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Ethanol Lowers Glutathione in Rat Liver and Brain and Inhibits Methionine Synthase in a Cobalamin-dependent Manner

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Abstract

Background—Methionine synthase (MS) is a ubiquitous enzyme that requires vitamin B12 (cobalamin) and 5-methyl-tetrahydrofolate for methylation of homocysteine to methionine. Previous studies have shown that acute or chronic ethanol (ETOH) administration results in inhibition of MS and depletion of glutathione (GSH), and it has been proposed that GSH is required for synthesis of methylcobalamin (MeCbl).

Methods—We measured GSH levels and investigated the ability of different cobalamin cofactors (cyano- (CNCbl), glutathionyl- (GSCbl), hydroxo- (OHCbl), and MeCbl) to support MS activity in liver and brain cortex from control and ETOH-treated rats.

Results—In control animals, MS activity was higher in liver than cortex for all cobalamins and MeCbl-based activity was higher than for other cofactors. S-Adenosylmethionine (SAM) was required for OHCbl, CNCbl, and GSCbl-based activity, but not for MeCbl. Feeding an ETOH-containing diet for four weeks caused a significant decrease in liver MS activity, in a cobalamin-dependent manner (OHCbl ≥ CNCbl > GSCbl > MeCbl). In brain cortex, OHCbl, CNCbl and GSCbl-based activity was reduced by ETOH treatment, but MeCbl-based activity was unaffected. GSH levels were reduced by ETOH treatment in both liver and cortex homogenates, and addition of GSH restored OHCbl-based MS activity to control levels. Betaine administration had no significant effect on GSH levels or MS activity in either control or ETOH-fed groups.

Conclusions—The ETOH-induced decrease in OHCbl-based MS activity is secondary to decreased GSH levels and a decreased ability to synthsize MeCbl. The ability of MeCbl to completely offset ETOH inhibition in brain cortex, but not liver, suggests tissue-specific differences in the GSH-dependent regulation of MS activity.

Keywords

Alcoholism; betaine; cirrhosis; methylation; vitamin B-12

Introduction

Vitamin B12 and 5-methyl-tetrahydrofolate are essential cofactors for MS, which converts homocysteine (HCY) to methionine as part of a methylation cycle involving ATP-dependent formation of S-adenosylmethionine (SAM). SAM, the major intracellular methyl donor, is

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converted to SAH by methyltransferases, forming S-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to HCY and adenosine by SAH hydrolase (Fig. 1). MS activity therefore supports methylation, while MS inactivation increases HCY and SAH accumulation, promoting HCY diversion to the transsulfuration pathway and cysteine synthesis (Finkelstein, 2007). Cysteine levels are rate-limiting for synthesis of GSH, the major cellular antioxidant (Stipanuk, 2004), and MS activity therefore plays a key role in regulating GSH synthesis and cellular redox status.

Cobalamin directly participates in MS-mediated methyl transfer, and its Cbl(I) state, generated upon methionine formation, is exceptionally vulnerable to oxidation, depending upon prevailing redox conditions (Bandarian et al., 2003). To reactivate enzyme activity after oxidation of Cbl(I) to Cbl(II), a carboxy-terminal SAM-binding domain carries out reductive methylation of Cbl(II), in conjunction with donation of an electron by methionine synthase reductase, forming MeCbl and re-establishing the primary cycle of methylfolate-dependent HCY methylation (Bandarian et al., 2002). However, recent studies in cultured neuronal cells reveal that MS activity is completely dependent upon GSH or on the provision of GSCbl (Waly et al.). GSCbl is readily formed from OHCbl in the presence of GSH (Xia et al., 2004) and its level could therefore reflect GSH levels as wellas cellular redox status. In a *in vitro* model system, formation of MeCbl from GSCbl has been demonstrated in the presence of either SAM or methyliodide and a second thiol (Pezacka et al., 1990). It has also been reported that GSH assists in cobalamin dealkylation, catalyzed by a cobalamin chaperone known as CblC protein or MMACHC (methylmalonic aciduria type C and homocystinuria) (Hannibal et al., 2009; Kim et al., 2009), which also carries out decyanation of CNCbl (Kim et al., 2008). Thus GSH can potentially affect MS activity via its influence on cobalamin status.

