Osmotic regulation of hepatic betaine metabolism

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Running title
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Abstract
Betaine critically contributes to the control of hepatocellular hydration and provides protection of the liver from different kinds of stress. To investigate how the hepatocellular hydration state affects gene expression of enzymes involved in the metabolism of betaine and related organic osmolytes we used qRT-PCR gene expression studies in rat hepatoma cells as well as metabolic and gene expression profiling in primary hepatocytes of both wild-type and 5,10-methylenetetrahydrofolate reductase (MTHFR) deficient mice. Anisotonic incubation caused co-ordinated adaptive changes in the expression of various genes involved in betaine metabolism, in particular of betaine homocysteine methyltransferase, dimethylglycine dehydrogenase, and sarcosine dehydrogenase. The expression of betaine-degrading enzymes was downregulated by cell shrinking and strongly induced by an increase in cell volume under hypotonic conditions. Metabolite concentrations in the culture system changed accordingly. Expression changes were mediated through tyrosine kinases, cyclic nucleotide–dependent protein kinases and JNK-dependent signalling. Assessment of hepatic gene expression using a customised microarray chip showed that hepatic betaine depletion in Mthfr<sup>-/-</sup> mice was associated with alterations that were comparable to those induced by cell swelling in hepatocytes. In conclusion, the adaptation of hepatocytes to changes in cell volume involves the co-ordinated regulation of betaine synthesis and degradation and concomitant changes in intracellular osmolyte concentrations. The existence of such a well-orchestrated response underlines the importance of cell volume homeostasis for liver function and of methylamine osmolytes such as betaine as hepatic osmolytes.

Key words
Choline, betaine, dimethylglycine dehydrogenase, compatible organic osmolytes
Glossary

BHMT, Betaine-homocysteine methyltransferase
CBS, cystathionine beta-synthase
CCTα, CTP:phosphocholine cytidylyltransferase (Gene: PCYT1A)
CHDH, choline dehydrogenase
CHK, choline kinase
DMG, N,N-dimethylglycine
DMGDH, dimethylglycine dehydrogenase
GPC, sn-glycero-3-phosphocholine
MS, methionine synthase (Gene: MTR)
MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase
MTHFR, 5,10-methylenetetrahydrofolate reductase
PCYT1A, phosphate cytidylyltransferase 1, choline, alpha isoform
PEMT, phosphatidylethanolamine N-methyltransferase
SAH, S-adenosyl-L-homocysteine
SAM, S-adenosyl-L-methionine
SARDH, sarcosine dehydrogenase
Introduction

Betaine (N,N,N-trimethylglycine) plays a key role in the control of hepatocellular hydration and hepatoprotection from different kinds of stress (17). Hepatocellular betaine is a product of the choline dehydrogenase (CHDH) catalysed catabolism of choline and can subsequently be further metabolised to dimethylglycine, sarcosine and glycine. These steps are catalysed by betaine-homocysteine methyltransferase (BHMT), dimethylglycine dehydrogenase (DMGDH), and sarcosine dehydrogenase (SARDH), respectively. Figure 1 displays the network of choline metabolism and adjacent metabolic pathways and shows how betaine as osmolyte and methyl group donor tightly connects organic osmolyte synthesis with the one-carbon cycle.

Some tissues, in particular liver and kidney in humans, can use betaine as an alternative to 5-methyltetrahydrofolate in the regeneration of methionine from homocysteine (5). 5,10-Methylenetetrahydrofolate reductase (MTHFR) knockout (Mthfr/−) mice present with long-term betaine and choline depletion and serve as a model for malfunctioning organic osmolyte metabolism (23).

Changes in the cellular hydration state are induced by shifts in ambient osmolarity or by hormones, amino acids and oxidative stress (18, 20). Small variations in cell volume activate signal transduction cascades that contribute to the control of cellular functions at the levels of gene expression, metabolism, and stress tolerance (21). Whereas a release of betaine from liver in response to hypotonic perfusion has been documented before (27), knowledge about a potential cell volume dependent regulation of the expression of enzymes involved in betaine metabolism is missing.

We have recently shown that the expression of BHMT, which catalyzes the remethylation of L-homocysteine to L-methionine, is highly sensitive to cell volume changes (16).
Cells and tissues exposed to anisosmotic conditions are frequently used as an experimental paradigm to study the influence of cellular hydration changes on metabolism and gene expression. Here, we studied key steps of betaine metabolism under hypo- and hypertonic conditions in H4IIE rat hepatoma cells and primary mouse hepatocytes from wild-type as well as MTHFR deficient animals to investigate the role and extent of cell volume mediated regulation of hepatic osmolyte metabolism.
Material and Methods

Animals
Mice with a transgenic disruption of the Mthfr gene and bred on a C57BL/6 background were kindly provided by Prof. Rima Rozen (McGill University, Montreal, Canada). Mice were bred and utilised at the Animal Care Facility of the University of Duesseldorf in accordance to guidelines of the Heinrich-Heine-University Medical Centre and Institutional Animal Care and Use Committee. Mthfr/− mice were used as a model for chronic betaine and choline depletion and a disturbance of organic osmolyte metabolism (22). Mice were fed ad libitum with standard chow. Drinking water was supplemented with 2% (w/v) betaine for the first 2 weeks of life to ensure survival, according to previous experimentation (23) All animal studies were performed with the approval of the Heinrich-Heine-University Institutional Animal Care and Use Committee.

