Osmotic regulation of betaine homocysteine-S-methyltransferase expression in H4IIE rat hepatoma cells

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Abstract

Cell hydration changes critically affect liver metabolism and gene expression. In the course of gene expression studies using Nylon cDNA-arrays we found that hyperosmolarity (405mosmol/l) suppressed the betaine-homocysteine methyltransferase (BHMT) mRNA expression in H4IIE rat hepatoma cells. This was confirmed by Northern blot and real time quantitative RT-PCR analysis, which in addition unravelled a pronounced induction of BHMT mRNA expression by hypoosmotic (205mosmol/l) swelling. Osmotic regulation of BHMT mRNA expression was largely paralleled at the levels of BHMT protein and enzymatic activity. Like hyperosmotic NaCl, hyperosmotic raffinose but not hyperosmotic urea suppressed BHMT mRNA expression, suggesting that cell shrinkage rather than increased ionic strength or hyperosmolarity per se is the trigger. Hypoosmolarity increased the expression of a reporter gene driven by the entire human BHMT promoter, whereas destabilization of BHMT mRNA was observed under hyperosmotic conditions. Osmosensitivity of BHMT mRNA expression was impaired by inhibitors of tyrosine kinases and cyclic nucleotide-dependent kinases. The osmotic regulation of BHMT may be part of a cell volume-regulatory response and additionally lead to metabolic alterations which depend on the availability of betaine-derived methyl groups.

Key words: cell volume, osmolytes, gene expression, methylation, liver

Abbreviations: BHMT, betaine-homocysteine methyltransferase

Running title: Osmotic regulation of BHMT
**Introduction**

Betaine (trimethylglycine) is a compatible organic osmolyte that is available for cells by either choline oxidase-catalyzed synthesis (43) or concentrative uptake by the betaine-/aminobutyric acid (GABA) transport system BGT-1(4). The intracellular accumulation of betaine protects cells from different kind of stress including long lasting dehydration (4), ischemia/reperfusion (41) and heat shock (33). Betaine has been considered a “chemical chaperon” because it prevents protein denaturation under adverse conditions (17), a property that likely contributes to the cytoprotective effects of this osmolyte.

Betaine is metabolically eliminated by betaine homocysteine S-methyltransferase (BHMT, EC 2.1.1.5), an enzyme that catalyzes a methyl group transfer from betaine to homocysteine to form dimethylglycine (DMG) and methionine (43). BHMT is expressed at high levels in human liver and kidney and rat liver (34), and present knowledge suggests that its activity is largely regulated at the expression level (24). Remethylation of homocysteine through BHMT competes with the alternative methylfolate-dependent remethylation, catalyzed by methionine synthase. BHMT activity becomes essential when folate-dependent remethylation is impaired due to nutritional deficiencies (folate or cobalamin deficiency) or genetic defects of 5,10-methylenetetrahydrofolate reductase (MTHFR), cystathionine-β-synthase (CBS), or cobalamin processing, respectively (30-32). Betaine supplementation is effective in the treatment of human homocystinuria (40). In mouse models of homocystinuria due to MTHFR- or CBS-deficiency, hyperhomocysteinemia and betaine depletion were associated with fatty liver, which was preventable by betaine supplementation (30; 32). BHMT gene expression is severely decreased in rat liver cirrhosis (9), providing more evidence for an association between disrupted betaine metabolism and liver disease.

The cellular hydration state is dynamic despite the presence of cell volume regulatory mechanisms that prevent excessive cell swelling or shrinkage. Cell hydration
changes induced by either anisoosmolarity or under isoosmotic conditions by hormones, substrates or oxidative stress contribute to the regulation of cellular metabolism and gene expression (12; 15; 28). Cell swelling, in general, supports anabolic metabolism and cell protection whereas cell shrinkage favours catabolism and insulin resistance, and sensitizes cells to different kinds of stress (12). The metabolism of betaine under anisoosmotic conditions has not yet been thoroughly studied. Only recently, high salt intake of NaCl was shown in vivo to decrease BHMT activity in liver and kidney but not pancreas in guinea pig (7).

Cells and tissues exposed to anisoosmolarity are frequently used as experimental paradigm to study the dependence of metabolism and gene expression on cell hydration. Here, the osmosensitivity of BHMT was studied in H4IIE rat hepatoma cells and mouse hepatocytes. The results demonstrate a clear osmosensitive regulation of BHMT. The hyperosmotic downregulation of BHMT expression was related to cell shrinkage rather than to an elevation of ionic strength or osmolarity per se.
Materials and Methods