A number of studies, utilizing different experimental models, have shown that ETOH treatment inhibits MS activity (Barak et al., 2002; Halsted et al., 1996; Halsted et al., 2002a; Halsted et al., 2002b), and it has also been demonstrated that chronic ETOH treatment depletes intracellular GSH in rat liver and brain (Calabrese et al., 1998). Since MeCbl is synthesized in a GSH and SAM-dependent manner (Pezacka et al., 1990), these observations raise the possibility that MS inhibition by ETOH could reflect a limitation in its reactivation due to reduced availability of GSCbl.

In liver, but not brain, there is an alternative pathway for HCY methylation, mediated by betaine homocysteine methyltransferase (BHMT), which utilizes trimethyl glycine (betaine) as a methyl donor for methylation of HCY (Fig. 1). Betaine administration increases hepatic SAM production and lowers HCY and SAH levels (Barak et al., 2003;Barak et al., 1996a), indicating activation of a betaine-dependent methionine cycle. Betaine administration may therefore be beneficial in off-setting ethanol inhibition of MS activity (Kharbanda, 2009).

In the current studies we investigated the ability of MeCbl or other cobalamins to support MS activity in rat liver and brain cortex after chronic (4-weeks) ETOH treatment. We also investigated the ability of betaine, administered during ETOH exposure, to affect the level of MS inhibition. Our results demonstrate tissue-specific differences in the ability of MeCbl to support MS activity after ETOH, suggesting differences in the mode of MS reactivation in these two tissues.

Materials and Methods

Chemicals

All reagents were purchased from Sigma Chemical (St. Louis, MO) except [methyl-^{14}C] -5-methyl-tetrahydrofolate, which was purchased from GE Healthcare. Diet formulation:

Nutritionally adequate Lieber DeCarli control and ethanol liquid diets (Lieber and DeCarli, 1989) were purchased from Dyets, Inc. (Bethlehem, PA). The ethanol-containing diet consisted of 18% of total energy as protein, 35% as fat, 11% as carbohydrate, and 36% as ethanol. In the control diet, ethanol was replaced isocalorically with carbohydrate such that both ethanol-fed and control rats consumed identical amounts of all nutrients except carbohydrates.

Ethanol and betaine feeding procedure

Male Wistar rats (Charles River Laboratories, Wilmington, MA) weighing 180 to 200 g were weight-matched and divided into four groups as detailed earlier (Kharbanda et al., 2005). Group 1 was fed the control diet. Group 2 was fed the same diet as Group 1 except betaine was added in the amount of 1% (w/v). Group 3 was fed the ethanol diet and Group 4 was fed the ethanol diet containing 1% betaine. Rats in groups 1–3 were fed the amount of diet consumed by rats in group 4. Overall, each group consisted of 6–12 rats that were fed the appropriate diet for 4 weeks. To further minimize variations in feeding patterns between the various groups, during 24 h before sacrifice, the rats were given the liquid diets in three portions: 25% at 0900 h, 50% at 1600 h, and the final 25% at 0700 h. At the time of sacrifice, the liver and brain cortex were removed and frozen in liquid nitrogen immediately and stored at −80°C until used.

Tissue homogenization

A portion of rat cortex or liver tissues (0.5 g) were homogenized in 10 mL of 100 mM potassium phosphate buffer (pH 7.2) by a glass-Teflon homogenizer with an ice-cold jacket. After centrifugation (100,000 × g) of tissue homogenates at 4°C for 1 h, the supernatant was used for determination of MS activity and measurement of intracellular GSH level.

