Dual Effects of Hypertonicity on Aquaporin-2 Expression in Cultured Renal Collecting Duct Principal Cells

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The driving force for renal water reabsorption is provided by the osmolarity gradient between the interstitium and the tubular lumen, which is subject to rapid physiologic variations as a consequence of water intake fluctuations. The effect of increased extracellular tonicity/osmolarity on vasopressin-inducible aquaporin-2 (AQP2) expression in immortalized mouse collecting duct principal cells (mpkCCD$_{cl4}$) is investigated in this report. Increasing the osmolarity of the medium either by the addition of NaCl, sucrose, or urea first decreased AQP2 expression after 3 h. AQP2 expression then increased in cells exposed to NaCl- or sucrose-supplemented hypertonic medium after longer periods of time (24 h), while urea-supplemented hyperosmotic medium had no effect. Altered AQP2 expression induced by both short-term (3 h) and long-term (24 h) exposure of cells to hypertonicity arose from changes in AQP2 gene transcription because hypertonicity did not modify AQP2 mRNA stability nor AQP2 protein turnover. On the long-term, vasopressin (AVP) and hypertonicity increased AQP2 expression in a synergistic manner. Hypertonicity altered neither the dose-responsiveness of AVP-induced AQP2 expression nor cAMP-protein kinase (PKA) activity, while PKA inhibition did not reduce the extent of the hypertonicity-induced increase of AQP2 expression. These results indicate that in collecting duct principal cells: (*1*) a short-term increase of extracellular osmolarity decreases AQP2 expression through inhibition of AQP2 gene transcription; (*2*) a long-term increase of extracellular tonicity, but not osmolarity, enhances AQP2 expression *via* stimulation of AQP2 gene transcription; and (*3*) long-term hypertonicity and PKA increases AQP2 expression through synergistic but independent mechanisms.

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Aquaporin (AQP) water channels facilitate osmotically-driven water movement across renal epithelial cells by forming aqueous pores across the plasma membrane. Currently, 10 members of the AQP family have been identified in mammals and at least 7 of these are expressed in the kidney (1,2). In the collecting duct (CD), the site of final adjustment of water excretion, water permeability is chiefly controlled by the antidiuretic hormone [8-arginine]vasopressin (AVP), which binds to vasopressin V_2 receptors of CD principal cells, leading to $G_s\alpha$/adenylyl cyclase activation, increased intracellular cAMP concentration, and cAMP-protein kinase (PKA) activation (2). An acute increase in plasma AVP concentration induces rapid AQP2 translocation from intracellular storage vesicles to the apical membrane (3) responsible for enhanced apical water permeability. Water exits the cells *via* basolateral AQP3 and AQP4 (4,5). Diminishing levels of circulating AVP leads to endocytotic retrieval of apical AQP2 and to reduced water permeability (3). Conversely, sustained increases in circulating AVP increase AQP2 and AQP3 abundance (6) and consequently maximal CD water permeability.

Water excretion is dependent on the corticopapillary osmotic gradient which arises from interstitial accumulation of both urea and NaCl. This gradient decreases during water diuresis and increases under conditions of antidiuresis (2). Consequently, renal medullary cells are subject to fluctuations of interstitial osmolarity. Renal epithelial cells protect themselves from the effects of hypertonicity-induced cell shrinkage and osmotic stress chiefly by activating specific, immediate, early-regulated genes (7), heat shock genes (7,8), and genes that enhance accumulation of small organic solutes, known as compatible osmolytes, which in turn lower cellular ionic strength toward isotonic levels (9). Because water restriction increases AQP2 abundance in rat kidney despite chronic administration of V_2-receptor antagonists (10), interstitial hyperosmolarity may regulate AQP2 expression as well. The aim of this study was to investigate the influence of increased extracellular tonicity/osmolarity on AQP2 abundance in the immortalized mouse collecting duct principal cells (mpkCCD$_{cl4}$) cell line. These cells, derived from microdissected collecting ducts of an SVPK/Tag transgenic mouse (11), develop into tight and highly differentiated epithelium when grown on permeable filters (11–14). In addition, we have shown that mpkCCD$_{cl4}$ cells maintain high levels of native AVP-inducible AQP2 expression, which can be exploited for analysis of intracellular mechanisms governing long-term AQP2 expression (15,16).

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Materials and Methods

Cell Culture

mpkCCD$_{C14}$ cells (passages 22 to 31) were grown in defined medium (DMEM:Ham's F_{12}, 1:1 vol/vol; 60 nmol/L sodium selenate; 5 μg/ml transferrin; 2 mmol/L glutamine; 50 nmol/L dexamethasone; 1nmol/L triiodothyronine; 10 ng/ml EGF; 5 μg/ml insulin; 20 mmol/L D-glucose; 2% fetal calf serum; and 20 mmol/L HEPES, pH 7.4) (11) at 37°C in 5% CO_2, 95% air atmosphere. Experiments were performed on confluent cells seeded on permeable filters (0.4 μm pore size, 1 cm^2 growth area; Transwell, Corning Costar, Cambridge, MA). Unless specified, cells were grown in defined medium until confluent, and then in serum-free and hormone-deprived defined medium 24 h before experiments. Isoosmotic medium (300 mOsm/L) was made hyperosmotic (350 to 500 mOsm/L) by replacing a fraction of apical and basal medium (37.5 to 150 μl/600 μl apical medium and 75 to 300 μl/1200 μl basal medium) with NaCl-, sucrose-, or urea-enriched medium.

Western Blot Analysis

After incubation, mpkCCD$_{C14}$ cells were homogenized in 150 μl ice-cold lysis buffer (20 mmol/L Tris-HCl; 2 mmol/L EGTA; 2 mmol/L EDTA; 30 mmol/L NaF; 30 mmol/L $Na_4O_7P_2$; 2 mmol/L Na_3VO_4; 1 mmol/L [4-(2-aminoethyl)benzenesulfonylfluoride] (AEBSF); 10 μg/ml leupeptin; 4 μg/ml aprotinin; 1% Triton X-100; pH 7.4). Equal amounts of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilion-P, Millipore, Bedford, MA). AQP2 and Na,K-ATPase α-subunit were detected by Western blot using polyclonal rabbit antibodies as described (17,18). The antigen-antibody complexes were detected by the Super Signal Substrate method (Pierce, Rockford, IL). Bands were quantified using a video densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Real-Time PCR Analysis

After incubation, total RNA from mpkCCD$_{C14}$ cells was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity was measured by ultraviolet spectrophotometry. One μg of RNA was used to synthesize cDNA using SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed on 10 μl of cDNA diluted 1:20 (vol/vol) using 3 ng of each primer and 12.5 μl of SYBR Green Master Mix (Applied Biosystems) to obtain a final reaction volume of 25 μl. Triplicate amplification reactions were performed with an ABI prism 7000 Sequence Detection System (Applied Biosystems). Primers used for detection of mouse acidic ribosomal phosphoprotein P_0 were 5′-AATCTCCAGAGGCACCATTG-3′ and 5′-GTTCAGCATGTTCAGCAGTG-3′; primers for AQP2 were 5′-CTTCCTTCGAGCTGCCTTC-3′ and 5′-CATTGTTGTGGAGAGCATTGAC-3′; and primers for aldose reductase were 5′-AGTGCGCATTGCTGAGAACTT-3′ and 5′-GTAGCTGAGTAGAGTGGCCATGTC-3′. Data were analyzed using ABI Prism software (Applied Biosystems) and P_0 was used as an internal standard. Fold difference in cDNA abundance (F) was calculated using the formula $F = 2^{(Ct1 - Ct2)}$, where Ct1 and Ct2 are the number of cycles required to reach the threshold of amplicon abundance for experimental and control conditions, respectively.

