Modulation of Hypertonicity-Induced Aquaporin-1 by Sodium Chloride, Urea, Betaine, and Heat Shock in Murine Renal Medullary Cells

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Aquaporin-1 (AQP1) expression is induced by hypertonicity in renal medullary cells. The purpose of the present study was to elucidate the role of sodium chloride (NaCl), urea, betaine, and heat shock on hypertonicity-induced AQP1 expression in cultured murine renal medullary-K2 (mIMCD-K2) cells. AQP1 expression was maximally induced under mild hypertonic medium supplemented with 100 mM NaCl (N100), whereas severe hypertonic medium supplemented with 150 mM NaCl (N150) caused little AQP1 induction. The reduction of AQP1 expression in N150 was associated with reduced cell viability. When cells were exposed continuously to N100, hypertonicity-induced AQP1 expression was elevated, whereas the return to isotonic medium reduced AQP1 expression in a time-dependent manner. The half-life of AQP1 protein in isotonic conditions was approximately 4 h, whereas hypertonicity markedly increased its half-life. These results indicate that hypertonicity plays an important role in AQP1 induction, stability, and degradation. On the contrary, urea inhibited hypertonicity-induced AQP1 expression in a dose-dependent manner. The addition of organic osmolyte betaine in N150 enhanced hypertonicity-induced AQP1 expression, whereas it decreased AQP1 expression in N100. This suggests that the excessive accumulation of betaine may counteract hypertonic stress and thus attenuate hypertonicity-induced AQP1 expression. Heat shock treatment promoted hypertonicity-induced AQP1 expression and heat shock protein 70 (HSP70) expression in both N100 and N150, suggesting an effect on the stability of hypertonicity-induced AQP1 expression. Taken together, NaCl, urea, betaine, and heat shock that regulate hypertonicity-induced AQP1 expression are potentially important factors in urinary concentration and contribute to the steady-state level of AQP1 expression.


The renal medullary cells, unlike most cells in mammals, are routinely exposed to extremely high concentrations of sodium chloride (NaCl) and urea. The adaptation of medullary cells to hyperosmotic stress involves acute cellular efflux of water, cell shrinkage by NaCl, chronic accumulation of compatible organic osmolytes, and acute activation of immediate-early and heat shock genes (1–4). Moreover, hypertonicity with solutes that are impermeable to the plasma membrane elevates the expression of a number of genes, including the sodium/myo-inositol cotransporter (5), sodium/chloride/betaine cotransporter (6), aldose reductase (7), and urea transporter (8). In addition, aquaporin-1 (AQP1), which provides a pathway of water movement across plasma membranes in renal cells, is a water channel protein that is induced by hypertonicity (9,10). Urea, which is permeant to the plasma membrane, elevates the expression of several immediate-early genes, including Egr-1 (11). Thus, NaCl, water, organic osmolytes, heat shock proteins (HSP), and urea may play an important role in the adaptation of renal medullary cells during urine concentration.

AQP1 plays an important role in constitutive water reabsorption in the epithelial cells of the renal medulla. Specifically, AQP1 expression in the descending limb of Henle and vasa rectae of the renal medulla is critical in generating and maintaining an axial osmotic gradient through the medulla (12). AQP1 expression in the epithelial cells of the renal medulla has been studied using cultured murine renal medullary-3 (mIMCD-3) cells. In these studies, AQP1 expression was demonstrated to be upregulated by hypertonicity and its induction to be mediated by mitogen-activated protein kinase pathways (ERK, p38, and JNK) and a hypertonicity response element in the AQP1 gene (9,13). Furthermore, both transcriptional and posttranscriptional regulation was found to be involved in hypertonicity-induced AQP1 expression (9), and a previous report suggested that protein stability was important to AQP1 induction by hypertonicity in mouse BALB/c fibroblasts (14). Thus, hypertonicity is crucial on AQP1 expression and urinary concentration.