Methionine synthase assay

MS activity in the tissue homogenates was determined by measuring incorporation of radiolabel from [methyl-^{14}C]methyltetrahydrofolate into methionine, as previously described (Waly et al., 2004). The reaction mixture contained: 100 mmol/L potassium phosphate buffer (pH 7.2), 500 μM D,L-HCY, 152 μM SAM, 2 mM titanium citrate, 0.1 μCi [methyl-^{14}C] methyltetrahydrofolate, 10 μM cobalamin and 380 μl of tissue supernatant in a total volume of 500 μL. Enzyme assays were performed under anaerobic conditions by bubbling nitrogen gas through stoppered vials for 1 hour at 37°C and terminated by heating at 98°C for 2 min after which samples were cooled on ice. Radiolabeled methionine was separated from unreacted radiolabeled methylfolate by passing the reaction mixture through an anion exchange column of DOWEX 1×8 (chloride form). The column was washed with 2 ml of water and the aqueous samples were collected in scintillation vials, to which 7 ml of scintillation fluid was added and the radioactivity was counted. All reported values are normalized to protein content and corrected for the counts observed in control assays in which sample enzyme was omitted.

GSH measurement

A 100 μL aliquot of supernatant was transferred to a fresh tube and 2 μL of monochlorobimane (25 mmol/L) and 2 μL of glutathione-S-transferase reagent was added, as provided by a commercial kit (Biovision, Mountain View, CA). After a 30 min incubation at 37°C, fluorescence was read at 380 nM excitation and 460 nm emission. GSH content was determined by comparison with values from a standard curve using freshly prepared GSH.

Data analysis and statistics

Data were normalized to the protein content of tissue homogenates, using the Lowry Protein Assay, and is expressed as mean ± standard error of the mean (S.E.M.) of three or more independent observations. Statistical analyses were conducted using Student's t-test and $p < 0.05$ was considered to be significant.

Results

Influence of ETOH on MS activity in rat liver

MS activity was measured in the presence of OHCbl, CNCbl, GSCbl or MeCbl using liver and cortex homogenates from rats receiving an ETOH-containing diet or control rats receiving an isoenergetic diet. As illustrated in Fig. 2A, MeCbl supported the highest level of MS activity in liver of control animals, followed by GSCbl, OHCbl and CNCbl. MS activity in ETOH-treated animals was significantly reduced compared to control animals for all cobalamins (Fig. 2A). However, there were significant differences in the extent of the ETOH-induced decrease for different cobalamins. The decrease was greatest for OHCbl (91%), followed by CNCbl (70%), GSCbl (65%) and MeCbl (51%). Thus ETOH treatment lowers MS activity in rat liver irrespective of the cobalamin cofactor utilized, but the decrease was least for MeCbl and greatest for OHCbl, suggesting that ETOH treatment might interfere with conversion of OHCbl to MeCbl.

Since OHCbl is readily converted to GSCbl in the presence of GSH (Xia et al., 2004), the large ETOH-induced decrease in OHCbl-based activity could reflect a limitation in GSH availability. In an *in vitro* study, Pezacka et al. (1990) reported the conversion of GsCbl to MeCbl, although this finding has not been confirmed by others. We therefore measured GSH levels in ETOH-treated rat liver compared to control levels. As illustrated in Table 1, ETOH treatment lowered GSH by 30%, which may restrict the ability of MS to utilize OHCbl as a co-factor.

To further assess the role of GSH, MS activity of ETOH-treated liver was measured in the presence of GSH (1 mM). As shown in Fig. 2A, addition of GSH to the MS assay significantly augmented OHCbl-based activity, but did not affect activity for other cobalamins. This result suggests that a substantial portion of the inhibitory effect of ETOH treatment on OHCbl-based MS activity results from its reduction of GSH levels, additional mechanisms also contribute.

Reactivation of MS requires MeCbl, which can either be produced by SAM-dependent methylation of Cbl(I) following reduction of Cbl(II) by methionine synthase reductase or by exchange of Cbl(II) with exogenous MeCbl. To evaluate the importance of SAM-dependent cobalamin methylation, MS activity of liver from ETOH-treated animals was measured in its presence (152 μM) or its absence. Deletion of SAM from the assay completely eliminated activity for all cobalamins except MeCbl, which showed a marginally significant 24% decrease (Fig. 2B). This indicates the requirement of SAM-dependent methylation for reactivation of MS in the presence of GSCbl, OHCbl and CNCbl, but not MeCbl.

