OSMOTIC REGULATION OF RENAL GENE AND PROTEIN EXPRESSION

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Aquaporin-1 knock out (AQP1KO) mice exhibit a reduced urine concentrating ability and fail to generate a hyperosmotic medullary interstitium in the presence of V_2 receptor stimulation by the peptide hormone vasopressin (AQP). The goals of this study were two-fold: (i) to identify the direct effects of vasopressin on renal gene and protein expression in AQP1KO mice which lack an osmotic gradient; and (ii) to evaluate the relative expression of several proteins implicated in ER stress response pathways in wild-type (WT) and AQP1KO mice. Following 6 day AVP-infusion mRNA and protein expression of AQP2 and UTA1 were increased in the cortex of AQP1KO mice. AVP infusion did not increase mRNA expression of AQP2 in the inner medulla of AQP1KO mice. AVP infusion was shown to increase AQP3 mRNA in the cortex of AQP1KO mice, decrease AQP3 mRNA in the medulla of AQP1KO mice, and have no effect on AQP3 protein expression in the cortex or the medulla of AQP1KO mice. AVP infusion increased collagen expression in WT mice and to an even greater extent in AQP1KO mice. Comparing the expression of several key ER stress response proteins in untreated AQP1KO mice and WT mice revealed that GRP78, eIF2α and the proportion of phosphorylated eIF2α found in AQP1KO mice compared to WT mice is not significantly different. Total GCN2 and the proportion of phosphorylated GCN2 were found to be elevated in AQP1KO mice relative to WT mice.

Role of aquaporins in renal function. After plasma is filtered at the glomerulus, filtrate enters the proximal tubule, then the thin descending limb of the loop of Henle. The majority of water reabsorption is passive and occurs via the aquaporin-1 (AQP1) water channel protein, which is constitutively expressed in both the apical and basolateral plasma membranes of the proximal tubule and the thin descending limb of the loop of Henle (1, 2). The proximal tubule and the thin descending limb of the loop of Henle, while permeable to water, are impermeable to solutes, resulting in a high-osmolarity filtrate at the bottom of the loop of Henle. The filtrate then enters the thick ascending limb of the loop of Henle, which is impermeable to water, where the NKCC2 (sodium, potassium, 2-chloride) cotransporter accounts for the majority of solute movement out of the filtrate. The intracellular sodium concentration in cells of the thick ascending limb is kept low relative to the filtrate sodium concentration by Na⁺K⁺ATPase, which constantly pumps sodium out of the cell and across the basolateral membrane. Apical transport of sodium down its concentration gradient on the apical membrane via NKCC2 supplies the driving force for cotransport of potassium and chloride. The sequence of water reabsorption in the proximal tubule and the thin descending limb of the loop of Henle followed by solute reabsorption in the thick ascending limb of the loop of Henle is necessary to establish the renal osmotic gradient crucial to effective water reabsorption (3).

Following solute transport in the thick ascending limb of the loop of Henle, the filtrate is rendered hypoosmotic relative to the surrounding cortical interstitium as it enters the collecting duct. The collecting duct is normally impermeable to water. However, collecting duct permeability can be induced when the peptide hormone vasopressin stimulates the insertion of the aquaporin-2 (AQP2) water channel protein into the apical membrane (4). The hypoosmotic state of the filtrate when entering the collecting duct ensures that water reabsorption out of the collecting duct via AQP2 occurs passively.
Vasopressin is released by the neurohypophysis when blood osmolarity is increased or when blood volume is low, and it binds to the V₂ receptor found on the basolateral membrane of renal collecting duct cells (8, 9). The V₂ receptor is coupled to the G-protein, Gᵣ, and upon stimulation activates adenylyl cyclase type IV resulting in an increase in cAMP (4, 10). cAMP, in turn, causes intracellular vesicles containing AQP2 to fuse with the apical plasma membrane resulting in a vasopressin-induced increase in collecting duct water permeability (11). Long term elevation of circulating vasopressin results in an increase in the maximum water permeability of the collecting duct due to an increase in AQP2 transcription and translation (4). Increases in AQP2 mRNA have been shown to occur following both vasopressin infusion and water restriction and are concomitant with an increase in collecting duct water permeability in the cortex and medulla (6, 7, 8).

