Osmoregulatory Alterations in Myo-Inositol Uptake by Bovine Lens Epithelial Cells
Part 4: Induction Pattern of Na⁺–Myo-Inositol Cotransporter mRNA Under Hypertonic Conditions Denoting an Early-Onset, Interactive, Protective Mechanism Against Water Stress

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Purpose. To examine the effect of hypertonicity on the induction of the Na⁺–myo-inositol (Na⁺–MI) cotransporter(s) in cultured bovine lens epithelial cells (BLECs).

Methods. Na⁺–MI cotransporter 626-bp reverse transcription-polymerase chain reaction product amplified from lens cell RNA and aldose reductase (AR) cDNA probes were used to measure respective mRNA content by Northern blot analysis.

Results. Northern blot analysis of BLEC mRNA hybridized to Na⁺–MI cotransporter cDNA showed that Na⁺–MI cotransporter mRNA increased when secondary cultures of BLECs were exposed to physiological medium supplemented with 116 mmol/1 NaCl. A time course further revealed a maximal increase in Na⁺–MI cotransporter mRNA by 8 hours. Thereafter, the level of Na⁺–MI cotransporter mRNA steadily declined for the duration of the 72-hour incubation period despite continuous exposure of BLECs to hypertonicity. AR mRNA levels maximally increased by 24 h of cell exposure to hypertonic condition. Unlike Na⁺–MI cotransporter mRNA, AR mRNA remained elevated throughout the duration of the experiment. Hypertonic exposure resulted in a steady state accumulation of myo-inositol and sorbitol for 6 days. Inhibition of sorbitol formation prompted the intracellular myo-inositol content to a higher level.

Conclusions. These data suggest that enhanced MI transport and accumulation, as an adaptive osmoregulatory response to hypertonicity in cultured BLECs, is a primary, early-onset, protective mechanism against water stress, succeeded by, enhanced sorbitol formation and accumulation, a secondary, late-onset protective mechanism. The lens appears to respond to the preliminary stages of hyperosmotic stress by induction of Na⁺–MI cotransporter mRNA, indicating that the myo-inositol carrier protein(s) play an initial responsive role in the management of osmotic stress. Lens water stress management is interactive because myo-inositol and sorbitol levels are regulated in concert. Invest Ophthalmol Vis Sci. 1994;35:4118–4125.

The lens must be able to withstand variations in extralenticular osmolality, such as glucose-derived osmotic stress. As part of this adaptive response, the lens must compensate for water stress by accumulating osmotically active, but chemically inert, nonperturbing organic solutes termed osmolytes.¹ The compatible organic osmolytes comprise three classes of compounds: sugar alcohols or polyols, neutral free amino acids and related solutes, and methylamines plus urea. At least three putative compatible osmolytes have been identified in cultured lens epithelial cells, namely, sorbitol (a polyol produced by the cell),⁴ myo-inositol,³⁴ and taurine.⁵ The latter are a polyol and an amino acid derivative, respectively. Doubtless, other
nonperturbing lens osmolytes will be characterized. The intracellular accumulation of these compatible organic osmolytes operates to maintain osmotic balance and to protect the cell against the perturbing effects of high intracellular concentrations of electrolytes that might otherwise adversely affect protein structure and function.

Osmotically stressed cultured dog lens epithelial cells reportedly respond with an increase in aldose reductase (AR) mRNA of approximately twofold by 24 hours, with a maximum increase of between fourfold and eightfold by 48 hours. Because it appears to take several days for the activity of AR to reach a maximum, the lens cell must manifest an early-onset response to protect itself from acute hypertonic stress. Enhanced myo-inositol uptake activity is that early adaptive mechanism in cultured bovine lens epithelial cells. The accumulation of myo-inositol in cultured BLECs, resulting from exposure to hypertonic medium, has been attributed to increased uptake activity from the extracellular medium to the cell, originating from increased maximal velocity (V_{max}) of both the high- and low-affinity Na\(^+\)--MI cotransporters without significant change in Michaelis constant (K_m). Those data suggested that hypertonicity increased the number of cotransporter carrier proteins in the plasma membrane. Indeed, we have demonstrated the necessity for protein synthesis as an integral component of the adaptive response to hypertonicity. The present study extends that observation by demonstrating that Na\(^+\)--MI cotransporter mRNA is also elevated under hypertonic conditions. The pattern of Na\(^+\)--MI cotransporter mRNA induction indicates a rapid, early-onset response to acute osmotic insult, in contrast to the later induction of AR mRNA.