Influence of ETOH on MS activity in rat cortex

In control animals, MS activity was lower in cortex than liver for all cobalamins, and MeCbl yielded a higher activity than other cobalamins (Fig. 3A). MS activity in cortex was significantly reduced by ETOH treatment compared to control animals for all cobalamins except MeCbl. The ETOH-induced decrease was greatest for CNCbl (75%), followed by OHCbl (72%) and GSCbl (25%), while the decrease for MeCbl (13%)was not significant.

Thus in both liver and cortex, ETOH treatment caused a greater decrease in OHCbl-based MS activity than MeCbl-based activity.

Basal levels of GSH were 26% lower in cortex than in liver, while ETOH treatment caused a 31% decrease in GSH levels in cortex, similar to its effect in liver (Table 1). Moreover, addition of GSH to the MS assay fully restored MeCbl-based, GSCbl-based and OHCbl-based enzyme activity of ETOH-treated cortex to the level found in untreated control animals (Fig. 3A). Thus the inhibitory effect of ETOH treatment on MS activity in cortex is entirely attributable to its reduction GSH levels, although in liver GSH only partially reversed the effect of ETOH. This indicates a tissue-specific difference in the inhibitory influence of ETOH on MS activity.

Deletion of SAM from the assay medium eliminated MS activity in the cortex of ETOH-treated animals for all cobalamins except for MeCbl (Fig. 3B). Thus SAM-dependent conversion of all cobalamins to MeCbl is absolutely required for MS activity in cortex, similar to liver.

Effect of betaine on methionine synthase activity in rat liver and cortex

BHMT activity provides an alternative pathway for HCY methylation, although it is only expressed in a limited number of tissues (e.g. liver and kidney in man, liver only in rat (McKeever et al., 1991)). Thus betaine administration is expected to increase the BHMT-mediated HCY methylation in liver, providing support for methionine cycle activity. We examined the influence of betaine administration on MS activity in control and ETOH-treated rats in both liver and cortex. As shown in Figures 4A and 4B, betaine administration did not introduce any significant change in either OHCbl-based or MeCbl-based MS activity in rat liver and cortex tissues as compared to their respective control or ETOH-treated groups. Thus ETOH-mediated inhibition of MS is not corrected by enhanced BHMT-mediated HCY remethylation by betaine supplementation.

Discussion

The ability of ETOH to inhibit MS activity has been well-documented (Barak et al., 2002; Halsted et al., 1996; Halsted et al., 2002a; Halsted et al., 2002b), although the underlying mechanism has remained obscure. Decreased MS activity results in accumulation of SAH and inhibition of more than 150 SAM-dependent methylation reactions, with broad cellular consequences (Clarke and Banfield, 2001). In particular it has been proposed that the steatohepatitis associated with chronic alcoholism (alcoholic fatty liver) might result from impaired MS activity, and that supplementation with betaine might provide a benefit by augmenting HCY remethylation via an alternative synthetic pathway (Barak et al., 1996b). A causative role for impaired MS activity in alcoholic fatty liver is supported by the observation that a methyl-deficient diet induces non-alcoholic steatohepatitis (NASH) (Kirsch et al., 2003; Pogribny et al., 2009), and the extent of liver damage depends upon the pre-existing methylation status (Pogribny et al., 2009). Notably, both alcoholic fatty liver and NASH are associated with inflammation, oxidative stress and a reduction in GSH levels (Garcia-Ruiz and Fernandez-Checa, 2006; Lieber, 2004), suggesting that lower GSH might be a common factor contributing to impaired MS activity.