Two different pathways are responsible for the cAMP-dependent upregulation of AQP2 mRNA and the insertion of AQP2-containing vesicles into the membrane. Cloning of the 5' flanking region of AQP2 has revealed the presence of a cAMP-responsive element (CRE) by which cAMP may induce the increase in AQP2 mRNA expression (12, 13, 14). Vasopressin binding of the V₂ receptor can also result in an increase in cytosolic Ca²⁺ concentration (15, 16, 17, 18) via stimulation of the phospholipase-c/inositol trisphosphate systems (19). This increase in Ca²⁺ concentration has been shown to be a prerequisite for an increase in collecting duct water permeability to occur in response to vasopressin stimulation. Calmodulin, a Ca²⁺ regulated protein, has also been shown to be required for the increase in collecting duct water permeability to occur. Subsequently, it has been postulated that an increase in the intracellular Ca²⁺ concentration activates calmodulin-dependent cellular processes that result in the insertion of AQP2 containing vesicles into the apical membrane of the collecting duct (20).

Two additional water channel proteins, aquaporin-3 (AQP3) and aquaporin-4 (AQP4), are constitutively expressed on the basolateral membrane of the collecting duct. However, only AQP3 expression is increased by vasopressin, as demonstrated by both vasopressin infusion and water restriction models (21, 22). Unlike in the AQP2 gene, no CREs have been identified in the AQP3 gene promoter sequence; however, two cis-regulatory elements associated with cAMP-mediated transcriptional regulation, Sp1 and AP2, have been found within the AQP3 gene promoter sequence (23, 24, 25, 26). While some evidence has been found localizing a small fraction of the total AQP3 population to intracellular vesicles similar to those containing AQP2, it is likely that these represent proteins in transit from the Golgi apparatus to the basolateral membrane and not stores of AQP3 protein waiting to be inserted into the membrane (21).

In addition to the renal osmotic gradient set up by water and solute reabsorption in the loop of Henle, movement of filtrate solutes out is the collecting duct and into the medullary interstitum further facilitates the osmotically driven reabsorption of water out of the collecting duct. Vasopressin has been shown to increase urea permeability and urea transporter A1 (UTA1) expression in the inner medullary collecting duct (27). An increase in urea permeability and UTA1 expression is achieved by vasopressin-stimulated phosphorylation of UTA1 by the cAMP-dependent kinase, protein kinase A (PKA) (28, 29).

In addition to inducing changes in the expression of AQP2, AQP3 and UTA1, vasopressin stimulation of the V₂ receptor dramatically increases the interstitial osmolarity in the renal medulla. Unlike any other cell type, collecting duct cells are exposed to a hyperosmotic environment that may play a role in the regulation of gene and protein expression. However, it has been difficult to delineate the role an increase in osmolarity plays in renal gene and protein expression from those brought about directly by vasopressin stimulation. Several studies have shown that hyperosmolarity may play an important regulatory role in the vasopressin-induced increases of both AQP2 and AQP3 (30, 31, 32, 33). However, treatment with furosemide, a loop-diuretic that “washes out” the renal osmotic gradient producing a hypoosmotic medullary interstitium, fails to prevent the vasopressin-induced increase in aquaporin-2 and aquaporin-3 in the collecting duct—suggesting that changes in interstitial osmolarity are not
necessary for increases in AQP2 and AQP3 expression to take place (34).

**AQP1 knockout mice.** AQP1 knockout (AQP1KO) mice provide a unique model for differentiating the effects of vasopressin and high osmolarity on renal gene and protein expression. AQP1KO mice exhibit a significant decrease in transepithelial water permeability in the proximal tubule and thin descending limb of the loop of Henle (35) and increased urine output (2). Severely limited water reabsorption in the thin descending limb of the loop of Henle effectively “washes out” the renal concentration gradient in AQP1KO mice due to the inability to generate a hypertonic medullary interstitum via normal countercurrent multiplication (36). When given ample access to water, AQP1KO mice are surprisingly normal. However, when access to water is restricted, AQP1KO mice are unable to conserve fluid by concentrating their urine, and become severely dehydrated (36). Long-term treatment with dDAVP, a V2 receptor agonist, and water restriction both fail to increase the urine osmolarity or generate a hyperosmotic medullary interstitium in AQP1KO mice (36). Therefore, AQP1KO mice provide a unique model with which to separate the regulatory effects of osmolarity from the direct effects of vasopressin on renal gene and protein expression.

The medullary interstitium of AQP1KO mice also provides a unique environment in which to study the effects of osmotic stress. In AQP1KO mice, the direct effects of vasopressin on the osmotic stress response can be delineated from the secondary effects of an increase in interstitial osmolarity.

