S-Adenosylmethionine prevents hepatic tocopherol depletion in carbon tetrachloride-injured rats

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ABSTRACT

In various experimental models, S-adenosylmethionine (SAMe) has been shown to reduce liver injury by preventing depletion of glutathione, one of the antioxidant systems that plays a critical role in defence against oxidative stress. On the other hand, α-tocopherol may be decreased in liver diseases, and treatment with this vitamin reduces liver injury in CCl4-treated rats. Since there is a close relationship among the different antioxidant systems (mainly glutathione, α-tocopherol and ascorbic acid), we have assessed whether, as well as restoring hepatic glutathione content, SAMe has any effect on liver α-tocopherol and ascorbic acid levels in CCl4-injured rats. Four groups of seven male Wistar rats treated for 9 weeks were studied: rats induced to cirrhosis with CCl4, rats induced to cirrhosis plus SAMe administration (10 mg [kg]−1 [day]−1) and their respective controls. Liver samples were obtained for measuring levels of glutathione, α-tocopherol, ascorbic acid and thiobarbituric acid-reactive substances (TBARS), and hydroxyproline concentration as an index of collagen content. The hydroxyproline content was higher in CCl4-injured rats than in the control group (4.4 ± 1.8 and 1.1 ± 0.3 μmol/g respectively; P < 0.05). In CCl4-injured rats, SAMe administration decreased collagen content (2.7 ± 1.0 μmol/g; P < 0.05) and TBARS, and corrected glutathione depletion. α-Tocopherol was significantly lower in CCl4-injured rats than in controls (17.3 ± 4.9 and 23.0 ± 4.0 μmol/g respectively; P < 0.05). By contrast, α-tocopherol levels were similar (23.8 ± 5.1 μmol/g) in CCl4-injured rats receiving SAMe and in controls. In CCl4-injured rats, liver ascorbic acid was decreased in comparison with controls (4.9 ± 1.8 and 8.2 ± 1.0 μmol/g respectively; P < 0.05), levels which were not replenished by SAMe (4.6 ± 0.4 μmol/g). In conclusion, SAMe not only decreases fibrosis and protects against hepatic glutathione depletion, but has a further antioxidant effect of preventing α-tocopherol depletion in CCl4-injured rats.

INTRODUCTION

S-Adenosylmethionine (SAMe), a cellular metabolite resulting from the activation of methionine by SAMe synthetase, is the principal methylating agent in a variety of transmethylation reactions [1,2] and plays a key role in cellular sulphur metabolism, as well as being a precursor of glutathione (GSH) [3]. In various experimental models SAMe has been shown to decrease liver injury by preventing depletion of reduced GSH [4–7], one of the

Key words: ascorbic acid, CCl4, glutathione, lipid peroxidation, vitamin C, vitamin E.
Abbreviations: SAMe, S-adenosylmethionine; TBARS, thiobarbituric acid-reactive substances.
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Antioxidant systems other than GSH may also play a role in preventing lipid peroxidation under experimental and clinical conditions. In this respect, $\alpha$-tocopherol (vitamin E) may be decreased in liver diseases [14–18], particularly in alcoholics [15,16], and treatment with this vitamin reduces liver injury in CCl$_4$-treated rats [19]. Ascorbic acid (vitamin C) is also a powerful antioxidant [20]. Both $\alpha$-tocopherol and ascorbic acid are naturally occurring free-radical scavengers [21] which protect cell membranes against toxic agents such as CCl$_4$ and alcohol. They are active in different media: while tocopherol is located in the lipid membrane environment, ascorbate is hydrophilic. Moreover, ascorbic acid can restore the antioxidant properties of oxidized vitamin E, suggesting that a major function of vitamin C is to recycle the vitamin E radical [21]. In this respect, an in vivo sparing action of vitamin C has been demonstrated [22]. GSH also maintains tissue ascorbic acid levels [23].

Given the close relationship among these antioxidant systems (GSH, $\alpha$-tocopherol and ascorbic acid), we have assessed whether, as well as correcting hepatic GSH levels, SAMe has any effect on the liver content of $\alpha$-tocopherol and ascorbic acid in CCl$_4$-injured rats.