In the current studies we confirm that chronic ETOH treatment impairs MS activity and lowers GSH levels in rat liver and we extend these observations to the brain. In addition we show that MS activity is substantially (liver) or completely (brain) restored when MeCbl is provided instead of OHCbl, or when GSH is provided in the presence of SAM, which allows MeCbl to be synthesized. These results indicate that a reduction in GSH-dependent MeCbl synthesis is important factor for the ETOH-induced decrease in MS activity. Lower MS

activity leads to a decrease in the SAM to SAH ratio, and the resultant decrease in methylation of DNA and histones can alter gene expression via epigenetic mechanisms. A significant role for ETOH-induced epigenetic changes in gene expression has increasingly been recognized (Bonsch et al., 2004; Esfandiari et al., 2010; Ouko et al., 2009; Shukla et al., 2008), and our findings help to clarify the molecular mechanism by which these changes occur. Notably, DNA methylation in the brain has been implicated in memory ((Franklin and Mansuy, 2010; Gupta et al., 2010; Miller et al., 2010), implying that ETOH might produce memory impairments via its inhibition of MS activity.

MS activity is most commonly assayed in the presence of OHCbl or CNCbl, however, earlier studies with apo-MS indicated that all cobalamins must be initially converted to Cob(II) and then to the active cofactor MeCbl before they can support MS activity (Kolhouse et al., 1991). CNCbl is decyanated to Cbl(II) by a chaperone protein, known as cblC protein or MMCHC, followed by SAM-dependent conversion of Cbl(II) to MeCbl by MS in conjunction with its reduction to Cbl(I) by methionine synthase reductase (Banerjee et al., 2009; Kim et al., 2008). A second pathway for MeCbl formation has been described in preliminary *in vitro* studies, in which OHCbl can be initially converted to GSCbl, which is then converted to MeCbl in a SAM-dependent reaction (Pezacka et al., 1990). However, the role of the latter pathway remains to be fully elucidated. Our results suggest that four weeks of ETOH treatment causes a decrease in MS activity by interfering with GSH-dependent MeCbl synthesis in both liver and brain. Since SAM was an absolute requirement for MS activity in all treatment groups, GSCbl may be converted to MeCbl via MS itself, which has the capacity to bind both SAM and cobalamins. Further studies are needed to determine if indeed MS plays a role in GSH-dependent MeCbl formation.

Conversion of SAH to HCY by S-adenosylhomocysteine hydrolase is thermodynamically reversible (De La Haba and Cantoni, 1959), and maintaining the forward reaction critically depends upon HCY conversion to either methionine or cystathionine (Fig. 1). Decreased MS activity, such as that caused by ETOH, results in an increase in SAH, as well as a decrease in SAM formation. Together these cause a large decrease in the SAM to SAH ratio, resulting in impaired methylation. In an earlier study we showed that ETOH treatment of cultured human neuronal cells reduced MS activity, inhibited N-methylation of phosphatidylethanolamine (PE) and also blocked growth factor-stimulated DNA methylation (Waly et al., 2004). Several studies have shown that a decrease in the formation of phosphatidycholine (PC) via PE N-methyltransferase (PEMT) leads to steatohepatitis (Li et al., 2006; Zhu et al., 2003) [For a review see (Li and Vance, 2008).]. We previously demonstrated that PEMT-mediated PC formation in liver is decreased following the same ETOH treatment regimen employed in the current study, indicating that lower MS activity and altered SAM to SAH ratios lead to impaired PEMT activity. PEMT is also important for normal brain development (Blusztajn et al., 1985; da Costa et al., 2010), and its activity is decreased in Alzheimer's disease (Guan et al., 1999). Thus lower MS activity following ETOH treatment will also lead to impaired brain PEMT activity.