Materials

Cell culture media and fetal calf serum were from Gibco Life Technologies (Gaithersburg, MD). The STET – lysis buffer (pH 8) used for the metabolite analysis was from Fluka (Buchs, Switzerland). Actinomycin D, EDTA, sodium vanadate, NP-40, Triton X-100, Dithiothreitol, DMG, and betaine anhydrous were obtained from Sigma (Munich, Germany). Genistein, daidzein, 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine · 2HCl (H7), N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isooquinolinesulfonamide· 2HCl (H89), Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132), wortmannin, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), 2´-Amino-3´-methoxyflavone (PD098059) and 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4´-pyridyl)1H-imidazole (SB203580) were from Calbiochem (Darmstadt, Germany) and 1,9-Pyrazoloanthrone (SP600125) was from Biomol (Hamburg, Germany). Gö6850 was a gift from Gödecke AG (Freiburg, Germany). HPLC grade methanol and water, ammonia solution (25%) and all other chemicals were from Merck (Darmstadt, Germany). d6-DMG was from CDN Isotopes (Quebec, Canada). d11-betaine was purchased from Cambridge Isotope Laboratories (Andover, USA). Protein content was determined using the BCA protein assay kit (Pierce, Rochford, USA). Polyclonal antibodies were raised in rabbits against highly purified human BHMT (21). The anti-GAPDH antibody was from Biodesign International (Cologne, Germany). Densitometric analysis was performed with the E.A.S.Y RH system (Herolab, Wiesloch, Germany) and the Scion Image software.
Plasmids

The isolation of genomic DNA encoding exons 1 and 2 of the human BHMT gene, and a large segment of 5'-flanking sequence, and the subsequent cloning of this DNA fragment into vector pBluescript II KS (Stratagene, La Jolla, CA) to form plasmid pEP8 have previously been described (23). The DNA comprising region -3179 to -28 of the 5' untranslated region of the human BHMT gene was isolated from pEP8 by the following steps. First, a Sal I digest of pEP8 gave an ~10 kb fragment that was gel purified and further digested with Kpn I. The resulting ~3.2 kb product then was gel purified and ligated into pGL3-Basic (Promega, Madison, WI), which had been linearized with Kpn I and Xho I. The ligase reaction product was then transformed into E. coli strain XL1-Blue (Stratagene, La Jolla, CA) and the resulting construct verified by sequencing. This promoter construct is referred to as the Kpn I construct. The DNA comprising region -424 to -28 composing what is referred to as the 400nt construct was generated from the Kpn I construct as follows. First, the Kpn I construct was digested with Stu I, and following a sufficient incubation period the restriction enzyme was heat inactivated and the DNA ends blunt ended using T4 DNA polymerase (New England Biolabs, Ipswich, MA) following the manufacturers’ instructions. The resulting fragment containing the pGL3-Basic vector plus the ~400 nt promoter sequence was gel purified and its blunt ends ligated together. The integrity of the 400nt construct was verified by sequencing.

Cell culture and experimental treatment of the cells

H4IIIE-C3 rat hepatoma cells (A.T.C.C. CRL 1600) were maintained in Cluster six dishes (Greiner bio-one, Frickenhausen, Germany) containing DMEM-F12 media supplemented with 5 mM glucose and 10% FCS. Hepatocytes were prepared from livers of male B57CL/6 mice fed at libitum with a standard diet by a collagenase perfusion technique as described previously (26). Cells were grown at 37°C in 5%
CO₂. Hepatocytes and H4IIE cells were washed with Dulbecco’s PBS and the culture was continued in 1.5 ml serum-free medium for an additional 15 - 24 h. Hyperosmolarity (405 mosmol/l) and hypoosmolarity (205 mosmol/l) were adjusted by dilution with medium of elevated NaCl concentration or without NaCl, respectively. In the normoosmotic control (305 mosmol/l), an identical volume of normoosmotic medium was added.

**Custom Nylon Array hybridization**

Custom nylon arrays (Atlas™ rat toxicology 1.2 arrays, Clontech Laboratories, Palo Alto, CA, U.S.A.) were used according to the manufacturers`’s recommendations. 5µg total RNA was reversely transcribed in the presence of α-³²P-dATP using gene specific primers. Unincorporated nucleotide was removed by two rounds of column chromatography (BioSpin 30, Bio-Rad, Hercules, CA, USA.). The array membranes were prehybridized for 30 min in ExpressHyb (Clontech, Mountain View, CA) and 100 µg/ml sheared salmon testes DNA (Eppendorf, Waterbury, NY, U.S.A). The probe was chemically denatured and membranes were hybridized at 68°C. The arrays were washed four times for 30 min each in 2X SSC, 1% SDS at 68°C, once with 0.1X SSC, 0.5% SDS for 30 min at 68°C, then briefly rinsed in room temperature 2X SSC. After washing, the membranes were exposed to a phosphoimager screen and were scanned with an Fuji FLA 8000-Scanner (Raytest, Straubenhardt, Germany) with the following parameters: excitation 532 nm, emission filter 570 nm, resolution 10 µm, scan speed 200 mm/s, photomultiplier voltage 100 %.
Northern-blot analysis of BHMT mRNA expression