In the present study, urea, organic osmolytes, and HSP are proposed to be important factors in modulating AQP1 expression in renal medulla under hypertonic conditions. Urea, in...
contrast to NaCl, is permeant to cell membrane. When a high concentration of urea is present, it denatures protein and provides harmful effects in the medulla (2). However, the combination of NaCl and urea enhances the survival rate of cells compared with NaCl or urea alone at the same osmolality (15). Chronic adaptation by hypertonicity leads to accumulation of compatible organic osmolytes such as betaine (16). The accumulation of such osmolytes protects the cells from hypertonic stress by lowering cellular inorganic salts. Thus, the uptake and accumulation of organic osmolytes allow medullary cells to maintain intracellular ion concentrations and to stabilize cellular proteins during hypertonic conditions. The HSP family, which is also called molecular chaperones, stabilizes macromolecules (17). It has been demonstrated that increased HSP70 expression or overexpression of HSP70 protects cells from apoptosis in hyperosmotic conditions (18,19). To date, the effect of NaCl, urea, organic osmolytes, and HSP on hypertonicity-induced AQP1 expression in renal medullary cells has not been studied. The objective of the present study therefore was to examine (1) whether hypertonicity affects the induction and stability of AQP1; (2) whether urea alters hypertonicity-induced AQP1 expression; (3) whether betaine, which is one of the major organic osmolytes, alters hypertonicity-induced AQP1 expression; and (4) whether heat shock, which induces HSP, is involved in hypertonicity-induced AQP1 expression.

Materials and Methods

Betaine, cycloheximide, protease inhibitor cocktail, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO).

Cell Line and Culture Condition

Murine renal medullary mIMCD-K2 cells (20), derived from the initial half of the inner medulla, were cultured at 37°C and 5% CO2 in Opti-MEM medium (Life Technologies, Gaithersburg, MD) that contained 10% FBS. This cell line was chosen because the cell attachment on culture dishes in a severe hypertonic environment (isotonic medium) revealed the highest induction of AQP1. A previous study showed that the cell viability was not significantly reduced under isotonic medium supplemented with 100 mM NaCl for 16 h followed by treating with 20 μg/ml cycloheximide (Sigma) for 1 h to block newly synthesized proteins. The cells were incubated further in isotonic or hypertonic medium supplemented with 100 mM NaCl containing 20 μg/ml cycloheximide for various time periods. At each time point, cells were homogenized in extraction buffer and protein was immunoblotted with anti-AQP1 antibody as described above. For comparing the amount of β-actin in each sample as a loading control, the membrane was stripped and incubated with anti-β-actin antibody (Sigma).

Statistical Analyses

All values represent means ± SEM. Statistical analysis was performed by unpaired t test. P < 0.05 was considered significant.

Results

AQP1 Induction by Hypertonicity

To investigate AQP1 expression in media with different osmolalities, murine renal medullary-K2 (mIMCD-K2) cells were treated by the addition of NaCl (100 to 300 mM) or urea (200 to 600 mM) to the isotonic medium. After 16 h, total cell extract was isolated and immunoblot analysis was performed to detect AQP1 protein. As shown in Figure 1A, the addition of N100 to the isotonic medium revealed the highest induction of AQP1. The addition of N150 or higher concentrations of NaCl showed little AQP1 expression. AQP1 expression was dramatically decreased by 93 ± 3% for N150 as compared with N100. Urea had no effect on AQP1 induction. Moreover, the addition of 200 mM glycerol and DMSO did not induce AQP1 expression (data not shown). These results suggest that a hypertonic gradient, not hyperosmolality, is necessary for AQP1 induction. To investigate whether AQP1 expression by hyperosmolality was associated with changes in cell viability, we performed MTT assay. The addition of NaCl or urea to the medium caused significant cell death in a dose-dependent manner (Figure 1B). Therefore, it was concluded that the reduction of AQP1 expression in N150 was associated with reduced cell viability. Next, to determine the maximum AQP1 induction by NaCl, cells were incu-
bated with hypertonic medium supplemented with 25, 50, 75, 100, 125, or 150 mM NaCl for 16 h. As shown in Figure 1C, N100 showed the highest AQP1 expression. To examine AQP1 induction by different impermeable osmolytes, cells were incubated in medium supplemented with NaCl or impermeable osmolytes (raffinose, glucose, sorbitol, sucrose, or mannitol) for 16 h. The expression level of AQP1 protein was significantly increased by the addition of all impermeable osmolytes tested. The relative expression level was NaCl > mannitol > raffinose > sorbitol > glucose > sucrose (Figure 1D). The result indicates that impermeable osmolytes also induce AQP1 expression.