**The unfolded protein response.** A DNA microarray study of dDAVP infused AQP1KO mice performed previously in our lab revealed that V2 receptor activation also upregulates key components of stress response pathways in the renal medulla (55). Similarly, recent studies in plants and yeast have shown that over-expression of key proteins in the unfolded protein response pathway can protect against drought and osmotic stress.

The unfolded protein response is activated when protein production demands on the endoplasmic reticulum (ER) exceed the organelle’s capacity to efficiently produce correctly folded proteins. When this occurs, incorrectly folded proteins accumulate causing ER stress. In order to correct this imbalance in ER homeostasis, the cell invokes the unfolded protein response, which reduces protein synthesis within the cell, selectively increases the production of ER proteins critical to protein processing, and upregulates protein degradation in the ER (43). ER stress can be caused by a host of cellular stresses, including nutrient deprivation, hypoxia, disturbances in Ca2+ mobilization and events that cause the secretory demands of the cell to exceed the folding capacity of the ER (43, 44). If left uncorrected, ER stress incurred from an accumulation of misfolded proteins is lethal to the cell and can result in caspase-mediated apoptosis (45).

Three main signaling pathways are activated in response to the accumulation of unfolded proteins, all of which are regulated by the chaperone protein GRP78 (glucose regulated protein 78) (46). PERK (double-stranded RNA activated protein kinase-like ER kinase), IRE1 (high inositol requiring endonuclease 1) and ATF6 (activating transcription factor 6) are all intramembrane ER proteins bound to GRP78 which are inactive under unstressed conditions (43). When ER stress occurs, GRP78 dissociates from PERK, IRE1 and ATF6 in order to decrease high levels of misfolded proteins and activate the unfolded protein response (43).

Following dissociation from GRP78, PERK dimerizes in the membrane, resulting in autophosphorylation. Activated PERK phosphorylates eIF2α (eukaryotic initiation factor 2α). The net effect of the eIF2α kinase pathway is to globally inhibit translation of non-critical proteins in order to prevent the accumulation of newly synthesized proteins in the ER and to increase production of proteins critical to ensuring proper ER function (43). To do this, eIF2α enhances translation of activating transcription factor 4 (ATF4) which increases expression of GADD153 (growth arrest and DNA damage-inducible protein 153), GADD34 (growth arrest and DNA damage-inducible protein 34) and ATF3 (activating transcription factor 3), all of which play a role in directing transcription of stress response proteins critical to attenuating the accumulation of unfolded proteins (43). GADD153 also plays a role in mediating ER stress induced apoptosis, and
GADD34 provides a regulatory feedback mechanism for the eIF2α kinase pathway (44).

eIF2α can also be phosphorylated by phosphorylated-GCN2 (general control non-depressible protein 2) in response to amino acid deprivation within the cell (47). In yeast, phosphorylation of eIF2α, prevents the inhibitory action of unphosphorylated eIF2 on translation of GCN4 (a transcriptional activator of genes responsible for amino acid synthesis) (39, 47).

Following dissociation from GRP78, ATF6 and IRE1 also play critical roles in initiating the unfolded protein response. ATF6 is targeted to the nucleus to aid in regulation of genes critical to the unfolded protein response (48, 49) including enhanced transcription of GRP78 and GADD153 (43). IRE1 oligomerizes and autophosphorylates, resulting in enhanced RNAse activity. IRE1 cleavage of a short sequence from the x-box binding protein 1 (XBP1) mRNA transcript results in the upregulation of bZIP (basic leucine zipper transcription factor), which also plays a role in enhanced GRP78 production (43, 50, 51, 52, 53).

Several proteins involved in ER stress response pathways have been demonstrated to play a crucial role in protecting cells against osmotic stress. Overexpression of GRP78 has been shown to increase resistance to ER stress (41, 42) and protect plants from the effects of drought and water restriction (38). High salinity in yeast has been shown to upregulate the expression of phosphorylated GCN2 and subsequent activation of the eIF2α pathway and translation of GCN4. Interestingly, elevated levels of GCN2 have been demonstrated to confer reduced tolerance to high salt exposure (39).

Given the hyperosmotic environment found in the medullary interstitium of the kidney and the ability of collecting duct cells to adapt to this type of exposure, it is possible that proteins instrumental in protecting against ER stress may play a crucial role in the adaptation of inner medullary cells to environments of high osmolarity. The AQP1KO mouse model, in which inner medullary cells are not exposed to a hyperosmotic environment, provides a unique model in which to study the role that ER stress response proteins play with regards to osmotic stress tolerance.

