Corticoadrenal activity in rat regulates betaine-homocysteine *S*-methyltransferase expression with opposite effects in liver and kidney

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Betaine-homocysteine *S*-methyltransferase (BHMT) is an enzyme that converts homocysteine (Hcy) to methionine using betaine as a methyl donor. Betaine also acts as osmolyte in kidney medulla, protecting cells from high extracellular osmolarity. Hepatic BHMT expression is regulated by salt intake. Hormones, particularly corticosteroids, also regulate BHMT expression in rat liver. We investigated to know whether the corticoadrenal activity plays a role in kidney BHMT expression. BHMT activity in rat kidneys is several orders of magnitude lower than in rat livers and only restricted to the renal cortex. This study confirms that corticosteroids stimulate BHMT activity in the liver and, for the first time in an animal model, also up-regulate the BHMT gene expression. Besides, unlike the liver, corticosteroids in rat kidney down-regulate BHMT expression and activity. Given that the classical effect of adrenocortical activity on the kidney is associated with sodium and water re-absorption by the distal tubule leading to volume expansion, by promoting lesser use of betaine as a methyl donor, corticosteroids would preserve betaine for its other role as osmoprotectant against changes in the extracellular osmotic conditions. We conclude that corticosteroids are, at least in part, responsible for the inhibition of BHMT expression and activity in rat kidneys.

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1. Introduction

Homocysteine (Hcy), a sulphur-containing non-protein amino acid, is an intermediary in the metabolism of methionine. The elevation of plasma Hcy levels, known as hyperhomocysteinemia, is a recognized risk factor for coronary and cerebrovascular atherosclerosis and venous thrombosis (Adachi *et al.* 2002; Albert *et al.* 2002; Sundström *et al.* 2003). Methionine is incorporated with the diet and is S-adenosylated, leading to *S*-adenosylmethionine, which can transfer its methyl group to cellular acceptors, becoming *S*-adenosyl homocysteine. *S*-adenosyl homocysteine is then converted in homocysteine. The intracellular Hcy may be remethylated to methionine or condensed with serine to form cystathionine. This latter reaction is catalysed by cystathionine β-synthase. Remethylation of Hcy to methionine occurs through two separate paths, one is catalysed by 5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase) and the other by betaine-homocysteine methyltransferase (BHMT; E.C. 2.1.1.5). The first enzyme, widely distributed in animal tissues, requires as a cofactor and methyl donor 5′-methyltetrahydrofolate and vitamin B12 respectively. On the other hand, BHMT, the only known enzyme that uses betaine as a substrate, mediates the transfer of a methyl group from betaine (*N,N,N*-trimethylglycine) to Hcy, forming methionine and *N,N*-dimethylglycine (Sunden *et al.* 1997; Millian and Garrow 1998). Methionine synthase is

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ubiquitously expressed (Chen *et al.* 1997) but the distribution of BHMT is more restricted (Delgado-Reyes *et al.* 2001). BHMT has been shown to be expressed at high levels in the livers of all vertebrate species tested. In mammalian liver, BHMT represents 1% or more of the total soluble protein (Garrow 1996). In addition to being present in the liver, BHMT activity also is present at significant levels in the pancreas of ruminants and guinea pigs, and in the kidney cortex of guinea pigs, pigs and primates (Delgado-Reyes *et al.* 2001; Delgado-Reyes and Garrow 2005). In humans, pigs and guinea pigs, BHMT has been specifically localized to the proximal tubules of kidney. In contrast, BHMT activity in rat kidney has been reported to be either absent (McKeever *et al.* 1991) or very low (Finkelstein *et al.* 1971). Delgado-Reyes *et al.* (2001) and Delgado-Reyes and Garrow (2005) found that whole rat kidney homogenates have about 1–2% of the BHMT-specific activity as that found in liver. Betaine, a body requirement, is found at high levels in certain foods (De Zwart *et al.* 2003; Zeisel *et al.* 2003) and is absorbed directly from the diet via non-specific transporters in the gut. Betaine can also be endogenously produced in the liver and kidney cortex from its metabolic precursor choline (Zhang *et al.* 1992; Zeisel and Blusztajn 1994; Fischer *et al.* 2005). In addition to its important role as a methyl donor in one-carbon metabolism, betaine, together with myo-inositol, sorbitol, taurine and glycerophosphorylcholine, plays a vital function as an organic osmolyte that is accumulated by the cells of the inner medulla of animal and human kidneys to maintain osmotic balance (Bagnasco *et al.* 1986; Garcia-Perez and Burg 1991; Sizeland *et al.* 1995). Betaine regulates cell volume by counteracting changes in extracellular tonicity and stabilizing macromolecules against a variety of physiological perturbations (Häussinger 1996; Schliess and Häussinger 2002; Wehner *et al.* 2003). BHMT expression and activity plays a key role in regulating betaine concentrations (Finkelstein *et al.* 1982) and determines the fate of betaine between its two divergent biological functions: metabolized to provide a methyl group for homocysteine methylation or stored to control cellular osmolarity. In a recent work, Teng *et al.* (2011) showed that deletion of BHMT in mice resulted in betaine accumulation in most tissues. A number of nutritional factors were shown to play a role in the regulation of methyl group and Hcy metabolism by specifically altering the expression of key enzymes. BHMT activity and gene expression in rat liver are affected by the supply of dietary betaine, choline and methionine (Finkelstein *et al.* 1982; Park *et al.* 1997; Park and Garrow 1999). However, unlike the rat liver enzyme, rat kidney BHMT is refractory to treatment with low dietary methionine and choline (Delgado-Reyes *et al.* 2001; Slow and Garrow 2006). In addition to diet, BHMT expression in liver is influenced by osmotic stress (Delgado-Reyes and Garrow 2005; Schäfer *et al.* 2007) and also various hormones, including corticosteroids, insulin, estradiol and testosterone (Finkelstein *et al.* 1971; Ratnam *et al.* 2006). In the rat hepatoma cell line (H4IIE cells), the synthetic glucocorticoid triamcinolone increases the level and rate of BHMT mRNA synthesis (Ratnam *et al.* 2006).