RNA samples (10 μg) were electrophoresed in 0.8% agarose / 3% formaldehyde and then RNA’s were blotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) with 20xSSC (3 M NaCl, 0.3 M sodium citrate). After brief rinsing with water and cross-linking (Hoefer UV-crosslinker 500; Hoefer, San Francisco, CA, U.S.A.), the membranes were inspected under UV light to determine RNA integrity and the location of the 28 S and 18 S rRNA spots. Blots were then subjected to a 3 h prehybridisation at 43°C in 50% deionized formamide in sodium phosphate buffer (0.26 M, pH 7.2) containing 0.25 M NaCl, 1 mM EDTA, 100 μg/ml salmon sperm DNA and 7% SDS. Hybridization was performed in the same solution with approx. 10^6 cpm/ml [α-32P]dCTP cDNA probe (Rediprime labelling kit; Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K). Membranes were washed three times in 2xSSC/0.1 % SDS for 15 min, twice in sodium phosphate buffer (25 mM, pH 7.2)/EDTA (1mM)/0.1% SDS for 10 min and twice in sodium phosphate buffer 10 min at 53°C. Blots were then exposed to Kodak AR X-Omat film (Eastman Kodak Company, Rochester; NY, U.S.A.) at -70°C with intensifying screens.

The BHMT cDNA fragment used as a probe was synthesized from 1 μg total RNA through the 1st Strand cDNA kit for RT-PCR (AMV) from Roche Molecular Biochemicals (Mannheim, Germany) with Oligo-dT primers. The following primers were used to amplify a 430 bp fragment according to the rat BHMT nucleotide sequence (GenBank accession no. AF038870): 5′-TTGCAGGAGGTGTGAGTCAG-3′ (upstream primer) and 5′-AATCCCTGTTTGCCACAGTC-3′ (downstream primer). The primers were chosen using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) and synthesized by the MWG Biotech AG (Ebersberg, Germany).

The PCRs were performed in 50 μl of the following reaction mixture: 1x reaction buffer, 2.5 mM MgCl₂, 0.2 mM dNTP mix, 0.2 pM each primer, 1.25 U Goldstar DNA-
polymerase (Eurogentec, Seraing, Belgium) and 100ng cDNA. The reaction was performed in a PE Biosystems GeneAmp PCR System 2400. The following program was used: 5 min 95°C; 40 cycles (40 s 95°C; 50 s 60°C; 1 min 72°C); 7 min 72°C. The PCR products were separated by agarose gel electrophoresis and eluted by using the Qiaquick Gel extraction kit (Qiagen, Hilden, Germany). Sequence analysis confirmed the specificity of the PCR amplification and was performed by the Biologisch-Medizinisches Forschungszentrum (Heinrich-Heine-University Düsseldorf, Germany).

RT-PCR-analysis of BHMT mRNA expression

To determine the expression of BHMT mRNA we used real-time PCR. CoxIV mRNA expression was unaffected by our experimental design and was used as a reference. One sense and two antisense primers were designed for each transcript with the help of FastPCR program (R. Kalendar, Institute of Biotechnology / Helsinki http://www.biocenter.helsinki.fi/bi/bare-1.html/download.htm) with a Tm= 58-63°C and a GC content of 50-60 % (Table 1).

We isolated total RNA from H4IIE cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Quality and quantity of total RNA was determined spectrometrically. Reverse transcription and PCR were done in a single step procedure using the QuantiTect SYBR Green™ RT-PCR Kit from Qiagen (Hilden, Germany) with 150 ng of total RNA as template. Real-time PCR was performed on the Applied Biosystems 7700 Sequence Detection System in Micro Amp 96-well optical reaction plates capped with MicroAmp optical caps (Applied Biosystems, Foster City, CA, USA). The thermal protocol was subdivided into 3 stages. Stage 1: 30 min at 50°C; stage 2: 15 min at 95°C; stage 3: 15 s 95°C, 60 s 60°C, 60s 72°C for 40 cycles. The specificity of amplification was determined after the last cycle of the PCR reaction by dissociation curve analysis. Each reaction showed a single amplification product.
Real-time PCR data were plotted as fluorescence signal versus cycle number. $R_n$ was calculated using the equation $\Delta R_n = (R_n^+) - (R_n^-)$, where $R_n^+$ is the fluorescence signal of the product at any given time and $R_n^-$ is the fluorescence signal of the baseline emission during cycles 6-15. An arbitrary threshold was set at the midpoint of log $R_n$ versus cycle number plot. The Cycle threshold (Ct) value is defined as the cycle number at which the $R_n$ crosses this line. The relative difference in target cDNA concentration was determined by $\Delta_{\text{conc}} = 2 \exp(Ct_A - Ct_B)$, where A is the experimental sample and B represents the respective control at isoosmolar conditions. $Bhmt$ mRNA/cDNA concentration was normalized for the concentrations of Cox IV mRNA/cDNA in the same sample.