**MATERIALS AND METHODS**

**Cell Culture**

Bovine (*Bos taurus*) eyes obtained from a local slaughterhouse were brought on ice to the laboratory where the lenses were removed aseptically. Bovine lens epithelial cells were isolated and cultured as previously described. Thawed cells were used to establish monolayers in 150-cm\(^2\) culture flasks (representing second-passage cells). Northern Blot Analysis

The cells from three subconfluent 150-cm\(^2\) flasks exposed for 12, 24, and 48 hours under hypertonic conditions, as described, were dispersed in Ca\(^{2+}\)--Mg\(^{2+}\)-free, serum-free, high-ambient sodium chloride medium containing 0.125% trypsin–0.05% EDTA, washed 1× with the same medium, and pelleted by centrifugation at 1500g for 7 minutes. Total lens epithelial cell RNA was extracted using RNAzol B kit (Bioteck Laboratories, Houston, TX). Poly (A)\(^+\)RNA from 10 subconfluent 150-cm\(^2\) flasks of cultured bovine lens epithelial cells exposed from 0 to 72 hours under the same experimental conditions as described was isolated by using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA).

**Northern Blot Analysis**

Purified lens epithelial cell total RNA or poly (A)\(^+\) RNA was separated by electrophoresis in 1.0% agarose–formaldehyde denaturing gels as described and blotted to a nylon membrane (Schleicher and Schuell, Keene, NH) for 16 hours in 20 × SSC. The gel containing total RNA was supplemented with two μg per lane of ethidium bromide (Gibco BRL, Gaithersburg, MD). After blotting, the nylon membrane was baked at 80°C for 1/2 hours under vacuum. The poly (A)\(^+\) blot was then subjected to hybridization with \(^{32}\)P-oligolabeled 626-bp lens cell Na\(^+\)--MI cotransporter cDNA using Random Primed DNA Labeling Kit (Boehringer
Mannheim, Indianapolis, IN), AR cDNA, or β-actin genomic DNA probes according to previously described procedures. The total RNA blot was subjected to hybridization with 32P-labeled β-actin genomic DNA or an 18S ribosomal RNA oligo probe, again according to standard procedures. The characterization of the 626-bp cDNA portion of an Na+-MI cotransporter amplified from lens epithelial RNA was recently reported. After hybridization, the blot was subjected to a stringent wash procedure. The blot was first washed twice with 2× SSC with 0.1% SDS at room temperature for 15 minutes and subsequently was washed in 0.1× SSC with 0.1% SDS at 42°C for 30 minutes with shaking. Afterward, the blot was subjected to fluorography using an intensifying screen and Hyperfilm (Amersham, Arlington Heights, IL) at −70°C. The density of the lens cell Na+-MI cotransporter mRNA, AR mRNA, and β-actin mRNA was determined by use of Quantity One, an image-processing and computer analysis program for the Unix SparcStation developed by Protein Data Imaging (Huntington Station, NY).

For hypertonicity-reversal studies, the cultured cells were maintained in high-ambient sodium chloride medium for 24 hours; thereafter, conditioned high-ambient sodium chloride medium was replaced with fresh physiological medium. Poly(A)+ RNA was isolated as described after 4, 8, 12, and 24 hours of medium reversal and subsequently was subjected to Northern blot analysis using the Na+-MI cotransporter and β-actin probes described earlier. In parallel experiments, myo-inositol and sorbitol levels were determined.

RESULTS

Measurement of Na+-MI Cotransporter mRNA and AR mRNA

Using reverse transcription-polymerase chain reaction (RT-PCR), we amplified a 626-bp cDNA portion for an Na+-MI cotransporter from cultured lens epithelial cell RNA. Our chosen PCR primers were designed to permit the amplification of a PCR product from an established Na+-MI cotransporter from Madin-Darby canine kidney cell cDNA. The cDNA probe aided in the identification and calculation of the relative content of Na+-MI cotransporter mRNA in hyperosmotically stressed lens epithelial cells.

