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

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Osmotic regulation of betaine homocysteine-S-methyltransferase expression in H4IIE rat hepatoma cells. Am J Physiol Gastrointest Liver Physiol 292: G1089–G1098, 2007. First published January 11, 2007; doi:10.1152/ajpgi.00088.2006.—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 (405 mosmol/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 unraveled a pronounced induction of Bhmt mRNA expression by hypoosmotic (205 mosmol/l) swelling. Osmotic regulation of Bhmt mRNA expression was largely paralleled at the levels of Bhmt protein and enzymatic activity. Like hyperosmotic NaCl, hyperosmotic rifensin and 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 hypoosmotic 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 that depend on the availability of betaine-derived methyl groups.

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/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 chaperone” 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 the human liver and kidney and the 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 because of nutritional deficiencies (folic or cobalamin deficiency) or genetic defects of 5,10-methylenetetrahydrofolate reductase, cystathionine-β-synthase, or cobalamin processing, respectively (30–32). Betaine supplementation is effective in the treatment of human homocystinurias (40). In mouse models of homocystinuria due to methylenetetrahydrofolate reductase or cystathionine-β-synthase 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 anisosmolarity 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 favors catabolism and insulin resistance and sensitizes cells to different kinds of stress (12). The metabolism of betaine under anisosmotic conditions has not yet been thoroughly studied. Only recently, high salt intake of NaCl was shown in vivo to decrease Bhmt activity in the liver and kidney but not pancreas in the guinea pig (7).

Cells and tissues exposed to anisosmolarity 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 demonstrated 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.

Schäfer C, Hoffmann L, Heldt K, Lornejad-Schäfer MR, Brauers G, Gehrmann T, Garrow TA, Häussinger D, Mayatepek E, Schwahn BC, Schliess F. Osmotic regulation of betaine homocysteine-S-methyltransferase expression in H4IIE rat hepatoma cells. Am J Physiol Gastrointest Liver Physiol 292: G1089–G1098, 2007. First published January 11, 2007; doi:10.1152/ajpgi.00088.2006.—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 (405 mosmol/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 unraveled a pronounced induction of Bhmt mRNA expression by hypoosmotic (205 mosmol/l) swelling. Osmotic regulation of Bhmt mRNA expression was largely paralleled at the levels of Bhmt protein and enzymatic activity. Like hyperosmotic NaCl, hyperosmotic rifensin and 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 hypoosmotic 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 that depend on the availability of betaine-derived methyl groups.

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MATERIALS AND METHODS

Materials. Cell culture media and FCS were from GIBCO Life Technologies (Gaithersburg, MD). The STEET-lysis buffer (pH 8) used for the metabolite analysis was from Fluka (Buchs, Switzerland). Actinomycin D, EDTA, sodium vanadate, Nonidet (NP-40), Triton X-100, diithiothreitol, DMG, and betaine anhydrides were obtained from Sigma (Munich, Germany). Genistein, daidzein, 1-(5-isooquinolinesulfonyl)-2-methylpiperazine·2HCl (H7), N-2-[2-(p-bromocinnamyl)amino]ethyl]-5-isooquinolinesulfonamide·2HCl (H89), wortmannin, 2-(4-morpholino)-8-phenyl-1H-I-benzopyran-4-one (LY294002), 2′-a-mino-3′-methoxyflavone (PD098059), and 4-(4-flourophenyl)-2-(4-methylsulfinylphenyl)-5-(4′-pyridyl)1H-imidazole (SB203580) were from Calbiochem (Darmstadt, Germany), and 1,9-pyrazoloanthrone (SP600125) was from BioMol (Hamburg, Germany). Go6850 was a gift from Goedcke (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). Protein content was determined by using the BCA protein assay kit (Pierce, Rockford, IL). Polyclonal antibodies were raised in rabbits against highly purified human BHMT (21). The anti-GAPDH antibody was from Biodesign (Cologne, Germany). Densitometric analysis was performed with the E.A.S.Y RH system (Herolab, Wiesloch, Germany) and 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 pBlue-script 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 SalI digest of pEP8 gave an ∼10-kb fragment that was gel purified and further digested with KpnI. The resulting ∼3.2-kb product was then gel purified and ligated into pGL3-Basic (Promega, Madison, WI), which had been linearized with KpnI and XhoI. The ligase reaction product was then transformed into Escherichia coli strain XLI-Blue (Stratagene, La Jolla, CA), and the resulting construct was verified by sequencing. This promoter construct is referred to as the KpnI construct.