**Western Blot analysis of BHMT expression**

At the end of the experimental treatment, medium was removed from the culture and cells were immediately lysed at 4°C using 10 mM Tris/HCl buffer (pH 7.4) containing 1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF and 1 tablet/50 ml of protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The homogenized lysates were centrifuged at 20,000 g at 4°C for 10 min. To perform SDS polyacrylamide gel electrophoresis and Western Blot analysis the supernatant was added to an identical volume of 2x gel loading buffer containing 200 mM dithiothreitol (DTT, pH 6.8). After heating to 95°C for 5 min, proteins were subjected to SDS-PAGE (60 µg protein/lane). Following electrophoresis, gels were equilibrated with transfer buffer (39 mM glycine, 48 mM Tris/HCl, 0.03% SDS, 20% methanol). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Pharmacia, Freiburg, Germany). Blots were blocked overnight in 5% bovine serum albumin solubilized in 20 mM Tris/HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20 and then incubated for 3-4 h with the anti-BHMT diluted 1:3,000. Following washing and incubation for 2h
with horseradish peroxidase-coupled anti-rabbit-IgG antibody (1:10,000-1:20,000, 4°C) the blots were washed again and developed using enhanced chemiluminescent detection (Amersham Biosciences Europe, Freiburg, Germany).

Transfection procedure and reporter gene assay of BHMT promoter activity

For magnetofection (MF), H4IIE cells were seeded 1 day before magnetofection in 6-well-plates (200,000/well) and grown in 2ml DMEM:F12 containing 10% FCS. Before magnetofection, at a confluency of about 80%, medium was removed and 1.5ml of serum-free DMEM was added. For preparation of the MF mixture, the desired amount of pDNA (2μg DNA/well, ratio 10:1 experimental vector:control plasmid) was mixed with 500μl DMEM, followed by addition of PolyMAG (Chemicell, Berlin) and gentle mixing by pipeting up and down 5 times. The PolyMAG to pDNA ratio was 1:1. For controls, PolyMAG alone was added to 500μl DMEM. After incubation at room temperature for 20 minutes, DNA/PolyMAG-solution was added to the cells. For magnetofection, 6-well plates were placed in a magnetic field provided by 3 Nd-Fe-B magnets, each 25 mm in diameter (IBS Magnete, Berlin) for 30 minutes at room temperature. Medium was removed after transfection and fresh DMEM with an ambient osmolarity of 205, 305, or 405 mosmol/l, respectively, was added. Osmolarity was adjusted by different concentrations of NaCl.

Twenty-four hours after magnetofection, luciferase expression was measured with the Dual-Luciferase Reporter System (Promega). Cells were thoroughly washed once with PBS and 100 μl of passive lysis buffer (Promega) were added. Cells were lysed for 15 min at room temperature and 20μl of each sample were measured in an Ascent Luminometer (Labsystems, Thermo, Germany). For quantitation of luciferase-activity 100μl luciferase assay puffer was injected to each sample and light emission over 10 seconds was measured. After 2 seconds lag time, 100μl stop and glo reagent (Promega) was added to each sample to measure renilla luciferase activity for another 10 seconds. The background of each well was measured before injection.
and subtracted from the reported values. Each experiment was done in triplicate and measured twice. Means of three independent experiments were used to calculate mean and SEM.

**Determination of BHMT activity**

Protein lysates were analyzed for BHMT activity as described previously (10).

**Determination of betaine and dimethylglycine**

Betaine and dimethylglycine in cell lysates and medium were measured by high performance liquid chromatography – tandem mass spectrometry (LC-MS/MS). A Waters 2795 Alliance HPLC system (Waters, Milford, UK), equipped with a thermostated autosampler, a column oven and a degasser, was used for solvent delivery and sample introduction. Lysate samples deproteinised with TCA 10% were placed in a cooled sample tray and 30μl were injected into an Agilent Zorbax 300 SB-C8 column (Agilent, Waldbronn, Germany). DMG and betaine were eluted with solution A (0.005% ammonia in water, pH 9.0) and solution B (0.65 M acetic acid, pH 2.0) at a flow rate of 0.8ml/min with the following steps: 0-0.5min, 100% A; 0.5 – 3.0min 100% B; 3.0 – 10.0min 100% A. One half of the eluate was delivered into a Quattro micro API tandem mass spectrometer (Micromass, Cambridge, UK) with an ESI probe in the positive-ion mode. The injection interval was 12min. Nitrogen was used as drying gas at a flow rate of 650l/h. The collision energy using argon as collision gas was 12eV for DMG and DMG d6 and 16eV for betaine and d11-betaine. The declustering potential was 18V for DMG and 31V for betaine, the ion source temperature was 100°C. Compounds were detected in the multiple reaction monitoring mode with the following transitions: DMG, m/z 103.8 → 57.7; d6-DMG,
m/z 109.8 → 63.6; betaine, m/z 117.9 → 58.5; d11-betaine, m/z 128.9 → 67.6. Data were acquired and analysed using MassLynx NT v4.0 software (Micromass, UK). We determined endogenous DMG and betaine concentrations in pooled human plasma by HPLC as described before (16) and found 3.0 μmol/l DMG and 29.2 μmol/l betaine. Plasma was spiked with DMG and betaine to produce standards in the range of 3.0 – 120.0 μmol/l for DMG and 29.2 – 150 μmol/l for betaine. Another standard series was obtained by spiking lysates of H4IIE cells. These standards were used to assess linearity and sensitivity of our method. Concentrations of samples were calculated from peak-area ratios of DMG to d6-DMG and of betaine to d11-betaine.