Cell culture of H4IIE rat hepatoma cells, media and antibodies
Cell culture media and FCS were from GIBCO Life Technologies (Gaithersburg, MD). We used STET-lysis buffer (pH 8.0) from Fluka (Buchs, Switzerland) for metabolite analysis. Actinomycin D, EDTA, sodium vanadate, Nonidet (NP-40), Triton X-100, dithiothreitol, DMG, and anhydrous betaine were purchased from Sigma (Munich, Germany). Genistein, daidzein, 1-(5-isooquinolinesulfonyl)-2-methylpiperazine 2HCl (H7), N-[2-[(p-bromocinnamyl)amino]ethyl]-5-isooquinoline sulfonamide 2HCl (H89), Wortmannin, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), 2-amino-3-methoxyflavone (PD098059), and 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-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% v/v), and all other chemicals were from Merck (Darmstadt, Germany). DMG-d6 was from CDN Isotopes (Quebec, Canada); betaine-d11 was purchased from Cambridge Isotope Laboratories (Andover, UK). Protein content was determined by using the BCA protein assay kit (Pierce, Rockford, IL). Polyclonal antibodies against highly purified human DMGDH and SARDH were kindly provided by Prof. Roderich Brandsch (Institute of Biochemistry and Molecular Biology, University of Freiburg, Germany). The anti-GAPDH antibody was purchased from Santa Cruz Biotechnologies).

**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/F12 media supplemented with 5 mM glucose and 10% v/v FCS. Primary mouse hepatocytes were prepared from livers of male C57BL/6 mice, fed *ad libitum* with a standard diet, by a collagenase perfusion technique as described previously (15). Cells were grown at 37°C in 5% v/v 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. Hypertonicity (405 mosmol/l) and hypotonicity (205 mosmol/l) were adjusted by adding medium of elevated NaCl concentration or without NaCl, respectively. In the isotonic control (305 mosmol/l), an identical volume of isotonic medium was added. Pharmaceutical agents (inhibitors) were added to the respective stimulation medium to start inhibitor incubation and osmotic change simultaneously.
RNA preparation and quality control

Total RNA from cells was prepared with the RNeasy Midi Kit (Qiagen GmbH, Hilden, Germany). The amount and quality of the RNA was tested spectrometrically (absorption 220nm – 350nm) and by capillary electrophoresis (Bioanalyzer, Agilent Technologies Inc., Palo Alto, CA, U.S.A.).

RT-PCR-analysis of mRNA expression

To determine mRNA expression levels, we used real-time PCR. Cytochrome C Oxigenase subunit IV (Cox IV) mRNA expression was unaffected by our experimental design and was used as a reference. Primers were designed for each transcript with the help of the “FastPCR” program (8). RT and PCR were done in a single-step procedure using the QuantiTect SYBR Green RT-PCR kit from Qiagen (Hilden, Germany) with 20ng of total RNA as a template and with a final primer concentration of 8pmol/µl. Real-time PCR was performed on the Applied Biosystems 7700 sequence-detection system (Applied Biosystems, Foster City, CA). The specificity of amplification was determined by dissociation curve analysis. Cycle threshold (Ct) values were calculated with SDS 2.3 (Applied Biosystems, Weiterstadt) using default parameters. The relative difference in target cDNA concentration was determined by $\Delta\text{Conc} = 2^{(\text{Ct}_A - \text{Ct}_B)}$, where A is the experimental sample and B represents the respective control. A and B each represents the mean from three independent measurements. All mRNA/cDNA concentrations were normalized for concentrations of Cox IV mRNA/cDNA in the same sample. Differences in expression were displayed as log-ratio according to:
**SDS-Page and Western blot analysis of SARDH and DMGDH**

H4IIE cells were incubated for 24h and 48h with different osmolarities (205, 305 and 405 mosmol/l). After removing the incubation medium the cells were washed with PBS and immediately lysed at 4°C by the use of STET buffer pH 8.0 (Fluka, Germany) with 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 2x 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 (8mg protein/lane) on a precast 7.5% w/v acrylamide gel. Proteins were transferred to PVDF membranes using a wet blot transfer apparatus (Bio-Rad, Germany). Blots were blocked for 1h in 5% w/v BSA solubilized in 20 mM Tris HCl (pH 7.5) containing 150 mM NaCl and 0.1% v/v Tween 20 and then incubated overnight with anti-DMGDH (dilution 1:1,000 ), anti-SARDH (dilution 1:1,000 ) or anti-GAPDH (FL-335, Santa Cruz Biosciences, dilution 1:5,000) antibodies, respectively. After removal of the first antibody, membranes were washed for 2-3h and incubated for 2h with horseradish peroxidase-coupled anti-rabbit- IgG antibody (dilution 1:10,000) at room temperature. Finally, the blots were washed again and developed by enhanced chemiluminescent detection (Amersham Biosciences Europe, Freiburg, Germany).

**Synthesis of fluorescent-labelled cDNA**
50µg of total RNA served as template for synthesis of the fluorescence labelled cDNA. The direct labelling with Cye3-dUTP (GE Healthcare Europe, München, Germany) was performed with Oligo-dT primers through the CyeScribe-Kit (GE Healthcare Europe, München, Germany). Unincorporated dye was removed with QIAquick PCR columns (Qiagen GmbH, Hilden, Germany).

