

POLYENYLPHOSPHATIDYLCHOLINE CORRECTS THE ALCOHOL-INDUCED HEPATIC OXIDATIVE STRESS BY RESTORING S-ADENOSYLMETHIONINE

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Abstract — Aims: Since the late stages of alcoholic liver injury are associated with decreased activity of methionine adenosyltransferase (MAT), we wondered whether this already occurs at the early stages and what is the mechanism involved. **Methods:** Sprague–Dawley rats ($n = 32$) were pair-fed ethanol (36% of energy) or isocaloric carbohydrates (control) in Lieber–DeCarli liquid diets, with or without polyenylphosphatidylcholine (PPC). **Results:** After 2 months, there was a striking depletion of S-adenosylmethionine (measured by high-performance liquid chromatography) from 68.2 ± 5.1 to 36.2 ± 3.4 nmol/g, associated with a reduction in hepatic reduced glutathione (GSH) from 4.95 ± 0.20 to 4.09 ± 0.08 $\mu\text{mol/g}$, and an increase from 0.24 ± 0.02 to 0.47 ± 0.07 nmol/g of 4-hydroxynonenal (4-HNE), a reliable marker of lipid peroxidation. Hepatic S-adenosylmethionine (SAME) correlated positively with GSH ($r = 0.5916$) and negatively with 4-HNE ($r = -0.6375$). Feeding PPC corrected all values and MAT activity did not differ significantly between groups. **Conclusions:** SAME depletion occurs already after 8 weeks of alcohol feeding and is fully corrected by PPC, in parallel with the prevention by PPC of the alcohol-induced oxidative stress. Since phosphatidylcholines (PCs) are produced in the liver via methylation of phosphatidylethanolamine by SAME, it is likely that PPC, by providing PCs, decreases the utilization of SAME and thereby contributes to its restoration, with replenishment of GSH and correction of the alcohol-induced oxidative stress.

INTRODUCTION

In chronic alcoholic liver disease (ALD), the synthesis of S-adenosylmethionine (SAME) is significantly impaired (Horowitz *et al.*, 1981; Barak and Beckenhauer, 1988; Martin-Duce *et al.*, 1988; Lieber *et al.*, 1990), but it is not known whether this occurs already at the early fatty liver stage. ALD is characterized by the excessive generation of free radicals contributing to oxidative stress, resulting in a decrease of hepatic reduced glutathione (GSH), a major intracellular antioxidant (Shaw *et al.*, 1981; Jewell *et al.*, 1986). Synthesis of GSH *de novo* includes cysteine as the rate-limiting amino acid, and SAME provides cysteine via a transsulfuration pathway, serving as GSH's ultimate precursor (Fernandez-Checa *et al.*, 1990). Chronic alcohol consumption also leads to the depletion of hepatic SAME in experimental animals (Lieber *et al.*, 1990; Barak *et al.*, 1993) but, at early stages of ALD, data are controversial (Barak *et al.*, 1987). The depletion of SAME in ALD could result either from decreased formation, e.g. associated with impairment of methionine adenosyltransferase (MAT) (EC 2.5.1.6), also called SAME-synthetase, as observed in patients with cirrhosis (Martin-Duce *et al.*, 1988), or from increased SAME consumption for transsulphuration (e.g. GSH synthesis) or for various transmethylation.

A mixture of polyenylphosphatidylcholines (PPC) corrects the oxidative stress and the hepatic GSH depletion resulting from long-term alcohol consumption in non-human primates (Lieber *et al.*, 1997) and at earlier stages of ALD in rats (Aleynik *et al.*, 2000), but whether PPC affects hepatic levels of SAME remains unknown. In the present study we explored a rat model of alcohol-induced fatty liver to answer these questions.

MATERIALS AND METHODS

Materials

SAME, S-adenosylmethionine, GSH, butylated hydroxytoluene, O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride, N,N-diisopropylethylamine and N,N-dimethylformamide were purchased from Sigma Chemical Co. (St Louis, MO, USA); Sylon BFT kit [solution of N-bis(trimethylsilyl)-trifluoroacetamide with 1% of trimethylchlorosilane] was purchased from Supelco (Bellefonte, PA, USA); N-(1-pyrenyl) maleimide was purchased from Aldrich (Milwaukee, WI, USA); phospholipase C from *Bacillus cereus* was purchased from Boehringer (Mannheim, Germany); and pure standards of phosphatidylcholine (PC) molecular species were purchased from Avanti Lipids (Alabaster, AL, USA). All other organic solvents were obtained from J. T. Baker Co. (Phillipsburg, NJ, USA). Deuterated 4-hydroxynonenal (4-HNE) ($^2\text{H}_3$ -4-HNE) was a kind gift from Dr F. J. G. M. van Kuijk. The PPC was kindly donated by Rhône-Poulenc Rorer & Co. (Cologne, Germany).