In the present study, we investigated the role of the corticoadrenal activity to regulate BHMT in the liver and kidney of an *in vivo* rat model. Our aim was to determine whether corticosteroids are involved in the regulation of kidney BHMT expression.

2. Material and methods

2.1 *Chemicals and reagents*

Betaine hydrochloride, L-Hcy thiolactone hydrochloride, Dowex 1-X4 (OH−; 200–400 mesh) resin, protease inhibitor cocktail and 3,3′-diaminodbenzidine were purchased from Sigma-Aldrich. [^{14}C-methyl]-betaine (57 mCi/mmol) was obtained from Moravek Biochemicals. Scintillation cocktail Optiphase Hisafe 3 was obtained from Perkin Elmer. Anti-BHMT polyclonal antibodies (rabbit) were kindly provided by Dra. María A Pajares Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Madrid, Spain. Goat anti-rabbit IgG horseradish peroxidase, peroxidase-labelled goat anti-rabbit IgG secondary antibody, primers, *Taq* DNA polymerase and SYBR Green PCR Master Mix, were supplied by Promega. All other chemicals were of the highest purity available from commercial sources.

2.2 *Animals*

Adult male Sprague–Dawley rats weighing 210 to 230 g were obtained from the Institute of Biology and Experimental Medicine Animal Facility (NIH Assurance Certificate # A5072-01) and were housed under controlled temperature (22°C) and humidity (50%) with 12 h/12 h light/dark cycles (lights on at 7:00 a.m.). Rats were fed with free access to standard chow and tap water (or saline after adrenalectomy) and randomized into study and control groups. Animals were 12 weeks old when sacrificed. All animal experiments followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Ethical Committee of the Institute of Biology and Experimental Medicine. All efforts were made to minimize animal suffering and to reduce the number of rats used. Rats were euthanized by carbon dioxide asphyxiation (Slow and Garrow 2006) according to protocols for animal use, in agreement with NIH guidelines for care and use of experimental animals and approved by the local ethical committee (IBYME-CONICET).

2.3 *Hormonal treatment and study procedure*

After 1 week of acclimatization, 20 rats were divided in two groups. Under Nembutol anaesthesia (60 mg/kg i.p.), one group was bilaterally adrenalectomized (ADX) and the other was sham operated as adrenal-intact controls. The day after operation five rats of each group received i.m. injections of methylprednisolone (MP) (Riedel-de Haën, Germany), 8 mg/kg/day in 0.2 mL of corn oil. Controls (n=5) received vehicle only. The rats were sacrificed on day 14 after the first injection, and tissue samples – liver and kidney – were rapidly dissected from each animal, individually wrapped in aluminium foil and flash frozen in liquid nitrogen.