Oligonucleotide Array Production

280 mouse oligonucleotide probes and 4 negative controls were designed using OligoWiz (14) and synthesized from Operon (Cologne, Germany). The probes are 70-mers with an average Tm = 79°C and 5'-amino group with a C6 spacer. They were dissolved in Genetix Micro Array Spotting Solution (Genetix, New Milton, UK) to a final concentration of 50pmol/µl and were printed as 2x2 replicates with a QArray 2 (Genetix, New Milton, UK) at 55% relative humidity and room temperature on Corning UltraGAPS (Corning, Lowell MA, USA). The amino-modified probes were covalently bound to the surface by UV crosslinking (600mJ) according to the protocol from Corning. The quality of the printing process was proofed with test hybridization using SpotCheck (Genetix, New Milton, UK).

DNA Microarray hybridization and fluorescence determination

The fluorescence labelled cDNA was dissolved in hybridization buffer, denaturated and injected in a Tecan HS400pro hybridization station (Tecan Deutschland GmbH, Crailsheim, Germany). Hybridized oligonucleotide arrays were visualized with a Fuji FLA 8000-Scanner (Raytest, Straubenhardt, Germany). The image analysis was computed with AIDA Array Analysis 3.5 (Raytest, Straubenhardt, Germany). The fluorescence intensities from each spot were integrated and the background
intensities derived from weighted image regions were subtracted. The normalization was performed using a global approach:

\[
N(\text{clone}) = \frac{I(\text{clone}) - B(\text{clone})}{\sum_i (I(\text{clone}_i) - B(\text{clone}_i))}
\]

\(N(\text{clone})\), normalized intensity; \(I(\text{clone})\), measured intensity; \(B(\text{clone})\), local background fluorescence.

Only the 50% upper values were used. The mean value of the four replicates from one array was calculated. Clear artefacts resulting from dust, salt crystals etc were deleted. In a next step, the median normalized intensity for a specific condition was calculated from four arrays. Genes with a signal of less than 10 under all experimental conditions, implying a signal to noise ratio below 2.5, were declared as “not detectable”. The difference in the expression is:

\[
\text{Ratio} = \log_{10} \left( \frac{N(\text{condition})}{N(\text{condition}_0)} \right)
\]

\(N(\text{condition})\), median of normalized intensity

This ratio was used to define five classes of genes. A ratio in the range \(\pm 0.18\) was declared as unregulated. A ratio from \(\pm 0.18\) to \(\pm 0.30\) indicates a moderate regulation. Genes with a ratio >0.30 were upregulated or with a ratio < -0.30 were downregulated.

HPLC- ESI tandem mass spectrometry

Metabolites in cell and tissue lysates and in the medium were measured by HPLC-ESI tandem mass spectrometry as described before (16). Compounds were detected
in the multiple reaction monitoring mode. Data were acquired and analyzed using MassLynx NT v4.0 software (GV Instruments, Manchester, UK).
Results

Variations in ambient tonicity induce co-ordinated changes in expression levels of genes involved in betaine metabolism in cultured H4IIE hepatoma cells

Consistent with our previous results (16) hypotonic swelling increased Bhmt mRNA levels, whereas hypertonic dehydration had the opposite effect. Hypotonic incubation increased Bhmt mRNA concentration significantly after 8h (Fig. 2a). Bhmt expression reached maximum levels after 16h, consistent with the reported expression profile over 24h (16). Hypertonic incubation of H4IIE cells resulted in a significant reduction in mRNA expression already after 4h, reaching a minimum level at 8h, which remained constant up to 24h.

Furthermore, we found that Dmgdh mRNA expression was highly sensitive to cell volume variation (Fig. 2b). Hypotonic incubation induced a significant upregulation of Dmgdh mRNA expression up to 5.1-fold (log ratio 0.70) after 24h compared to the respective mRNA levels found in the isotonically treated cells. Hypertonic incubation led to a downregulation of Dmgdh mRNA expression to 0.2-fold (log ratio –0.76) after 8h (Fig. 2b). We observed a modulation of Dmgdh mRNA expression already after 4h of anisotonic treatment.

Sardh mRNA expression (Fig. 2c) showed a similar but weaker pattern of osmosensitivity: A small but significant upregulation of Sardh mRNA (1.3-fold or log-ratio 0.12) was noted after 16h of hypotonic treatment and a significant decrease in Sardh mRNA levels already after 4h.

Choline kinase (CHK) catalyses the first step of phosphatidylcholine synthesis converting choline to phosphocholine (Fig. 1). Expression studies of Chk mRNA (Fig. 2d) revealed a fast and transient response to anisotonic exposure in H4IIE cells.
Hypotonic incubation induced a significant decrease (0.2-fold expression, log ratio – 0.68) of Chk mRNA concentration after 4h with a restoration of normal Chk mRNA expression already after 8h. Chk mRNA expression increased significantly 2.1-fold (log ratio 0.32) after 4h of hypertonic treatment and returned to control levels after 8h.

The mRNA expression of choline-phosphate cytidyltransferase 1 alpha (PCYT1a), encoding the enzyme CTP:phosphocholine cytidylytransferase (CCTα) that catalyses the conversion of phosphocholine to CDP-choline, showed no significant osmosensitivity within 24h of anisotonic treatment (Fig. 2e). Moreover, mRNA expression of Pemt, encoding phosphatidylethanolamine N-methyl transferase (PEMT) which generates phosphatidylcholine from phosphatidylethanolamine in three consecutive reactions (25), was essentially insensitive to changes in ambient tonicity (Fig. 2f).