Animals and diets

Thirty-two male rats of a Sprague–Dawley strain [CRL:CR(SD)BR], divided into four groups, were fed Lieber–DeCarli diets containing either ethanol or dextrose, with PPC or safflower oil (2.1 g/1000 kcal) for 8 weeks. The diets were purchased from Dyets Inc. (Bethlehem, PA, USA) and contained 1 kcal/ml, with 18% of energy as protein, 35% as fat, and 47% as carbohydrate in the controls, or 11% as carbohydrate in the rats fed ethanol (36% of energy). One rat escaped at the end of the experiments. PPC was given in the diet at a concentration of 3 g/1000 kcal. PPC contained 42% of dilinoleoyl-PC (18:2-18:2 PC), 24% of palmitoyl-PC (16:0-18:2 PC), 10% of oleoyllinoleoyl-PC (18:1-18:2 PC), 8% of linolenoyllinoleoyl-PC (18:3-18:2 PC), 6% of stearoyllinoleoyl-PC (18:0-18:2 PC), about 2% of palmitoyl-oleoyl-PC (16:0-18:1 PC) and stearoylarachidonoyl-PC (18:0-20:4 PC), as well as

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several other minor fractions, each less than 1%. The day before death, the animals received one-third of their daily ration in the morning and two-thirds in the evening.

SAMe assay

SAMe was measured in the liver by a high-performance liquid chromatographic (HPLC) method (Wise *et al.*, 1997) with some modifications, including the use of *S*-adenosylethionine as an internal standard.

MAT assay

Liver cytosol was obtained by conventional differential centrifugation of liver homogenates and was incubated with 5 mM methionine according to Huang *et al.* (1998). The formed SAMe was measured by the HPLC procedure of Wise *et al.* (1997). MAT activity was expressed as the amount of SAMe produced per milligram of protein per minute.

Parameters of oxidative stress

4-HNE was measured by the GC/MS method of van Kuijk *et al.* (1995) with slight modifications. GSH was determined by HPLC with fluorometric detection according to Winters *et al.* (1995).

Statistical analysis

Statistical significance was assessed by *t*-test and one-way analysis of variance (ANOVA) with *post hoc* Student–Newman–Keuls multiple comparison test. To estimate correlations, Pearson’s linear coefficients were calculated. A *P* value of <0.05 was considered significant.

RESULTS

As described before, a fatty liver developed in animals consuming alcohol as part of a standard liquid diet (Lieber and DeCarli, 1970), an effect shown to be largely prevented by PPC (Navder *et al.*, 1997). This was accompanied by a striking depletion of hepatic SAMe from a control value of 68.2 ± 5.1 to 36.2 ± 3.4 nmol/g ($P < 0.001$, Fig. 1), and PPC

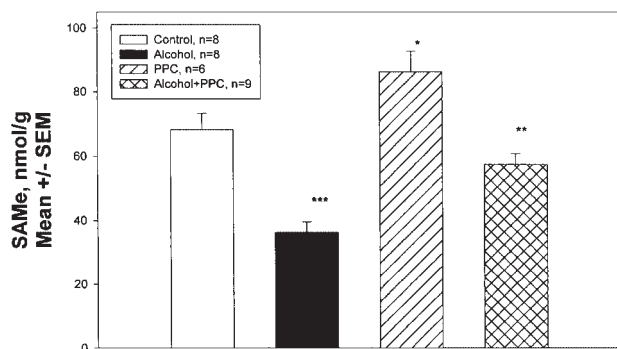


Fig. 1. Effect of alcohol and/or polyenylphosphatidylcholine (PPC) on hepatic S-adenosylmethionine (SAMe).

In rats fed a liquid diet with 36% of energy as ethanol for 8 weeks, hepatic SAMe was significantly decreased. Addition of PPC to the diet attenuated the ethanol effect. Given with the control diet, PPC also resulted in a significant increase of SAMe. **P* < 0.05, ****P* < 0.001 vs control; ***P* < 0.01 vs alcohol.

prevented this effect (57.4 ± 3.4 nmol/g, not significantly different from control). PPC, given without alcohol, also significantly increased hepatic SAMe to 86.2 ± 6.6 nmol/g ($P < 0.05$, Fig. 1). GSH was reduced from 4.95 ± 0.20 to 4.09 ± 0.08 μ mol/g ($P < 0.01$) in alcohol-fed rats, and its level was restored by PPC to 4.86 ± 0.15 μ mol/g. PPC feeding significantly increased GSH in control animals to 5.55 ± 0.21 μ mol/g ($P < 0.05$, Fig. 2).