**Analysis of results**

Results are from 3 independent experiments in triplicate and are expressed as mean ± SEM. Results were compared using one-way or two-way ANOVA, as appropriate, followed by Bonferroni’s multiple comparison test or single t-tests. A p<0.05 was considered statistically significant.
Results

BHMT mRNA steady state concentration is sensitive to ambient osmolarity

cDNA Array
To study osmosensitivity of gene expression H4IIE rat hepatoma cells were exposed to normo- (305 mosmol/l) or hyperosmolarity (405 mosmol/l) adjusted by addition of 50 mmol/l NaCl) for 8h. RNA was isolated and used to prepare probes for the hybridization of commercially available cDNA-Arrays. These studies unravelled a suppression of Bhmt mRNA expression by hyperosmolarity (Figure 1).

Northern blot
We further studied the osmosensitivity of Bhmt mRNA expression by Northern blot analysis. As shown in Figure 2A hypoosmolarity (280 – 205 mosmol/l) adjusted by removal of NaCl gradually increased Bhmt mRNA expression up to 2.30 ± 0.10 fold. Already slight hyperosmolarity (355 mosmol/l) effectively suppressed Bhmt mRNA towards a 0.47 ± 0.10-fold expression (one way ANOVA for osmolarity: p<0.0001).

Quantitative real-time RT - PCR
We confirmed the osmosensitive Bhmt mRNA expression by real-time quantitative RT-PCR (Figure 2B). With this method, we detected a 4.32 ± 0.66 fold upregulation of Bhmt by hypoosmolarity (205mosmol/l) compared to normo-osmolarity, whereas hyperosmolarity downregulated the relative expression to 0.09 ± 0.01, i.e. by more than 90% at the 24h time point. Osmotic modulation of Bhmt mRNA expression was easily detected after only 4h of treatment, with the maximum responses obtained at 8 and 16h for the hyperosmotic and hypoosmotic conditions, respectively. (Figure 2B). The osmosensitivity of Bhmt mRNA expression was also observed in cultured mouse hepatocytes (Figure 2C).
Pharmacological characterization of the osmotic modulation of *Bhmt* mRNA expression

Signals mediating the osmosensitivity of *Bhmt* mRNA expression were analyzed by pharmacological means (Figure 3). Genistein, a tyrosine kinase inhibitor (1) but not daidzein, its inactive analogue, impaired the hypoosmotic stimulation of *Bhmt* mRNA expression but was largely without effect on hyperosmotic *Bhmt* mRNA suppression. H89, a cAMP-dependent protein kinase (PKA) inhibitor (8) and the broad spectrum kinase inhibitor H7 at dose which is within the range of Kᵢ values for PKA, cGMP-dependent protein kinase (PKG) and protein kinase C (PKC) (13) impaired the hyperosmotic suppression of *Bhmt* mRNA expression. H89 in addition inhibited the hypoosmotic induction of *Bhmt* mRNA. On the other hand, Gö68960, a broad specificity PKC inhibitor (20), had only minor effects, indicating that PKC is probably not involved. Also the phosphoinositide 3-kinase (PI 3-kinase) inhibitors wortmannin and LY 294002 (5; 6) at concentrations which blunted PI 3-kinase dependent insulin signaling in H4IIE cells (18), and the highly specific mammalian target of rapamycin (mTOR) inhibitor rapamycin (36) at concentrations, which blocked insulin signaling towards the mTOR downstream targets 4E-BP1 and p70 S6-kinase in H4IIE cells (18) affected the osmosensitivity of *Bhmt* mRNA expression only to a lesser degree.

Hypoosmolarity in H4IIE cells does not activate mitogen-activated protein (MAP)-kinases whereas hyperosmolarity activates extracellular signal-regulated kinases (Erk-1/Erk-2), c-Jun-N-terminal kinase (JNK) and the p38-type MAP-kinase in these cells (29; 42). PD098059, SB220025 and SP600125 inhibit signaling via Erk-1/Erk-2, p38, and JNK, respectively (2; 3; 14) and were used to study a potential role of MAP-kinases in hyperosmotic *Bhmt* mRNA suppression. Although these compounds are effective in H4IIE cells (18; 19; 29) they do not affect the hyperosmotic suppression of *Bhmt* mRNA expression.

The data point to an involvement of tyrosine kinases and cyclic nucleotide-dependent
protein kinases but to only minor or no contributions of PKC-, PI 3-kinase- and MAP-kinase-dependent signaling in mediating osmosensitivity of Bhmt mRNA expression.

**Osmolyte dependence of hyperosmotic Bhmt mRNA suppression**

Like hyperosmotic NaCl, increasing osmolarity by the impermeable osmolyte raffinose to 405mosmol/l reduced Bhmt mRNA levels after 24h (Figure 4). However, adjusting hyperosmolarity with urea, a solute rapidly penetrating liver cells (35), was ineffective to decrease Bhmt mRNA expression levels in H4IIE cells. Similar to hypoosmolarity, urea rather increased Bhmt steady state mRNA levels (Figure 3). This may be explained by the earlier report that urea in some respects mimicks the hepatocellular response to hypoosmolarity (11). Overall, our findings indicate that cell shrinkage rather than increased concentrations of Na$^+$ and Cl$^-$ or an upward shift of osmolarity per se triggers suppression of Bhmt mRNA expression levels.