**MATERIALS AND METHODS**

**Chemicals**

Vitamin E ($\alpha$-tocopherol), tocopheryl acetate, ascorbic acid, o-phthaldehyde and hydroxyproline were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ascorbate oxidase spautula and reduced glutathione were from Boehringer Mannheim G.m.b.H. (Mannheim, Germany). SAMe, in the stable form of sulphate $p$-toluenesulphonate, was kindly provided by Europharma (Madrid, Spain). Tocopherol and tocopheryl acetate standards were adjusted for concentration by spectrophotometry, and the method was initially calibrated by means of the standard reference material SRM 968b from the National Institute for Standards and Technology (Gaithersburg, MD, U.S.A.). All other reagents were of analytical grade or better.

**Animals and experimental design**

The study was performed in 28 male Wistar rats, of body weight $250 \pm 3.5$ g, which were fed a standard diet ad libitum (Purina Chow A03; Panlab, Barcelona, Spain). Animals were housed in metabolic stainless steel cages in a room with a controlled 12 h light/dark cycle at 22 °C. Cirrhosis was induced in two groups of seven rats by intraperitoneal injection of 0.5 ml of CCl$_4$, diluted 1:1 (v/v) in vegetal oil, twice a week for 9 weeks. One group of these rats also received SAMe (intramuscular; 10 mg/kg body weight; daily) during the study. Two additional groups of seven rats, a control group and another which received SAMe only, were also studied. The animals received humane care in compliance with our institution’s criteria for the care and use of laboratory animals.

At the end of the study, rats were killed by cardiac puncture and exsanguination. The liver was removed immediately and prepared for analytical measurements or frozen immediately in liquid nitrogen and stored at −80 °C for further determinations. Some samples were fixed in 4% formaldehyde and embedded in paraffin for histological analysis. Other liver samples were processed for measurement of levels of $\alpha$-tocopherol, ascorbic acid, GSH and thiobarbituric acid-reactive substances (TBARS) (as a measure of lipid peroxidation), and of hydroxyproline content as a measure of liver collagen. Plasma samples were also collected from each animal.

**Biochemical determinations**

Liver $\alpha$-tocopherol concentrations were measured by HPLC after tissue homogenization in cold acetone according to the method of Shearer [24]. Total ascorbic acid, including ascorbic acid and dehydroascorbic acid, was measured by HPLC using the method of Speck et al. [25] after o-phenylenediamine derivatization to allow fluorimetric detection. The total GSH concentration was determined by the method of Hissin and Hill as described previously [26], and the TBARS concentration in liver homogenates was measured by HPLC after deproteinization of tissue homogenates with phosphoric acid according to the method of Bird et al. [27]. The hydroxyproline concentration in liver homogenates was measured by Pico-Tag HPLC (Waters, Milford, MA, U.S.A.) using phenylisothiocyanate to produce the phenylthiothiocabamyl amino acids after overnight tissue hydrolysis in 6 mol/l HCl [28]. All liver measurements were expressed per g of wet liver tissue. HPLC analyses were performed on a Waters Chromatographic system (Waters, Milford, MA, U.S.A.) equipped with two M510 pumps, a gradient AGL 560 controller, a WISP 712 automatic sampler, and a M440 fixed-wavelength detector or a M480 fluorescence detector. The chromatograms were recorded and calculated using the computer-based program Baseline (Waters). Standard liver function tests, including aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase activities, were measured as an index of hepatic cellular necrosis,
using a DAX-72 analyser (Bayer Corp., Barcelona, Spain).

**Histological analysis**
Sections of 4 μm thickness were stained with haematoxylin/eosin and Masson’s trichrome, coded for blind reading and classified as normal liver, fibrosis and cirrhosis. Fibrosis was graded from 0–3 as follows: 0, no fibrosis; 1, sinusoidal or perivenular fibrosis; 2, fibrotic septa between portal tracts; 3, cirrhosis.

**Statistical analysis**
Results are expressed as means ± S.E.M. One-way ANOVA was used to assess differences between groups, and the Student–Newman–Keuls test was used for multiple comparisons. Fisher’s exact test was used to analyse differences in categorical variables. Correlations between continuous variables were evaluated by linear regression analysis. A P value of ≤ 0.05 was considered significant.