Hypertonicity-Induced AQP1 Expression Is Decreased by Returning to Isotonic Medium

To investigate whether a continuous hypertonic stress is required for hypertonicity-induced AQP1 expression, cells were treated with N100 for 16 h to induce AQP1, and then replaced the medium with isotonic or continuous N100. When the medium was returned to isotonic, hypertonicity-induced AQP1 expression was reduced after 8 h of incubation and its expression level decreased by 50% after 20 h of incubation. On the contrary, continuous exposure of cells to N100 elevated hypertonicity-induced AQP1 expression by 2.5-fold in a time-dependent manner (Figure 2). These data suggest that a continuous hypertonic stress is necessary for the steady-state level of AQP1 expression.

Comparison of AQP1 Protein Stability in Isotonic versus Hypertonic Conditions

To compare the stability of AQP1 in isotonic versus hypertonic conditions, cells were incubated with N100 for 16 h to induce AQP1 followed by treating with cycloheximide for 1 h to block newly synthesized proteins. Cycloheximide-treated cells were incubated further in isotonic or N100 with cycloheximide for various time periods. As shown in Figure 3, the half-life of AQP1 protein in isotonic conditions was approximately 4 h. In contrast, AQP1 protein in N100 was still sustained at a level of >70% of the protein after 8 h of incubation. Cycloheximide did not affect the protein level of β-actin in the experiment. This result indicates that hypertonicity enhances AQP1 protein stability.

Effect of Urea on Hypertonicity-Induced AQP1 Expression

Previous studies have shown that under severe hypertonic conditions (>600 mOsm/kg), apoptosis is induced and cell death occurs (22), whereas under mild hypertonic conditions (<600 mOsm/kg), apoptosis is blocked by activated p53, cell cycles cease, and the DNA repair mechanism is activated (23–25). Nevertheless, it has been reported that a combination of NaCl and urea enhances cell survival compared with NaCl or urea alone at the same osmolality (15). Therefore, we investigated whether urea promoted or prevented hypertonicity-induced AQP1 expression. Cells were pretreated with N100 or N150 for 16 h, and then urea (100 to 300 mM) was added to the hypertonic medium and incubated for 24 h. As shown in Figure 4A, urea inhibited hypertonicity-induced AQP1 expression in a
dose-dependent manner. However, the addition of urea in both N100 and N150 did not significantly decrease cell viability compared with N100 or N150 alone (Figure 4B). Our data indicate that urea counteracts AQP1 expression under both mild and severe hypertonic conditions.

Effect of Betaine on Hypertonicity-Induced AQP1 Expression

In hypertonic conditions, the accumulation of organic osmolytes such as betaine protects cells from hypertonic stress (16). To investigate whether betaine influences hypertonicity-induced AQP1 expression under mild and severe hypertonic conditions, cells were incubated with N100, or N150 that contained 0, 5, or 10 mM betaine for 16 h. The medium used in this study contained no betaine. There was no detectable AQP1 in the presence of betaine under isotonic conditions (data not shown). Hypertonicity-induced AQP1 expression in N100 significantly decreased in the presence of betaine in a dose-dependent manner. On the contrary, AQP1 expression in N150 was strongly enhanced in the presence of betaine (Figure 5A). The addition of betaine in both N100 and N150 did not significantly increase cell viability (Figure 5B). These results suggest that the additional accumulation of betaine under mild and severe hypertonic conditions inversely influences hypertonicity-induced AQP1 expression.