Alcohol resulted in an increase of hepatic 4-HNE from 0.24 ± 0.02 to 0.47 ± 0.07 nmol/g ($P < 0.001$) and this effect was completely abolished by PPC (0.22 ± 0.03 nmol/g, $P < 0.001$, Fig. 3). In contrast, MAT activity remained unchanged in all groups of animals (Fig. 4). In addition, there was a significant negative correlation of hepatic SAMe with 4-HNE ($r = -0.64$, $P = 0.0001$) (Fig. 5A) and a positive correlation with GSH ($r = 0.59$, $P = 0.0005$) (Fig. 5B).

DISCUSSION

The present study revealed that already at an early stage of ALD, hepatic SAMe was decreased, without any reduction in

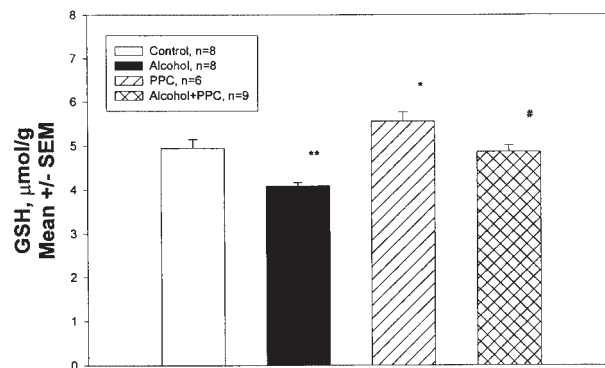


Fig. 2. Effect of alcohol and/or polyenylphosphatidylcholine (PPC) on hepatic reduced glutathione (GSH).

Ethanol feeding increased the oxidative stress, documented by a decrease of hepatic GSH. Incorporation of PPC in the diet fully restored GSH levels. PPC also significantly increased GSH in the liver of control animals. **P* < 0.05, ***P* < 0.01 vs control; #*P* < 0.01 vs alcohol.

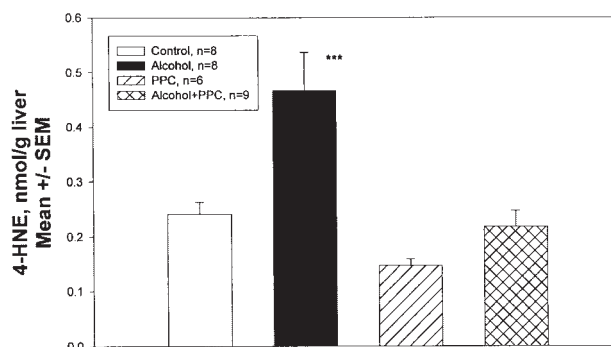


Fig. 3. Effect of alcohol and/or polyenylphosphatidylcholine (PPC) on hepatic 4-hydroxynonenal (4-HNE).

Alcohol feeding for 8 weeks increased lipid peroxidation, as assessed by hepatic 4-HNE. Addition of PPC to the diet prevented this rise. ****P* < 0.001 vs control or alcohol + PPC.

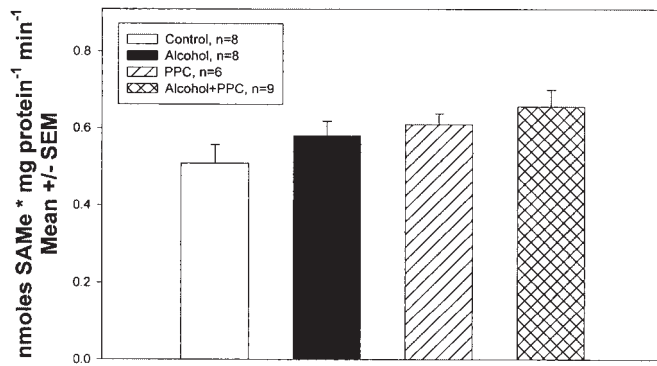


Fig. 4. Effect of alcohol and/or polyenylphosphatidylcholine (PPC) on methionine adenosyltransferase (MAT) activity in the liver.

Rats were fed control or alcohol containing liquid diets, with or without PPC, for 8 weeks. MAT activity was not affected by either ethanol, PPC or their combination.

MAT activity. Levels of SAME correlated significantly with parameters of oxidative stress, such as 4-HNE (Fig. 5A) and GSH (Fig. 5B), suggesting a causal link, corroborated by the fact that PPC administration corrected both the ethanol-induced oxidative stress and the SAME depletion.