Therefore, the goals of this study are two-fold: (i) to identify the direct effects of vasopressin on renal gene and protein expression in mice lacking an osmotic gradient; and (ii) to evaluate the relative expression of several proteins implicated in ER stress response pathways in wild-type and AQP1KO mice.

MATERIALS AND METHODS

Animals. AQP1KO mice were generated by homologous recombination in embryonic stem cells as previously reported (15). Mice were bred and maintained in the animal facility of the University of Arizona Health Sciences Center under National Institutes of Health guidelines. AQP1 genotypes designated (+) for wild-type alleles and (−) for the targeted allele were determined by PCR analysis of genomic DNA isolated from tail biopsies. F2 generation AQP1 −/− and ++ mice derived from crosses of AQP1 +/− were used for this study. Mice were 8 wk old and received regular food and water ad libitum. For the AVP portion of this study, osmotic mini pumps were loaded with AVP and subcutaneously implanted in mice under light anesthesia. The infusion rate of AVP was 50 ng/h for 6 days.

RNA isolation, amplification and cDNA purification. Full methodology for the RNA purification, amplification and cDNA production has been previously published (36). RNA was isolated from the cortex and inner medulla using a RNeasy Mini Kit (Qiagen cat. no. 74104) according to the manufacturer’s protocol for isolation from tissue. All samples were kept as individual samples. 2 µg of total RNA was transcribed to cDNA using an EndoFree RT kit (Ambion cat. no. 1740) according to the manufacturer’s protocol.

Real-time Quantitative PCR. Real-time quantitative PCR was carried out using the RotorGene RG3000 (Corbett Research) sequence detection system and SYBR Green reagents from a Quantitect Sybr Green PCR kit (Qiagen cat. no. 20414). Primers were designed using Primer3 software (54) and are listed in Table 1 along with the gene accession number for the target gene. The cDNA was diluted to 8 ng/µl, and the PCR reaction mixture contained 5
µl of Sybr master mix, 0.4 µl 25mM MgCl₂, 0.6 µl RNase-free water, 4 pmol of forward and reverse primers, and 16 ng cDNA, in a volume of 10 µl. Each reaction was performed in triplicate at 95°C for 5 min and then at 95°C for 15 s, 65°C for 30 min for 40 cycles. This was followed by a melt cycle that consisted of a stepwise increase in temperature from 72°C to 99°C. In the dissociation curve of each gene, a single predominant peak was observed, supporting the specificity of the PCR product. Threshold values (count numbers) set within the exponential phase of the PCR reaction were used to calculate the expression levels of the genes of interest before being normalized to endogenous cellular beta-actin mRNA. The level of beta-actin RNA was measured in parallel samples using beta-actin-specific primers.

Protein sample preparation, SDS-PAGE, and Western blot. Tissue from inner medullas and cortices were separated and homogenized in ice-cold isolation solution (250 mM sucrose and 10 mM triethanolamine, pH 7.6, containing 1 mg/ml leupeptin and 0.1 mg/ml phenylmethylsulfonfyl fluoride) using a tissue homogenizer (Omni 1000 fitted with a microsawtooth generator) at maximum speed for three 15 s intervals. Total protein concentrations were measured using a BCA kit (Pierce cat. no. 23227) according to the manufacturer’s protocol. Samples were solubilized in Laemmli sample buffer at 60°C for 15 min. To confirm that protein loading of the gels was equal, preliminary 12% polyacrylamide gels were stained with Coomassie blue. Densitometry (Bio-Rad Chemi-Doc™ EQ Densitometer and Bio-Rad Quantity One software) was performed on three major bands to ensure equal loading (within 5% variation relative to the mean). Proteins were separated on 6%, 10%, 12% or 15% SDS-PAGE gels and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore cat. no. IPVH0010). Membranes were blocked for 1 h at room temperature with 5% non-fat dry milk and then incubated overnight at 4°C with primary antibodies (AQP2, AQP3 and UTA-1) polyclonal antibodies were generously provided by Dr. M. A. Knepper (National Institutes of Health); anti-GCN2, anti-phosphorylated-GCN2 (Thr 898), anti-eIF2α, and anti-phosphorylated-eIF2α (Ser 51) were purchased from Cell Signalling Technology (Danvers, MA), followed by incubation with horse radish peroxidase-linked secondary antibody for 1 hr at room temperature. HRP was visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Protein Research cat. no. 34077). Band densities were determined by the BioImaging System (UVP, Upland, CA) or using Bio-Rad Quantity One software and normalized to control values.