2.4 *BHMT assay*

Kidney cortex and liver were homogenized at 4°C in 50 mM Tris HCl buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid, 5 mM β-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride and centrifuged at 20,000g at 4°C for 30 min. BHMT activity was measured as described by Finkelstein and Mudd (1967). DL-Hcy was prepared fresh by a procedure described by Duerre and Miller (1966). Hcy thiolactone hydrochloride (15.4 mg) was dissolved in 400 µL of 2 N sodium hydroxide. The solution was allowed to sit for 5 min at room temperature. The reaction was then neutralized by the addition of 600 µL of a saturated solution of monopotassium phosphate and used immediately in the BHMT assay. The standard assay contained 5 mM DL-Hcy, 2 mM for livers or 0.25 mM for kidneys (in order to increase the assay sensitivity) betaine, 0.05–0.1 mCi ^{14}C-betaine, and one aliquote of homogenate. The final reaction volume was 0.5 mL, completed with the homogenization buffer. Incubations were started by transferring the tubes into a 37°C water bath. Following 1 h incubation, samples were chilled in ice water, and 2.5 mL of ice-cold water was added. The samples were applied to a Dowex 1-X4 (OH^-; 200–400 mesh) column (0.6×3.8 cm), and the unreacted betaine was washed from the column with water (3×5 mL). Dimethylglycine and methionine were eluted with 3 mL of 1.5 N HCl and 1 mL was transferred into scintillation vials containing 2.5 mL of scintillation mixture and counted. Blanks contained all of the reaction components except for the enzyme, and their values were subtracted from the sample values. All samples were assayed in duplicate. Enzyme activity was expressed as pmol/mg protein/min.

2.5 *Western blot analysis of BHMT expression*

Kidney cortex or liver were homogenized at 4°C (1:20 w/v) in 50 mM Tris HCl buffer (pH 8.0) containing 0.1% β-mercaptoethanol, 1 mg/mL lysozyme and (1 mL/20 g tissue) protease inhibitor cocktail, and centrifuged at 20,000g at 4°C for 30 min. After being heated to 95°C for 5 min, proteins were subjected to SDS-PAGE (30 and 100 µg protein/lane for liver and kidney respectively). Following electrophoresis, gels were equilibrated with transfer buffer (39 mM glycine, 48 mM Tris-HCl, 0.03% SDS, and 20% methanol). Proteins were transferred to nitrocellulose membranes using mini Trans–Blot Cell (Bio Rad). The blots were blocked overnight with 5% non-fat dried milk, and incubated for 1 h with anti-BHMT diluted 1:20,000. This was followed by a wash and incubation for 1 h with anti-BHMT diluted 1:20,000. Following a wash and incubation for 2 h with horseradish peroxidase-coupled anti-rabbit-IgG antibody (1:1,000, 4°C), the blots were washed again and visualized by 3,3-diaminobenzidine tetrahydrochloride substrate. Anti-β-actin antibodies were used to detect β-actin protein in order to verify that similar amount of protein had been loaded in each lane. In both BHMT assay and Western blot, protein concentrations were determined by Bradford (1972) using BSA as standard.

2.6 *RT-PCR analysis of BHMT mRNA expression*

To determine the expression of *BHMT* mRNA, we used real-time PCR. Rat kidney cortex and liver total RNA isolation was performed using the SV total RNA system kit (Promega) according to the manufacturer's instructions. RNA was quantified by spectrophotometry and stored at −80°C.The specific primers were designed using the sequence deposited in Genbank (access AF038870) with the help of Primer-Express software (version 2.0, ABI). The oligonucleotide primers used for real-time PCR amplification were as follows: BHMT 5′-TAGCGGCTACCATGTGCATC-3′ (sense primer), 5′-ATCCCATCTGGTGGCAACTC-3′(antisense primer); β-Actin 5′-CGGAACCGCTCATTGCC-3′ (sense primer), 5′-ACCCACACTGTGCCCAT CTA-3′ (antisense primer). Total RNA (2 µg) was reversed-transcribed using 0.5 nmol of the BHMT antisense primer or β-actin antisense primer, 10 mM dNTPs, 1 Unit RT Improm, 25 mM $MgCl_2$, Buffer 5X, H2O. The RT reaction was performed using an Eppendorf Mastercycler Personal. The thermal protocol was 5 min at 25°C, 60 min at 42°C and 15 min at 70°C. The cDNA generated in the RT reaction was used as the template in real-time PCR analysis. PCR were performed using a 7500 Real Time PCR System (Applied Biosystem). Triplicate samples of each template were analysed. Individual reactions were carried out in 20 µL volume containing 10 µL 2x SYBR Green PCR Master Mix (Applied Biosystem), 0.4 µL 50 µM sense and antisense primers of BHMT or β-actin, 2 µL sample cDNA and 7.2 µl steril deionized water. Following pre-incubation for 2 min at 94°C, the thermal protocol was 10 s at 96°C, 15 s

at 63°C; 20 s at 72°C, 1 s at 78°C and 10 min at 72°C for 45 cycles. The specificity of amplification was determined by analysing the melting curve after 40 cycles and agarose gel electrophoresis. Each reaction showed a single amplification product. Real-time PCR was performed in duplicate on a 7500 Real Time PCR System (Applied Biosystem). The relative abundance of amplified cDNA was calculated as $2^{-\Delta Ct}$, where ΔCt (change in cycle threshold) equals Ct of BHMT target Ct of β-actin loading control. Results are expressed as mean relative BHMT mRNA/β-actin mRNA values. The mean fold increase and SE were calculated from analyses of four rats per group.

