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Research Article

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Medium Tonicity Regulates Expression of the Na^+- and Cl^--dependent Betaine Transporter in Madin-Darby Canine Kidney Cells by Increasing Transcription of the Transporter Gene

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Abstract

Betaine is one of the major compatible osmolytes accumulated by kidney derived Madin-Darby canine kidney cells cultured in hypertonic medium. Betaine is accumulated by Na^+- and Cl^--dependent uptake from the medium. To gain insight into the mechanism by which hypertonicity evokes an increase in the V_{max} of the betaine transporter in Madin-Darby canine kidney cells, we measured the relative abundance of mRNA for the transporter in cells shifted to a hypertonic medium and found parallel increases in mRNA abundance and cotransporter activity. The increase in mRNA levels preceded the increase in transporter activity slightly. Transcription of the gene for the transporter rose rapidly and to the same relative extent as mRNA abundance in cells shifted to hypertonic medium, indicating that transcription of the gene for the cotransporter plays a major role in regulating the accumulation of betaine in response to hypertonicity. (*J. Clin. Invest.* 1993. 91:1604–1607.) Key words: hypertonicity • cell volume regulation • cotransport • organic osmolytes

Introduction

The renal medulla, the site of final urine formation, is the only tissue in mammals that is normally exposed to varying concentrations of sodium that range from near that in plasma during diuresis to more than three times plasma levels during antidiuresis. To balance the high extracellular osmolarity, cells in the medulla (1, 2), like cells in a variety of nonmammalian species exposed to hypertonicity, accumulate small nonperturbing organic solutes, termed osmolytes, rather than high concentrations of electrolytes that perturb the function of cell macromolecules (3). Some kidney derived cell lines accumulate osmolytes when shifted to hypertonic culture media (4), and are valuable models for studying osmolyte accumulation (5–8).

Glycine betaine (betaine) is a major osmolyte in the hypertonic renal medulla (2) and in kidney derived cells (4) when they are cultured in a hypertonic medium. Betaine is accumulated in kidney-derived Madin-Darby canine kidney (MDCK)[1] cells by uptake on a Na^+- and Cl^--dependent carrier located in the basolateral plasma membrane (9). Transport is increased as the result of an increase in V_{max} with no change in K_m (6, 10). Studies in which expression of the betaine transporter in *Xenopus laevis* oocytes was greater after injection of poly(A)$^+$RNA from hypertonic cells than it was after injection of poly(A)$^+$RNA from isotonic cells led to the suggestion that expression of the transporter was increased in hypertonic cells as the result of an increase in the abundance of transporter mRNA (11). That conclusion was confirmed recently after the cloning of the cDNA for the MDCK cell betaine transporter (10).

The purpose of this study was to gain further understanding of the regulation of expression of the transporter by hypertonicity. Using ribonuclease protection assays, we found a high correlation between mRNA abundance and the expression of transporter activity. Using nuclear run-on assays, we found that exposure of cells to hypertonicity stimulates transcription of the gene for the transporter. We conclude that the activity of the transporter is regulated at the transcriptional level, and that changes in mRNA abundance lead to changes in transporter activity.

Methods

Cell culture. MDCK cells were purchased from the American Type Culture Collection (Rockville, MD) and grown in defined medium (7). The defined medium contained 200 μM *myo*-inositol and 64 μM choline, which support the accumulation of the osmolytes *myo*-inositol and glycerophosphorylcholine in MDCK cells in hypertonic medium (4–6). The culture medium did not contain betaine to maximize the expression of the betaine transporter (6). Cells were grown on tissue culture plastic dishes. Where indicated, medium tonicity was increased by adding raffinose.

Measurement of betaine transporter activity. All experiments were performed with cells grown on tissue culture plastic dishes. Although the transport response to hypertonicity is greater in MDCK cells grown on filters, we studied the transport response of cells grown on plastic tissue culture dishes, since those cultures are more suitable for harvesting material for studies of mRNA abundance and rate of gene transcription. MDCK cells were grown in dishes (Cluster 6; Costar Corp., Cambridge, MA) to measure betaine uptake. Cells were rinsed three times with PBS, and then incubated for 10 min at 37°C in uptake solution (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes/Tris, pH 7.4, containing 50 μM [^{14}C]betaine (58 mCi/mmol) (New England Nuclear, Boston, MA/National Institutes of Health, Bethesda, MD a kind gift of M. B. Burg NIH). Previous studies have shown that betaine uptake is linear with time for 30 min, and

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1. *Abbreviations used in this paper:* betaine, glycine betaine; betaine transporter, Na^+- and Cl^--coupled betaine transporter; MDCK, Madin-Darby canine kidney (cells).