The DNA comprising region −424 to −28, comprising what is referred to as the 400-nt construct, was generated from the KpnI construct as follows. First, the KpnI construct was digested with SstI, and, following a sufficient incubation period, the restriction enzyme was heat inactivated and the DNA ends were blunted by using T4 DNA polymerase (New England Biolabs, Ipswich, MA) following the manufacturer’s instructions. The resulting fragment containing the pGL3-Basic vector plus the ∼400-nt promoter sequence was gel purified, and its blunt ends were ligated together. The integrity of the 400-nt construct was verified by sequencing.

Cell culture and experimental treatment of the cells. H4IIE-C3 rat hepatoma cells (American Type Culture Collection CRL 1600) were maintained in Cluster six dishes (Greiner bio-one, Frickenhausen, Germany) containing DMEM-F-12 media supplemented with 5 mM glucose and 10% FCS. Hepatocytes were prepared from livers of male B57CL/6 mice fed ad 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 of serum-free medium for an additional 15–24 h. Hyperosmolality (405 mosmol/l) and hypoosmolality (205 mosmol/l) were adjusted by dilution with medium of elevated NaCl concentration or without NaCl, respectively. In the normosmotic control (305 mosmol/l), an identical volume of normosmotic medium was added.

Custom nylon array hybridization. Custom nylon arrays (Atlas rat toxicology 1.2 arrays, Clontech Laboratories, Palo Alto, CA) were used according to the manufacturer’s recommendations. Total RNA (5 μg) was reverse transcribed in the presence of [α-32P]dATP by using gene-specific primers. Unincorporated nucleotide was removed by two rounds of column chromatography (BioSpin 30, Bio-Rad, Hercules, CA). 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). The probe was chemically denatured and membranes were hybridized at 68°C. The arrays were washed four times for 30 min each in 2× SSC and 1% SDS at 68°C, once with 0.1× SSC and 0.5% SDS for 30 min at 68°C, and then briefly rinsed in room temperature 2× SSC. After being washed, the membranes were exposed to a phosphoimager screen and were scanned with an Fuji FLA 8000-scanner (Raytest, Straubinghardt, Germany) with the following parameters: excitation 532 nm, emission filter 570 nm, resolution 10 μm, scan speed 200 mm/s, and multiplier voltage 100%.

Northern blot analysis of Bhmt mRNA expression. RNA samples (10 μg) were electrophoresed in 0.8% agarose-3% formaldehyde and then RNAs were blotted onto Hybond-N nylon membranes (Amerham Pharmacia Biotech Europe, Freiburg, Germany) with 20× SSC (3 M NaCl, 0.3 μM sodium citrate). After being briefly rinsed with water and cross-linked (Hoefner UV-cross-linker 500; Hoefner, San Francisco, CA), the membranes were inspected under UV light to determine RNA integrity and the location of the 28S and 18S rRNA spots. Blots were then subjected to a 3-h prehybridization at 43°C in 50% denitized 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 ∼105 counts·min−1·ml−1 [α-32P]dCTP cDNA probe (Re-diprime labeling kit; Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were washed three times in 2× SSC-0.1% SDS for 15 min, twice in sodium phosphate buffer (25 mM, pH 7.2)-EDTA (1 mM)-0.1% SDS for 10 min, and twice in sodium phosphate buffer for 10 min at 53°C. Blots were then exposed to Kodak AR X-Omat film (Eastman Kodak, Rochester, NY) at −70°C with intensifying screens.

