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# Controlled aquaporin-2 expression in the hypertonic environment

# **Udo Hasler**

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**Hasler U.** Controlled aquaporin-2 expression in the hypertonic environment. *Am J Physiol Cell Physiol* 296: C641–C653, 2009. First published February 11, 2009; doi:10.1152/ajpcell.00655.2008.—The corticomedullary osmolality gradient is the driving force for water reabsorption occurring in the kidney. In the collecting duct, this gradient allows luminal water to move across aquaporin (AQP) water channels, thereby increasing urine concentration. However, this same gradient exposes renal cells to great osmotic challenges. These cells must constantly adapt to fluctuations of environmental osmolality that challenge cell volume and incite functional change. This implies profound alterations of cell phenotype regarding water permeability. AQP2 is an essential component of the urine concentration mechanism whose controlled expression dictates apical water permeability of collecting duct principal cells. This review focuses on changes of AQP2 abundance and trafficking in hypertonicity-challenged cells. Intracellular mechanisms governing these events are discussed and the biological relevance of altered AQP2 expression by hypertonicity is outlined.

osmolality; vasopressin; collecting ducts; nuclear factor- $\kappa B$ ; mitogen-activated protein; kinase; TonEBP

MOST MAMMALIAN CELLS are exposed to an isoosmotic environment (e.g., 280-295 mosmol/kgH<sub>2</sub>O in humans) under normal physiological conditions, largely as a result of renal regulatory mechanisms that maintain water and electrolyte body fluid composition within a very narrow range. There are however numerous instances of prevailing osmolalities occurring outside of this range. Such aniosmotic environments play important physiological roles under both normal and pathological conditions. Water movement across animal cell membranes is determined by osmotic gradients across the cell membrane. In the absence of aquaporin (AQP) water channels, diffusional permeability is low in lipid bilayers exposed to an osmotic gradient. AQPs, of which 13 isoforms have so far been reported in human tissue (92, 162), dramatically increase water permeability across the lipid bilayer. The presence of these proteins in endo- and epithelia was confirmed by the identification of the first water channel AQP1, initially termed channel-like integral membrane protein of 28 kDa (CHIP28) (34), that earned Dr. Peter Agre the 2003 Nobel Prize in chemistry. An established osmotic gradient and the presence of AQP water channels in membranes of glands and epithelia together form the biological basis for facilitated fluid secretion. The underlying relevance of these combined factors in various processes such as immunity (54, 56), food absorption (53, 63, 154), sperm maturation (30, 136, 155), sight (171), hearing (106, 159), salivation (33), sweat secretion (126), and fluid exchange in the central nervous system (160) is beginning to be fully appreciated. This review will first outline basic biological processes affected by hypertonicity. The effects of hypertonicity on AQP2 transcription and trafficking and the resulting

effects on renal water reabsorption will then be described in greater detail. Notably, intracellular mechanisms that govern these events are described and a protective role for AQP2 against hypertonicity-induced cell shrinkage is evoked.

#### Renal Corticomedullary Osmolality Gradient

Of all regions of the body, environmental osmolality is by far highest in the renal medulla. The corticomedullary osmolality gradient that drives water reabsorption in the kidney increases along the tubule with a maximum osmolality (1,200 mosmol/kgH<sub>2</sub>O in humans) at the tip of the inner medulla (83). The gradient arises from active NaCl reabsorption in the thick ascending limb of Henle (TAL) via Na-K-Cl cotransporter (NKCC2) and by passive reabsorption of NaCl and urea by the thin ascending limb of Henle (tAL) and the inner medullary collecting duct (IMCD), respectively. It additionally depends on the water impermeability of both TAL and tAL together with low blood flow through the vasa recta. Vasopressin (VP) plays a fundamental role in establishing and maintaining the hyperosmotic environment of the kidney medulla by controlling the expression levels of key proteins present in distinct regions of the nephron that together mediate the countercurrent concentration mechanism. VP controls interstitial NaCl accumulation by stimulating NKCC2 abundance and cell surface expression in the TAL (8, 55, 90) and by promoting the coordinated expression of the epithelial sodium channel (ENaC) and Na<sup>+</sup>-K<sup>+</sup>-ATPase at the cell surface of collecting duct (CD) principal cells (58, 117, 122). VP also increases urea permeability by acutely increasing UT-A1 cell surface expression (45) and UT-A3 mRNA abundance in terminal IMCD cells (40) and by increasing UT-A2 expression in thin descending limbs of Henle (139, 173). Finally, VP tightly regulates osmotically driven water reabsorption by mediating transcriptional activity as well as apical cell surface expression of AQP2