> 95% is Na^+ dependent (6). After the uptake period, cells were rinsed three times with 3 ml of ice-cold wash solution (160 mM choline chloride, 5 mM Hepes/Tris-buffered to pH 7.4). The cells were then solubilized in 1 ml of 0.25 N NaOH, and aliquots were taken for liquid scintillation counting and protein determination (Protein Assay Kit; Bio-Rad Laboratories, Richmond, CA). Uptake assays of MDCK cells adapted to hypertonic medium were performed in uptake and wash solutions made appropriately hypertonic by addition of mannitol.

Ribonuclease protection assay. Total RNA was extracted using LiCl precipitation and acid-phenol extraction as described (11). An antisense RNA probe of 374 bp (nucleotides 988–1,361 of the BGT-1 cDNA [10]) was prepared by subcloning the BspMII fragment of pBGT-1 (containing 1,361 bp from the 5′ end of the cDNA insert) into the BspMII site of pSPORT1. The subclone was digested with BglII and antisense RNA synthesized using T7 RNA polymerase and [α-^{32}P]-CTP (800 Ci/mmol) (Amersham Corp., Arlington Heights, IL).

10 μg of total RNA was hybridized with 10^5 cpm of the probe in 20 μl of hybridization solution (80% formamide, 40 mM piperazine-*N*,*N*′-bis (2-ethane-sulfonic acid) pH 6.4, 400 mM Na acetate, and 1 mM EDTA) at 45°C overnight. After digestion with RNases A and T1, samples were electrophoresed on a 6% polyacrylamide gel containing 7 M urea. The areas corresponding to the protected bands were identified by autoradiography, excised from the gel, and their radioactivity was measured by liquid scintillation counting.

Nuclear run-on assays. Assays were performed using standard techniques (12). Briefly, nuclei were isolated by centrifugation of the cell homogenate through a 2-M sucrose cushion and stored at −80C in 25% glycerol until use. Run-on transcripts were labeled for 30 min at 25°C in a transcription solution containing 2 mM each of ATP, GTP, and UTP and 0.7 μCi/μl of [α-^{32}P]CTP (3,000 Ci/mmol, Amersham Corp.). Transcripts were isolated by standard phenol extraction and ethanol precipitation after DNase and proteinase K treatments of the nuclei. To quantify run-on transcripts of the betaine transporter and β-actin genes, plasmids carrying the corresponding cDNAs were immobilized on nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) and hybridized to the labeled transcripts. The same amount (cpm) of transcripts were added to each hybridization reaction, and hybridized transcripts were quantified by measuring ^{32}P bound to the nitrocellulose membranes using liquid scintillation counting. The amount of hybridization was corrected for hybridization efficiency, which was determined for each reaction by simultaneous hybridization of [^{3}H]cRNA made from the plasmids.

Hybridizations were performed in 50% formamide, 5× SSC, 0.1% SDS, 20 mM Tris-Cl, pH 8.0, 5× Denhardt's solution, and 100 μg/ml salmon sperm DNA, and washed three times at 50°C in 0.1 × SSC and 0.1% SDS.

Results

Regulation by medium tonicity of betaine transporter activity and betaine transporter mRNA abundance. When medium tonicity was increased from 300 to 500 mosmol/kg H_2O, betaine transport activity increased, reaching a peak 24–30 h after the switch (Fig. 1), as previously described (6, 9). In preliminary Northern and dot blot hybridization analyses (data not shown), we found that 24 h of hypertonicity elicited a marked increase in betaine transporter mRNA and little or no change in β-actin mRNA, extending earlier observations (10). Because of the low abundance of transporter mRNA in isotonic cells, we used ribonuclease protection assays to quantify transporter mRNA levels throughout this study.