Northern blot analysis of poly(A)+ RNA from BLECs showed some faint minor bands and one prominent band; the predominant BLEC Na+-MI cotransporter mRNA measured approximately 10.5 kb (Fig. 1). The size of the Na+-MI cotransporter mRNA agreed with that previously reported for the Madin-Darby canine kidney cell Na+-MI cotransporter mRNA transcript. Cells transferred to hypertonic medium responded with a transient inductive profile of Na+-MI cotransporter mRNA, demonstrated by Northern blot analysis (Fig. 2A). The Na+-MI cotransporter mRNA was maximally expressed at approximately 8 hours. Thereafter, the cotransporter mRNA steadily declined to near control levels (Fig. 2) for the duration of the 72-hour exposure to hyperosmotic insult. Consistent results were repeatedly observed in several similar experiments. After hybridization using the 626-bp Na+-MI cDNA probe, the blot was stripped and reprobed with the AR cDNA (Fig. 2B). Unlike the early-onset pattern of induction of the Na+-MI cotransporter mRNA, the level of AR mRNA was not appreciably elevated above physiological control until 24 hours of incubation under hypertonic condition. However, once induced, unlike the Na+-MI cotransporter mRNA, the level of AR mRNA remained elevated for the duration of the 72-hour experiment. Our data for AR expression in hypertonically treated bovine lens epithelial cells is entirely consistent with previously reported data on AR induction in dog lens epithelial cultures.

To determine that amounts of poly(A)+ RNA loaded in each lane were comparable, the blots were again stripped and subsequently hybridized with a genomic probe for β-actin. The relative level of β-actin was elevated with increasing duration of exposure to...
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Figure 2. Time course of expression of Na\(^+\)-MI cotransporter mRNA by Northern blot analysis of bovine lens epithelial poly (A)\(^+\) RNA. (A) RNA from lens cells in physiological medium (lane 1) later switched to high-ambient sodium chloride medium for 2 hours (lane 2), 4 hours (lane 3), 6 hours (lane 4), 8 hours (lane 5), 10 hours (lane 6), 12 hours (lane 7), 24 hours (lane 8), 48 hours (lane 9), and 72 hours (lane 10). (B) The blot was stripped and subsequently probed with AR cDNA. (C) The same blot was reprobed with genomic \(\beta\)-actin. Five micrograms of poly (A)\(^+\) RNA was applied to each lane.

Figure 3. Northern blot analysis of total bovine lens epithelial cell RNA. Total RNA was extracted from lens cells exposed to high-ambient sodium chloride medium for 12 hours (lane 1), 24 hours (lane 2), and 48 hours (lane 3). (A) The gel stained with ethidium bromide showed the prominent 18S ribosomal RNA bands (arrow) as visualized with ultraviolet light. (B) The blot of the same gel with 18S ribosomal RNA oligo probe. (C) Northern analysis of the blot probed with genomic \(\beta\)-actin. Twenty micrograms of total RNA was applied to each lane.

hypertonic condition, most clearly evident by 24 hours. The levels of \(\beta\)-actin remained elevated for the duration of the experiment, 72 hours (Fig. 2C), suggesting induction of \(\beta\)-actin. This, however, did not compromise the interpretation of the results. It was evident from Figure 2A that Na\(^+\)-MI cotransporter mRNA levels increased and declined when AR mRNA was not induced, that is, the increase in Na\(^+\)-MI cotransporter mRNA preceded the increase in AR mRNA. Nor could the data have been the result of a lane loading problem or Figures 2A and 2B would have increased in parallel. A similar mRNA inductive profile was observed with a cDNA probe of a second "housekeeping gene," glyceraldehyde-3-phosphate dehydrogenase, indicative of the difficulty we encountered in finding an appropriate "control" gene that does not respond to osmotic stress. Other workers, using rat liver perfused with hypotonic medium\(^{14}\) or isolated rat hepatocytes exposed to hypotonic and hypertonic incubation media\(^{15}\) have shown that actin mRNA levels can be unstable, which is attributed to cellular response to the persistent mechanical pressures of water stress.