The Bhmt cDNA fragment used as a probe was synthesized from 1 μg total RNA through the first 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′-TTGCAGAGGAGGTGAGTCTCAG-3′ (upstream primer) and 5′-AATCCCCTTTTGGCATGC-3′ (downstream primer). The primers were chosen by using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/ primer3.cgi) and were synthesized by MWG Biotech (Ebersberg, Germany).

The PCRs were performed in 50 μl of the following reaction mixture: 1× reaction buffer, 2.5 mM MgCl2, 0.2 mM dNTP mix, 0.2 μM each primer, 1.25 units GoTaq DNA-polymerase (Eurogentec, Seraing, Belgium), and 100 ng cDNA. The reaction was performed in a PE Biosystems GeneAmp PCR System 2400. The following program was used: 5 min at 95°C, 40 cycles (40 s at 95°C, 50 s at 60°C, and 1 min at 72°C); and 7 min at 72°C. The PCR products were separated by agarose gel electrophoresis and eluted by using the Qiagel 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. Cyclooxygenase (Cox) IV 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. Kalander, Institute of Biotechnology/Helsinki; http://
In target cDNA concentration was determined by respective control at isoosmolar conditions. Experimental treatment, medium was removed from the culture and cDNA in the same sample. The homogenized lysates were centrifuged at 20,000 g, proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). (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 per 50 ml of protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The homogenized lysates were centrifuged at 20,000 g at 4°C for 10 min. To perform SDS PAGE and Western blot analysis, the supernatant was 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, and 20% methanol). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Pharmacia, Freiburg, Germany). Blots were blocked overnight in 5% BSA 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 anti-BHMT diluted 1:3,000. Following a wash and incubation for 2 h with horseradish peroxidase-coupled anti-rabbit IgG antibody (1:10,000–1:20,000, 4°C), the blots were washed again and developed by enhanced chemiluminescent detection (Amersham Biosciences Europe, Freiburg, Germany).

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 by use of 10 mM Tris·HCl buffer (pH 7.4) containing 1% Trit X-100, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, and 1 tablet per 50 ml of protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The homogenized lysates were centrifuged at 20,000 g at 4°C for 10 min. To perform SDS PAGE and Western blot analysis, the supernatant was added to an identical volume of 2× gel loading buffer containing 200 mM DTT (pH 6.8). After being heated 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, and 20% methanol). Proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Pharmacia, Freiburg, Germany). Blots were blocked overnight in 5% BSA 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 anti-BHMT diluted 1:3,000. Following a wash and incubation for 2 h with horseradish peroxidase-coupled anti-rabbit IgG antibody (1:10,000–1:20,000, 4°C), the blots were washed again and developed by 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 MF in six-well plates (200,000/well) and grown in 2 ml DMEM-F-12 containing 10% FCS. Before MF, at a confluency of about 80%, medium was removed and 1.5 ml of serum-free DMEM-F-12 was added. For preparation of the MF mixture, the desired amount of pDNA (2 μg DNA/well, ratio 10:1 experimental vector-control plasmid) was mixed with 500 μl of DMEM, followed by addition of PolyMAG (Chemicell, Berlin) and gentle mixing by pipetting up and down five times. The PolyMAG to pDNA ratio was 1:1. For controls, PolyMAG alone was added to 500 μl of DMEM. After an incubation at room temperature for 20 min, DNA/PolyMAG solution was added to the cells. For MF, six-well plates were placed in a magnetic field provided by three Nd-Fe-B magnets, each 25 mm in diameter (IBS Magnete, Berlin, Germany), for 30 min at room temperature. Medium was removed after transfection and fresh DMEM-F-12 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 MF, 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 20 μl of each sample were measured in an Ascent Luminometer (Labsystems, Thermo, Germany). For quantification of luciferase activity, 100 μl of luciferase assay buffer were injected to each sample, and light emission over 10 s was measured. After a 2-s lag time, 100 μl of stop and glo reagent (Promega) were added to each sample to measure Renilla luciferase activity for another 10 s. The