MTHFR catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which is required as methyl group donor for the methionine synthase dependent remethylation of homocysteine (Fig. 1). Mthfr mRNA expression was largely insensitive to hypotonic cell swelling but was potently upregulated up to 1.7-fold (log ratio 0.22) after 8h in hypertonic conditions (Fig. 2g).

Expression of mRNA encoding methionine synthase (MS or MTR), which catalyzes the methyl transfer from 5-methyltetrahydrofolate onto homocysteine to form methionine (Fig. 1), showed no consistent response to changes of ambient osmolarity (Fig. 2h). Cystathionine-β-synthase (CBS) initiates the degradation and removal of homocysteine from the methionine regeneration cycle (Fig. 1). Cbs mRNA expression showed a slight reduction under hypertonic conditions while hypotonic swelling was apparently without effect on its expression levels (Fig. 2i).
Variations in ambient tonicity induce co-ordinated changes in expression levels of genes involved in betaine metabolism in primary hepatocytes of wild-type and Mthfr⁻/⁻ mice

To prove the transferability of results from immortalised rat H4IIE hepatoma cells to a more physiological model and to extend our investigations, we measured expression levels of mRNAs encoding a wider range of enzymes involved in betaine metabolism in wild-type (Mthfr⁺/+), primary murine hepatocytes after exposure to anisotonic media for 24h. In addition, we used primary hepatocytes prepared from livers of mice homozygous for a complete disruption of the Mthfr gene (Mthfr⁻⁻) to test whether a deficiency in transferable methyl groups due to MTHFR deficiency does affect the regulation of betaine metabolism.

As shown in Figure 3a, the sensitivity of Bhmt mRNA expression to cell volume changes was affected by MTHFR deficiency in cultured primary hepatocytes. The response to hypotonic stress in wild-type mice (increase in expression 2.6-fold, log ratio 0.41) further increased significantly to 4.3-fold (log ratio 0.64) in Mthfr⁻⁻ mice. The hypertonic decrease (0.5-fold, log ratio –0.25 in wild-type and 0.4 fold, log ratio 0.35 in Mthfr⁻⁻ mice) did not differ between the genotypes.

Dmgdh mRNA levels (Fig. 3b) in primary hepatocytes of both genotypes were significantly upregulated in a hypotonic environment 2.5-fold (log ratio 0.39) in wild-type and 2.7-fold (log ratio 0.43) in Mthfr⁻⁻ mice, respectively. Unlike in H4IIE cells, we found no effect of hypertonic stimulation on Dmgdh mRNA expression in primary hepatocytes.

Correlating to the changes of Sardh mRNA expression induced by anisotonic incubation of H4IIE cells, we found a significant reduction of Sardh mRNA expression to 0.6-fold (log ratio –0.29) after 24h of hypertonic stimulation in wild-type primary
hepatocytes. We found no significant changes in Sardh mRNA expression in Mthfr\(^{-/-}\) cells however (Fig. 3c).

Choline dehydrogenase (CHDH) that catalyzes betaine synthesis from choline (Fig. 1) was not affected by hypotonic stress. It was however significantly upregulated 2.0-fold (log ratio 0.29) and 1.5-fold (log ratio 0.16) under hypertonic conditions in hepatocytes from wild-type and Mthfr\(^{-/-}\) mice, respectively (Fig. 3d).

Hypotonic exposure resulted in a significantly decreased Pemt mRNA expression in wild-type and Mthfr\(^{-/-}\) mice. Pemt mRNA expression was largely insensitive to rises in ambient osmolarity in both genotypes but for a trend (p=0.258 in wild-type and p=0.075 in Mthfr\(^{-/-}\)) towards increased expression (Fig. 3e).

Choline kinase (Chk) mRNA concentrations in wild-type and Mthfr\(^{-/-}\) hepatocytes did not change during hypotonic exposure. Wild-type, but not Mthfr\(^{-/-}\) hepatocytes, showed a significant increase (1.4-fold or log ratio 0.14) of Chk mRNA concentration after a 24h of hypertonic incubation (Fig. 3f).

In contrast to results in H4IIE cells, the expression of Cbs mRNA was significantly increased by hypotonic incubation in both wild-type (2.1-fold, log ratio 0.32) and Mthfr\(^{-/-}\) (1.6-fold, log ratio 0.20) hepatocytes (Fig. 3g). Hypertonic conditions led to a decrease in Cbs expression in both genotypes (0.6-fold, log ratio –0.23 in wild type and 0.4-fold, log ratio –0.44 in Mthfr\(^{-/-}\) cells). In both anisotonic environments, the expression of Cbs mRNA was significantly lower in Mthfr\(^{-/-}\) hepatocytes compared to wild-type cells.

Hypotonic incubation increased methionine synthase (Mtr) mRNA levels in wild-type (1.5-fold expression, log ratio 0.16) but not clearly in Mthfr\(^{-/-}\) hepatocytes (Fig. 3h). In hypertonic conditions, Mtr mRNA levels became downregulated in wild-type (to 0.6-fold, log ratio –0.23) and in Mthfr\(^{-/-}\) hepatocytes (to 0.4-fold, log ratio –0.39). Similar to the observed expression changes of Cbs, Mtr mRNA levels in Mthfr\(^{-/-}\) hepatocytes
were less upregulated under hypotonic and significantly more downregulated under
hypertonic conditions compared to wild-type cells.