Measurement of PKA Activity

After incubation, mpkCCD$_{cl4}$ cells were homogenized in extraction buffer (25 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L β-mercaptoethanol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Protein kinase A (PKA) activity was then measured using the SignaTECT® cAMP-Dependent Protein Kinase Assay System (Promega, Madison, WI) according to the manufacturer's instructions.

Statistical Anaylses

Results are given as the mean ± SEM from n independent experiments. Each experiment was performed on mpkCCD$_{cl4}$ cells from the same passage. Statistical differences were assessed using the Mann-Whitney U test or the Kruskal-Wallis test for comparison of two groups or more, respectively. $P < 0.05$ was considered significant.

Results

Increasing Extracellular Tonicity Exerts a Biphasic Effect on AQP2 Expression

We first investigated the effect of increased extracellular tonicity on AQP2 expression in mpkCCD$_{cl4}$ cells. Cells were pretreated or not pretreated for 24 h with 10^{-10} mol/L AVP followed by 3 or 24 h of incubation in isotonic (300 mOsm/L) or NaCl-supplemented medium (350 to 500 mOsm/L) before Western blot analysis. AQP2 protein was revealed by a narrow 28 kD band and a more diffuse band of about 35 kD representing the nonglycosylated and glycosylated forms of AQP2, respectively (19). In AVP-pretreated cells, AQP2 expression first decreased after 3 h in a NaCl concentration–dependent manner (Figure 1, A and B, left panel). Similar results were obtained after 3 h from experiments in which NaCl supplementation was restricted to either the apical or basolateral medium (data not shown). In contrast, AQP2 expression increased after 24 h of incubation in hypertonic medium with a maximal effect observed at 400 mOsm/L (Figure 1, A and B, right panel). Increasing extracellular tonicity to 500 mOsm/L reduced AQP2 expression after 24 h to levels comparable to those of cells incubated in isotonic medium. NaCl supplementation also exerted a biphasic effect characterized by a short-term (3 h) decrease followed by a long-term (24 h) increase of AQP2 protein content in cells not treated with AVP (Figure 1, C and D). Unfortunately, the effect of NaCl supplementation restricted to either the apical or basolateral medium on AQP2 expression could not be tested after long periods of time (>6 h). Indeed, the osmolarities of apical and basolateral medium equilibrated several hours after NaCl was added to either medium, as revealed by measurement of medium osmolarity, due to water movement across Transwell filters. Comparison of Western blotting and RT-PCR analysis revealed coordinated expression of AQP2 protein and mRNA. AQP2 mRNA first decreased after 3 h of hypertonic challenge and then increased 24 h later in a concentration-dependent manner both in the absence and presence of AVP (Figure 2A), the only difference being that AQP2 mRNA did not decrease when exposed to 500 mOsm/L medium for 24 h. To demonstrate the specificity of the biphasic effect of extracellular hypertonicity on AQP2 expression, we assessed the expression levels of three major tonicity-inducible genes responsible for intracellular accumulation of the compatible osmolytes sorbitol, betaine, and myo-inositol in renal medulla, *i.e.*, aldose reductase (AR), the sodium- and chloride-coupled betaine/γ-aminobutyric acid transporter (BGT1), and the sodium/myo-inositol cotransporter (SMIT) (20–22), Results showed that mRNA expression of AR (Figure 2B), BGT1, and

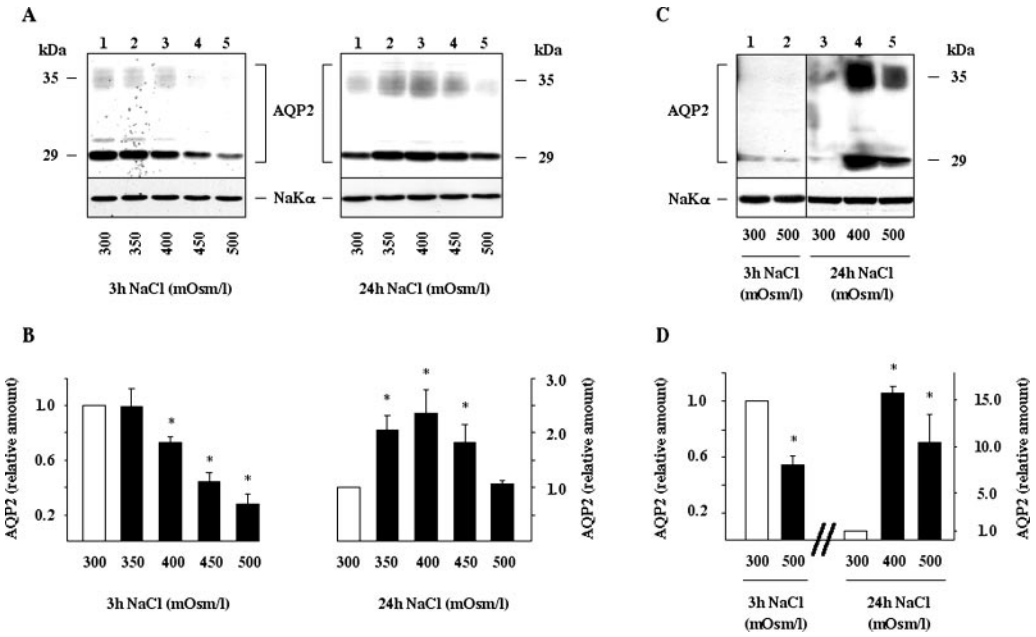

Figure 1. Hypertonicity modulates aquaporin-2 (AQP2) protein expression in a time-dependent manner. Cultured mouse collecting duct principal cells (mpkCCD$_{cl4}$) cells were incubated 24 h in the absence or presence of 10^{-10} mol/L vasopressin (AVP) and then for an additional 3 or 24 h in either isotonic (300 mOsm/L) or NaCl-supplemented hypertonic (350 to 500 mOsm/L) medium containing or not containing AVP. Total protein extracts (40 μg) of AVP-pretreated cells (A and B) and untreated cells (C and D) were separated by 10% SDS-PAGE. A and C, representative immunoblots are shown. AQP2 (*upper panel*) was detected using a polyclonal anti-AQP2 antibody. The Na,K-ATPase α subunit (*lower panel*) was used as a loading control and was detected using a polyclonal anti-α Na,K-ATPase antibody. In C, the AQP2 signal was overexposed as compared with that shown in A to visualize the low levels of AQP2 protein expression. B and D, densitometric quantification of AQP2 protein expressed as the ratio of optical density values measured at each experimental condition and those measured after 3 and 24 h under isotonic conditions (white bars) and in the presence (B) or absence (D) of AVP. Bars are mean ± SEM from five independent experiments. *$P < 0.01$.

SMIT significantly increased after 3 and 24 h of hypertonic challenge both in the absence and presence of 10^{-10} mol/L AVP (data not shown). It should be mentioned that while AQP2 mRNA expression was strongly induced after 3 h and further increased after 24 h of AVP treatment (by approximately 20- and 40-fold, respectively), AR mRNA expression was not influenced by AVP (Figure 2B). These results indicate that AQP2 expression is regulated specifically by hypertonicity in a time-dependent manner. The fact that AQP2 protein, but not AQP2 mRNA, content decreased in cells subjected to 500 mOsm/L hypertonic medium for 24 h as compared with that of cells subjected to 400 mOsm/L hypertonic medium suggests that high osmolarity (500 mOsm/L) disturbs AQP2 protein processing in mpkCCD$_{cl4}$ cells.