**RESULTS**
After 9 weeks of treatment, all CCl4-injured rats had developed cirrhosis, while this was only observed in two out of seven CCl4-injured rats treated with SAMe (P = 0.02). The remaining CCl4-injured rats receiving SAMe had different degrees of liver damage and fibrosis, but not cirrhosis (sinusoidal or perivenular fibrosis in two cases, and septal fibrosis in three cases). Liver histology was normal in the control groups. These results were consistent with the hepatic hydroxyproline levels, which were significantly increased in CCl4-injured rats in comparison with control rats. SAMe treatment significantly decreased hepatic hydroxyproline levels in CCl4-injured rats (Figure 1). Aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase levels were also increased in CCl4-injured rats, effects which were partially prevented by SAMe (Table 1).

The hepatic GSH concentration was significantly diminished in CCl4-injured rats with respect to controls, but normal levels were observed in rats receiving CCl4 and SAMe (Table 1). Hepatic TBARS were increased in CCl4-injured rats, effects which were neutralized by SAMe (Table 1). Moreover, the hydroxyproline content was correlated inversely with GSH concentration (r = −0.75, P < 0.001), and directly with TBARS levels (r = 0.63, P = 0.001).

The liver α-tocopherol concentration was significantly lower in rats induced to cirrhosis than in controls (17.3 ± 4.9 and 23.0 ± 4.0 μmol/g respectively; P < 0.05). By contrast, the liver α-tocopherol level was similar in CCl4-injured rats treated with SAMe (23.8 ± 5.1 μmol/g) and in control rats. SAMe administration was not associated with increased α-tocopherol levels in non-injured rats (Figure 2). Furthermore, the α-tocopherol level was correlated directly with GSH content (r = 0.38, Table 1).

**Figure 1** Effects of SAMe on liver fibrosis, measured as hepatic hydroxyproline content, in CCl4-injured and control rats
F = 13.4, P < 0.0001; *P < 0.05 compared with other groups.

**Figure 2** Effects of SAMe on hepatic α-tocopherol content in CCl4-injured and control rats
F = 3.04, P = 0.04; *P < 0.05 compared with other groups.

