental cholestasis induced by α-naphthylisothiocyanate (ANIT) (100 mg/kg, po) were monitored in male mice for 1 week. We also examined the effects of betaine supplementation (1% in drinking water) for 2 weeks on the hepatotoxicity and changes in the sulfur amino acid metabolism induced by ANIT treatment. Acute ANIT challenge elevated the serum alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities, and total bilirubin contents from 5 h after the treatment, reaching a peak at t = 48–72 h. Hepatic S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) levels were decreased significantly in a manner almost inversely proportional to the changes in serum parameters measured to determine the ANIT-induced toxicity. Hepatic glutathione and cysteine levels were elevated at t = 120 h after the treatment. Betaine supplementation blocked or significantly attenuated induction of the hepatotoxicity by ANIT. The decrease in SAM and SAH levels was also inhibited by betaine intake. The results indicate that betaine supplementation may antagonize the induction of experimental cholestasis and changes in the metabolism of sulfur amino acids associated with ANIT treatment. The underlying mechanism and pharmacological significance of its action are discussed.

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1. Introduction

Normal bile formation and secretion are essential for a number of important physiological functions including fat digestion, assimilation of lipid soluble nutrients in the small intestine, protection of the small intestine from oxidative insults, excretion of endogenous and xenobiotic compounds, and maintenance of the homeostasis of cholesterol and bile acids (Tuchweber et al., 1996). Cholestasis, an arrest or stagnation in the bile flow, is one of the most common and devastating manifestations of both hereditary and acquired liver diseases, ultimately leading to cirrhosis and liver failure (Aboutwer et al., 2003). In mammals the liver has a central role in the metabolism of sulfur amino acids. The sulfur amino acid metabolism occurs primarily via the transsulfuration pathway, which results in the transfer of methionine

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANIT, α-naphthylisothiocyanate; AST, aspartate aminotransferase; BHMT, betaine homocysteine methyltransferase; GSH, glutathione; MAT, methionine adenosyltransferase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

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sulfur to serine to form cysteine (Fig. 1). The abnormal plasma clearance of methionine in human liver cirrhosis appears to be related to a decrease in hepatic levels of cysteine and its derivatives (Horowitz et al., 1981; Chawla et al., 1984). It has been suggested that the reduction of methionine adenosyltransferase (MAT) activity is responsible for the blockage of methionine metabolism in cirrhotic livers (Duce et al., 1988). It was also shown that impairment of the transsulfuration of homocysteine at the level of cystathionine degradation resulted in elevation of serum cystathionine levels in patients with hepatic cirrhosis (Look et al., 2000). Therefore, alterations in the sulfur amino acid metabolism in cirrhosis may arise at multiple levels in the transsulfuration pathway. But changes in the sulfur amino acid metabolism in intrahepatic cholestasis remain largely undefined.

Betaine, or trimethylglycine, is involved in the synthesis of methionine from homocysteine in liver. This reaction, catalyzed by betaine homocysteine methyl-transferase (BHMT), has an important role in the maintenance of hepatic concentration of methionine in mammals, especially when dietary intake of this amino acid is limited (Finkelstein et al., 1982). It has been reported that betaine has protective effects against hepatic steatosis and necrosis in experimental animals (Barak et al., 1993; Kim et al., 1998; Kim and Kim, 2002). We previously showed that betaine administration affects the metabolism of sulfur amino acids significantly (Kim et al., 2003a), which could account for the hepatoprotective effect of this substance.

In the present study we first determined the changes in hepatic sulfur amino acid metabolism for 1 week following ANIT administration to mice. ANIT has been accepted as a model hepatotoxin that produces intrahepatic cholestasis in a reproducible, dose-dependent manner in experimental animals, which is pathologically similar to drug-induced cholangiolic hepatitis in man (Goldfarb and Singer, 1962). Secondly, we examined the effects of betaine intake on the changes in the sulfur amino acid metabolism and experimental cholestasis induced by ANIT.

2. Materials and methods

2.1. Animals and treatments

Adult male ICR mice (Dae Han Laboratory Animal, Seoul, Korea), weighing 25–35 g, were used throughout the study. The use of animals was in compliance with the guidelines established by the Animal Care Committee of this institute. Animals were acclimated to temperature (22 ± 2°C) and humidity (55 ± 5%) controlled rooms with a 12-h light/dark cycle (light: 07.00–19.00, dark 19.00–07.00) for at least 1 week before use. Laboratory chow and tap water were allowed ad libitum. Betaine-dissolved tap water (1%) replaced regular tap water for 2 weeks prior to ANIT treatment (100 mg/kg, po). ANIT was dissolved in corn oil. Betaine supplementation continued until sacrifice. Daily betaine consumption calculated from the volume of water intake was approximately 3.8 g/kg body weight. Betaine supplementation had no significant effects on the body weight gains, food consumption or water intake throughout the experimentation.