Betaine hydroxymethyltransferase (BHMT) provides an alternative source of methionine synthesis in liver and kidney. By aiding in the remethylation of HCY, betaine administration lowers HCY and SAH, while increasing the level of SAM, thereby normalizing the SAM to SAH ratios and reversing ETOH-induced steatosis and reversing oxidative stress (Kharbanda, 2009; Kharbanda et al., 2007; Kharbanda et al., 2005). However, we found that betaine treatment did not alter either the ETOH-induced reduction in GSH levels or the extent of MS inhibition (Table 1, Fig. 4). This suggests that betaine exerts its beneficial effects by helping to restore methylation capacity when MS is inhibited, but does not reverse the decrease in GSH which leads to MS inhibition. By serving as the methyl donor for BHMT-mediated conversion of HCY to methionine, betaine supports methylation at the

expense of transsulfuration (Fig. 1). Thus it may not be surprising that GSH levels and MS activity are not normalized by betaine. While betaine is considered a promising treatment approach for alcoholic steatohepatitis (Kharbanda, 2009), a recent clinical study failed to find benefit in treatment of nonalcoholic steatohepatitis (Abdelmalek et al., 2009), suggesting that an underlying GSH deficit might be important in NASH, in addition to a deficit in methyl metabolism (Li and Vance, 2008; Zeisel, 2008).

In summary, GSH levels in rat liver and cortex are significantly decreased after 4 weeks feeding of an ETOH-containing diet, in association with decreased MS activity, as measured with OHCbl. However, MS in the presence of MeCbl was significantly higher, and was less sensitive to ETOH inhibition, especially in cortex. These findings indicate the importance of GSH in sustaining MS activity, and suggest that supplementation with MeCbl, rather than other forms of cobalamin, might be beneficial in maintaining or restoring methylation capacity during or following alcohol ingestion.

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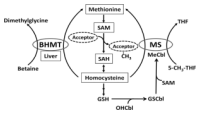

Figure 1. The methionine methylation cycle

In the liver, homocysteine is converted to methionine by two enzymes: (1) methionine synthase (MS), which utilizes methylcobalamin (MeCbl) as a cofactor and acquires methyl groups from 5-methyltetrahydrofolate (5-CH_3-THF). (2) betaine-homocysteine methyltransferase (BHMT), which uses betaine as a methyl donor. BHMT is only active in liver and kidney, not in brain. Methionine is converted to S-adenosylmethionine (SAM), which supplies methyl groups to various acceptor molecules such as DNA, RNA, phospholipids and proteins via the action of methyltransferases. Methyltransferases convert SAM to S-adenosylhomocysteine (SAH), which is reversibly converted to HCY. HCY is either remethylated to methionine or converted to cysteine by the transsulfuration pathway, which supports glutathione (GSH) synthesis. GSH reacts readily with hydroxocobalamin (OHCbl) to form glutathionylcobalamin (GSCbl), which is converted to MeCbl in a SAM-dependent reaction.

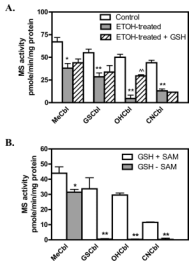

Figure 2. Cobalamin-dependent MS activity of rat liver from control and ETOH-fed rats

(A) MS activity of liver homogenates was measured in the presence of different cobalamin derivatives under standard assay conditions or under the same conditions supplemented with GSH (1 mM). (B) MS activity of a liver homogenate from ETOH-fed rats was measured in the presence of different cobalamin derivatives under standard assay conditions containing SAM (152 μM), supplemented with GSH (1 mM), or in the absence of SAM. Data is expressed as mean ± S.E.M. Significant decrease from control: * P<0.05, ** P<0.01. Significant increase from ETOH only: ^^ P<0.01.