If an increase in mRNA abundance is responsible for the increase in transporter activity, the time course of the increase in mRNA abundance should be similar to or precede the time course for the increase in transporter activity in response to

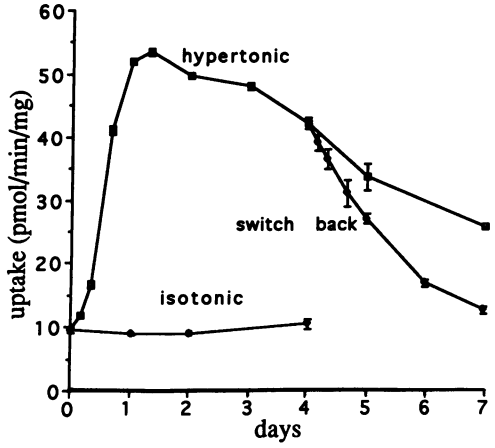

Figure 1. Time course of change in the activity of the betaine transporter after shift in medium tonicity. On day 0, cultures grown to confluence in isotonic medium were switched to medium made hypertonic (500 mosmol) by adding raffinose (*open squares*) or refed with isotonic medium (*filled circles*). On day 4, some of the hypertonic cultures were switched back to isotonic medium (*filled circles*). Each point is the mean of three determinations, brackets indicate standard errors. In some cases, standard errors were smaller than the symbol for the mean and are not indicated.

hypertonicity. The experiments in Figs. 1 and 2 summarize the time courses of the two responses after shift of cells to hypertonicity and their return to isotonicity. The rate of betaine transport reached peak of about five- to sixfold the isotonic rate 24–30 h after the shift to hypertonic medium. The rate declined slowly thereafter, remaining greater than the rate in isotonic cells throughout the period of study. When cells were switched back to isotonic medium, the transport rate decreased to isotonic levels in ~ 3 d (Fig. 1), as reported previously (8).

To determine the temporal relationship between mRNA levels for the transporter and transporter activity, mRNA was

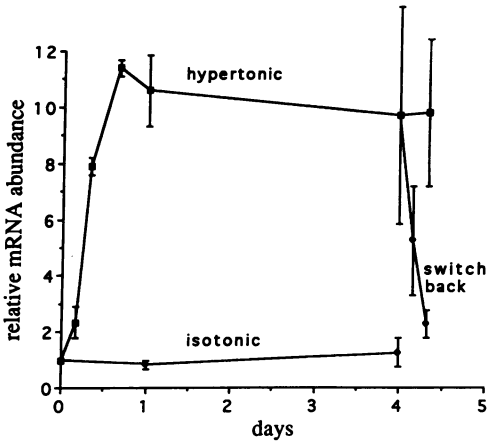

Figure 2. Time course of change in betaine transporter mRNA abundance after shift in medium tonicity. Protocol and symbols as in Fig. 1. Values obtained in ribonuclease protection assays are expressed as fold increase compared to the isotonic level at time 0. Each point is the mean of three experiments, each bracket indicates the standard error of the mean.

collected at times similar to those at which transporter activity was assayed after medium tonicity was changed from isotonic to hypertonic and from hypertonic to isotonic (Fig. 2). 16 h after medium tonicity was increased, the abundance of transporter mRNA reached a peak ~ 12-fold isotonic levels, and then remained at about that level. The rise in transporter mRNA levels was rapid and sufficient to explain the increase in transporter activity. When cells were switched back from hypertonic to isotonic medium, the level of transporter mRNA fell rapidly. The slower fall in transporter activity presumably reflects a slower turnover of the transporter protein.

Hypertonicity activates transcription of mRNA for the transporter. To determine whether hypertonicity regulates transporter mRNA abundance by regulating the rate of its transcription, we performed nuclear run-on assays using nuclei isolated from cells that had been switched from isotonic to hypertonic medium or from hypertonic to isotonic medium. The data in Fig. 3 indicate that transcription of the gene for the betaine transporter rose after medium tonicity was increased, and reached a peak at ~ 16 h after the switch, a pattern compatible with the interpretation that the increase in transcription is primarily responsible for the increase in mRNA abundance. Transcription of the gene for β-actin was not changed 24 h after the switch (Fig 3., *inset*), indicating that activation of betaine transporter gene-1 transcription by hypertonicity was not part of a generalized induction of transcription. When cells were switched back to isotonic medium after 4 d in hypertonic medium, transcription of the gene for the betaine transporter fell very rapidly.

Discussion

These experiments establish the key role of increased transcription and the resulting increase in mRNA abundance in the induction of increased activity of the betaine transporter in MDCK cells in response to hypertonicity. The time courses and relative changes are compatible with the interpretation that the increase in transcription is primarily responsible for the increase in betaine transporter mRNA, which in turn is primarily responsible for the increase in activity of the transporter (presumably the amount of transporter protein). Our results do not rule out the possibility that tonicity exerts a minor regulatory effect at a later step, such as the stability of the mRNA for the betaine transporter. That will be difficult to study because of the very low abundance of transporter mRNA in isotonic MDCK cells. Similarly, there may be minor translational or posttranslational regulation by tonicity. We are preparing antibodies to the betaine cotransporter to evaluate those possibilities.