**Effect of anisoosmolarity on BHMT promoter activity and Bhmt mRNA stability**

In order to study osmosensitivity of BHMT promoter activity, a plasmid containing the entire promoter of the human BHMT gene fused to the luciferase reporter gene was transfected into H4IIE cells. As shown in Figure 5, hypoosmolarity increased the activity of the human BHMT promoter about 5-fold. Hypoosmolarity was less efficient to stimulate transcription driven by a truncated 400 bp fragment of the BHMT promoter (Figure 5A), indicating additional responsive elements to be localized between the positions -3179 and -400 of the BHMT promoter. Hyperosmolarity tended to decrease BHMT promoter activity under control of the longer fragment while the shorter fragment slightly increased its activity. The findings indicate that hypoosmotic swelling affects BHMT expression at the transcriptional level.
In order to study a potential effect of anisoosmolarity on BHMT mRNA stability experiments with the transcription inhibitor actinomycin D were performed (Figure 5B). After a hypoosmotic pre-stimulation of Bhmt mRNA expression cells were incubated for another 30, 60, or 120 min in presence of actinomycin D under hypo-, normo-, or hyperosmotic conditions, respectively. As shown in Figure 5B, actinomycin D consistently leads to a decay of BHMT mRNA, irrespective of the ambient osmolarity. 120 min following actinimycin D addition Bhmt mRNA levels are significantly decreased under hyperosmotic conditions, compared to the Bhmt mRNA levels found under the respective normo- or hypoosmotic condition. This suggests that Bhmt mRNA destabilization could contribute to the suppression of Bhmt mRNA expression by hyperosmolarity.

BHMT protein concentrations follow changes in gene expression

Western blot analysis (Figure 6) shows that the osmosensitivity of BHMT mRNA expression is also reflected at the protein level. After 24h BHMT expression was increased 1.53 ± 0.21 -fold under hypo- and decreased to 0.50 ± 0.13 -fold expression under hyperosmotic conditions (n=4).

BHMT enzymatic activity and concentrations of its substrate betaine and its product DMG are altered under anisoosmotic conditions

Activity of BHMT in lysates of H4IIE cells was 5.3 ± 2.3 U/mg protein at ambient osmolarity of 305mosmol/l. BHMT activity was 9.1 ± 1.3 U/mg protein after 24h incubation in 205mosmol/l and 2.3 ± 1.2 U/mg protein in 405mosmol/l (n=3). Both effects were statistically significant (Figure 7).
In addition, we measured intra- and extracellular concentrations of betaine and DMG, the substrate and the product of the BHMT-catalyzed reaction, respectively (Figure 8). Hypoosmolarity (24h) decreased intracellular and increased extracellular betaine concentrations, indicating a volume-regulatory release of betaine. Simultaneously, both, intra- and extracellular DMG concentrations were decreased, suggesting its further metabolism by the DMG dehydrogenase. Hyperosmolarity (24h) significantly decreased medium betaine concentration. In view of the initial absence of betaine in the medium, the relatively low medium concentration of choline (its metabolic precursor) and the lack of hyperosmotic BGT-1 induction in H4IIE cells (38) this may largely reflect a reduced net release of betaine. Intracellular betaine concentrations were not significantly changed by hyperosmolarity, whereas both, intra- and extracellular DMG concentrations were significantly reduced. The intracellular ratio DMG/betaine was significantly elevated in cells exposed to hypoosmolarity and slightly but not significantly decreased under hyperosmotic conditions, which would correspond to the osmosensitive expression of BHMT. The data are consistent with the view that betaine acts as an osmolyte in H4IIE cells and that an increase of BHMT activity contributes to the reduction of betaine levels in H4IIE cells under hypoosmotic conditions, whereas a decrease of BHMT activity may contribute to save intracellular betaine under hyperosmotic conditions.
**Discussion**

This study demonstrates that BHMT expression in H4IIE rat hepatoma cells is strongly regulated by changes in ambient osmolarity. *Bhmt* mRNA and protein expression and BHMT enzyme activity are upregulated under hypoosmotic but downregulated under hyperosmotic conditions. Increased net release and catabolic elimination of betaine decrease the intracellular concentration of betaine and may facilitate the adaptation to a hypoosmotic extracellular ambience. Hyperosmolarity induces the opposite response.

BHMT expression and activity were decreased also *in vivo* in liver and kidney of NaCl-loaded guinea pigs (7). Interestingly, NaCl treatment was without effect on pancreatic BHMT activity (7). Serum osmolarity was not monitored in this study and indirect mechanisms triggered e.g. by altered cytokine or hormone patterns on BHMT expression can not be excluded in this model. The use of cultured cells exposed to anisoosmotic media in the study presented here avoids such confounding variables possibly caused by secondary effects of high salt animal treatment. As shown here (Figure 4), cell shrinkage as induced by addition of NaCl or raffinose was sufficient to downregulate BHMT expression, whereas urea was ineffective. Thus, neither elevation of ionic strength, nor an osmolarity increase *per se* accounts for downregulation of BHMT. Osmotic regulation of *Bhmt* mRNA expression was found in both, hepatoma cells and cultured hepatocytes (Figure 2), suggesting that it is not related to cell transformation.