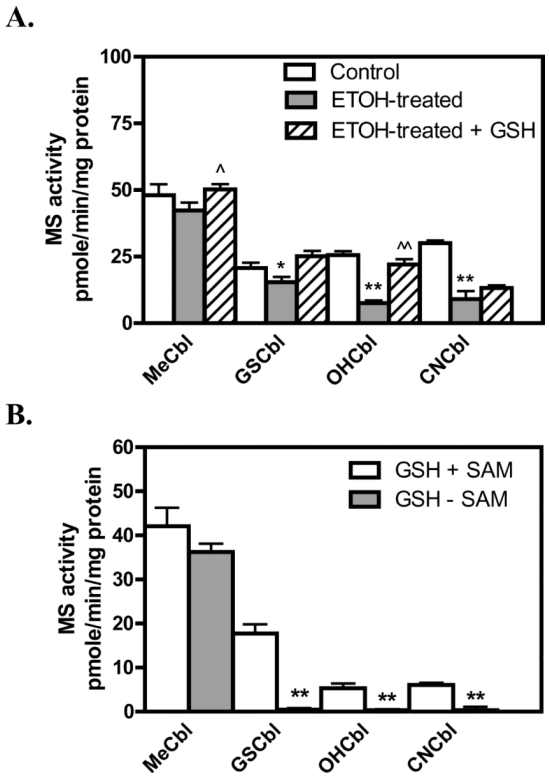

Figure 3. Cobalamin-dependent MS activity of rat brain from control and ETOH-fed rats

(A) MS activity of cortical homogenates was measured in the presence of different cobalamin derivatives under standard assay conditions or under the same conditions supplemented with GSH (1 mM). (B) MS activity of a cortex homogenate from ETOH-fed rats was measured in the presence of different cobalamin derivatives under standard assay conditions containing SAM (152 μM), supplemented with GSH (1 mM), or in the absence of SAM. Data is expressed as mean ± S.E.M. Significant decrease from control: * P<0.05, ** P<0.01. Significant increase from ETOH only: ^ P<0.05, ^^ P<0.01.

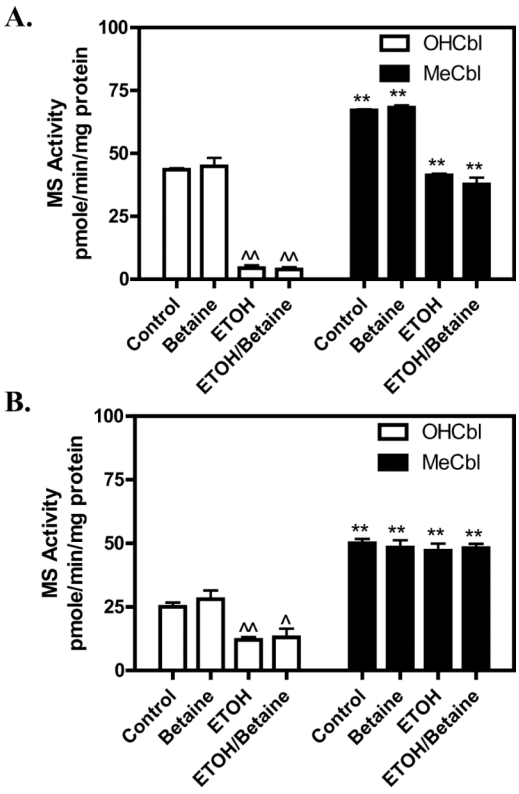

Figure 4. Effect of betaine on MS activity in rat liver and brain from control and ETOH-fed rats

(A) MS activity of liver homogenates was measured in the presence of OHCbl or MeCbl. (B) MS activity of cortex homogenates were measured in the presence of OHCbl or MeCbl. Data is expressed as mean ± S.E.M. Significant decrease from OHCbl group: ** P<0.01. Significant decrease from Control or Betaine-only group: ^ P<0.05, ^^ P<0.01.

Table 1

GSH level (nmol/mg protein) in rat liver and brain cortex:

Group	Liver tissue	Cortex tissue
Control	17.41 ± 1.6	12.88 ± 0.41
ETOH-fed rats	12.16 ± 0.44*	8.90 ± 0.19*
Betaine-fed rats	15.81 ± 0.80	12.6 ± 0.17
Betaine/ETOH-fed rats	10.97 ± 0.90*	8.92 ± 0.16*

Data are presented as the mean ± S.E.M.

*Significantly different from Control or Betaine-fed group ($p < 0.05$).