2.7 *Statistical analysis*

All values are presented as means±SE. The differences were considered to be significant at $p<0.05$. For multiple comparisons statistical analyses were performed using analysis of variance (one-way ANOVA) and *post hoc* Tukey test. Two groups were compared with an unpaired Student's *t*-test. SPSS/PC (Version 10.0) software program was used.

3. Results

3.1 *Effect of adrenalectomy and methylprednisolone on BHMT activity*

Liver of ADX rats had about one-half of the BHMT-specific activity of the liver of Sham-operated animals (452±78 vs 887±30 pmol/mg protein/min, respectively), and liver of MP-treated ADX rats showed a significant recuperation of the BHMT activity respect to ADX (756±27 pmol/mg protein/min). The MP treatment of Sham rats induced a non-significant increase on hepatic BHMT activity with respect to untreated Sham animals. However, this synthetic corticoid induced the highest difference (1.05±0.03 nmol/mg protein/min) with respect to ADX rats, the two extreme conditions in the corticosteroid status (figure 1A). As it was demonstrated previously (Finkestein *et al.* 1971; Delgado-Reyes *et al.* 2001; Delgado-Reyes and Garrow 2005), the renal cortex presented a very low BHMT activity. Kidney cortex of MP-treated Sham rats did not show any difference on BHMT activity compared to untreated Sham animals (1.8±0.4 vs 1.9±0.1 pmol/mg protein/min, respectively) while adrenalectomy resulted in a significant increase of 54% (2.9±0.3 pmol/mg protein/min) in BHMT activity with respect to untreated Sham rats. The treatment with MP resulted in a non-significant reduction on the BHMT activity (2.50±0.45 pmol/mg protein/min) in kidney cortex of ADX animals with respect to untreated ADX rats (figure 2A).

3.2 *Effect of adrenalectomy and methylprednisolone on BHMT protein levels*

We performed Western blot analyses on BHMT in liver and kidney homogenates to determine whether the modification of BHMT activities in these organs were due to changes in BHMT protein content. Adrenalectomy decreased about 50% the hepatic BHMT protein levels compared with the Sham group, and while MP treatment did not change the hepatic BHMT protein abundance in Sham-operated rats, this treatment resulted in a significant increase of about 60% of BHMT protein content in liver of ADX rats (figure 1B). The abundance of renal cortex BHMT was significantly increased in about 100% after adrenalectomy, and MP treatment decreased the BHMT content in ADX rats. No effect on BHMT abundance was observed in kidney after MP treatment in Sham-operated rats (figure 2B).

3.3 *Effect of adrenalectomy and methylprednisolone on BHMT mRNA levels*

BHMT mRNA levels in liver and kidney cortex were measured with real-time RT-PCR. When corrected for β-actin mRNA, liver BHMT mRNA of ADX rats was reduced to one third from BHMT mRNA of Sham operated rat livers. In MP-treated rats, *BHMT* gene expression was 11-fold higher than in controls (figure 3A). On the other hand, after adrenalectomy, kidney cortex BHMT mRNA was 6-fold higher than in Sham-operated rats, and compared with controls, in kidney of MP-treated rats, BHMT mRNA levels decreased 66% (figure 3B).

4. Discussion

Previous studies have shown that the specific activity and expression of hepatic BHMT varies with dietary intake of sulphur amino acids, choline, and betaine (Finkelstein *et al.* 1982; Finkelstein and Martin 1986; Park *et al.* 1997; Park and Garrow 1999). Also, the endocrine system is involved in its regulation. Corticosteroids, insulin, estradiol and testosterone were the hormones studied on rat liver and a rat hepatoma cell line (Finkelstein *et al.* 1971; Ratnam *et al.* 2006). Our results demonstrated that adrenalectomy produce in liver of rat a marked and significant reduction (~50%) in BHMT activity when compared with Sham-operated rats. In addition, treatment with MP induces a significant recovery of the enzyme activity in these ADX rats. The BHMT protein abundance, in rat liver measured by Western blots, showed the same pattern than the enzymatic activity, after ADX and MP treatment. Also, the transcription of hepatic *BHMT* gene, increased significantly after MP treatment. Here we show for the first time the action of a steroid hormone on the expression of the *BHMT* gene in an *in vivo*