SAME synthesis from methionine and ATP is catalysed by MAT. Unlike the diminished activity reported in the advanced cirrhotic stage of ALD (Martin-Duce *et al.*, 1988), we found no decrease in MAT activity at the earlier stage of liver disease in the model studied here (Fig. 4), suggesting that alcohol-induced SAME depletion was caused by another mechanism, possibly increased SAME consumption. Indeed, lipid peroxidation plays a major role in the development of ALD,

and enhanced oxidative stress increases GSH consumption. As a compensatory mechanism, more SAME is converted to cystathionine and subsequently to cysteine to form GSH (*vide supra*). However, since SAME synthesis, as assessed by the MAT reaction, remained unchanged, net SAME depletion may have resulted (Fig. 1). The strong positive correlation between SAME and GSH and the negative one between SAME and 4-HNE (Fig. 5) are consistent with, and strengthen, the possibility that it is the alcohol-induced oxidative stress which results, at least in part, in an increase of GSH consumption, associated with SAME depletion. Some other data are also corroborative. It is known that cystathionine β -synthase (EC 4.2.1.22) is activated by oxidative stress. In such a case, more SAME will be metabolized through transsulphuration, resulting in a decrease of the SAME pool (Taoka *et al.*, 1998).

Synthesis of GSH is highly dependent on availability of intracellular cysteine and, in the liver, cysteine originating from transsulphuration of SAME is of particular importance. In chronic liver disease, the decrease in GSH was attributed, in part, to diminished synthesis of SAME (Lu, 1998) and supplementation with SAME normalized GSH levels in both animal models and patients (Lieber *et al.*, 1990; Corrales *et al.*, 1992; Mato *et al.*, 1997). Therefore, one could anticipate a parallelism in the changes of SAME and GSH. This hypothesis is strengthened by known effects of alcohol and/or PPC on hepatic GSH levels in rats (Aleynik *et al.*, 2000) and, at more advanced stages of ALD, in baboons (Lieber *et al.*, 1997). In the present study, changes of SAME levels in the various experimental groups (Fig. 1) show the same trend as for GSH (Fig. 2). Furthermore, hepatic SAME and GSH levels were strongly correlated (Fig. 5B) and the feeding of PPC resulted in a significant blockade of the alcohol-induced SAME decrease (Fig. 1).

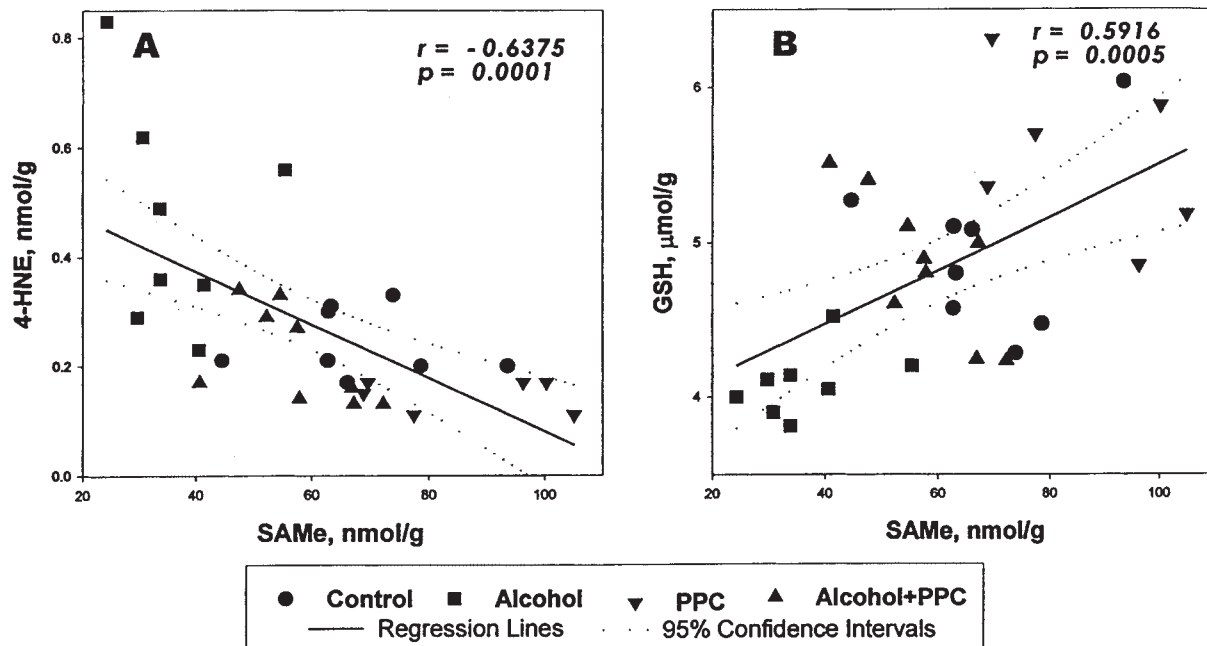


Fig. 5. Correlation of hepatic *S*-adenosylmethionine (SAME) with indices of oxidative stress.