Respective Effects of Increased Osmolarity and Tonicity on AQP2 Expression

Hypertonic medium, established by the addition of NaCl or sucrose, induce osmotic mpkCCD$_{cl4}$ cell shrinkage (23) whereas hyperosmotic medium, established by the addition of urea which easily crosses the plasma membrane, does not appreciably alter cell volume (Hasler, unpublished results). We studied the respective influence of hypertonic challenge, after the addition of sucrose, and hyperosmotic challenge, after the addition of urea, on AQP2 expression. Western blot analysis showed that supplementation of the cell medium with either sucrose or urea decreased AQP2 protein expression to similar extents and in a concentration-dependent manner after 3 h in AVP-pretreated cells (Figure 3, A and B). Consistent with Western blot analysis, RT-PCR experiments showed that AQP2 mRNA content decreased in cells incubated in either sucrose- or urea-supplemented medium for 3 h (Figure 3C, left panel). Sucrose, but not urea, supplementation increased AR mRNA expression (Figure 3C, right panel), indicating that in contrast to hypertonic sucrose, hyperosmotic urea does not induce a typical hypertonicity-dependent response. Because PKA is a major stimulatory factor that enhances AQP2 gene expression (24), we wondered whether PKA inhibition might explain the decreased levels of AQP2 expression occurring in mpkCCD$_{cl4}$ cells exposed to short periods (3 h) of hypertonic challenge. This is unlikely because, in agreement with the results of previous work (25), 3 h of hypertonic NaCl stimulation slightly but significantly increased PKA activity in a concentration-dependent manner.

The respective roles of hypertonicity and hyperosmolarity in the long-term increase of AQP2 expression was analyzed by exposing AVP-pretreated mpkCCD$_{cl4}$ cells to sucrose- or urea-supplemented medium for 24 h. Western blot and RT-PCR analysis showed that while hypertonic sucrose increased both AQP2 protein and mRNA content, no significant increase in AQP2 expression was observed after addition of hyperosmotic

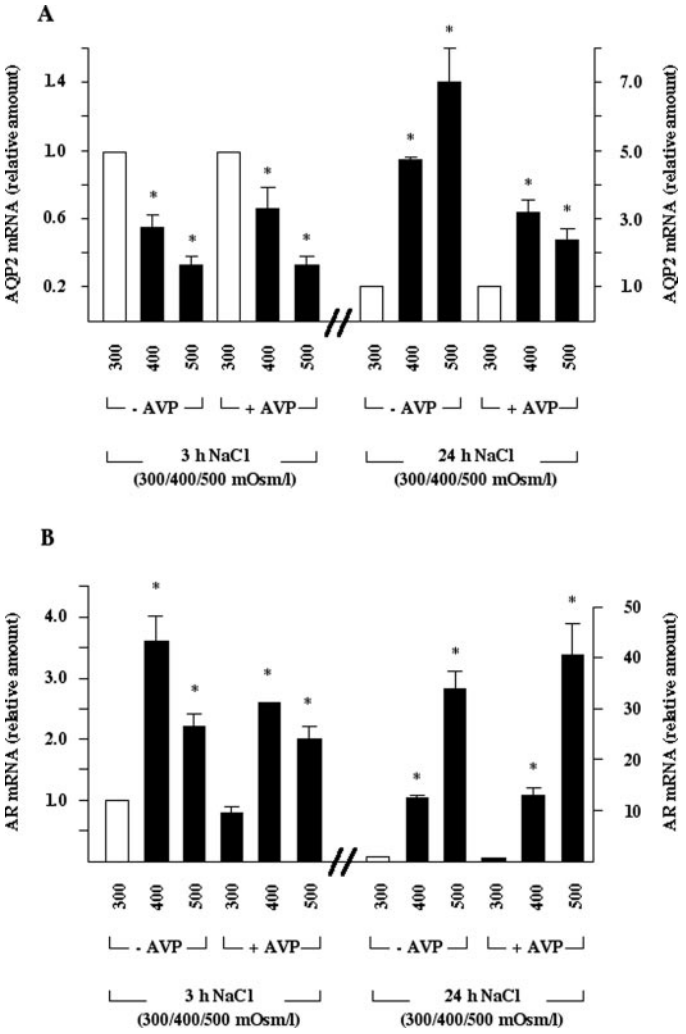

Figure 2. Hypertonicity modulates AQP2 mRNA expression in a time-dependent manner. Cultured mpkCCD$_{cl4}$ cells were incubated in the absence or presence of 10^{-10} mol/L AVP for 24 h and then for an additional 3 h or 24 h in either isotonic (300 mOsm/L) or NaCl-supplemented hypertonic (350 to 500 mOsm/L) medium containing or not containing AVP. RNA extraction and reverse transcription were performed as described in Materials and Methods. Real-time PCR (RT-PCR) was performed using primers specific for AQP2 (A) and aldose reductase (AR) (B). For AQP2, results are expressed as the percentage of control values (white bars) determined after 3 or 24 h incubation in isotonic medium. For AR, results are expressed as the percentage of control values (white bars) determined after 3 h incubation in isotonic medium in the absence of AVP. Bars are mean ± SEM from five independent experiments. *$P < 0.01$.

urea (Figure 4, A and C). Similarly, hypertonic sucrose, but not hyperosmotic urea, increased AR mRNA expression after 24 h (Figure 4C). Finally, 24-h exposure of cells to hypertonic NaCl did not alter PKA activity as compared with that of cells exposed to isotonic medium (Figure 4D).

Overall, these results indicate that the short-term decrease of AQP2 mRNA and protein expression is related to an osmotic effect, whereas the long-term increase of AQP2 mRNA and protein expression is part of the cellular response to hypertonic stress, both events being independent of variations of PKA activity.

Increased Extracellular Tonicity Does Not Alter AQP2 mRNA and Protein Stability

Because AQP2 protein and mRNA content varied to similar extents in mpkCCD$_{cl4}$ cells, extracellular hypertonicity might be expected to directly alter AQP2 gene transcription. However, a posttranscriptional effect, *i.e.*, altered stability of AQP2 mRNA and/or protein, could not be ruled out. We have previously shown that AQP2 protein degradation in mpkCCD$_{cl4}$ cells is principally achieved by the lysosomal pathway (15). We investigated whether short-term exposure of cells to increased extracellular osmolarity alters AQP2 protein degradation by exposing AVP-pretreated mpkCCD$_{cl4}$ cells for 3 h in medium supplemented or not supplemented with NaCl and in the absence or presence of the lysosomal inhibitor chloroquine. Adding chloroquine to NaCl-supplemented medium did not alter the decrease in AQP2 protein expression observed after 3 h of incubation (Figure 5, A and B). To determine whether downregulated AQP2 mRNA expression in response to short-term hypertonic medium was the result of decreased AQP2 gene transcription or of decreased AQP2 mRNA stability, AVP-pretreated mpkCCD$_{cl4}$ cells were incubated or not incubated in NaCl-supplemented medium for 1 or 3 h in the presence of the transcriptional inhibitor actinomycin D. Actinomycin D reduced AQP2 mRNA levels to similar extents in both unstimulated and hypertonic-challenged cells (Figure 5C).

To determine whether long-term exposure of mpkCCD$_{cl4}$ cells to hypertonic medium altered AQP2 protein degradation, AVP pretreated cells were exposed to medium supplemented or not supplemented with NaCl in the absence or presence of chloroquine for 9 h. Adding chloroquine to NaCl-supplemented medium did not modify the extent of the increase in AQP2 protein expression after 9 h (Figure 6, A and B). To assess AQP2 mRNA stability in response to long-term exposure of cells to hypertonic medium, AVP-pretreated mpkCCD$_{cl4}$ cells were sequentially incubated or not incubated in NaCl-supplemented medium for 24 h and then with actinomycin D for an additional 3 or 8 h. Actinomycin D reduced AQP2 mRNA levels to similar extents in both unstimulated and hypertonic-challenged cells (Figure 6C).