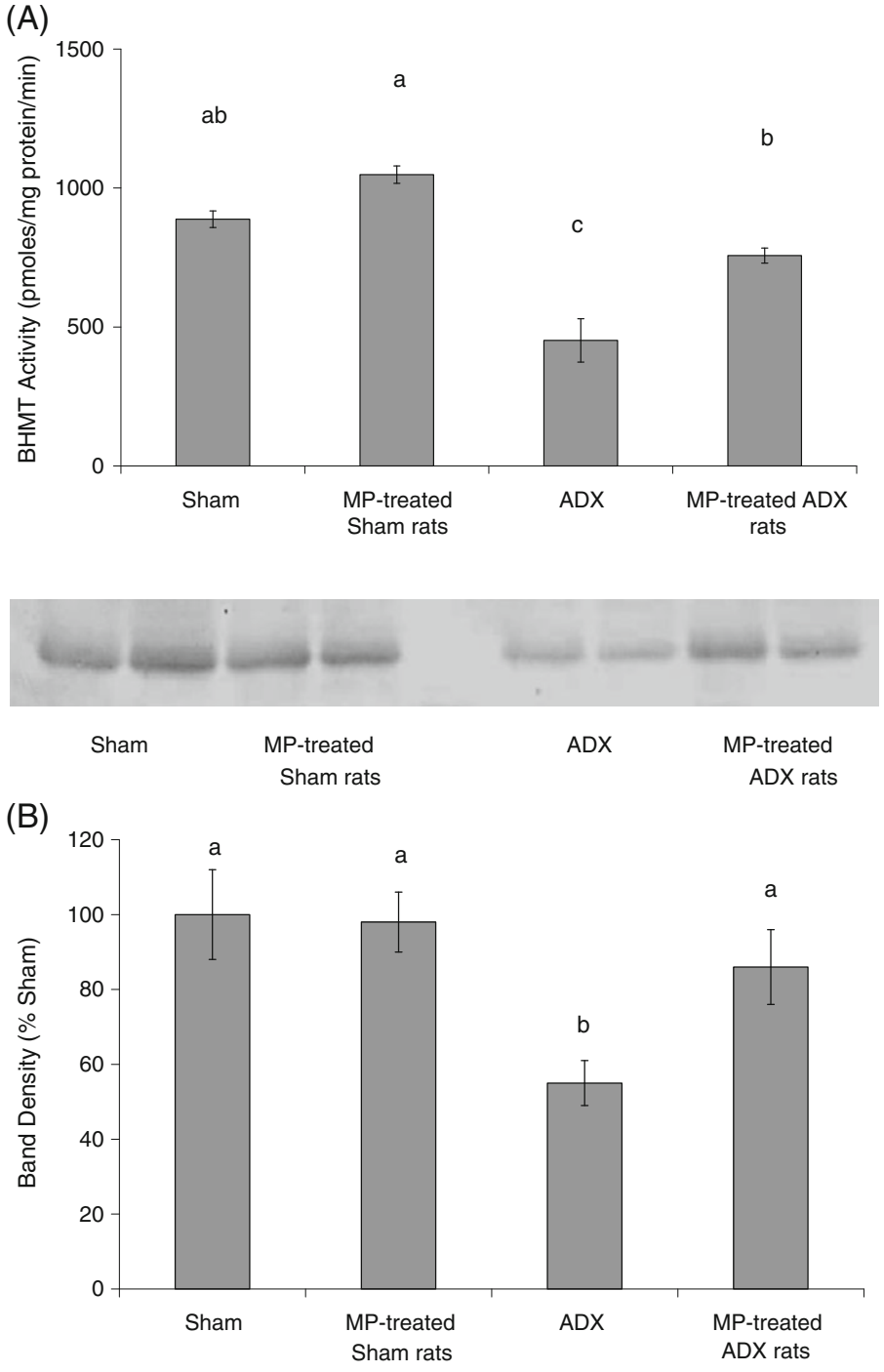

Figure 1. Effects of bilateral adrenalectomy (ADX) and methylpredisolone (MP) treatment on betaine-homocysteine methyltransferase (BHMT) in liver of rats. Sham-operated and ADX male Sprague–Dawley rats were treated with a daily dose of MP (8 mg/kg body weight/day) for 14 days. Liver samples were removed and analysed for BHMT activity and abundance as described in the Materials and Methods section. Data are means±SE from three separate experiments with $n=5$ per group; bars with different letters differ, $P<0.05$. (**A**) BHMT enzyme activity in Sham-operated and ADX rats, and after administration of MP or corn oil. (**B**) Western blot analysis of BHMT abundance in Sham-operated and ADX rats, and after administration of PM or corn oil. A polyclonal IgG-purified antibody against rat BHMT was used, and a representative immunoblot is shown.