2.2. Chemicals

Drugs and chemicals such as NADPH, betaine anhydrous, amino acid standards, GSH, glutathione disulfide, p-nitrophenol, aminopyrine, S-adenosylmethionine (SAM) iodide, S-adenosylhomocysteine (SAH), cystathionine, and ANIT were purchased from Sigma Chemical (St. Louis, MO, USA). p-Nitroanisole was obtained from Aldrich-Chemie (Steinheim, Germany). All other chemicals and solvents used were reagent grade or better.

2.3. Determination of ANIT-induced hepatotoxicity

Blood sample was collected by cardiac puncture from mice under light ether anesthesia. The blood samples were allowed to clot at room temperature followed by centrifugation. Activities of aspartate aminotransferase
(AST) and alanine aminotransferase (ALT) in serum were determined by using the method of Reitman and Frankel (1957). Total bilirubin contents and alkaline phosphatase (ALP) activity were measured using commercially available kits (Youngdong Pharmaceutical, Seoul, Korea). The lipid peroxidative damage in liver tissue was determined employing a thiobarbituric acid method (Ohkawa et al., 1979).

2.4. Determination of GSH, SAM, SAH, cysteine and MAT activity in liver

The liver was homogenized in a cold 1 M perchloric acid solution. Denatured protein was removed by centrifugation at 10,000 g for 10 min. Hepatic GSH, SAM, SAH and cysteine levels were determined using the methods previously employed (Kim et al., 2003b). Briefly, total GSH concentration was determined using a HPLC equipped with a fluorescence detector and 3.5 μm Symmetry C18 column (4.6 × 75 mm; Waters, Milford, MA, USA). Cysteine levels were estimated by the acid-ninhydrin method (Gaitonde, 1967). For determination of SAM and SAH, the supernatant was directly applied to a HPLC system equipped with a UV detector and TSK-GEL ODS-80TM column (4.6 × 250 mm; Tosoh, Tokyo, Japan).

The activity of MAT was estimated by quantifying SAM and SAH production (Kim et al., 2003b). The liver was homogenized in a three-fold volume of an ice-cold buffer consisting of 0.154 M KCl/50 mM Tris–HCl and 1 mM EDTA (pH 7.4). The homogenate was centrifuged at 10,000 g for 20 min. The supernatant was further centrifuged at 104,000 g for 60 min. The 104,000 g supernatant fraction was used to determine the enzyme activity. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

2.5. Determination of hepatic microsomal metabolizing enzyme activities

Hepatic microsomal drug metabolizing enzyme activities were determined using the methods described elsewhere (Kim and Kim, 1996). The concentration of cytochrome P-450 was estimated from the CO difference spectrum. The microsomal enzyme activities of p-nitrophenol hydroxylase, aminopyrine N-demethylase, and p-nitroanisole O-demethylase were determined by measuring the formation of p-nitrocatechol, formaldehyde, and p-nitrophenol, respectively.

2.6. Data analysis

All results expressed as the mean ± SEM were analyzed by a two-tailed Student’s t-test or one-way analysis of variance (ANOVA) followed by Dunnett’s test or Newman–Keuls multiple range test. The acceptable level of significance was established at p < 0.05 except when otherwise indicated.

3. Results

3.1. ANIT-induced hepatotoxicity in mice

Changes in the serum parameters were determined for 1 week following ANIT administration to mice (Fig. 2). Total bilirubin contents were increased rapidly, reaching a peak at t = 72 h (Fig. 2A). There was a significant increase in ALP activity at t = 24 h, and the increase was further enhanced until 72 h after the treatment. AST and ALT activities were also increased from t = 5 h with a maximum at t = 48–72 h (Fig. 2B). The serum parameters measured to estimate the ANIT-induced hepatotoxicity returned to normal levels at t = 168 h.

Hepatic levels of thiobarbituric acid reactive substances were measured to estimate the lipid peroxidative damage associated with ANIT treatment. There was a significant difference in the malondialdehyde formation...
between control and ANIT-treated mice (control, 120 ± 21 nmol/g liver; ANIT-treated group, 182 ± 34 nmol/g liver, \( p < 0.01 \)) when measured at 24 h after the treatment.