**Table 1** Effects of SAMe on biochemical parameters of liver disease and on hepatic glutathione and TBARS levels in CCl4-injured and control rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Control + SAMe</th>
<th>CCl4</th>
<th>CCl4 + SAMe</th>
<th>F; P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (units/l)</td>
<td>46 ± 5</td>
<td>69 ± 7</td>
<td>2351 ± 876*</td>
<td>932 ± 140</td>
<td>5.9; &lt; 0.01</td>
</tr>
<tr>
<td>ALT (units/l)</td>
<td>29 ± 4</td>
<td>41 ± 4</td>
<td>1179 ± 506†</td>
<td>557 ± 86</td>
<td>4.9; &lt; 0.01</td>
</tr>
<tr>
<td>AP (units/l)</td>
<td>252 ± 24</td>
<td>270 ± 16</td>
<td>540 ± 98*</td>
<td>370 ± 37</td>
<td>5.8; &lt; 0.01</td>
</tr>
<tr>
<td>Glutathione (μmol/g)</td>
<td>5.9 ± 0.6</td>
<td>6.5 ± 0.9</td>
<td>4.2 ± 0.4*</td>
<td>5.8 ± 0.5</td>
<td>15.9; &lt; 0.0001</td>
</tr>
<tr>
<td>TBARS (nmol/g)</td>
<td>36.4 ± 12.9</td>
<td>23.5 ± 6.3</td>
<td>60.7 ± 7.5*</td>
<td>35.1 ± 5.9</td>
<td>18.5; &lt; 0.0001</td>
</tr>
</tbody>
</table>
The results of the present study indicate that, in CCl₄-injured rats, as well as decreasing liver fibrosis and lipid peroxidation and increasing hepatic GSH levels (as reported in other studies [29,30]), SAMe administration prevents hepatic vitamin E depletion without having any effect on liver vitamin C content. Therefore, in rats induced to cirrhosis, SAMe administration results in normal levels of major known natural antioxidants, such as GSH and vitamin E, which are commonly diminished in acute and chronic liver damage generated by various toxic agents. By contrast, SAMe had no effect on ascorbic acid levels in injured animals, although it increased the levels of this vitamin in control rats, thus suggesting that this agent may enhance the antioxidant reserves under non-toxic conditions. Indeed, apart from the beneficial effects of SAMe in CCl₄-injured rats, this agent also had some effect in normal rats, since the lipid peroxidation levels (measured as TBARS) were significantly lower and vitamin C levels significantly higher, with a trend of higher GSH levels, in normal rats treated with SAMe. These effects of SAMe on the antioxidant systems in the liver may explain in part the favourable effects on liver fibrosis, since, in addition to the well known association between GSH and lipid peroxidation, close correlations have been found between both vitamin E and vitamin C and TBARS, thus indicating that the protective effect on liver fibrosis is secondary to the reduced lipid peroxidation. In this respect, α-tocopherol can inhibit stimulation of collagen synthesis by fibroblasts through decreasing lipid peroxidation [31]. On the other hand, low GSH [8,32] and vitamin E levels have been found in chronic liver diseases, particularly in cirrhosis of different aetiologies [14–18]. Low blood vitamin C levels have also been found associated with liver diseases [33–35]. Moreover, vitamin E-deficient animals are more susceptible to oxidative damage [36]. In CCl₄-induced liver damage, several studies have demonstrated that a high hepatic vitamin E content may prevent the acute liver damage induced by the toxin [19,37]. In addition, several clinical and experimental studies suggest that a vitamin E intake greater than the recommended dietary allowance may be of benefit in the prevention or treatment of several pathological conditions [38]. It is also known that, following dietary supplementation, the liver content of vitamin E increases only in hepatocytes and not in non-parenchymal cells [39]. The major role of this vitamin involves its ability to protect cell membranes from damage mediated by lipid peroxidation. This concept is supported by a large number of studies showing that α-tocopherol, as well as its synthetic derivatives, is able to prevent the onset of cell damage consequent to the induction of oxidative stress [19,40–42]. In fact, because of its lipophilic properties, vitamin E is expected to be highly effective in protecting against membrane lipid peroxidation by reacting with lipid peroxyl and alkoxyl radicals [43].

Ascorbic acid serves as both an antioxidant and a pro-oxidant. In fact, excess amounts of vitamin C may act as a pro-oxidant in the presence of metals such as iron and copper by generating cofactors of activated oxygen radicals during the promotion of lipid peroxidation. Indeed, ascorbate is related to the promotion of collagen synthesis in some experimental models. As an antioxidant, vitamin C exerts a sparing effect on the antioxidant action of vitamin E and selenium. GSH acts as an antioxidant by serving as a substrate for several enzymes that reduce hydrogen peroxide and organic peroxides, and by mediating the reduction of dehydroascorbate [44,45] and the oxidized forms of α-tocopherol. Thus GSH deficiency would be expected to produce effects that also result from deficiency of ascorbate and α-tocopherol. Furthermore, GSH deficiency decreases tissue ascorbate. On the other hand, ascorbate protects against GSH deficiency and spares GSH in various rat models. Likewise, ascorbic acid, which alone is a moderately effective antioxidant, may interact with vitamin E, thus enhancing the antioxidant activity of the latter. The lack of an effect of SAMe on ascorbate levels in CCl₄-injured rats could be explained in terms that ascorbate is used for regenerating α-tocopherol. In this respect, SAMe administration was associated with increased ascorbate levels in healthy rats.

Our findings confirm that SAMe may lead to an
increase in GSH levels, which could prevent inactivation of SAMe synthetase and inhibit lipid peroxidation, and consequently attenuate the development of liver fibrosis and cirrhosis. Moreover, SAMe prevents hepatic vitamin E depletion in this model of CCl4-induced liver damage, thus supporting the view that SAMe not only increases GSH levels, but also spares α-tocopherol, in the livers of rats induced to liver injury by CCl4 intoxication. This may be an additional means of preventing lipid peroxidation and liver fibrosis, and provides a rationale for using SAMe as a treatment for chronic liver disease, particularly in alcoholic liver disease which is characterized by low hepatic levels of antioxidants such as vitamin E and GSH.

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