The pharmacological characterization performed in this study (Figure 3) supports the involvement of genistein-sensitive tyrosine kinases and cyclic nucleotide-dependent protein kinases in mediating the osmosensitivity of *Bhmt* mRNA expression. Interestingly, cyclic nucleotide-dependent protein kinases but not MAP-kinases, PKC, PI 3-kinase and mTOR were suggested in H4IIE cells to play also role in mediating the osmosensitivity of phosphoenolpyruvate carboxykinase (*Pepck*) mRNA.
expression, which, in contrast to Bhmt mRNA (this paper) is suppressed under hypo-
and induced under hyperosmotic conditions (22; 37).
The data presented in Figure 5 support the view that hypoosmolarity increases Bhmt mRNA levels by stimulation of transcription, whereas destabilization of Bhmt mRNA may play a major part in its decrease under hyperosmotic conditions. In order to facilitate the examination of the Bhmt mRNA decay, Bhmt mRNA levels were increased by a hypoosmotic treatment prior to the addition of actinomycin D. Therefore, at this stage it can not be excluded that the hypoosmotic pretreatment per se modifies the osmosensitivity of Bhmt mRNA stability. Further, indirect effects of actinomycin D (e.g. by preventing expression of Bhmt mRNA (de)stabilizing proteins) could be taken into consideration.
What functional role could the osmotic regulation of BHMT activity play in liver? In the liver the parenchymal cells synthesize betaine, which is utilized by the non parenchymal endothelial cells (39), Kupffer cells (44), and stellate cells (25) as an osmolyte. Thus, inactivation of parenchymal BHMT under dehydrating conditions may make higher amounts of betaine available for protection of the non-parenchymal cells. On the other hand, a hypoosmotic stimulation of betaine catabolism may contribute to a regulatory volume decrease. Besides its role in volume regulation, betaine has an important metabolic role in hepatic homocysteine metabolism. Betaine catabolism indirectly provides methyl groups for many essential transmethylation reactions in the body. Cell swelling is associated with increased anabolism, whereas cellular dehydration confers a catabolic situation (12). Increased BHMT activity under conditions of cell swelling may lead to an increased provision of betaine-derived methyl groups for synthetic pathways. On the other hand, hyperosmotic dehydration sensitzes hepatocytes and H4IIE cells to different kinds of stress including apoptotic stimuli (19; 27) and this may be supported by a deficiency of betaine-derived methyl groups due to BHMT inactivation.
Acknowledgements

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Legends to Figures

**Figure 1. Hyperosmotic suppression of Bhmt mRNA expression**

H4IIE cells were exposed for 8h to normo- (305mosmol/l) or hyperosmotic (405mosmol/l) medium. At the end of the experimental treatment cell lysates were collected for RNA preparation. Nylon cDNA arrays were probed with $^{32}$P-labeled cDNAs prepared from the mRNA samples. Arrows indicate the signals representing Bhmt mRNA expression (position C07j). Visual inspection indicated that hyperosmolarity decreases Bhmt mRNA expression. For comparison, the signals representing expression of the ribosomal proteins S19 and L15 are also indicated (positions C13m and C13l, respectively). Expression of S19 and L15 remain largely unaffected by hyperosmolarity. A representative of 5 independent experiments is shown.

**Figure 2. Osmosensitivity of Bhmt mRNA expression**

Cells were exposed to the normoosmotic (305 mosmol/l) control medium or to anisoosmotic media for the time periods indicated. RNA was prepared and analyzed for the presence of Bhmt mRNA by either Northern Blot or quantitative RT-PCR. (A) Osmolarity dependence in H4IIE cells. For densitometric quantification of the Northern Blots normalized Bhmt mRNA expression (ratio Bhmt mRNA to ribosomal 18S RNA) under the normoosmotic control condition was taken as onefold expression. (B) Time course in H4IIE cells. Mean and SEM of 3 independent experiments is shown. Results were normalized to the expression of Cox IV and are shown relative to the respective isoosmotic condition. *, Bhmt mRNA expression significantly different from that found under the normoosmotic control condition. (C) Osmotic regulation of Bhmt mRNA expression in cultured mouse hepatocytes (n=3). Analyzed by RT-PCR as described in (B).
Figure 3. Pharmacological characterization of the osmosensitivity of Bhmt mRNA expression

H4IIE cells were exposed for 8h to ambient hypo-, normo- or hyperosmolarity without further treatment or following a 20 min pre-treatment with genistein (Gen, 50 µmol/l). daidzein (Dai, 50 µmol/l), H89 (10 µmol/l), H7 (20 µmol/l), Gö6850 (10 µmol/l), wortmannin (Wo, 500 nmol/l), LY294002 (LY, 20 µmol/l), rapamycin (Rapa, 500 nmol/l), SB220025 (SB, 20 µmol/l), SP600125 (SP, 50 µmol/l) or PD098059 (PD, 20 µmol/l), respectively. The respective inhibitors remained present during the entire experimental incubation. Samples were prepared and analyzed for relative Bhmt mRNA expression levels by RT-PCR as described in the legend to Figure 2. *, Bhmt mRNA expression differs significantly from the respective normoosmotic control condition; §, Bhmt mRNA induction differs significantly from that found in absence of the inhibitor under the respective condition. (n=3).