These results indicate that the changes in AQP2 mRNA and protein expression levels following increased extracellular tonicity are not due to altered stability of AQP2 mRNA or protein.

AVP and Long-Term Hypertonicity Synergistically Increase AQP2 Expression

We next investigated the influence of long-term hypertonic challenge on AVP-induced AQP2 expression by pretreating or not pretreating cells for 24 h with NaCl-supplemented medium and then by exposing or not exposing cells to 10^{-10} mol/L AVP for 3 or 8 h before RNA and protein extraction. As shown earlier (Figure 2), long-term hypertonic-challenge alone in-

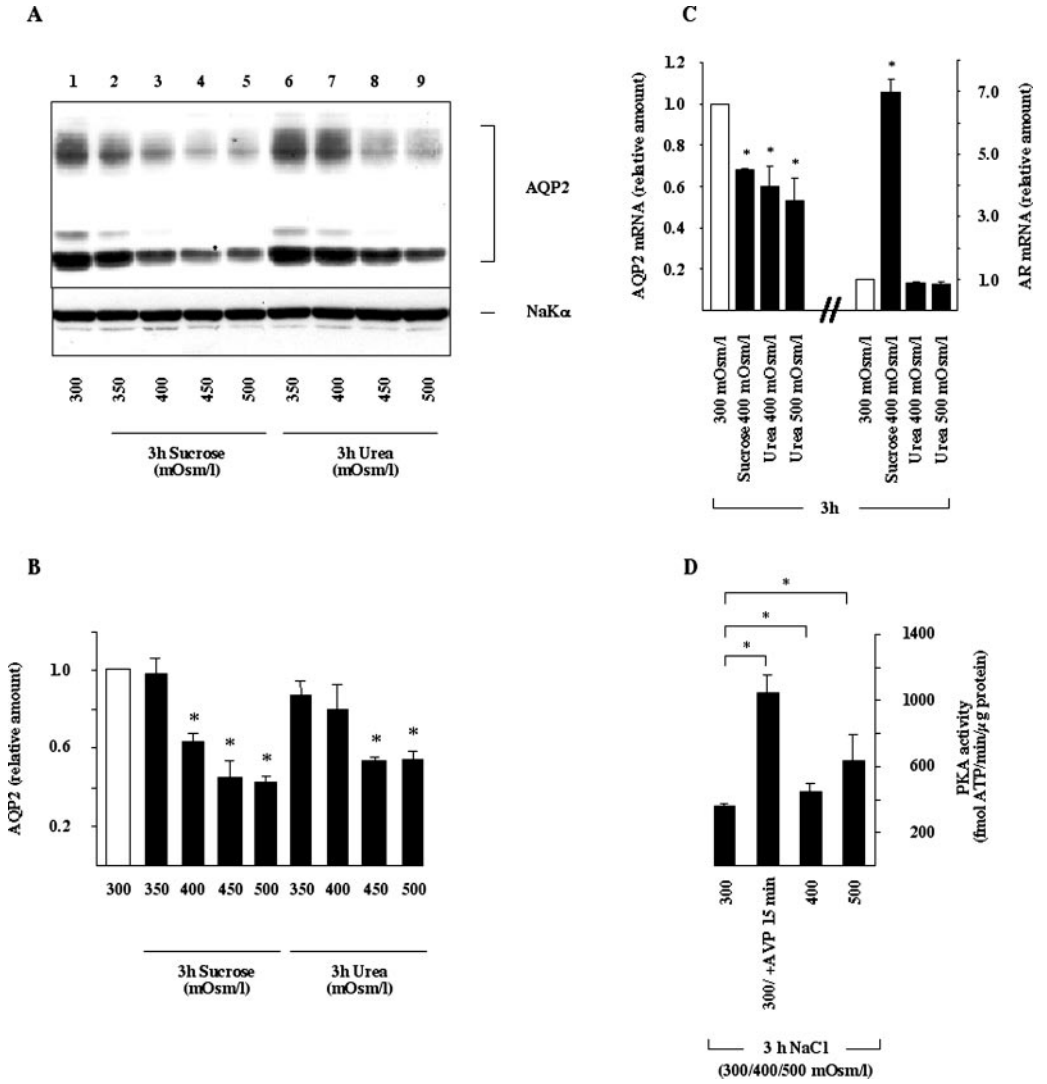

Figure 3. Short-term hyperosmotic challenge downregulates AQP2 expression. A through C, cultured mpkCCD$_{cl4}$ cells were incubated in the presence of 10^{-10} mol/L AVP for 24 h and then for an additional 3 h in either isotonic (300 mOsm/L) or sucrose- or urea-supplemented hyperosmotic (350 to 500 mOsm/L) medium supplemented with AVP before protein or mRNA extraction. A, a representative immunoblot is shown. AQP2 was detected as in Figure 1 (*upper panel*) and Na,K-ATPase α subunit (*lower panel*) was used as a loading control. B, densitometric quantification of AQP2 protein was expressed as the ratio of optical density values measured at each experimental condition and was measured after 3 h under isotonic conditions (white bar). Bars are mean ± SEM from four independent experiments. *$P < 0.01$. C, RT-PCR was performed using primers specific for AQP2 (left panel) or AR (right panel). Results are expressed as the percentage of control values (white bars) determined after 3 h in isotonic medium. Bars are mean ± SEM from four independent experiments. *$P < 0.01$. D, cultured mpkCCD$_{cl4}$ cells were either incubated in isotonic medium (300 mOsm/L) for 3 h or were subjected to AVP 10^{-10} mol/L stimulation for 15 min or to hypertonic-challenge (400 or 500 mOsm/L) in the absence of AVP for 3 h, before cell homogenization and measurement of cAMP-protein kinase (PKA) activity. Bars are mean ± SEM from five independent experiments. *$P < 0.05$.

creased AQP2 protein (Figure 7, A and B) and mRNA (Figure 7C) expression in the absence of AVP. These increased expression levels induced by hypertonicty were much more pronounced when cells where co-incubated with AVP. This effect was not additive but rather synergistic, suggesting that hypertonicity potentiates the effect of AVP on AQP2 gene transcription. We assessed the possibility that hypertonicity enhances AVP-induced PKA activation by measuring PKA activity of cells exposed for 24 h to isotonic or hypertonic medium before addition of 10^{-10} mol/L AVP for an additional 3 h or without addition of 10^{-10} mol/L AVP (Figure 7D). Stimulation of PKA activity in response to AVP was not further increased after hypertonic challenge, suggesting that hypertonicity does not stimulate AQP2 expression *via* PKA activation.