### 3.2. Changes in sulfur amino acid metabolism in ANIT-treated mice

Effects of ANIT treatment on hepatic levels of SAM, SAH, GSH and cysteine are shown in Fig. 3. Both SAM and SAH levels in liver were decreased in mice, reaching lowest levels at 48 h following ANIT administration (Fig. 3A and B). The hepatic MAT activity was decreased significantly from 0.17 ± 0.02 to 0.10 ± 0.01 nmol/min/mg protein \( (p < 0.01) \) at 24 h after the treatment, which appears to be responsible for the reduction in SAM and SAH levels. Hepatic GSH levels remained relatively constant, but increased markedly at \( t = 120 \) h, during the recovery phase from the liver injury (Fig. 3C). Cysteine levels in liver were increased transiently at 5 h following ANIT treatment, which returned to control at 10 h (Fig. 3D). Like GSH levels, hepatic cysteine was increased significantly at \( t = 120 \) h.

### 3.3. Effect of betaine supplementation on ANIT-induced hepatotoxicity

Effects of betaine supplementation prior to the ANIT challenge are summarized in Table 1. The ANIT-induced elevation of ALP activity and bilirubin contents was completely blocked by betaine when determined at 48 h after the treatment. The increase in ALT and AST activities was also reduced markedly.

Alterations in the levels of major metabolites in the transsulfuration pathway were determined (Table 2). Betaine intake elevated hepatic SAM and SAH levels markedly. Concentration of hepatic GSH was not altered. In mice treated with ANIT hepatic SAM and SAH levels were reduced significantly, but hepatic GSH was not affected. Betaine supplementation blocked the reduction in SAM and SAH levels induced by ANIT. The ratio of hepatic SAM/SAH was lower only in the group of mice fed betaine without ANIT challenge. Since SAM is the principal biological methyl donor and also the precursor of aminopropl groups utilized in the synthesis of polyamines (Lu, 2000), it appears that the excess SAM availability could lead to enhanced utilization of this substance via the alternative pathways.

As the ANIT-induced cholestatic injury is associated with metabolic activation of this chemical (El-Hawari and Plaa, 1977), the effects of betaine supplementation on the cytochrome P450-mediated metabolic activities were examined (Table 3). There were no differences in the contents of microsomal protein, total cytochrome P450, activities of \( p \)-nitrophenol hydroxylase, \( p \)-nitroanisole \( O \)-demethylase or aminopyrine \( N \)-demethylase between control and betaine-fed mice.

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**Fig. 3.** SAM, SAH, GSH and cysteine levels in liver following ANIT (100 mg/kg, po) administration. Value given is the mean ± SEM for more than 4 mice. **, ***Significantly different from each corresponding control (Student’s \( t \)-test, \( p < 0.01 \), 0.001, respectively).**
4. Discussion

It has been reported that administration of a sulfur amino acid, such as SAM or taurine, is effective in the treatment of cholestasis (Almasio et al., 1990; Muriel et al., 1994; Galan et al., 1999; Milkiewicz et al., 1999; Kim and Kim, 2002; Muhlfeld et al., 2003), although the mechanism of its beneficial action remains unknown. We previously demonstrated that acute treatment of mice and rats with betaine resulted in significant changes in the metabolism of sulfur amino acids (Kim et al., 1998, 2003a). In these studies betaine rapidly enhanced the transsulfuration reactions in the methionine cycle, leading to an increase in hepatic levels of methionine, SAM, and SAH, while reducing the condensation of homocysteine with serine into cystathionine. It was also shown that hepatic SAM levels were increased in rats treated with betaine-containing liquid diet for 4 weeks (Barak et al., 1993). Therefore, it was of interest to examine the effects of betaine supplementation on the changes in hepatic sulfur amino acid metabolism and experimental cholestasis induced by ANIT treatment.

In the present study acute ANIT administration resulted in a significant and prolonged decrease in hepatic SAM levels. The MAT activity was decreased to 60% of control at 24 h following ANIT treatment, which would account for the decrease in SAM levels. It has been shown that nitric oxide and reactive oxygen species switch hepatic MAT isozymes (MAT I and MAT III) to an inactive conformation though $S$-nitrosylation and oxidation of a single cysteine residue in position 121 (Avila et al., 1997; Sanchez-Gongora et al., 1997). In this study hepatic levels of thiobarbituric acid reactive substances, used as a marker of lipid peroxidation, were increased significantly in ANIT-treated mice at $t = 24$ h, which is consistent with the results reported by Ohta et al. (1999). The present results suggest strongly that oxidative stress is involved in the ANIT-induced inhibition of the MAT activity.