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>Bhmt</td>
<td>ACC CAG AGT TGC CAC CAG ATG G</td>
<td>TGG ACA TCG AAG GAT TGC ACG GTC TGC</td>
</tr>
<tr>
<td></td>
<td>TCC AGG GTT TGC TGG GCA TGT CC</td>
<td></td>
</tr>
<tr>
<td>Cox IV</td>
<td>TGT TGA TCG GGC TGA CTA CC</td>
<td>TGG AAA GCC TGC TGC AGT CG</td>
</tr>
<tr>
<td></td>
<td>ATE GCT GGA AAG GCT GCT CC</td>
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Bhmt, betaine homocysteine S-methyltransferase; Cox IV, cyclooxygenase IV.
Fig. 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. Means and SEs of 3 independent experiments are shown. Results were normalized to the expression of cyclooxygenase IV (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). Results were analyzed by RT-PCR as described in B.
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 means and SE.

**Determination of Bhmt activity.** Protein lysates were analyzed for Bhmt activity as described previously (10).

**Determination of betaine and DMG.** Betaine and DMG in cell lysates and medium were measured by HPLC-tandem mass spectrometry. 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 deproteinized with TCA (10%) were placed in a degasser, was used for solvent delivery and sample introduction. 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.

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molarity (205 mosmol/l) compared with normoosmolarity, whereas hyperosmolarity downregulated the relative expression to 0.09 ± 0.01, i.e., by more than 90% at the 24-h time point. Osmotic modulation of Bhmt mRNA expression was easily detected after only 4 h of treatment, with the maximum responses obtained at 16 h in the hyperosmotic and hypoosmotic conditions, respectively (Fig. 2B). The osmosensitivity of Bhmt mRNA expression was also observed in cultured mouse hepatocytes (Fig. 2C).

Pharmacological characterization of the osmotic modulation of Bhmt mRNA expression. Signals mediating the osmosensitivity of Bhmt mRNA expression were analyzed by pharmacological means (Fig. 3). Genistein, a tyrosine kinase inhibitor (1), but not daidzein, its inactive analog, impaired the hypoosmotic stimulation of Bhmt mRNA expression but was largely without effect on hyperosmotic Bhmt mRNA suppression. H89, a PIA inhibitor (8), and the broad-spectrum kinase inhibitor H7, at a dose that was within the range of Ki values for PIA, PKG, and PKC (13), impaired the hyperosmotic suppression of Bhmt mRNA expression. H89, in addition, inhibited the hypoosmotic induction of Bhmt mRNA. On the other hand, Go 69860, a broad-specificity PKC inhibitor (20), had only minor effects, indicating that PKC is probably not involved. Also, the phosphoinositide 3-kinase (PI3K) inhibitors wortmannin and LY294002 (5, 6), at concentrations that blunted PI3K-dependent, insulin signaling in H4IIIE cells (18), and the highly specific mammalian target of rapamycin (mTOR) inhibitor rapamycin (6a) at concentrations that

Fig. 5. Anisoosmotic effects on BHMT promoter activity and Bhmt mRNA stability. A: osmosensitive regulation of the human BHMT promoter. H4IIIE cells cotransfected with the respective reporter construct and a control construct were exposed to hypoosmotic (Hypo), normoosmotic (Normo), or hyperosmotic (Hyper) medium for 24 h. BHMT promoter activity was assessed 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 means and SEs of 3 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 anisoosmolarity on Bhmt mRNA stability. Bhmt mRNA expression was increased by a 24 h 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, 10 μg/ml). Bhmt mRNA expression levels were analyzed by RT-PCR as described in the legend to Fig. 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.
blocked insulin signaling toward the mTOR downstream targets 4E-BP1 and p70 S6-kinase in H4IIE cells (18), affected the osmosensitivities of Bhmt mRNA expression only to a lesser degree.

Hypoosmolarity in H4IIE cells does not activate MAPKs, whereas hyperosmolarity activates ERK-1/ERK-2, JNK, and p38-type MAPK 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 MAPKs 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-, PI3K-, and MAPK-dependent signaling in mediating osmosensitivity of Bhmt mRNA expression.