Statistics. Data is presented in the following format: mean ± SEM. T tests were used to determine significance and P-values of less than 0.05 were considered significant.

RESULTS

Effects of vasopressin infusion on urine osmolarity in AQP1KO mice. Short-term infusion of the V2 receptor agonist dDAVP and water restriction have been shown to have no effect on the urine osmolarity of AQP1KO mice (36). In order to investigate the effects of long-term increases in circulating AVP levels in the absence of the renal concentration gradient, AVP was infused (50 ng/hr) into AQP1KO mice for 6 days. AVP infusion for 6 days resulted in a small but significant increase in urine osmolarity compared to saline-infused AQP1KO mice (887±21 vs 736±29 mOsm/KgH₂O, P<0.05) as shown in Figure 1.

Effects of AVP infusion on AQP2, AQP3 and UTA-1 mRNA and protein expression. Short- and long-term increases in AVP are known to increase expression of AQP2, AQP3 and UTA-1 in the renal collecting duct in the presence of a hyperosmotic medullary interstitum (4, 21, 22, 27). However, the role played by the hyperosmotic medullary interstitium in the expression of these proteins is unclear. In order to evaluate the effect of long-term AVP elevation in the absence of an osmotic gradient, AVP was delivered at 50 ng/hr for 6 days to AQP1KO mice. mRNA expression of all three genes was evaluated using real-time PCR, and western blot analysis was performed to assess changes in protein expression.

AVP significantly increased AQP2 mRNA in the cortex (1.68±0.14 vs 1.00±1.07, P<0.05) but did not effect a change in AQP2 mRNA expression in the inner medulla (1.17±0.06 vs. 1.00±0.14, P≥0.05) as shown in
Figure 2a. This was accompanied by an increase in AQP2 protein expression in both the cortex and inner medulla (cortex: 143±13.9 vs 100±6.6%, P<0.05; inner medulla: 187±12.5 vs 100±9.1%, P<0.05) as shown in Figure 2b.

AVP significantly increased UTA1 mRNA (2.09±0.10 vs 1.00±0.22, P<0.05) and protein (184±30 vs 100±18.1%, P<0.05) in the inner medulla as demonstrated in Figure 3a and Figure 3b, respectively.

AVP significantly increased AQP3 mRNA expression in the cortex (1.24±0.07 vs 1.00±0.09, P<0.05), yet significantly decreased AQP3 mRNA expression in the inner medulla (0.7±0.07 vs 1.0±0.1, P<0.05) as shown in Figure 4a. No significant changes in AQP3 protein expression were observed in either the cortex or inner medulla (cortex: 83±22 vs 100±25.1%, P≥0.05; inner medulla: 117±10.1 vs 100±6.7%, P≥0.05) as shown in Figure 4b.

**Effect of vasopressin infusion on collagen mRNA expression in the kidney of AQP1KO mice.** Previous studies in our lab have shown that long-term infusion of dDAVP in AQP1KO mice resulted in increased cell proliferation in the renal medulla of AQP1KO mice suggesting that chronic activation of the V2 receptor may lead to kidney damage and fibrosis (55). To follow up this finding, collagen mRNA expression was evaluated in both AVP-infused AQP1KO and wild-type (WT) mice.

AVP significantly increased collagen1α1 mRNA expression in the cortex and inner medulla of AQP1KO mice (cortex: 1.66±0.21 vs 1.00±0.24, P<0.05; inner medulla: 1.78±0.23 vs 1.00±0.16, P<0.05) as shown in Figure 5a. An increase in collagen1α1 mRNA was observed in the cortex of wild-type mice (1.21±0.05 vs 1.00±0.12, P<0.05), but not in the inner medulla (0.86±0.46 vs 1.00±0.16, P≥0.05) as shown in Figure 5a.

AVP significantly increased collagen1α2 mRNA expression in the cortex of AQP1KO mice (1.28±0.10 vs 1.00±0.13, P<0.05) but not in wild-type mice (1.19±0.14 vs 1.00±0.08, P≥0.05) as shown in Figure 5b.

AVP significantly increased collagen3α1 mRNA expression in the cortex of AQP1KO mice (1.87±0.21 vs 1.00±0.23, P<0.05) and the cortex of wild-type mice, (1.47±0.11 vs 1.00±0.04, P<0.05) as shown in Figure 5c.