It was reported that induction of intrahepatic cholestasis was associated with elevation of hepatic GSH levels (Dahm and Roth, 1991; Dahm et al., 1991). In rats treated with ANIT the hepatic GSH levels were not affected initially, but elevated gradually at 20–24 h after

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Table 1
Effect of betaine supplementation on ANIT-induced cholestasis in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (units/serum µl)</th>
<th>AST (units/serum µl)</th>
<th>ALP (U/l)</th>
<th>Total bilirubin (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.7 ± 2.0a</td>
<td>68.2 ± 3.2a</td>
<td>115 ± 5a</td>
<td>0.65 ± 0.05a</td>
</tr>
<tr>
<td>Betaine</td>
<td>31.7 ± 3.0a</td>
<td>72.2 ± 11.2a</td>
<td>120 ± 10a</td>
<td>0.62 ± 0.03a</td>
</tr>
<tr>
<td>ANIT</td>
<td>1336.0 ± 341.0c</td>
<td>853.6 ± 199.5c</td>
<td>298 ± 42b</td>
<td>3.52 ± 0.31b</td>
</tr>
<tr>
<td>Betaine + ANIT</td>
<td>173.4 ± 38.1b</td>
<td>205.0 ± 31.9b</td>
<td>92 ± 4a</td>
<td>0.72 ± 0.03a</td>
</tr>
</tbody>
</table>

Mice were provided with 1% betaine in drinking water for 14 days prior to ANIT (100 mg/kg, po) administration. Animals were sacrificed 48 h after ANIT treatment. Each value represents the mean ± SEM for more than 5 mice. Values with different letters (a, b, c) are significantly different one from another (one-way ANOVA followed by Newman–Keuls multiple range test, $p < 0.05$).

Table 2
Effect of betaine supplementation on hepatic GSH, SAM and SAH levels in mice treated with ANIT

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (nmol/g liver)</th>
<th>SAM (nmol/g liver)</th>
<th>SAH (nmol/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7900 ± 260a</td>
<td>108.8 ± 4.2a</td>
<td>25.5 ± 1.2a</td>
</tr>
<tr>
<td>Betaine</td>
<td>7540 ± 290a</td>
<td>157.2 ± 3.9b</td>
<td>57.1 ± 6.3b</td>
</tr>
<tr>
<td>ANIT</td>
<td>7630 ± 830a</td>
<td>69.5 ± 1.9c</td>
<td>16.8 ± 0.2c</td>
</tr>
<tr>
<td>Betaine + ANIT</td>
<td>9220 ± 750a</td>
<td>131.2 ± 5.9a,b</td>
<td>30.2 ± 2.0a</td>
</tr>
</tbody>
</table>

Mice were provided with 1% betaine in drinking water for 14 days prior to ANIT (100 mg/kg, po) administration. Animals were sacrificed 48 h after ANIT treatment. Each value represents the mean ± SEM for more than 5 mice. Values with different letters (a, b, c) are significantly different one from another (one-way ANOVA followed by Newman–Keuls multiple range test, $p < 0.05$).

Table 3
Effect of betaine supplementation on hepatic microsomal enzyme system

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein (mg/g liver)</th>
<th>Cytochrome P-450 (nmol/mg protein)</th>
<th>$\alpha$-Nitrophenol hydroxylase (product nmol/min/mg protein)</th>
<th>Aminopyrine $N$-demethylase (product nmol/min/mg protein)</th>
<th>$\alpha$-Nitrophenol O-demethylase (product nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.3 ± 0.9</td>
<td>0.99 ± 0.05</td>
<td>3.20 ± 0.16</td>
<td>2.80 ± 0.16</td>
<td>3.77 ± 0.21</td>
</tr>
<tr>
<td>Betaine</td>
<td>11.2 ± 0.4</td>
<td>1.05 ± 0.03</td>
<td>3.59 ± 0.16</td>
<td>2.93 ± 0.21</td>
<td>3.97 ± 0.23</td>
</tr>
</tbody>
</table>

Mice were provided with 1% betaine in drinking water for 14 days prior to sacrifice. Value given is the mean ± SEM for 4 independent pooled samples each made of livers from 2 mice.
the treatment. The authors first hypothesized that the elevation of hepatic GSH by ANIT could be attributed to the inhibition of GSH efflux across the canalicular membrane of hepatocytes. However, it was observed in the following studies that ANIT treatment increased the bile GSH concentration as well (Jean and Roth, 1995; Jean et al., 1995). The authors suggested that the GSH-dependent biliary disposition of ANIT would have an important role in the induction of hepatotoxicity. The mechanism of GSH elevation by ANIT remained unexplained. In the present study the elevation of hepatic GSH and cysteine in mice was significant in a much later phase, only after the serum parameters for cholestasis and liver injury returned to normal control levels. The reason for this discrepancy in the temporal profile of GSH elevation is not known. It has been demonstrated that both the transsulfuration of methionine to cysteine and hepatic cysteine uptake from blood are enhanced in regenerating livers (Teshigawara et al., 1995; Freeman et al., 1999). Elevation of hepatic GSH and cysteine levels following partial hepatectomy has also been noted (Teshigawara et al., 1995). The present results suggest that the ANIT-induced elevation of hepatic GSH and cysteine is associated with hepatic regeneration and recovery following the liver injury.