Because we used a physiologic concentration of AVP (10^{-10} mol/L) that does not maximally stimulate AQP2 expression (15), we assessed the possibility that long-term hypertonic challenge sensitizes mpkCCD$_{cl4}$ cells to the effects of AVP, leading to increased AVP-induced AQP2 expression. Cells were first stimulated for 24 h with increasing

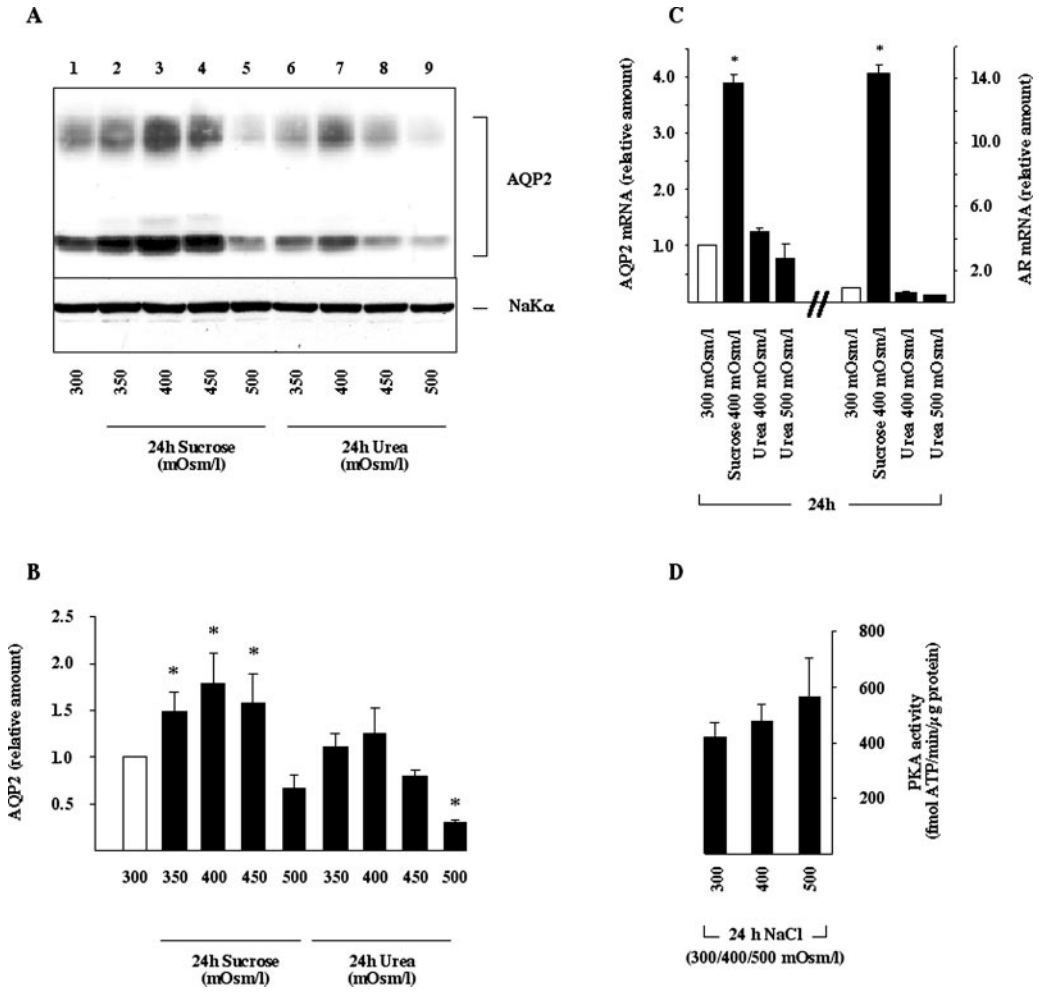

Figure 4. Long-term hypertonic challenge upregulates AQP2 expression. A through C, cultured mpkCCD$_{cl4}$ cells were incubated in the presence of 10^{-10} mol/L AVP for 24 h and then for an additional 24 h in either isotonic (300 mOsm/L) or sucrose- or urea-supplemented hyperosmotic (350 to 500 mOsm/L) medium supplemented with AVP before protein or mRNA extraction. A, a representative immunoblot is shown. AQP2 was detected (*upper panel*) and Na,K-ATPase α subunit (*lower panel*) was used as a loading control. B, densitometric quantification of AQP2 protein was expressed as the ratio of optical density values measured at each experimental condition and that measured after 24 h under isotonic conditions (white bar). Bars are mean ± SEM from four independent experiments. *$P < 0.01$. C, RT-PCR was performed using primers specific for AQP2 (left panel) or AR (right panel). Results are expressed as the percentage of control values (white bars) determined after 24 h in isotonic medium. Bars are mean ± SEM from four independent experiments. *$P < 0.01$. D, cultured mpkCCD$_{cl4}$ cells were either incubated in isotonic (300 mOsm/L) or hypertonic (400 or 500 mOsm/L) medium in the absence of AVP for 24 h before cell homogenization and measurement of PKA activity. Bars are mean ± SEM from five independent experiments.

concentrations of AVP in isotonic medium and then incubated for an additional 24 h in the continuous presence of AVP and in either isotonic or NaCl-supplemented hypertonic medium (Figure 8, A and B). Western blot analyses revealed that AQP2 protein content was significantly greater in cells subjected to both AVP and hypertonic challenge than in cells subjected to AVP alone over the entire range of AVP concentration tested. Half-maximal and maximal effects of AVP on AQP2 protein expression were not altered by exposure of cells to NaCl-supplemented medium, indicating that hypertonic challenge does not modify the sensitivity of AQP2 expression to AVP. Finally, inhibition of PKA by 10^{-5} mol/L myristoylated PKA inhibitor (PKI) (Merck Biosciences, Darmstadt, Germany), decreased AQP2 protein expression to similar extents in 24 h AVP-pretreated cells incubated in isotonic or hypertonic medium for 24 h (Figure 8, C and D). Control experiments have shown that myristoylated PKI efficiently inhibits PKA activity in mpkCCD$_{cl4}$ cells because the AVP-induced stimulation (10^{-10} mol/L for 15 min) of PKA activity was blunted in cells pretreated for 2 h with 10^{-5} mol/L PKI (as percent of control ± SEM: AVP, 186.8 ± 20.2%; PKI, 98.1 ± 8.6%; PKI + AVP, 123.5 ± 15.9%). These results suggest that hypertonicity does not stimulate AQP2 expression through modulation of AVP signaling. Rather, hypertonicity may activate a specific signaling pathway acting in conjunction with PKA and in a synergetic manner to enhance AQP2 expression.