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

Effect of anisoosmolarity on BHMT promoter activity and Bhmt mRNA stability. 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 Fig. 5, hypoosmolality increased the activity of the human BHMT promoter about fivefold. Hypoosmolality was less efficient to stimulate transcription driven by a truncated 400-bp fragment of the BHMT promoter (Fig. 5A), indicating additional responsive elements to be localized between the positions −3179 and −400 of the BHMT promoter. Hyperosmolality 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.

To study a potential effect of anisoosmolarity on Bhmt mRNA stability, experiments with the transcription inhibitor actinomycin D were performed (Fig. 5B). After a hypoosmotic prestimulation 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 Fig. 5B, actinomycin D consistently led to a decay of Bhmt mRNA, irrespective of the ambient osmolality. At 120 min after actinomycin D addition, Bhmt mRNA levels were significantly decreased under hyperosmotic conditions, compared with 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 hyperosmolality.

Bhmt protein concentrations follow changes in gene expression. Western blot analysis (Fig. 6) showed that the osmosensitivity of Bhmt mRNA expression is also reflected at the protein level. After 24 h, Bhmt expression was increased 1.53 ± 0.21-fold under hypoosmotic 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 osmolality of 305 mosmol/l. Bhmt activity was 9.1 ± 1.3 U/mg protein after 24 h incubation in 205 mosmol/l and 2.3 ± 1.2 U/mg protein in 405 mosmol/l (n = 3). Both effects were statistically significant (Fig. 7).

In addition, we measured intra- and extracellular concentrations of betaine and DMG, the substrate and product of the Bhmt-catalyzed reaction, respectively (Fig. 8). Hypoosmolality (24 h) 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. Hyperosmolality (24 h) 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 of DMG to 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. These 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 the 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 by, e.g., altered cytokine or hormone patterns on Bhmt expression cannot be excluded in this model. The use of cultured cells exposed to anisosmotic media in the study presented here avoids such confounding variables possibly caused by secondary effects of high-salt animal treatment. As shown here (Fig. 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 (Fig. 2), suggesting that it is not related to cell transformation.

The pharmacological characterization performed in this study (Fig. 3) supports the involvement of genistein-sensitive
tyrosine kinases and cyclic nucleotide-dependent protein ki-
nases in mediating the osmosensitivity of Bhmt mRNA expres-
sion. Interestingly, cyclic nucleotide-dependent protein kinases
but not MAPks, PKC, PI3K and mTOR were suggested in H4IIE
cells to also play role in mediating the osmosensitivity of phos-
phenoxypruvyrate carboxykinase mRNA expression, which, in
contrast to Bhmt mRNA (this study), is suppressed under hypo-
osmotic conditions and induced under hyperosmotic conditions
(22, 37).

The data presented in Fig. 5 support the view that hypoos-
molarity increases Bhmt mRNA levels by stimulation of tran-
scription, whereas destabilization of Bhmt mRNA may play a
major part in its decrease under hyperosmotic conditions. To
facilitate the examination of the Bhmt mRNA decay, Bhmt
mRNA levels were increased by a hypoosmotic treatment before
the addition of actinomycin D. Therefore, at this stage,
the hypoosmotic pretreatment per se modifies the osmosensitivity of Bhmt mRNA stability. Further-
more, 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 the liver? In the liver, parenchymal cells
synthesize betaine, which is utilized by nonparenchymal endo-
thelial cells (39), Kupffer cells (44), and stellate cells (25) as an
osmolyte. Thus inactivation of parenchymal Bhmt under de-
hydrating conditions may make higher amounts of betaine
available for protection of the nonparenchymal cells. On the
other hand, a hypoosmotic stimulation of betaine catabolism
may contribute to a regulatory volume decrease. Besides its
involvement in volume regulation, betaine has an important
metabolic role in hepatic homocysteine metabolism. Betaine
catabolism indirectly provides methyl groups for many essential trans-
methylation reactions in the body. Cell swelling is associated
with increased anabolism, whereas cellular dehydration con-
fers 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 sensitizes hepatocy-
cytes 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.

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