It remained necessary, however, to ascertain whether the temporal escalation of synthesis of the \(\beta\)-actin transcript observed in Figure 2C was, in fact, due to a variation of loading of poly (A)\(^+\) RNA in each lane of the gel. Reexamination of the expression of \(\beta\)-actin was repeated with lens cell cultures incubated for 12, 24, and 48 hours of sodium hypertonic exposure, with the exception that total lens epithelial cell RNA was collected. The difficulty with normalization on the basis of \(\beta\)-actin mRNA levels in Figure 2 was thus overcome in Figure 3 by correlation with rRNA levels, as determined by ethidium bromide staining and 18S ribosomal RNA probe. A consistent level of 18S ribosomal RNA, detected by ethidium bromide staining, demonstrated the invariability of our lane loading (Fig. 3A). Similarly, consistency in lane loading was apparent by the 18S ribosomal RNA bands (Fig. 3B), as demonstrated by hybridization of the 18S ribosomal probe. The \(\beta\)-actin transcript was elevated with increasing duration of exposure to hypertonic condition (Fig. 3C). Northern blot analysis of total RNA from lens epithelial cells using the lens Na\(^+\)-MI cotransporter 626-bp cDNA as a probe failed to detect any transcripts (data not shown), probably because of an insufficient amount of myo-inositol cotransporter...
FIGURE 4. Time course of turnover of Na⁺-MI cotransporter(s) by Northern blot analysis of bovine lens epithelial poly (A)⁺ RNA. (A) RNA from lens cells incubated in high-ambient sodium chloride medium for 24 hours (lane 1) and switched to physiological medium for 4 hours (lane 2), 8 hours (lane 3), 12 hours (lane 4), and 24 hours (lane 5) and probed with a Na⁺-MI cotransporter 626-bp cDNA. (B) The same blot reprobed with genomic β-actin. Five micrograms of poly (A)⁺ RNA was applied to each lane. The operational term “turnover” does not attempt to make a distinction between degradation of mRNA and changes in transcription rate.

Measurement of Intracellular Myo-Inositol and Sorbitol

Carbohydrate analysis showed that myo-inositol and sorbitol (Fig. 5, left panel) accumulated in hypertonicity-stressed cells. The intracellular free myo-inositol pool appeared to be regulated in accordance with sorbitol levels. When the lens cell cultures were exposed to hyperosmotic insult, myo-inositol accumulated, rapidly approaching steady state. However, as the intracellular level of sorbitol accumulated and approached steady state, myo-inositol concentration was maintained, but never exceeded, a plateau (Fig. 5, left panel). Inhibition of sorbitol formation with the aldose reductase inhibitor Sorbinil (Pfizer) prompted the intracellular myo-inositol content to reach a higher steady state (Fig. 5, right panel), to compensate for the lack of sorbitol. When cells hypertonically stressed for 24 hours were switched to physiological medium, both myo-inositol and sorbitol were dramatically reduced within 24 hours of medium reversal (Fig. 6).

DISCUSSION

It was recently reported that primary cultures of human lens epithelium exposed to hypertonic medium containing NaCl (600 mOsm) or cellobiose (500 mOsm) for 72 hours displayed increased myo-inositol concentrations of, respectively, 218% and 147% of control. Secondary cultures of bovine lens epithelium respond to hypertonic insult with an increase in Na⁺-MI cotransporter uptake activity and an accumulation of intracellular myo-inositol. In a recent report, we documented that BLEC also upregulate Na⁺-MI cotransporter mRNA when they are exposed to high-ambient NaCl. In the present study, we extend that preliminary observation by demonstrating that cellular synthesis of the Na⁺-MI cotransporter transcript vacillates with increasing duration of hypertonic exposure. As a result of 72 hours of continuous osmotic insult, the Na⁺-MI cotransporter mRNA was maximally induced by 8 hours. Thereafter, it steadily declined to physiological levels for the next 64 hours (Fig. 2). The transient induction profile of the Na⁺-MI cotransporter mRNA suggests that myo-inositol accumulation in the bovine lens cell must function in concert with other protective osmolytes in defense of sustained, chronic osmotic stress. Induction of AR mRNA was maximally induced by 24 hours. After that, the AR mRNA level remained in steady state for the duration of the experiment (Fig. 4B). Enhanced myoinositol accumulation in cultured lens cells, as an adaptive osmoregulatory response to hypertonicity, appears to be an early-onset protective mechanism against the effects of acute water stress and is closely followed by an enhanced capacity for sorbitol accumulation (i.e., increased aldose reductase activity resulting from AR mRNA induction), which represents a late-onset, secondary protective mechanism against the effects of chronic hypertonicity.