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in CD principal cells (10, 18, 129). In addition to VP, other hormones act on Na<sup>+</sup> and Cl<sup>-</sup> reabsorption in the TAL and CD. TAL Na<sup>+</sup> and Cl<sup>-</sup> reabsorption is increased by adrenaline, calcitonin, parathyroid hormone, and glucagon and decreased by prostaglandin  $E_2$  (3, 36, 174). In the CD, Na<sup>+</sup> and Cl<sup>-</sup> reabsorption is increased by aldosterone and decreased by atrial natriuretic peptide and prostaglandins (5, 9, 157, 166).

# Adaptation and Intracellular Signaling in Response to Hypertonicity

Kidney cells, and particularly medullary cells, are routinely exposed to conditions challenging cell volume as a consequence of urine concentrating disparity that in turn maintains whole body water homeostasis within a restricted range. An accumulation of poorly permeable electrolytes outside the cell gives rise to a hypertonic (>300 mosmol/kgH<sub>2</sub>O) environment. Cells exposed to such an environment are subjected to immediate water efflux causing cells to shrink. Conversely, osmotic water influx causes cells to swell when exposed to a hypotonic (<290 mosmol/kgH<sub>2</sub>O) environment. A fundamental requirement of all mammalian cells is to maintain a constant volume. Cells react to shrinkage by rapidly accumulating extracellular Na<sup>+</sup> and Cl<sup>-</sup> as part of the regulatory volume increase (RVI) mechanism that restores cell volume within minutes by passive water uptake (100, 121). In hypotonicitychallenged cells, cell swelling is reduced by osmotic water efflux as a consequence of intracellular ion efflux, mainly K<sup>+</sup> and Cl<sup>-</sup> and various organic molecules including amino acids and amines, as part of the regulatory volume decrease (RVD) mechanism (135). RVI occurs at the expense of perturbed protein function that results from elevated intracellular ionic strength. Depending on the extent of hyperosmotic challenge, cells either adapt to their new conditions of elevated osmolality or, when osmolality is too high, undergo apoptotic-mediated cell death. In the latter case, RVI does not occur, cells continue to shrink and undergo apoptosis within hours of exposure. In cultured renal cells grown in 290 mosmol/kgH<sub>2</sub>O medium, such a response is typically induced following an acute increase of environmental osmolality of  $>600 \text{ mosmol/kgH}_2\text{O}$ .

Hypertonicity induces cell cycle arrest providing the cell with time (hours) needed for its adaptation to conditions of higher osmolality. Hypertonicity additionally induces DNA breaks, an event that is well known to be associated with cell cycle arrest (38). Notwithstanding the many DNA breaks observed in renal inner medulla cells exposed to hypertonic medium, adapted cells grow normally but do not display the usual DNA damage response (38). Only a subset of proteins involved in the classical DNA damage response, including p53, ATM, Gadd45a, and Gadd153, is activated by hypertonicity (38). Hypertonic adaptation involves increased signaling of osmoprotective responses mediated by reactive oxygen species (19) and COX-2 induction (31). Increased p53 activity helps reduce hypertonicity-induced apoptosis by restricting DNA replication (23). Increased expression of Gadd45 contributes to protect the genomic integrity of renal inner medulla cells (110). Hypertonicity increases the expression levels of several heat shock proteins (HSPs) including HSP25, HSP70, HSP110, Osp90, and  $\alpha$ B-crystalin (19), which collectively enhance cell survival and prevent apoptosis. In addition to increased HSP expression, an important hallmark of hypertonic adaptation is the intracellular accumulation of small organic osmolytes that reduce intracellular ionic strength without affecting protein function. Tonicity-responsive enhancer binding protein (TonEBP or NFAT5) plays a key role in this process by stimulating transcription of aldose reductase (AR) (97), sodium-chloridebetaine cotransporter (BGT1) (120), sodium-myo-inositol cotransporter (SMIT) (143), and taurine transporter (82) that mediate intracellular accumulation of sorbitol, betaine, myo-inositol, and taurine, respectively. TonEBP additionally stimulates transcription of HSP70 (177).