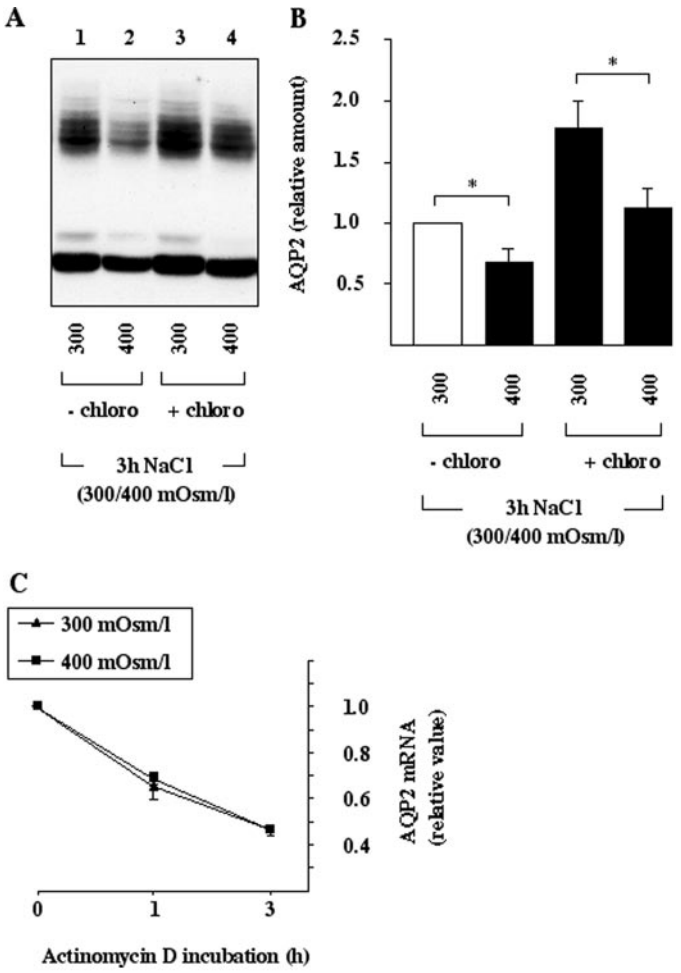

Figure 5. Short-term hypertonic challenge does not affect AQP2 protein and mRNA stability. A and B, cultured mpkCCD$_{cl4}$ cells were incubated 24 h in the presence of 10^{-10} mol/L AVP and then for an additional 3 h in the continuous presence of AVP and in either isotonic (300 mOsm/L) or NaCl-supplemented hypertonic (400 mOsm/L) medium containing or not containing 10^{-4} mol/L chloroquine before protein extraction and Western blot analysis. A, a representative immunoblot is shown. B, densitometric quantification of AQP2 protein was expressed as the ratio of optical density values measured at each experimental condition and that measured after 3 h under isotonic conditions (white bar). Bars are mean ± SEM from four independent experiments. *$P < 0.05$. C, cultured mpkCCD$_{cl4}$ cells were lysed for RNA extraction or were administered with 5×10^{-6} M actinomycin D for 30 min before 1 or 3 h of incubation in either isotonic (300 mOsm/L, △) or NaCl-supplemented hypertonic (400 mOsm/L, □) medium in the continuous presence of actinomycin D before RNA extraction. RT-PCR was performed using primers specific for AQP2. Results are expressed as the percentage of control values determined in isotonic medium in the absence of actinomycin D. Bars are mean ± SEM from three independent experiments.

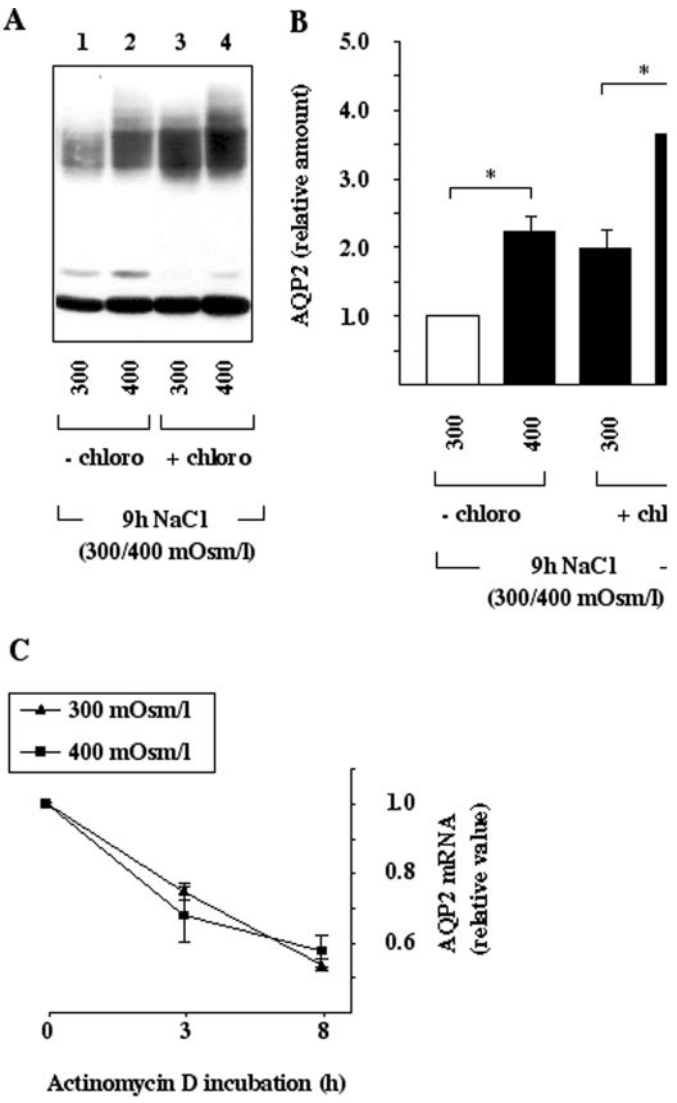

Figure 6. Long-term hypertonic challenge does not affect AQP2 protein and mRNA stability. A and B, cultured mpkCCD$_{cl4}$ cells were incubated 24 h in the presence of 10^{-10} mol/L AVP and then for an additional 9 h in the continuous presence of AVP and in either isotonic (300 mOsm/L) or NaCl-supplemented hypertonic (400 mOsm/L) medium containing or not containing 10^{-4} mol/L chloroquine before protein extraction and Western blot analysis. A, a representative immunoblot is shown. B, densitometric quantification of AQP2 protein was expressed as the ratio of optical density values measured at each experimental condition and that measured after 9 h under isotonic conditions (white bar). Bars are mean ± SEM from four independent experiments. *$P < 0.05$. C, cultured mpkCCD$_{cl4}$ cells were incubated in either isotonic (300 mOsm/L) or NaCl-supplemented hypertonic (400 mOsm/L) medium for 24 h. Cells were then lysed for RNA extraction or were incubated for an additional 3 or 8 h in the presence of 5×10^{-6} mol/L actinomycin D before RNA extraction. RT-PCR was performed using primers specific for AQP2. Results are expressed as the percentage of control values determined in isotonic (△) or hypertonic (□) medium after 24 h of incubation. Bars are mean ± SEM from four independent experiments.

Roles of ERK, p38 Kinase, and PI3K in the Modulation of AQP2 Expression by Hypertonicity

The mitogen-activated protein kinase family (26) and phosphatidylinositol 3-kinase (PI3K) (27) play a key role in hyperosmotic-inducible gene expression. We investigated the roles