As first indicated by Hohman and coworkers, myo-inositol uptake and accretion and sorbitol formation and accumulation are not redundant mechanisms acting superfluously in response to osmotic insult. Using cultured glomerular endothelial cells, they recognized the existing relationship between "early-
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onset" accumulation of myo-inositol followed by sorbitol in response to osmotic stress. Our data advance their initial observation by demonstrating the temporal variation in the early inductive profile of the Na⁺-MI cotransporter mRNA relative to the "late-onset" synthesis of the AR transcript. Moreover, Hohman and coworkers further identified an important association between myo-inositol and sorbitol accumulation, namely, that myo-inositol concentrations decreased once the aldose reductase transcript was induced and sorbitol accumulation reached its maximal level, suggesting that these two mechanisms acted in concert. Data reported in the present study confirm the contention that myo-inositol uptake and sorbitol accumulation in cultured bovine lens cells function in accordance. Na⁺-MI cotransporter mRNA was maximally expressed approximately 16 hours before maximal expression of AR mRNA under identical treatment conditions (Fig. 2). However, Na⁺-MI cotransporter mRNA returned to near physiological levels when AR mRNA continued to be maximally induced by hypertonic treatment. Medium reversibility experiments indicated that hypertonically induced Na⁺-MI cotransporter mRNA levels returned to control levels by 4 to 8 hours (Fig. 4A). The rapid turnover (decreased rate of synthesis, increased rate of degradation, or both) of the Na⁺-MI cotransporter mRNA transcript suggests an amazing capacity by the lens cell to osmoregulate in response to medium tonicity. Similar medium reversal experiments with rat mesangial cells and Chinese hamster ovary cells have previously shown that AR mRNA did not return to control levels until 48 hours. Moreover, myo-inositol reached steady state in hypertonic stressed BLECs and did not increase as intracellular sorbitol accumulation reached steady state (Fig. 5, left panel), yet myo-inositol content compensated for osmotic stress by attaining higher intracellular levels in the presence of an aldose reductase inhibitor (Fig. 5, right panel). Both myo-inositol and sorbitol decreased in parallel to near physiological concentrations when the cells were switched from a hypertonic to an isotonic medium (Fig. 6). Lens osmoregulatory systems appear to be designed to regulate the intracellular levels of myo-inositol and sorbitol in concert (intracellular concentration is linked to the

FIGURE 5. Sorbitol and myo-inositol intracellular content in cultured lens cells incubated in high-ambient NaCl medium (473 mOsm ± 6 mOsm) without (left) or with (right) 0.1 mmol/l Sorbinil for 1 to 6 days. Data represent mean ± SE taken from replicate determinations from individual flasks. When the SEM is not visible, it is because the error bars are smaller than the symbol. *Significant from the myo-inositol determinations by NaCl without sorbinil treatment using analysis of covariance (P < 0.05). SE = Standard error; SEM = standard error of the mean.

FIGURE 6. Sorbitol and myo-inositol intracellular content in cultured lens cells incubated in high-ambient NaCl medium (473 mOsm ± 6 mOsm) for 24 hours and then returned to physiological (isotonic) medium (257 mOsm ± 2 mOsm) for 24 hours. Data represent mean ± SE taken from triplicate determinations from individual flasks. SE = Standard error.
steady state equilibrium of each active osmolyte). The data further suggest that enhanced myo-inositol uptake in defense of acute osmotic stress is an early-onset protective mechanism, followed by enhanced aldose reductase activity, a late-onset protective mechanism, responding to chronic osmotic stress.