Effect of Heat Shock on Hypertonicity-Induced AQP1 Expression

Some of the HSP, including HSP70, are strongly induced by both heat shock and hypertonicity (3,26). A potential role of HSP70 in response to hypertonicity to stabilize protein conformation has been proposed. Also, increased HSP70 expression or overexpression of HSP70 protects cells from apoptosis in hyperosmotic conditions (18,19). To investigate whether heat shock influences AQP1 expression, cells were pretreated at 42°C for 30 min and then incubated them with isotonic, N100, or N150 for 16 h at 37°C. Heat shock did not induce AQP1 expression under isotonic conditions. However, AQP1 expression was significantly enhanced by heat shock under both mild and severe hypertonic conditions (Figure 6A). HSP70 was also increased under both hypertonic conditions as well as heat-shock treatment. Furthermore, its expression level in heat shock followed by hypertonic conditions was comparable to that in hypertonic shock alone (Figure 6B). Nevertheless, heat shock significantly reduced cell viability in both isotonic and hypertonic conditions (Figure 6C). This result suggests that heat shock enhances AQP1 expression under both mild and severe hypertonic conditions, although cell viability is not associated with hypertonicity-induced AQP1 expression. HSP70 may be associated with the stability of hypertonicity-induced AQP1 expression.
The role of arginine vasopressin (AVP) in urinary concentration in mammals is well known. However, maximal urinary concentration, as achieved during hyperosmolality associated with fluid deprivation, cannot be achieved by AVP alone, thus suggesting the involvement of other factors. The role of AVP in upregulating AQP2 water channel and enhancing AQP2 trafficking to the apical membrane of the collecting duct has been well defined (27). However, an important role of AQP1, which is located in the descending limb of Henle’s loop and vasa rectae in the renal medullary region (28), in urinary concentration in mice and men has been shown to be independent of AVP (29,30). Although AQP1 is not regulated by AVP, recent results from our laboratory and others have shown that AQP1 mRNA and protein are upregulated by hypertonicity (9,10).

Thus, AQP1 has emerged as a potentially independent AVP action in urinary concentration.

The renal medullary cells are exposed to a hyperosmotic environment during urinary concentration in which other cells would cause injury and death. Several adaptive mechanisms protect renal medullary cells against the deleterious effects of hyperosmolality. Therefore, the present study was undertaken to examine whether NaCl, urea, organic osmolyte betaine, and heat shock influence hypertonicity-induced AQP1 expression in renal medullary mIMCD-K2 cells. This cell line showed the maximal induction of AQP1 under a mild hypertonic condition supplemented with 100 mM NaCl, whereas a severe hypertonic condition supplemented with 150 mM NaCl caused little AQP1 induction. The reduction of AQP1 expression in N150 was associated with reduced cell viability. However, stepwise adaptation of cells to N150

**Figure 4.** Effect of urea on hypertonicity-induced AQP1 expression. (A) Cells were pretreated with hypertonic medium supplemented with 100 mM NaCl or 150 mM NaCl. After 16 h, cells were incubated further with 100, 150, 200, 250, or 300 mM urea for 24 h. Cells were harvested, and total protein was analyzed by immunoblot. Each protein blot was analyzed by densitometry. AQP1 expression in N100 was assigned as 100%. Values represent the mean ± SEM of three independent sets of experiments. (B) Cell viability. MTT assay was performed to assess cell viability as described in the Materials and Methods section. Cell viability in isotonic medium was assigned as 100%. Values represent the mean ± SEM of three independent sets of experiments.

**Figure 5.** Effect of betaine on hypertonicity-induced AQP1 expression. (A) AQP1 expression. Cells were incubated with N100 or N150 that contained 0, 5, or 10 mM betaine. After 16 h, cells were harvested and total protein was analyzed by immunoblot. Each protein blot was analyzed by densitometry. AQP1 expression in N100 was assigned as 100%. Values represent the mean ± SEM of three to five independent sets of experiments. (B) Cell viability. MTT assay was performed to assess cell viability as described in the Materials and Methods section. Cell viability in isotonic medium was assigned as 100%. Values represent the mean ± SEM of three independent sets of experiments.
also declined when returned to isotonic medium (31). These data suggest that hypertonicity may be associated with the stability of AQP1. Therefore, AQP1 stability was measured by cycloheximide treatment, which inhibits protein synthesis. We demonstrated that hypertonicity significantly enhanced AQP1 protein stability compared with the short half-life of 4 h in isotonic conditions. The similar observation was also reported by Leitch et al. (14). We conclude that hypertonicity enhances the induction and stability of AQP1.