**Dmgdh mRNA expression is highly sensitive to changes in ambient osmolarity**

The enzyme DMGDH catalyses the degradation of dimethylglycine to sarcosine and
prevents substrate inhibition of BHMT. When we further investigated the sensitivity of
*Dmgdh* mRNA expression in H4IIE cells to more subtle changes in ambient
osmolarity we found upregulation starting at 255mosmol/l and downregulation
starting at 371mosmol/l (Fig. 4). This surprisingly strong effect of a variation in cell-
volume on *Dmgdh* mRNA expression is even more profound than the effect on BHMT
that we found in our previous study (16).

**DMGDH and SARDH protein expression appears to follow changes in gene
expression in H4IIE cells**

In a previous study, we reported that the osmosensitive changes of *Bhmt* mRNA
expression in H4IIE cells were mirrored by respective changes in BHMT protein
expression and activity (16). In this study, we are able to provide preliminary
evidence that DMGDH protein levels also follow the changes in *Dmgdh* gene
expression (Fig. 5a). After 48h of incubation, *Dmgdh* expression was increased
under hypo- and decreased under hypertonic conditions in H4IIE cells. Moreover, the
osmosensitivity of *Sardh* mRNA expression (Fig. 2c) seems also be reflected at the
protein level, albeit to a lesser extent (Fig. 5b). Reliable quantification of protein
expression changes was however not possible.
Osmotic modulation of Dmgdh mRNA expression is mediated by Tyrosine kinases, cyclic nucleotide-dependent protein kinases and JNK-dependent signalling

Signal cascades relaying cell volume changes to Dmgdh mRNA expression were characterised by using varied pharmacological inhibitors (Fig. 6). The tyrosine kinase inhibitor genistein (1) prevented Dmgdh mRNA suppression in response to hypertonic incubation, whereas its inactive analogue daidzein was ineffective, supporting the specificity of the genistein effect. Neither genistein nor daidzein affected the increase in hypotonic medium. H89, a cAMP-dependent protein kinase (PKA) inhibitor (6) and the broad-spectrum kinase inhibitor H7 completely abolished both the hypertonic suppression and the hypotonic increase of Dmgdh mRNA expression levels. Gö68960, a PKC inhibitor with broad specificity (13), showed no effect, thus indicating that PKC is not involved in mediating the osmosensitivity of Dmgdh mRNA expression. Wortmannin, an inhibitor of the phosphatidylinositol 3 (PI3) kinase, had no effect on modulation of Dmgdh mRNA expression. In addition, the highly specific mammalian target of rapamycin (mTOR) inhibitor rapamycin, at concentrations, which were able to block insulin signalling towards the mTOR downstream targets 4E-BP1 and p70 S6-kinase in H4IIE cells (12), did not affect the osmosensitivity of Dmgdh mRNA expression in this model. It is known from previous experimentation that hypotonic exposure of H4IIE cells does not activate mitogen-activated protein kinases (MAPK) whereas hypertonic incubation activates extracellular signal-regulated kinases (Erk-1/Erk-2), c-Jun-N-terminal kinase (JNK) and the p38-type MAPK in these cells (19, 28). PD098059, SB220025 and SP600125 inhibit signalling via Erk-1/Erk-2, p38, and JNK, respectively (2, 4, 7) and were used to study a potential role of MAPKs in Dmgdh mRNA suppression through hypertonic incubation. Although all these compounds are known to be effective in
H4IIE cells (11, 12, 19) only SP600125 was able to affect the hypertonicity-related suppression of Dmgdh mRNA expression, whereas PD098059 and SB220025 were ineffective.

These data suggest an involvement of tyrosine kinases, cyclic nucleotide-dependent protein kinases and JNK-dependent signalling in mediating the osmosensitivity of Dmgdh mRNA expression.

Concentrations of betaine and related metabolites are altered in Mthfr<sup>−/−</sup> mouse liver and in H4IIE exposed to anisotonic medium

We had previously documented changes in betaine and DMG concentrations in H4IIE cells (16). To extend these observations we exposed H4IIE cells to anisotonic conditions for 24h and analysed concentrations of choline, homocysteine and methionine in cell lysate and supernatant by tandem mass spectrometry. As shown in Figure 7a, hypotonic ambient conditions led to a slight reduction of intracellular choline while extracellular choline concentrations were significantly higher than after isotonic incubation, indicating decreased uptake of choline by cells. Extracellular homocysteine concentrations changed under anisotonic conditions whereas intracellular homocysteine remained largely unaltered. H4IIE cells tended to release more homocysteine in response to hypotonic stimulation and released significantly less under the hypertonic condition (Fig. 7b). Intra- and extracellular concentrations of methionine tended to be reduced under hypotonic conditions whereas hypertonic incubation led to significantly increased intracellular methionine concentrations and a trend to higher concentrations in medium (Fig. 7c).