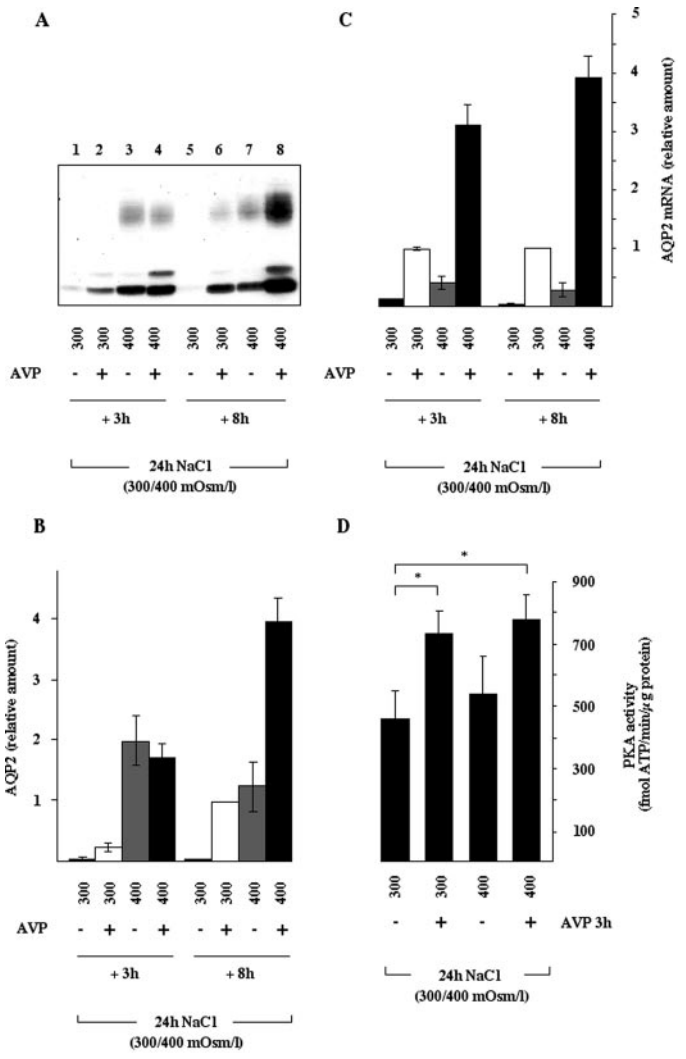

Figure 7. AVP and hypertonicity increase AQP2 mRNA and protein expression in a synergistic manner. A through C, cultured mpkCCD$_{cl4}$ cells were incubated in either isotonic (300 mOsm/L) or NaCl-supplemented hypertonic (400 mOsm/L) medium for 24 h and then in the absence or presence of 10^{-10} mol/L AVP for an additional 3 or 8 h before RNA or protein extraction. A, a representative immunoblot is shown. B, densitometric quantification of AQP2 protein expressed as the ratio of optical density values measured at each experimental condition and that measured after 8 h under isotonic conditions in the presence of AVP. Bars are mean ± SEM from four independent experiments. C, RT-PCR was performed using primers specific for AQP2. Results are expressed as the percentage of control values determined in isotonic medium after 8 h in the presence of AVP. Bars are mean ± SEM from four independent experiments. D, cultured mpkCCD$_{cl4}$ cells were incubated in either isotonic (300 mOsm/L) or NaCl-supplemented hypertonic (400 mOsm/L) medium for 24 h and then in the absence or presence of 10^{-10} mol/L AVP for an additional 3 h before cell homogenization and measurement of PKA activity. Bars are mean ± SEM from five independent experiments. *$P < 0.05$.

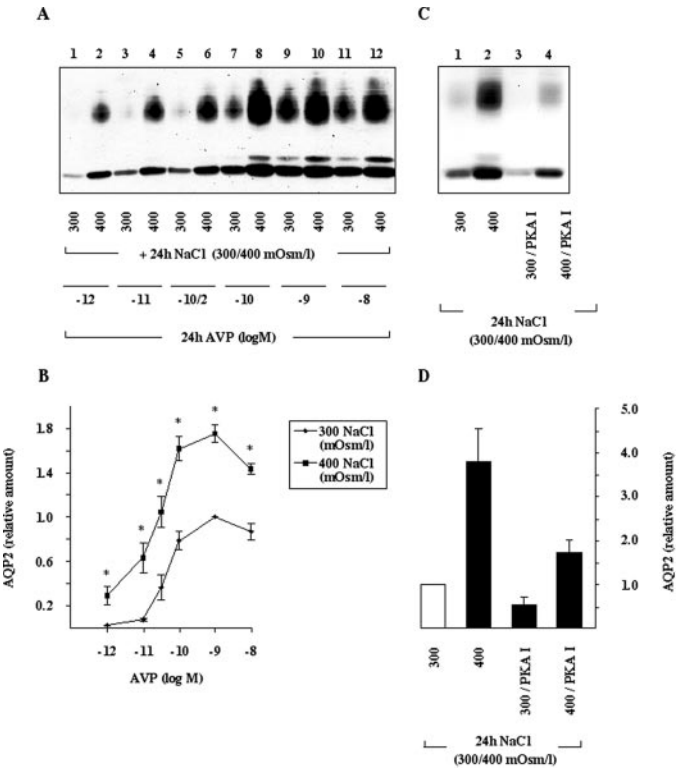

Figure 8. Hypertonicity does not enhance AQP2 expression *via* modulation of the AVP-PKA signaling pathway. A and B, cultured mpkCCD$_{cl4}$ cells were incubated in the presence of various concentrations of AVP (10^{-12} to 10^{-8} mol/L) for 24 h and then for an additional 24 h in the continuous presence of AVP and in either isotonic (300 mOsm/L, ○) or NaCl-supplemented hypertonic (400 mOsm/L, □) medium before protein extraction and Western blot analysis. A, a representative immunoblot is shown. B, densitometric quantification of AQP2 protein expressed as the ratio of optical density values measured at each experimental condition and that measured under isotonic conditions in the presence of 10^{-9} mol/L AVP. Bars are mean ± SEM from four independent experiments. *$P < 0.01$. C and D, cultured mpkCCD$_{cl4}$ cells were preincubated for 2 h in the presence or absence of 10^{-5} mol/L PKA inhibitor (PKI) after which time cells were treated with 10^{-10} mol/L AVP for an additional 2, after which they were incubated for 24 h in isotonic (300 mOsm/L) or NaCl-supplemented hypertonic (400 mOsm/L) medium before protein extraction and Western blot analyses. C, a representative immunoblot is shown. D, densitometric quantification of AQP2 protein expressed as the ratio of optical density values measured at each experimental condition and that measured under isotonic conditions in the absence of PKI (white bar). Bars are mean ± SEM from four independent experiments.

that extracellular signal-regulated protein kinase (ERK), p38 kinase, and PI3K might play in hypertonic-induced AQP2 down- and upregulation by exposing AVP-pretreated mpkCCD$_{cl4}$ cells to either 3 or 24 h of NaCl-supplemented medium in the absence or presence of the MAP kinase/ERK kinase (MEK) inhibitor UO126 (1 µmol/L), the p38 kinase inhibitor SB203580 (10 µmol/L), or the PI3K inhibitor wortmannin (0.1 µmol/L). We have previously confirmed the efficacy of all inhibitors in mpkCCD$_{cl4}$ cells (28). The extent of decreased AQP2 protein expression induced by 3 h of incubation of cells in NaCl-supplemented medium was not significantly altered by either

Table 1. Roles of ERK, p38 kinase, and PI3K in decreased AQP-2 expression mediated by short-term hypertonicity (3 h NaCl)

NaCl (mOsm/l)	Inhibitor	AQP-2 Protein (relative value)	AQP-2 mRNA (Relative Value)
300	—	100	100
300	UO	103.1 ± 7.7	
300	SB	85.9 ± 4.5	
300	Wort	80 ± 2.6	81 ± 4
400	—	62.7 ± 4.6	76 ± 5
400	UO	62.6 ± 6.7	
400	SB	48.1 ± 3.8	
400	Wort	83.4 ± 20.2	75 ± 1

Confluent mouse collecting duct principal cells ($mpkCCD_{cl4}$) cells were pre-incubated 2 h in the presence or absence of either 10^{-6} mol/L UO126 (UO), 10^{-6} mol/L SB203580 (SB), or 10^{-7} mol/L wortmannin (Wort), after which time cells were treated with 10^{-10} mol/L vasopressin (AVP) for an additional 24 h and then incubated for 3 h in isotonic (300 mOsm/L) or NaCl-supplemented hypertonic (400 mOsm/L) medium prior to Western blot or real-time PCR analyses. The results are expressed as the percentage of control values determined in isotonic medium in the absence of pharmacological inhibitors and means ± SEM from four independent experiments. ERK, extracellular-signal regulated kinase; PI3K, phosphatidylinositol 3-kinase; AQP-2, aquaporin-2.