Figure 4. Differential effects of hyperosmolarity adjusted by raffinose, urea or NaCl on Bhmt mRNA expression

H4IIE cells were exposed to hyperosmotic media. Hyperosmolarity of 405mosmol/l was adjusted by addition of 100mM raffinose, urea, or 50mM NaCl, respectively. After 24h cells were lysed for RNA preparation. Bhmt mRNA expression was determined by quantitative RT-PCR. The figure summarizes data from 3 different experiments. *, relative mRNA expression significantly different from the respective normoosmotic control.

Figure 5. Anisoosmotic effects on BHMT promoter activity and Bhmt mRNA stability

(A) Osmosensitive regulation of the human BHMT promoter. H4IIE cells cotransfected with the respective reporter construct and a control construct were exposed to hypo- normo- or hyperosmotic medium for 24h. BHMT promoter activity was addressed as described in Materials and Methods. Luciferase activity normalized
for transfection efficiency was taken as a measure for BHMT promoter activity and presented relative to that found under the normoosmotic control condition. Shown are mean and SEM of three independent experiments. *, Luciferase activity significantly different from the respective normoosmotic control or between constructs, respectively. The promoter constructs are schematized, for description see Materials and Methods. (B) Effect of anisoomolarity on Bhmt mRNA stability. BHMT mRNA expression was increased by a 24h exposure of the H4IIIE cells to hypoosmolarity. Then, medium was exchanged and cells were incubated for another 30, 60, or 120 min under hypo-, normo-, or hyperosmotic conditions in presence or absence of actinomycin D (act D, 10μg/ml). Bhmt mRNA expression levels were analyzed by RT-PCR as described in the legend to Figure 2. Bhmt mRNA levels at the zero time point under the normoosmotic control condition was set to 100%. *, relative Bhmt mRNA expression significantly different from that found under the respective osmotic condition in absence of act D. §, relative Bhmt mRNA expression under the “hyperosmolarity plus act D” condition differs significantly from that found under hypo- or normoosmotic conditions in the presence of act D.

Figure 6. Osmotic regulation of BHMT protein expression
H4IIIE cells were exposed to hypo- (205 mosmol/l), normo- (305 mosmol/l) or hyperosmotic (405 mosmol/l) medium for the time periods indicated. Total cell protein was analyzed for the presence of BHMT by probing Western Blots with an antibody specifically recognizing BHMT. A representative blot of 4 independent experiments is shown. For densitometric quantification the normalized BHMT expression (ratio BHMT to glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) under the normoosmotic control condition was taken as onefold. *, BHMT expression significantly (p<0.05) different from that found under the normoosmotic control condition.
Figure 7. Osmosensitivity of BHMT activity
H4IIE cells were exposed for 24h to hypo- (205 mosmol/l), normo- (305 mosmol/l) or hyperosmotic (405 mosmol/l) medium. BHMT activity was normalized for protein content of lysates. Shown are mean and SEM of three independent experiments. Expression of mean BHMT activity in the respective control samples was set to one. *, relative BHMT activity significantly different from that found under the normoosmotic control condition.

Figure 8. Intracellular concentrations of betaine and DMG in H4IIE cells exposed to anisoosmolarity
H4IIE cells were exposed for 24h to hypo- (205 mosmol/l), normo- (305 mosmol/l) or hyperosmotic (405 mosmol/l) culture medium, which was virtually free of betaine. Betaine and dimethylglycine (DMG) concentrations in cell lysates and the medium after 24h were determined by LC–tandem mass spectrometry as described in Materials and Methods. Betaine and DMG in lysates were normalised for protein content. Shown are mean and SEM of 5 independent experiments. The asterisk indicates a significant deviation from normoosmotic control conditions.
Table 1. Primers used for quantitative real-time PCR of the Bhmt and Cox IV rat genes

<table>
<thead>
<tr>
<th>Transcript</th>
<th>sense primer</th>
<th>antisense primer</th>
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<tbody>
<tr>
<td><strong>Bhmt</strong></td>
<td>ACC CAG AGT TGC CAC CAG ATG G</td>
<td>TGG ACA TCG AAG GAT TGT ACG GTC TGC</td>
</tr>
<tr>
<td><strong>Cox IV</strong></td>
<td>TGT TGA TCG GCG TGA CTA CC</td>
<td>TGG AAA GGC TGC TCC AGT CG</td>
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Figure 1
Figure 2A
Figure 2B
Figure 2C
Figure 3
Figure 4
Figure 5B
Figure 6
Figure 7
Figure 8