The consequences of MTHFR deficiency on the betaine-related metabolite profile in liver and plasma of wild-type, heterozygous, and Mthfr<sup>−/−</sup> mice has been previously...
We measured metabolite and organic osmolyte concentrations in a small number of experimental animals to confirm results under present experimental conditions. As expected, the concentration of betaine in livers of Mthfr<sup>−/−</sup> mice was significantly reduced (Fig. 8a), probably due to a compensatory increase in remethylation activity via BHMT, whereas plasma betaine concentrations remained largely unchanged (Fig. 9a). Choline concentrations in liver or plasma were not dependent on Mthfr genotype (Figs. 8b and 9b) which confirms previous results in the same animal model (22). The product of the BHMT-catalysed remethylation reaction is dimethylglycine (DMG). DMG concentrations in liver showed a trend towards lower levels with reduced MTHFR activity that was however not significant (Fig. 8c). Plasma metabolite concentrations showed no differences between genotypes (Fig. 9c). MTHFR deficiency in humans causes accumulation of high concentrations of homocysteine in plasma. Our results showed a pronounced accumulation of homocysteine in plasma in Mthfr<sup>−/−</sup> mice (Fig. 9d). However, homocysteine concentrations in homogenised liver tissue of Mthfr<sup>−/−</sup> mice remained constant, indicating efficient export out of liver cells (Fig. 8d). Methionine concentrations showed a trend towards lower values in Mthfr<sup>−/−</sup> animals in both compartments (Figs. 8e and 9e). Liver concentrations of sarcosine and glycine, representing the last two intermediate metabolites of betaine degradation, remained unaltered (Figs. 8h and 8g). The concentrations of the organic osmolytes taurine (Fig. 8f) and GPC (Fig. 8i) were not significantly altered in relation to the Mthfr genotype.

The betaine-depleted Mthfr<sup>−/−</sup> mouse reveals a gene expression profile in liver that resembles that of hepatocytes after hypotonic exposure

To study the metabolism of organic osmolytes on a wider range we used a custom designed microarray focused on hepatic osmolyte metabolism, with approximately
350 possible targets and compared the gene expression of male Mthfr^/- mice with that of their wild-type C57BL/6 littermates. A signal to noise ratio ≥ 2 was considered a detectable target and a total of 104 targets were detectable of which 38 were differentially expressed targets in Mthfr^/- mice (see Table 1). Gene expression levels were assessed using logarithmized ratios between Mthfr^/- and wild-type mice.

Bhmt and Sardh as well as Cbs mRNA expression were all upregulated in Mthfr^/- mice compared to wild-type. Serine hydroxymethyltransferase (Shmt) mRNA, encoding the enzyme responsible for interconversion of glycine to serine, was upregulated as well. Two more genes involved in homocysteine removal, adenosine kinase (Adk) and S-adenosylhomocysteine hydrolase (Sahh) were highly upregulated in Mthfr^/- mouse liver, probably reflecting increased homocysteine accumulation.

The genes coding for Carbamoyl-phosphate synthase 1 (Cps1) and Glutaminase 2 (Gls2), two enzymes related to ammonia detoxification in liver, were upregulated in Mthfr^/- mice, possibly indicating a response to increased amino acid degradation in liver. We interpret the increased mRNA expression of several heat shock proteins under betaine depletion as part of a compensatory stress response. Consistently, mRNAs coding for several cathepsins and saposin, were upregulated in livers of Mthfr^/- mice, indicating a pro-apoptotic state.
Discussion

In this paper, we are able to demonstrate for the first time that variations in ambient osmolality induce synergistically co-ordinated changes in the expression of genes that direct the metabolism of the organic osmolyte betaine in hepatocytes. We can show that the expression $Dmgdh$ and $Sardh$ is upregulated together with $Bhmt$ in hypotonic, but downregulated under hypertonic conditions. This supports previous suggestions that betaine is a relevant organic osmolyte in liver and that its availability is regulated to adjust to cell volume changes. In addition, the upregulation of mRNA expression of CHDH and CHK that catalyze the first step of betaine formation and GPC synthesis, respectively, together with a trend for increased expression of PEMT that promotes methylation of phosphatidylethanolamine for GPC production, all indicate a mechanism of co-ordinated cellular adaptation to a hypertonic environment and cell shrinking.

The synchronised regulation of the expression of mRNAs coding for enzymes related to betaine metabolism was observed in both hepatoma cells and cultured primary hepatocytes (Figs. 2 and 3), suggesting that it is not a phenomenon merely related to cell transformation. Pharmacological characterization as shown in Fig. 6 demonstrates that tyrosine kinases and cyclic nucleotide-dependent protein kinases as well as the JNK signalling pathway relay cell volume changes to regulate $Dmgdh$ expression, similarly to what we previously described for $Bhmt$ (16). The modulation of $Dmgdh$ expression through the same signalling pathways as $Bhmt$ supports the notion that betaine metabolism is co-ordinated at the level of gene expression.

Betaine is an important osmolyte in kidney where its intracellular concentration is mainly regulated by transport through the betaine transporter BGT1 (see 9 for a
Betaine concentration in liver tissue is relatively low under physiological conditions but can reach millimolar concentrations with betaine supplementation (23). In liver, betaine derives from choline in hepatocytes and seems to be predominantly stored in liver macrophages and sinusoidal endothelial cells from where it is released in response to hypotonic cell swelling. Hypertonic incubation does however not induce increased betaine uptake into cultured rat hepatocytes (27). It was recently demonstrated that BGT1 is strongly expressed in murine hepatocytes (29) suggesting a prominent role for betaine transport in liver that remains to be further characterized. In this study, we provide evidence that there is an important contribution of methylamine metabolism in hepatocytes to the adaptive provision of betaine to liver cells.