In the present study betaine supplementation for 2 weeks blocked the elevation of serum ALP activity and total bilirubin contents completely, and significantly inhibited the increase in ALT and AST activities induced by ANIT administration. Betaine also prevented the reduction of hepatic SAM and SAH induced by ANIT treatment. It has been demonstrated that SAM administration is beneficial in the treatment of various types of liver diseases including steatosis, cholestasis, and cirrhosis (Friedel et al., 1989; Almasio et al., 1990; Muriel et al., 1994; Galan et al., 1999; Milkiewicz et al., 1999; Mato et al., 1999; Aleynik and Lieber, 2000). The protective effect of betaine against the ANIT-induced liver injury demonstrated in this study appears to be associated with the restoration of hepatic SAM levels. The exact mechanism of protection conferred by SAM still remains unclear. One possibility is that, through its role in the phospholipid sequential methylation required for optimal membrane fluidity, SAM would protect critical functions of membrane such as maintenance of Na⁺, K⁺ ATPase and transport of bile acids (Almasio et al., 1990; Galan et al., 1999). Another possibility is that enhancement of SAM production would contribute to an increase in the supply of substrates needed for the synthesis of GSH that protects the cell from reactive metabolites and reactive oxygen species (Gonzalez-Correa et al., 1997). However, Dahm and Roth (1991) observed that pretreatment of animals with a GSH depletor, such as buthionine sulfoximine, phorone or diethylmaleate, prevented induction of cholestasis and liver injury by ANIT, suggesting a causal or permissive role of GSH in the ANIT-induced liver injury. In this study also the GSH levels were not affected by betaine intake, indicating that GSH did not play a role in the inhibition of ANIT-induced cholestasis in mice supplemented with betaine.

In the meanwhile we do not exclude a possibility that betaine has direct beneficial effects on liver cells. ANIT triggers hepatocellular damage in vitro by releasing neutrophil proteases as demonstrated by an increase in activity of serine proteases, cathepsin G and elastase (Hill and Roth, 1998), suggesting that inflammation is a prominent feature of the hepatotoxicity produced by ANIT. Betaine has been identified as an organic osmolyte in Kupffer cells, diminishing lipopolysaccharide-induced cyclooxygenase-2 and prostaglandin E2 formation in a normosmotic or hyperosmotic medium (Zhang et al., 1996). It has been shown that betaine may decrease ischemia-reoxygenation injury, presumably by inhibiting Kupffer cell activation (Barak et al., 1996; Wettstein and Haussinger, 1997). Recently we observed that administration of betaine inhibited the elevation of tumor necrosis factor-α and the liver injury induced by lipopolysaccharide (Kim and Kim, 2002), which also supports the speculation that the hepatoprotective effects of betaine may result from inhibition of Kupffer cell activation.

Betaine is widely distributed both in animal tissues and plants. Intake of this substance at high levels would be a common practice to a large number of populations. In patients with homocysteinuria, an inborn error of methionine metabolism, a daily dose of 6–9 g betaine is indicated (Wilcken et al., 1983). The daily consumption of betaine in this study was approximately 3.8 g/kg body weight, approximately 30 times greater than the dose consumed by human patients. But all the parameters determined in this study were unaffected by betaine intake, suggesting that normal physiology and biochemistry of mice are not likely to be influenced at the dose used.

In conclusion the present study shows that ANIT treatment causes significant alterations in the metabolism of sulfur amino acids in addition to induction of cholestatic and necrotic damage in liver, which are all blocked or depressed by betaine supplementation. Alleviation of the ANIT-induced liver injury in betaine-fed mice appears to be accounted for by the elevation of hepatic SAM levels. It is suggested that betaine may be used as a possible alternative to SAM in the treatment of liver diseases. SAM is highly expensive, unstable, and not easily taken up by hepatocytes. On the other hand, betaine is easily transported into hepatocytes through amino acid transporters, and also elevation of SAM by betaine is highly organ-selective because distribution of BHMT is mainly localized in liver (Mckeever et al., 1991). Further study is being conducted in this laboratory to clarify how betaine intake elevates hepatic
SAM levels, and also to examine the pharmacological significance of its effects on chronic liver injury.

References