Collagen4α1 mRNA expression was also increased in the cortex of AQP1KO mice in response to AVP (1.92±0.44 vs 1.00±0.08, P<0.05), whereas no significant change in collagen4α1 mRNA expression was observed in the cortex of wild-type mice (0.91±0.02 vs 1.00±0.14, P≥0.05) as shown in Figure 5d.

**ER stress protein expression in the inner medulla of wild-type vs AQP1KO mice.** Expression of GRP78, eIF2α, phosphorylated eIF2α, GCN2, and phosphorylated GCN2 in the inner medulla of wild-type mice and AQP1KO mice was measured using western blot analysis.

GRP78 expression was not significantly different between wild-type mice and AQP1KO mice (100±9.1 vs 105±12.2%, P≥0.05) as shown in Figure 6.

Total eIF2α expression was not significantly different between AQP1KO and wild-type mice (93±6.1 vs 100±1.6%, P≥0.05) as shown in Figure 7a. The proportion of phosphorylated-eIF2α relative to the total eIF2α expression was also not significantly different between AQP1KO and wild-type mice (76±27.6 vs 100±5.9%, P≥0.05) as shown in Figure 7b.

Total GCN2 expression was significantly increased in AQP1KO mice compared to wild-type mice (159±7.5 vs 100±5.4%, P<0.05) as shown in Figure 8a. The proportion of phosphorylated-GCN2 relative to the total GCN2 expression was also significantly increased in AQP1KO mice compared to wild-type mice (232±33.1 vs 100±25.1 %, P<0.05) as shown in Figure 8b.

**DISCUSSION**

In this study, AQP1KO mice were used to study two aspects of the renal medullary interstitium in the absence of hyperosmolarity.

First, the direct effects of long-term vasopressin elevation were delineated from the secondary effects of increased osmolarity by infusing AQP1KO mice, which lack the vasopressin-induced increase in osmolarity, with vasopressin over a six-day period. We found that mRNA and protein expression of AQP2 and UTA1 was increased in the cortex, a result that is also seen in wild-type mice (6, 7, 8, 28, 29).
Vasopressin regulation of AQP3 in the absence of an increase in medullary osmolarity, resulted in an increase in AQ3 mRNA in the cortex, a decrease in AQ3 mRNA in the medulla, and no significant changes in protein expression in either the cortex or the medulla. Long-term elevation of circulating vasopressin was also shown to increase collagen expression in wild-type mice and to a greater extent in AQP1KO mice.

Second, AQP1KO mice were used to evaluate the hypothesis that ER stress response pathways may potentially play a crucial role in the ability of inner medullary cells to survive constant osmotic stress brought about by vasopressin-mediated increases in medullary osmolarity. Expression of the molecular chaperone protein GRP78 and the PERK-dependent kinase eIF2α were not observed to be significantly different. Further there was no significant difference in the proportion of phosphorylated-eIF2α found in AQP1KO mice compared to wild-type mice. There was, however, an observed increase in the total amount of GCN2 found in AQP1KO compared to wild-type mice as well as an increase in the proportion of the GCN2 population which had been phosphorylated.

Vasopressin regulation of AQP2 and UTA1 is conserved in the absence of an osmotic gradient. Elevation of AVP in vivo, both by direct AVP infusion and through water restriction, has been shown to increase expression of both AQP2 and UTA1 in wild-type models (6, 7, 8, 27). While the mechanism by which AVP regulates the expression of these two proteins is different (AQP2 vesicles are inserted into the apical membrane of the collecting duct, whereas UTA-1 expression is regulated via cAMP-dependent phosphorylation via PKA), the presence of both AQP2 and UTA1 play an influential role in facilitating water reabsorption in the collecting duct (11, 28, 29).

While several previous in vitro studies have demonstrated that hyperosmolarity is a prerequisite for a vasopressin-induced increase in AQP2 expression (30, 31, 32, 33), this study suggests that increased osmolarity is not a critical element in vasopressin-induced AQP2 or UTA1 expression. AQP2 mRNA was significantly increased in the renal cortex, whereas AQP2 protein expression was significantly elevated in both the cortex and inner medulla in response to elevated vasopressin in AQP1KO mice. This confirms findings from in vivo studies using furosemide suggesting that vasopressin-induced AQP2 expression is not dependent upon the presence of a hypertonic medullary interstitium (34). Similarly, UTA1 mRNA and protein expression were both found to be increased nearly two-fold in the inner medulla in AVP-infused AQP1KO mice.