H4IIE cells in culture take up choline and release betaine into the medium. We have previously described the decrease of intracellular betaine concentrations after 24 hours of hypotonic stimulation that was caused by increased degradation and transport into the medium. We also found an increase in intracellular betaine concentration after hypertonic incubation due to decreased degradation and export (16). BHMT enzyme activity is strongly inhibited by excess accumulation of its product DMG (3). In this study, we found that intra- and extracellular DMG concentrations were generally low under both iso- and anisotonic conditions. DMG did not accumulate in the culture system even with increased betaine degradation, a fact that suggests its immediate further conversion to sarcosine and glycine, probably to minimize BHMT product inhibition. This view is corroborated by the increase in Dmgdh and Sardh expression, the genes coding for key enzymes responsible for the degradation of DMG and sarcosine to glycine.

In an earlier study, Lang et al. had found a low abundancy of Dmgdh mRNA but a significant expression of the protein in rat liver, indicating either low expression or an
instable Dmgdh mRNA (9). We performed a stability assay of Dmgdh mRNA originating from H4IIE hepatoma cells (data not shown) that did not indicate significant instability of this particular mRNA. Due to a poor yield of the intramitochondrial protein fraction, we could only provide preliminary evidence for corresponding changes of enzyme content under anisotonic conditions (Fig. 5). The observed changes in metabolite concentrations however do support changes in enzyme activity in accordance with changes in gene expression. A good correlation between mRNA and protein expression was shown previously for a number of enzymes in the choline metabolism pathways, including Chdh and Bhmt (24).

Hypotonic conditions stipulated a reduction in choline uptake and enhanced homocysteine export (Fig. 7). Since intracellular homocysteine is preferably exported, the increase of homocysteine concentration in medium after hypotonic incubation indicates an increase in homocysteine formation, probably due to increased anabolism and demand in transferable methyl groups as well as decreased availability of betaine for remethylation. The release of potentially cytotoxic homocysteine constitutes another mechanism that contributes to the well-known hepatoprotective effect of cell swelling (17).

Intra- and extracellular concentrations of methionine tended to be reduced under hypotonic conditions but were significantly increased in cell lysate after hypertonic exposure with a trend to higher concentrations in medium (Fig. 7c). Together with the homocysteine data, this suggests an increase in transmethylation activity and methionine consumption with hypotonic cell swelling and the opposite effect occurring with hypertonic cell shrinkage.

In addition to cell cultures, we used an Mthfr^{-/-} mouse model of severe hyperhomocysteinemia to study the expression of enzymes related to organic
osmolyte metabolism ex vivo. Mthfr<sup>−/−</sup> mice display a betaine depletion phenotype that to a certain extent simulates the intracellular condition after prolonged hypotonic incubation. Increased catabolism of choline and betaine could potentially lead to a reduction of choline availability. However, we found that Mthfr<sup>−/−</sup> animals largely maintain their intracellular choline pool despite increased demands for remethylation and increased production of alternative osmolytes, such as GPC (Fig. 8). We interpret this, in line with previous findings (27), as a mechanism to maintain cell volume regulatory capacity even under conditions of betaine depletion. It also reflects the large size of the choline pool. MTHFR deficient animals maintain normal intracellular homocysteine concentrations in liver, mostly by exporting excess homocysteine to prevent accumulation of toxic concentrations. Even though taurine is a relevant osmolyte in rat Kupffer cells (26), lowered taurine levels indicate a minor involvement in osmotic regulation strategies of liver parenchymal cells in the MTHFR deficient mouse.

In conclusion, our results are consistent with a synergistically co-ordinated regulation of betaine synthesis and degradation and concomitant changes in intracellular osmolyte concentrations to allow adaptation of the liver to cell volume variations that arise under physiological periprandial and under pathological conditions. The existence of such a well-orchestrated system underlines the importance of cell volume homeostasis for liver function and of methylamine osmolytes such as betaine as hepatic osmolytes.
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Authors’ contributions
LH, BCS, TG and FS designed experiments, analysed data, and wrote the paper. EM and DH supervised the project and approved the final manuscript. LH mainly performed the experiments with contributions from GB, TG, and BCS, respectively.

Current affiliations: F. Schliess; Profil Institut für Stoffwechselforschung GmbH, Neuss, Germany. B.C. Schwahn; Metabolic Department, Royal Hospital for Sick Children and University of Glasgow, United Kingdom.

Disclosures and ethics
As a requirement of publication authors have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and protection of animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication. The authors have no conflicts of interest to disclose.
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involves epidermal growth factor receptor-dependent CD95 tyrosine


Figure captions

Figure 1. Overview of hepatic methylamine osmolyte metabolism. 1) 5,10-Methylenetetrahydrofolate reductase (MTFHR), 2) Methionine synthase (MS or MTR), 3) Betaine-homocysteine methyltransferase (BHMT), 4) Dimethylglycine dehydrogenase (DMGDH), 5) Sarcosine dehydrogenase (SARDH), 6) Choline dehydrogenase (CHDH), 7) Cystathionine-β-synthase (CBS), 8) Phosphatidylethanolamine N-methyltransferase (PEMT), 9) CTP:phosphocholine cytidylytransferase (CCTα), 10) Choline kinase (CHK) Only enzymes mentioned in this study are shown and marked as grey circles with the respective number.