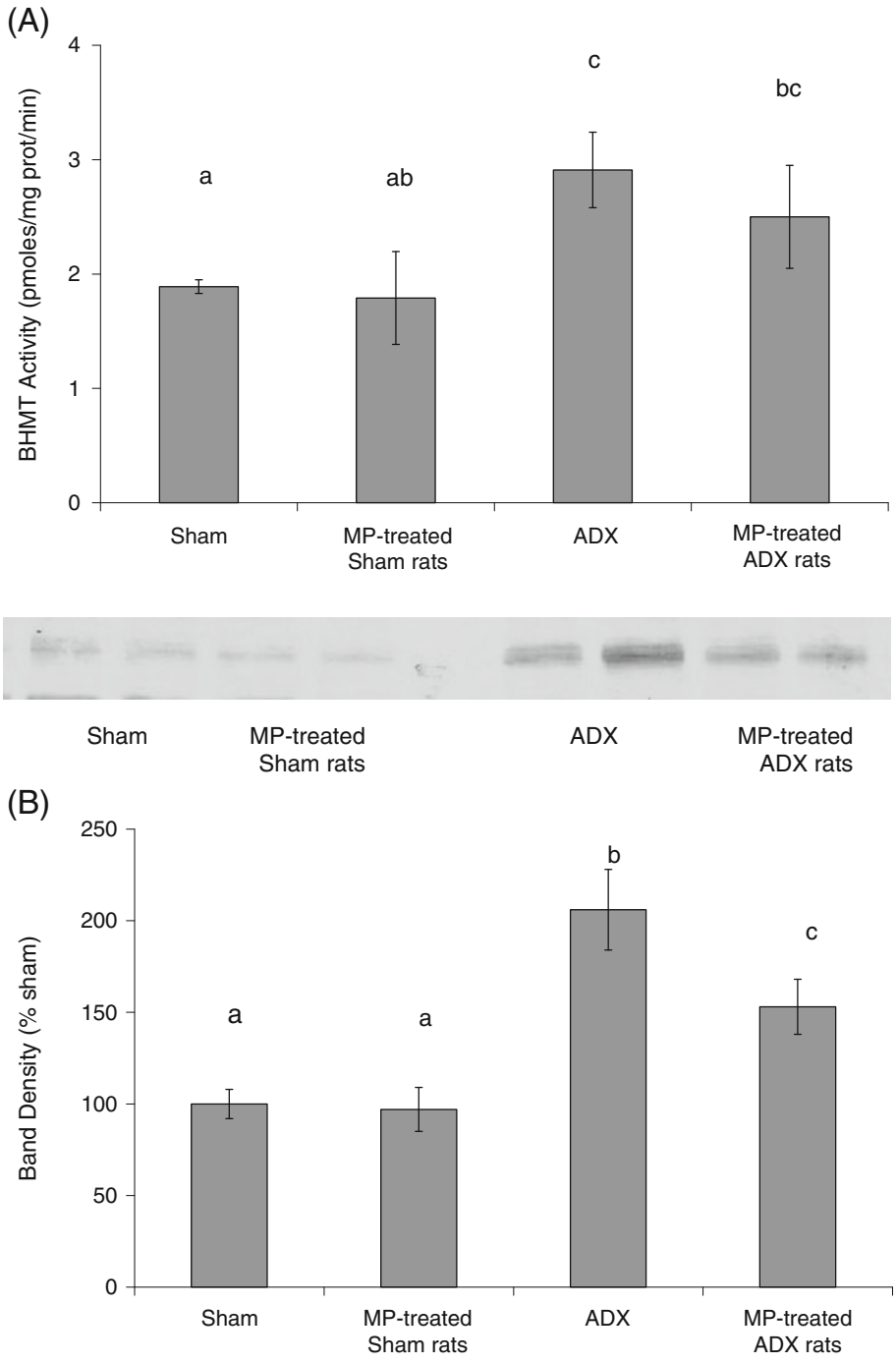

Figure 2. Effects of bilateral adrenalectomy (ADX) and methylpredisolone (MP) treatment on betaine-homocysteine methyltransferase (BHMT) in kidney cortex of rats. Sham-operated and ADX male Sprague–Dawley rats were treated with a daily dose of MP (8 mg/kg body weight/day) for 14 days. Liver samples were removed and analysed for BHMT activity and abundance as described in the Materials and Methods section. Data are means±SE from 3 separate experiments with $n=5$ per group; bars with different letters differ, $P<0.05$. (**A**) BHMT enzyme activity in Sham-operated and ADX rats, and after administration of MP or corn oil. (**B**) Western blot analysis of BHMT abundance in Sham-operated and ADX rats, and after administration of PM or corn oil. A polyclonal IgG-purified antibody against rat BHMT was used, and a representative immunoblot is shown.