Table 2. Roles of ERK, p38 kinase, and PI3K in increased AQP-2 expression mediated by long term hypertonicity (24 h NaCl)

NaCl (mOsm/l)	Inhibitor	AQP-2 Protein (Relative Value)
300	—	100
300	UO	57 ± 7
300	SB	85 ± 21
400	—	324 ± 47
400	UO	239 ± 7
400	SB	304 ± 32
300	—	100
300	Wort	81 ± 13
400	—	249 ± 33
400	Wort	245 ± 45

Confluent $mpkCCD_{cl4}$ cells were pre-incubated 2 h in the presence or absence of either 10^{-6} mol/L UO126, 10^{-6} mol/L SB203580, or 10^{-7} mol/L wortmannin, after which time cells were treated with 10^{-10} mol/L AVP for an additional 2 h and then incubated for 24 h in isotonic (300 mOsm/L) or NaCl-supplemented hypertonic (400 mOsm/L) medium prior to Western blot analysis. The results are expressed as the percentage of control values determined in isotonic medium in the absence of pharmacological inhibitors and means ± SEM from four independent experiments.

UO126 or SB203580 (Table 1). On the other hand, the decreased expression levels of both AQP2 protein and mRNA observed in cells treated with wortmannin alone were not further reduced by 3 h of hyperosmotic challenge (Table 1). The extent of increased AQP2 protein expression induced by 24 h of incubation in NaCl-supplemented medium was not significantly altered by either UO126, SB203580, or wortmannin, although a slight decrease was produced by UO126 in both isotonic and hyperosmotic-challenged cells (Table 2).

Altogether, our results suggest that of all pathways tested, only PI3K may participate in the downregulation of AQP2 expression immediately after hypertonic challenge. Our results further indicate that ERK, p38 kinase, and PI3K do not play a significant role in the increase of AQP2 expression induced by long-term hypertonicity.

Discussion

In our study, using cultured $mpkCCD_{cl4}$ cells as an experimental model, we show that hypertonicity exerts two specific effects on AQP2 expression. While AQP2 mRNA and protein content initially decreased after hypertonic challenge, longer exposure times (9 and 24 h) led to increased AQP2 mRNA and protein expression.

In renal epithelial cells, acute hypertonic challenge triggers a rapid increase in expression of genes involved in the accumulation of compatible osmolytes, such as SMIT, BGT1, or AR (20–22), which counteracts the damaging effects of elevated intracellular ion concentration (29). Other mechanisms also counteract hypertonic stress, such as upregulated expression of DNA damage-inducible 45 (GADD45) protein (30), cycloxygenase-2 (COX-2) (31), and several heat shock proteins (8). The increased expression level of most proteins in response to hypertonicity arises from increased transcription of their genes, with the notable exception of stabilized GADD45 mRNA (30). In this study, in contrast to BGT1, SMIT, and AR, AQP2 mRNA transiently decreased in cells exposed to hypertonic medium (Figure 2). This downregulation of AQP2 expression was independent of AQP2 mRNA stability and was most likely mediated by decreased transcription of the AQP2 gene.

Our results show that, in contrast to short-term hypertonic challenge which decreases AQP2 expression, long-term exposure of $mpkCCD_{cl4}$ cells to hypertonicity induces upregulation of AQP2 expression. PKA plays a key role in the transcriptional activation of the AQP2 gene *via* binding of adenosine 3′5′-cyclic monophosphate responsive element binding protein (CREB) to the CRE *cis*-element of the AQP2 promoter (32). However, several pieces of evidence indicate that increased AQP2 expression in response to long-term hypertonicity is not mediated *via* modulation of the AVP-PKA signaling pathway. Indeed, long-term hypertonicity did not significantly alter PKA activity in the absence or presence of AVP (Figures 4D and 7D), nor did it alter the sensitivity of AQP2 expression to AVP (Figure 8, A and B). Moreover, inhibition of PKA did not modify the extent of hypertonicity-induced AQP2 expression (Figure 8, C and D). In agreement with our results, sustained hypertonic challenge increased AQP2 mRNA expression without increasing CREB

phosphorylation in db-cAMP-treated inner medullary collecting duct cells (33). The synergistic effect of AVP and hypertonicity (Figure 7, A to C) suggests that hypertonicity potentiates PKA-dependent AQP2 gene transcription by affecting transcription factor(s) that act(s) on the AQP2 promoter together with phosphorylated CREB (pCREB). Our *in vitro* results are in good agreement with several observations from animal studies. Both medullary osmolarity and AQP2 expression are reduced in senescent rats despite unchanged papillary cAMP levels (34), while increasing medullary osmolarity restored AQP2 expression (35). Water restriction strongly increased AQP2 content in normal animals (36), returned AQP2 expression to normal levels in animals treated with the V_2-antagonist OPC-31260 (10), and increased AQP2 abundance to a much greater extent than 1-deamino-8-D-arginine vasopressin (DDAVP) in animals subjected to lithium treatment (37). Conversely, water loading decreased AQP2 content in normal animals (36) and reduced elevated AQP2 expression levels despite ongoing V_2 receptor stimulation (38). Altogether, these results suggest that as the cell adapts to the sustained hypertonic environment, a process achieved in part by increased expression of AR, BGT1, and SMIT, AQP2 expression increases as well and contributes to the general adaptive mechanism of the entire organism by decreasing water excretion.

Taken together, down- and upregulated AQP2 expression after hypertonic challenge suggests that, in mpkCCD$_{cl4}$ cells, hyperosmolarity may first activate an as yet unknown mechanism that specifically reduces AQP2 gene transcription and that would be eclipsed by a second hypertonicity-activated mechanism that promotes AQP2 transcription. Several observations hint that tonicity-responsive enhancer binding protein (TonEBP), which binds to specific tonicity-responsive enhancer (TonE) *cis*-elements (39), may play a role in upregulation of AQP2 by long-term hypertonicity. TonEBP participates in compatible osmolyte accumulation by upregulating AR (40,41), BGT1 (42,43), and SMIT (44) mRNA expression. In addition, TonEBP regulates expression of other genes such as UT-A and HSP70–2 (8,45). Enhanced AR, BGT1, and SMIT expression after hypertonic stimulation in mpkCCD$_{cl4}$ cells is indicative of TonEBP activity. It is therefore tempting to speculate that in response to sustained hypertonicity, *i.e.*, the situation prevailing in the renal medulla, TonEBP may bind to the highly conserved TonE *cis*-element situated about 600 base pairs upstream of the AUG start codon of the AQP2 gene and participate in the upregulated AQP2 expression.

Our results show that, at least in the short term (≤3 h), increased extracellular osmolarity can be sensed from both basolateral and apical poles of mpkCCD$_{cl4}$ cells. This result may suggest that similar sensing mechanisms may exist in native renal collecting duct principal cells. One can speculate that transient AQP2 downregulation in response to an acute increase of basolateral (interstitial) osmolarity may arise from cellular water loss and the need for intracellular osmolyte accumulation. AQP2 downregulation may consequently be part of the adaptive process necessary for renal epithelial cell survival. After the process of accumulating nontoxic "compatible" osmolytes is completed, the inhibitory influence would be released, allowing increased AQP2 expression and enhanced transcellular water flux. On the other hand, increased luminal osmolarity under conditions of osmotic diuresis, *i.e.*, during diabetes mellitus, would trigger downregulation of AQP2 expression that may reduce luminal water loss *via* AQP2 inserted in the apical membrane of CD principal cells. Whether increased apical or basolateral osmolarity trigger different adaptive responses in the long term remains to be investigated.

In conclusion, the results of this study indicate that increasing extracellular tonicity specifically first inhibits and then enhances AQP2 expression, most likely through transcriptional control of the AQP2 gene. The early decrease of AQP2 expression is mediated by hyperosmolarity while the later increase of AQP2 expression relies on hypertonicity, which acts synergistically with PKA, possibly *via* TonEBP.

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