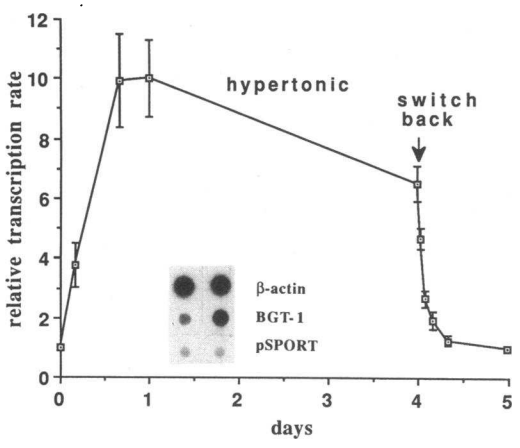

Figure 3. Time course of change in rate of transcription of the gene for the betaine transporter after shift in medium tonicity. Confluent cultures were switched to hypertonic medium on day 0 and returned to isotonic medium on day 4. Nuclear run-on assays were performed using nuclei harvested at the indicated times. Values are expressed as fold increase compared to the value at time 0. Each point is the mean of three or four experiments, the brackets are standard errors. The insert is an autoradiogram of dot blot hybridizations from nuclear run-on assays using nuclei collected 1 d after the switch to hypertonic medium (*right*) and from isotonic cells (*left*).

The accumulation of two other major organic osmolytes is also regulated by hypertonicity at a transcriptional level. In GRB-PAP1 cells derived from rabbit renal papilla, sorbitol is the major osmolyte (13, 14). The transcription of aldose reductase, which catalyzes the rate limiting step in sorbitol production, is increased in GRB-PAP1 cells by hypertonicity (15). Hypertonicity also stimulates MDCK cell accumulation of the compatible osmolyte *myo*-inositol that is the result of increased *myo*-inositol uptake (5). Recent studies (16) indicate that hypertonicity increases the transcription of the gene for the *myo*-inositol transporter in MDCK cells.

The mechanism by which hypertonicity increases transcription of the betaine transporter in MDCK cells remains to be determined. There is more information about regulation of betaine transport by hypertonicity in bacteria. Betaine is taken up by *Escherichia coli* and *Salmonella typhimurium* on transporters designated Pro P and Pro U, both identified originally as transport systems mediating uptake of proline. Both systems have higher affinity for betaine than for proline, and the activity of both is increased in bacteria shifted to hypertonic media, the former increasing by > 10-fold, and the latter increasing > 100-fold (17). Transcription from the Pro U promoter has been reconstituted in vitro and shown to be stimulated by direct action of high concentrations of potassium glutamate (18), the major intracellular salt of bacteria.

The intracellular salt concentration may also be important in activating transcription of genes involved in osmolyte accumulation in kidney cells. In GRB-PAP1 cells, hypertonicity increases transcription of aldose reductase mRNA with a time course (15) like that for the MDCK cell *myo*-inositol transporter (16) and that seen here for the betaine transporter. The increase in total cell aldose reductase activity was slower than the increase in activity of the betaine and *myo*-inositol transporters because the rate of degradation of aldose reductase appears to be much slower than the rate of degradation of the transporters, all assayed by activity. Induction of aldose reductase activity by hypertonicity correlated well with the sum of the intracellular concentrations of Na^+ plus K^+ (19). There is no information as to how the high cell concentration of Na^+ plus K^+ increases transcription of aldose reductase. It is likely that shift to a hypertonic medium causes a similar increase in the intracellular concentration of Na^+ and K^+ in MDCK cells, since their volume changes rapidly when they are shifted to anisotonic media (20). Hypertonicity affects the level of other mRNAs. The abundance of mRNA for heat shock protein 70, *Egr*-1, and c-*fos* was elevated when MDCK cells were shifted to hypertonic medium (21). The significance of those observa-

tions remains to be established. In view of the major effect of tonicity on transcription established in the present studies, we have begun to clone the gene for the betaine transporter to identify *cis*-acting regions that participate in regulation of transcription by medium tonicity.

Acknowledgments

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