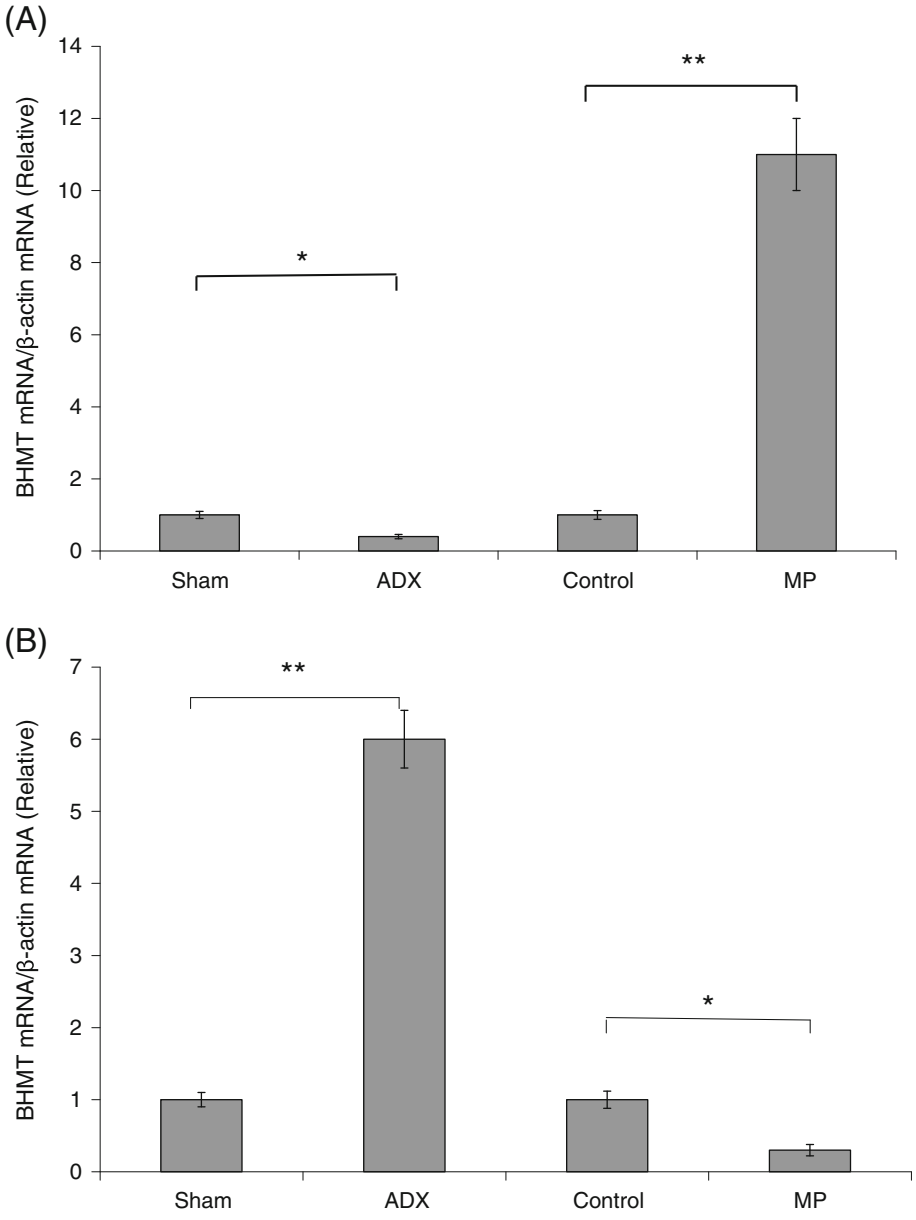

Figure 3. Effects of bilateral adrenalectomy (ADX) or methylpredisolone (MP) treatment on hepatic and renal steady-state mRNA levels. Male Sprague–Dawley rats were Sham-operated or bilaterally adrenalectomized and controls were treated with a daily dose of MP (8 mg/kg body weight/day) for 14 days. At the end of the treatment period, total RNA from liver (**A**) and kidney cortex (**B**) were isolated and reverse transcribed to cDNA; BHMT and β-actin (internal control) cDNAs were then quantified by real-time PCR, as described in the Materials and Methods section. Results are expressed as mean BHMT mRNA/ β-actin mRNA (SE), from two separate experiments with $n=4$ per group normalized relative to the control animals. *Significant difference at $P<0.05$. **Significant difference at $P<0.001$.