That myo-inositol uptake and accretion and sorbitol formation and accumulation are interactive osmo-regulatory mechanisms is further evidenced by our past studies. We previously reported that myo-inositol accumulation in cultured BLECs functions by a mechanism that includes at least two processes: a sodium-dependent, high-affinity transport system and a sodium-dependent, low-affinity transport system. Glucose competitively inhibits the high-affinity transport site and supports no apparent adverse effect on the low-affinity transport site; sorbitol inhibits the low-affinity transport site and has little apparent effect on the high-affinity transport site. Neither the high- nor the low-affinity transport sites are affected by galactose, whereas galactitol noncompetitively inhibits both the high-affinity transport site and the low-affinity transport site. In addition, intracellular galactitol formation and accumulation downregulates Na⁺-MI cotransporter mRNA expression. It would thus appear that sorbitol (or galactitol) and myo-inositol are active, nonperturbing, organic osmolytes in cultured BLECs and that the increased enzymatic formation of the former obviates a necessity to limit transport activity and accumulation of the latter (why accrue two osmolytes serving a similar function?) and accomplishes this through multiple mechanisms—modulation of myo-inositol uptake by polyol-mediated (partial) suppression of high- and low-affinity transport sites, modulation by polyol-mediated downregulation of Na⁺-MI cotransporter mRNA, and polyol-mediated modulation of myo-inositol efflux, an as yet poorly defined mechanism.

The direct impairment of the myo-inositol transport system and downregulation of Na⁺-MI cotransporter mRNA that results from lens cell exposure to high-ambient glucose (and galactose) or extracellular hypertonicity, therefore, both represent plausible mechanisms that could, at least in part, account for the depletion of intracellular myo-inositol associated with hyperglycemia or hyperosmotic insult. When extracellular glucose levels rise, as they do in diabetes, animal models of hyperglycemia, or induction of high-ambient glucose in physiologic surrogates such as the BLEC culture system, myo-inositol uptake through the high-affinity transport system is reduced and myo-inositol uptake through the low-affinity transport system likely proceeds normally only if an adequate concentration of extracellular myo-inositol is available. The situation is further exacerbatated if glucose is converted to sorbitol, suppressing the activity of the low-affinity transport system. Polyol accumulation further impacts on myo-inositol uptake by suppression of Na⁺-MI cotransporter mRNA levels and by polyol-driven efflux of myo-inositol from cell to medium.

Although hyperosmotic enhancement of myo-inositol accumulation results from increased uptake activity because of upregulation of Na⁺-MI cotransporter mRNA and ensuing de novo synthesis of the myo-inositol carrier protein(s), the early genetic response to hyperosmotic stress in eukaryotic cells remains to be identified. It is conceivable that immediate early gene expression encodes DNA-binding proteins that can modify the rate at which other genes are transcribed. Distinct immediate early gene profiles may, at least in part, form the underlying basis that governs the diverse programs of gene expression, leading to osmolyte accumulation in NaCl.

Hypertonicity then (by virtue of induction of AR mRNA), such as in the diabetic state (by virtue of increased substrate availability), elicits an increase in intracellular sugar alcohol, and, in analogous fashion, has an impact on the myo-inositol uptake system in the lens. Although it may be unlikely that the lens, the tissue from which these cells are derived, would be exposed to hypertonic conditions in situ, it is nevertheless intriguing to observe that in the cultured BLEC model, the act of concentrating one organic osmolyte (i.e., sorbitol) in the face of chronic osmotic insult—an evolutionarily ancient mechanism to protect against hypertonic stress—may itself promote depletion of another osmolyte (i.e., myo-inositol). The eventual loss of intracellular myo-inositol might be an underlying causative factor leading to diabetic complications. This may be particularly relevant for cases of sugar cataract development in the lens because the high concentration of intralenticular myo-inositol is probably derived primarily, if not exclusively, by active transport from aqueous humor through the anterior lens epithelium. That is, little to no ouabain-resistant (i.e., sodium-independent) passive uptake of myo-inositol occurs in cultured lens epithelium, and there is no appreciable synthesis of this metabolite in the lens.

**Key Words**

hypertonicity, induction, Na⁺-MI cotransporter mRNA, osmoregulation, cultured lens epithelial cells

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**References**

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