Urea, in contrast to NaCl, is permeant to cell membrane. When a high concentration of urea is present, it denatures protein and causes harmful effects to the medulla (2). However, the combination of NaCl and urea enhances the survival rate of cells exposed to hyperosmolality (15), suggesting that the coexistence with NaCl and urea protects medullary cells from the proapoptotic effect of NaCl (32). In the present study, an inhibitory effect of urea on hypertonicity-induced AQP1 expression was demonstrated. Urea in itself caused the adverse effect on AQP1 expression by NaCl. It also has been reported that urea inhibits hypertonicity-induced toxicity-responsive enhancer binding protein and aldose reductase (33). Thus, the upregulation of AQPs and osmotically responsive proteins by hypertonicity is likely to be suppressed in the presence of urea. Taken together, urea counteracts the action to increase AQP1 expression by NaCl.

In epithelial cells of renal medulla, five compatible organic osmolytes are mainly accumulated by prolonged exposure to hypertonicity: Betaine, myo-inositol, sorbitol, taurine, and glycero-phosphorylcholine. The accumulation of such osmolytes protects cells from hypertonic stress by relatively lowering cellular inorganic salts. When cells are exposed to hypertonicity or to the higher concentration of betaine (>5 mM) in the medium, they accumulate a large amount of organic osmolytes. A previous report demonstrated that the addition of betaine to N100 strongly inhibited hypertonicity-induced betaine transporter expression in MDCK cells (34). In the present study, we showed that the additional accumulation of betaine under N100 decreased hypertonicity-induced AQP1 expression, whereas it was strongly improved by the addition of betaine under N150. Although the uptake and accumulation of betaine protects cells from severe hypertonicity, the addition of betaine under mild hypertonic conditions counteracts hypertonicity-induced AQP1 expression. Thus, the addition of betaine under severe hypertonic conditions restores its expression because betaine prevents hypertonicity-induced cell damage and apoptosis (35).

Although the protective effect of betaine on hypertonicity-induced cell death after 48 h of incubation has been reported previously (36), our data showed that the adverse expression of AQP1 by the addition of betaine in N100 and N150 was not due to changes in cell viability. This was because there was no significant effect of betaine on cell viability after 16 h of incubation. This finding suggests that the excessive accumulation of betaine may simply counteract hypertonic stress and thus attenuate hypertonicity-induced AQP1 expression.

The HSP family stabilizes macromolecules (17). It was demonstrated previously that HSP70 was strongly induced by not only heat shock but also hypertonicity (3,26). In the present study, we showed that heat-shock treatment enhanced hyper-
tonicity-induced AQP1 expression in both mild and severe hypertonic conditions. It has been demonstrated that increased HSP70 expression or overexpression of HSP70 protects cells from apoptosis in hypertonic conditions (18,19). In such severe conditions, HSP may be needed to assist in folding and degradation of proteins. In vivo, dehydration elevates the expression of HSP70 in the inner medulla, which is able to adapt to very high concentrations of NaCl (37). Among seven members of the HSP70 family, HSP70.1 is induced by hypertonicity. When HSP70.1-deficient mice were exposed to osmotic stress, the renal medulla of these mice exhibited increased apoptosis, whereas wild-type mice did not (38). Thus, the HSP70 family may play a critical role in the tolerance to osmotic stress in the kidney. In addition, Osp94 and HSP110, which are the HSP110/SSE subfamily induced by heat and osmotic stress, are highest in the inner medulla (39). Our data suggest that HSP70 and other HSP family members such as Osp94 and HSP110, which are induced by hypertonicity, may be associated with the stability of hypertonicity-induced AQP1 expression.

In summary, hypertonicity with impermeant solutes such as NaCl but not the permeant solute urea induces and stabilizes AQP1 protein, whereas urea inhibits hypertonicity-induced AQP1 expression. Organic osmolyte betaine and heat shock treatment promote hypertonicity-induced AQP1 expression under severe hypertonic conditions. All of these important factors that regulate hypertonicity-induced AQP1 expression may contribute to the steady-state level of AQP1 during urinary concentration.

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References