In rats fed control diets with or without ethanol and/or polyenylphosphatidylcholine (PPC), hepatic SAME correlated negatively with 4-hydroxynonenal (4-HNE) (A) and positively with reduced glutathione (GSH) (B).

The degree of SAME depletion found in this study may lead to a decreased capacity to sustain normal levels of phosphatidylethanolamine *N*-methyltransferase (PEMT, EC 2.1.1.17)-dependent PC synthesis, as described in more advanced stages of ALD (Lieber *et al.*, 1994), providing an additional explanation for the alcohol-induced reduction in hepatic phospholipids, particularly arachidonate-containing PC species, as reported before (Aleynik *et al.*, 2000). Indeed, SAME is a universal methyl donor that provides a methyl group to phosphatidylethanolamine, thereby forming PC, the backbone of cellular membranes. This reaction is catalysed by PEMT, and represents an alternative pathway to that of cytidyldiphosphocholine for PC synthesis. It is particularly active in the liver (Sundler and Akesson, 1975), and generates PCs with long-chain polyunsaturated fatty acids containing a higher percentage of arachidonate (DeLong *et al.*, 1999). This pathway of PC synthesis requires a considerable amount of SAME, compared with other transmethylation reactions, since, for each mole of PC formed, 3 mol of SAME are needed. Therefore, we hypothesize that PPC, by providing unsaturated long-chain PCs, spares the substantial utilization of SAME for PC synthesis catalysed by PEMT. This could explain, at least in part, the PPC-induced GSH replenishment (Fig. 2) and also the correction of the alcohol-induced oxidative stress (Fig. 3). This hypothesis pre-supposes that, unlike in long-lasting chronic experiments (Lieber *et al.*, 1994), PEMT activity is not as yet decreased after only 8 weeks of alcohol feeding as carried out in the present study. In any event, the hypothesis may also explain the statistically significant increase of SAME and GSH levels above control values when PPC was given without alcohol (Figs 1 and 2).

The findings of this study have some practical implications. Some essential nutrients, including methionine, must first be activated in the liver before they can exert their key functions. This activating process, however, is altered by liver disease and, as a consequence, nutritional requirements change. Experimentally, it has been shown that even a 7-fold increase in the normal dietary methionine content fails to significantly alter hepatic SAME (Finkelstein and Martin, 1986). This is exacerbated when there is significant liver disease, which is commonly associated with impairment of the enzyme activating methionine to SAME (Martin-Duce *et al.*, 1988). Therefore, supplementation with methionine is useless in most such circumstances and may even result in toxicity, because of its accumulation as a result of non-utilization (Kinsell *et al.*, 1947). Indeed, elevated concentrations of circulating methionine in patients with liver disease have been reported (Iber *et al.*, 1957; Fischer *et al.*, 1974; Montanari *et al.*, 1988), and excess methionine was shown to have toxic effects (Finkelstein and Martin, 1986), including a decrease in hepatic ATP (Hardwick *et al.*, 1970). Accordingly, treatment with SAME instead of methionine has been advocated (reviewed in Lieber, 2002). However, it has been claimed that the liver does not take up SAME from the bloodstream (Hoffman *et al.*, 1980), but other studies indicate its uptake by isolated hepatocytes (Pezzoli *et al.*, 1978; Travers *et al.*, 1984; Engstrom and Benevenga, 1987). Results in baboons also clearly showed hepatic uptake of exogenous SAME *in vivo*, associated with beneficial effects on liver function and structure (Lieber *et al.*, 1990). In these baboons, correction of the ethanol-induced hepatic SAME depletion with oral SAME administration resulted in a corresponding attenuation of

ethanol-induced liver injury. Furthermore, a significant therapeutic success in ALD was achieved in a long-term randomized, placebo-controlled, double-blind, multicentre clinical trial of SAME in patients with alcoholic liver cirrhosis in whom SAME improved survival or delayed liver transplantation (Mato *et al.*, 1999). The results of the present study indicate that SAME should not only be used for the treatment of alcoholic cirrhosis (Mato *et al.*, 1999), but that it should now also be considered for the therapy of earlier stages of the disease, and that its beneficial effects could be potentiated by concomitant administration of PPC.

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