Vasopressin regulation of AQP3 is not conserved in the absence of an osmotic gradient. Contrary to the findings regarding AQP2, this study suggests that the vasopressin-induced increase in AQ3 expression is dependent upon the presence of a hyperosmotic medullary interstitium. AQ3 mRNA was significantly decreased in the inner medulla and significantly increased in the cortex in response to vasopressin infusion in AQP1KO mice. Both the cortex and the inner medulla demonstrated no significant change in AQ3 protein expression in response to vasopressin infusion in AQP1KO mice. This finding is interesting because it indicates that local osmolarity may have several distinct and mechanistically different roles in AQ3 regulation—at the level of transcription, at the level of translation and perhaps with regards to AQ3 protein degradation.

Elevated levels of vasopressin increase collagen expression in both the cortex and the medulla. Collagen mRNA levels in both the cortex and medulla of AQP1KO mice, and in some cases wild-type mice, were found to be elevated following vasopressin infusion. Previous studies in our lab have shown that increased levels of circulating dDAVP increase cell proliferation in the medulla of AQP1KO mice (55). Increased collagen expression and cell proliferation are both indicators of kidney damage and fibrosis.

Our data suggests that low interstitial osmolality and high levels of circulating vasopressin may lead to progressive kidney damage. However, increases in collagen1α1 (Figure 5a) and collagen3α1 (Figure 5c) in the cortex of wild-type mice suggest that a normally functioning renal concentrating mechanism may not completely protect the kidney from damage incurred from elevated vasopressin.
Clinically, vasopressin is elevated in many disease processes associated with the development of progressive kidney damage, including diabetes and congestive heart failure. Our study provides evidence that the high levels of circulating vasopressin present in patients with these conditions may contribute to the development of kidney damage. Further, treatment of these disorders often involves loop-diuretics such as furosemide, which “wash out” the renal concentration gradient—leaving a patient with reduced renal osmolarity and a potentially weakened defense against the progression of renal injury. The increased collagen expression found in this study in conjunction with prior evidence of collecting duct cell proliferation in response to dDAVP infusion in mice with a reduced renal osmolarity (55) suggests that further investigation of vasopressin-regulated pathways in situations of low renal osmolarity may provide clinically relevant insights into the development of kidney disease.

Involvement of ER stress proteins in the medullary tolerance of osmotic stress. Recently, several studies have demonstrated an increased tolerance to osmotic stress when several key ER stress response proteins, including GRP78, are overexpressed, both in vitro and in vivo in plants (38, 41, 42). Consequently, it has been hypothesized that ER stress response pathways may play a crucial role in the survival of inner medullary cells constantly exposed to the osmotic stress brought about by the hyperosmotic environment of the renal medulla. By comparing the expression of several key ER stress response proteins in AQP1KO mice and wild-type mice we sought to evaluate the proposed relationship between ER stress and osmotic stress.

Levels of expression of GRP78 and eIF2α were found to be unchanged in AQP1KO mice compared to wild-type mice. Similarly, the ratio of phosphorylated eIF2α to the total eIF2α concentration was not significantly different between the two groups. Phosphorylation of eIF2α occurs in two major ways: (i) in the presence of ER stress, GRP78 dissociates from PERK in response to an accumulation of unfolded proteins, resulting in PERK oligomerization and activation through autophosphorylation, resulting in eIF2α phosphorylation (43); and (ii) in response to amino acid deprivation, when the eIF2α kinase GCN2 is phosphorylated and activated (47). Given the general “house-keeping” properties of both GRP78 and eIF2α we would not expect to see changes in the global concentrations of these two proteins. However, under the hypothesis that ER stress proteins may protect against osmotic stress, we expected to see an increase in the proportion of phosphorylated eIF2α in the inner medulla of wild-type mice, which is exposed to a hyperosmotic medullary interstitium. This was not observed, suggesting that the branch of the ER stress response pathway regulated by PERK and eIF2α may not play a role in the adaptation of medullary cells to environments of high osmotic stress.