Figure 2. Steady-state mRNA expression analysis in H4IIE rat hepatoma cells. The cells were incubated for 4, 8, 16, or 24 hours, respectively, with various ambient osmolarity. Total RNA was purified and 100ng of total RNA was subjected to expression analysis by quantitative real-time PCR. Each bar represents three independent experiments measured in triplicate. CoxIV mRNA was used for normalization. Relative changes in mRNA concentration refer to the correspondent normo-osmolar control. Values are presented as log-ratio +/- SEM. Differences in mRNA concentrations with p<0.05 were considered statistically significant and marked as *.

Figure 3. Steady-state mRNA expression analysis in primary murine hepatocytes isolated from mice, either wild-type (white bars) or homozygous (dark grey bars) for a disruption of Mthfr. Total RNA was purified after 24 hours of stimulation and 100ng of
total RNA was subjected to expression analysis by quantitative real-time PCR. Each bar represents three independent experiments measured in triplicate. Relative changes in mRNA concentration refer to the correspondent isotonic control. Cox IV mRNA was used for normalization. Values are presented as log-ratio +/- SEM. Differences in mRNA concentrations with p<0.05 were considered statistically significant and marked as *. Significant changes between genotypes are marked as §.

**Figure 4.** Osmosensitivity of Dmgdh mRNA expression. H4IIE rat hepatoma cells were stimulated for 24 hours with media featuring the indicated osmolarities. Dmgdh mRNA expression was determined by quantitative Realtime PCR. The assay was performed in triplicate at three independent experiments and values are shown as log-ratio values +/- SEM. Relative changes with a p value p<0.05 were considered significant and marked as *.

**Figure 5.** Representative Western Blot analysis of a) DMGDH and b) SARDH protein expression in H4IIE cells after anisotonic incubation. The upper blots show the specific signal of DMGDH and SARDH while the lower blots show the GAPDH signal as a loading control. Cells were incubated in the indicated osmolarity for 24 hours or 48 hours, respectively. 8mg per lane were separated on a 7.5% polyacrylamide gel and transferred to a PVDF membrane for subsequent immunodetection. The secondary antibodies (goat-anti mouse) were conjugated with a horseradish peroxidase, able to generate a signal with an ECL-substrate. The signals were detected on an ECL-sensitive film and processed automatically.
Figure 6. Pharmacological characterization of the regulation of Dmgdh mRNA expression induced by cell volume changes. H4IIE cells were incubated for 8 hours in hypo- (205 mosmol/l light grey bars) and hypertonic (405 mosmol/l dark grey bars) medium and treated with Genistein (Gen, 50µmol/l), Daidzein (Dai, 50µmol/l), H89 (10µmol/l), H7 (20µmol/l), Gö6580 (10µmol/l), Wortmannin (Wort, 500nmol/l), Rapamycin (Rap, 500nmol/l), SB220025 (SB,20µmol/l), SP600125 (SP, 50µmol/l) PD098059 (PD, 50µmol/l). Controls remained untreated. The assay was performed in triplicate at three independent experiments and values are displayed as log-ratio +/- SEM. * marks significant changes on mRNA levels compared to the respective mRNA level under normo-osmolar conditions. § denotes significant changes to the respective osmolarity of the untreated control. Relative changes with a p value p<0.05 were considered significant. The difference between the active inhibitor Genistein and its inactive precursor Daidzein is indicated separately.

Figure 7. Metabolite concentrations in H4IIE rat hepatoma cells and supernatant medium after 24 hours of stimulation as measured by HPLC-MS/MS. Three experiments were performed, each in triplicate. Values are presented as mean values +/- SEM. Relative changes with a p value p<0.05 were regarded as significant and marked with *.

Figure 8. Concentrations of a) betaine, b) choline, c) dimethylglycine (DMG), d) homocysteine (Hcy), e) methionine, f) taurine, g) sarcosine, h) glycine and i) glycerophosphocholine (GPC) as determined by HPLC-MS/MS in samples of liver tissue of mice that were either wild-type (WT, n=4) or homozygous (KO, n=5) for a disruption of the Mthfr gene. Values are given as mean +/- SEM. Liver metabolite
concentrations are expressed relative to sample weight. Relative changes with a p value \( p<0.05 \) were considered significant and marked with *.

**Figure 9.** Concentrations of a) betaine, b) choline, c) dimethylglycine (DMG), d) homocysteine (Hcy) and e) methionine were determined by HPLC-MS/MS in plasma samples of mice that were either wild type (WT, \( n=4 \)) or homozygous (KO, \( n=5 \)) for a disruption of the *Mthfr* gene. Values are given as mean +/- SEM. Relative changes with a p value \( p<0.05 \) were considered significant and marked with *.
(a) 

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(b) 

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Table 1.
Comparison of cDNA array gene expression profiles from livers of mice, either wild type (WT) or homozygous for a disruption of the Mthfr gene (KO).

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<th>Name</th>
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DNA arrays were hybridized with labeled cDNA derived from mRNA extraction of n=7 animals for each genotype. Gene expression changes are expressed as logarithmic ratio $\log_{10}(\text{KO}/\text{WT})$ of the median of the background-corrected and normalized fluorescence signal. A ratio from $\pm 0.18$ to $\pm 0.30$ indicates a moderate regulation. Genes with a ratio $>0.30$ (bold) were upregulated or with a ratio $<-0.30$ were downregulated (bold cursive).