model. In their seminal work performed in rats, Finkelstein *et al.* (1971) only measured changes in the enzyme activity in response to steroids, while the most recent results of Ratnam *et al.* (2006) that show that increased synthesis of BHMT mRNA in response to triamcinolone were obtained with the H4IIE rat hepatoma cell line. The latter work, done in a cell culture, also indicates that corticosteroids regulate *BHMT* gene expression in the absence of other physiological factors. As it has been widely published, rat kidneys have much less BHMT activity than liver, and restricted to the renal cortex. In the kidney cortex of sham operated rats, we observed no effects on the activity and abundance of the enzyme after treatment with MP. However, the suppression of endogenous corticosteroids by bilateral adrenalectomy

resulted in increased BHMT protein and its enzymatic activity. Probably, the lack of response in kidney cortex to MP treatment was due to insufficient time and/or dose of treatment, or the inability to induce a further reduction in BHMT activity beyond that caused by endogenous corticosteroids in sham-operated rats. Regarding the transcription of the *BHMT* gene in the kidney, MP treatment produced a significant reduction, and adrenalectomy a marked and significant increase, indicating that corticosteroids act at the level of mRNA synthesis rate of BHMT in the kidney cortex. In summary, our studies showed that adrenocortical activity is responsible, at least in part, for maintaining these low levels of renal BHMT activity. In this work, we used MP, a synthetic glucocorticosteroid, to study the adrenocortical activity. The endogenous glucocorticoids (e.g. cortisol in humans and corticosterone in rat) and intermediates in corticosteroid biosynthetic pathways (e.g. deoxycorticosterone) also have mineralocorticoid properties being potential agonists of mineralocorticoid receptors (MR), particularly when circulating levels are high enough to overcome the protective 11-oxidation step that inactivates glucocorticoid hormones in MR target tissues (Quinkler and Stewart 2003). Mineralocorticoids are associated with sodium and water reabsorption by the distal tubule, leading to volume expansion at least in the short to medium term. Glucocorticoids also influence renal function independent of MR via glucocorticoid receptors (GR). GR-specific ligands affect glomerular filtration rate and proximal tubular function (Ali *et al.* 2000; Ortiz and Garvin, 2001), and GR activation may also influence sodium transport in the distal nephron (Gaeggeler *et al.* 2005). In the case of hypothalamo-pituitary-adrenal axis activation, both MR and GR processes can increase epithelial sodium channel activity (Bailey *et al.* 2009). Another issue that confounds the evaluation of glucocorticoid specific regulation of cardiovascular risk factors is that most glucocorticoids used in clinical medicine, such as prednisone, hydrocortisone and MP, have substantial mineralocorticoid effects when used in pharmacological doses. As such, physiological studies using high doses of these agents are, in fact, examining the combined effects of glucocorticoids and mineralocorticoids (Bruno *et al.* 1994; Mangos *et al.* 2000). Through our work, we have shown that corticosteroids represent factors with the ability to regulate BHMT in the liver. A novel finding from this research is that similar to *in vitro* studies with H4IIE cells, *in vivo* studies in rats with corticosteroids increase the *BHMT* gene expression and the abundance and activity of hepatic BHMT. Also, these parameters are reduced in the kidney of rats under corticosteroid action. Although we cannot dismiss other factors like allosteric and post-translational mechanisms, or an increase in BHMT degradation as contributing factors in the renal BHMT regulation by corticosteroids, it appears that the primary mechanism resides in decreased expression of the protein. In conclusion, this report shows that the expression of liver BHMT is up-regulated by adrenocortical activity, stimulating the use of betaine as a source of methyl groups and promoting the realization of a large number of intracellular processes that require methylation of macromolecules. Conversely, in the kidney, BHMT expression and activity are down-regulated by corticosteroids, thus decreasing the use of betaine as a methyl donor and favouring the preservation and accumulation of betaine as intracellular osmolyte to combat high extracellular osmolarity. The specific mechanism by which corticosteroids mediates their inhibitory effects on BHMT transcription remains to be determined.

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