There are two different, conflicting hypotheses regarding the role phosphorylated-GCN2 plays in cellular response to osmotic stress. On one hand, in yeast, phosphorylated-GCN2 is inversely correlated with tolerance to salt stress (cite). However, on the other hand, unpublished data from our lab has shown that phosphorylated-GCN2 activates the unfolded protein response in collecting duct cells in response to osmotic stress. Data from this study show that AQP1KO mice exhibited increased phosphorylated-GCN2 compared to wild type mice. Because AQP1KO mice lack a hyperosmotic medullary interstitium, our data supports previous findings in yeast that phosphorylated-GCN2 is inversely correlated with tolerance to osmotic stress. Given that very little is known about the role, if any, that phosphorylated-GCN2 plays in negating osmotic stress tolerance, further investigation into the role of phosphorylated-GCN2 in response to osmotic stress is certainly warranted.
REFERENCE LIST

44. Wek, R. C., and Cavener, D. R. (2007) ARS 9, 2357-2371
FIGURE LEGENDS

Fig. 1. Vasopressin infusion for 6 days causes a small but significant increase in urine osmolarity in AQP1KO mice (n = 4, *P<0.05).

Fig. 2. Vasopressin increases AQP2 expression in AQP1KO mice. (a) Vasopressin infusion for 6 days increased AQP2 mRNA expression in the cortex but not in the medulla of AQP1KO mice (*P<0.05, n=4; ns=not significant). (b) Vasopressin infusion for 6 days increased AQP2 protein expression in both cortex and medulla of AQP1 null mice (*P<0.05, n=5).

Fig. 3. Vasopressin increases UTA1 expression in AQP1KO mice. (a) Vasopressin infusion for 6 days increased the expression of UTA1 mRNA in the medulla of AQP1KO mice (*P<0.05, n=4;). (b) Vasopressin infusion for 6 days increased the expression of UTA1 protein in the medulla of AQP1KO mice (*P<0.05, n=5).

Fig. 4. Vasopressin regulates AQP3 mRNA but not protein expression in AQP1KO mice. (a) Vasopressin infusion for 6 days decreased expression of AQP3 mRNA in the inner medulla of AQP1KO mice and increased the expression of AQP3 mRNA in the cortex(*P< 0.05, n=4). (b) Vasopressin infusion for 6 days did not significantly change protein expression in either the inner medulla or cortex (ns=not significant, n=5)

Fig. 5. Vasopressin increases collagen expression in wild-type mice and AQP1KO mice. Vasopressin infusion for 6 days: (a) increases the expression of collagen1α1 mRNA in the cortex of wild-type and in both the cortex and the inner medulla of AQP1KO mice (*P<0.05, ns=not significant, n=4); (b) increases the expression of collagen1α2 mRNA in the cortex of AQP1KO mice (*P<0.05, ns=not significant, n = 4); (c) increases the expression of collagen3α1 mRNA in the cortex of both wild-type mice and AQP1KO mice (*P<0.05, n=4); (d) increases the expression of collagen4α1 mRNA in the cortex of AQP1KO mice (*P<0.05, ns=not significant, n=4).

Fig. 6. GRP78 protein expression is not significantly different in AQP1KO mice relative to wild-type mice. A significant difference in GRP78 expression was not observed in the inner medulla of AQP1KO mice compared to wild-type mice (ns = not significant, n_{WT} = 6, n_{AQP1KO} = 4).

Fig. 7. Total eIF2α and phosphorylated eIF2α are unchanged in AQP1KO mice compared to wild-type mice. (a) There is no significant difference in total eIF2α expression in the inner medulla of wild-type and AQP1KO mice. (b) There is no significant difference between the proportion of phosphorylated-eIF2α (relative to total eIF2α) in AQP1KO mice and that in wild-type mice (ns=not significant, n_{WT}=6, n_{AQP1KO}=4).

Fig. 8. Total GCN2 and phosphorylated-GCN2 are elevated in AQP1KO mice relative to wild-type mice. (a) There is a significant increase in the total GCN2 expression in the inner medulla of AQP1KO mice relative to wild-type mice. (b) There is a significant increase in the proportion of phosphorylated-GCN2 (relative to total GCN2) in AQP1KO mice compared to wild-type mice (*P<0.05, n_{WT}=6, n_{AQP1KO}=4).
Figure 1

![Graph showing urine osmolarity (mOsm/kgH2O) for control and AVP 6d conditions.](image)

- Control
- AVP 6d

Urine Osmolarity (mOsm/kgH2O)

* Indicates significant difference.
Figure 2

a)

b)
Figure 3

(a) Mean UTA1 mRNA Level Relative to Control

(b) Western blot of UTA1 in Inner Medulla

Control: 100 ± 18.1 %
AVP 6d: 184 ± 30.0 % *

kDa
114
97
Figure 4

a) 

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b) 

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Figure 5

a)

b)
Figure 6

**GRP78**

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Figure 7

a)

b)
Figure 8

a) 

b)
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