Hypertonicity induces the activation of a network of kinases in a variety of cell types (150). In yeast, adaptation to osmotic stress is dependent on the p38 kinase homolog high-osmolality glycerol (HOG1) kinase (15). Numerous studies have since demonstrated the pivotal role that p38 kinase plays in mammalian cells in numerous processes including transcriptional regulation, apoptosis, cytokine production, and cytoskeletal reorganization that characterize the adaptive response to osmotic stress (150). Transcriptional regulation by ERK kinases, via activation of targets such as *c-myc*, *c-jun*, and *c-fos* (32), may counter hypertonicity-induced apoptosis by enhancing cell survival and proliferation. Enhanced transcription of HSP70, COX-2, and the  $\gamma$ -subunit of Na<sup>+</sup>-K<sup>+</sup>-ATPase by JNK2 kinase may additionally contribute to kidney cell survival under hypertonic conditions (24, 175, 181). It should be noted that mitogenactivated protein (MAP) kinase activation appears to depend on the cell type. For instance, hypertonicity activates ERK1/2 in inner medullary collecting duct cells and in cells of the medullary TAL (7, 144, 167) but decreases ERK1/2 activity in NIH 3T3 fibroblasts (44). In addition to transcriptional activity, hypertonicity-induced MAP kinase signaling has been shown to modulate intracellular trafficking of a number of receptors and transporters. Cumulating evidence, gathered in both renal and nonrenal cells, supports a role for MAP kinases in synaptic receptor and transporter trafficking (61), as illustrated by the role that p38, ERK1/2, and JNK kinases may play in the cell surface expression of serotonin and dopamine transporters and the AMPA-sensitive glutamate receptor (123, 147, 186). In neurons, Kv2.1 potassium channel cell membrane insertion relies on p38 MAP kinase activity (142), whereas ERK-dependent inhibition of ENaC activity in renal cortical collecting duct (CCD) cells may result from reduced expression of ENaC at the cell surface (153). In addition to MAP kinases, hypertonicity alters the activities of numerous other signaling pathways, including phosphatidylinositol phosphate 5-kinase (PIP5K) kinase, which contributes to hypertonicity-induced phosphatidylinositol 4,5-bisphosphate-dependent cytoskeletal rearrangement (179), PI3K-1A, which activates TonEBP via PIP<sub>3</sub>-dependent ATM activation (80) and Src tyrosine kinases that mediate various cellular responses to hypertonicity including RVI, cytoskeletal reorganization (28), and TonEBP activation (96). Activation of the catalytic subunit of PKA (PKAc) by hypertonicity has been demonstrated in a liver cell line (HepG2) in the absence of an increase of intracellular cAMP (42). A similar increase of PKA activity was observed in cultured renal CD cells (70) in the absence of increased intracellular cAMP concentration (69). Of particular pertinence to the present review, PKAc has been shown to be part of a NF-KB-IkB-PKAc complex that dissociates following stimulation of the nuclear factor (NF)-kB pathway, leading to the release of active PKAc (185). Hypertonicity is one such factor that stimulates NF-κB activity in renal CD principal cells (see below).

Signaling pathways and downstream effectors induced by hypertonicity are being increasingly identified and understood. However, the specific osmosensor involved in initiating the hypertonicity response has yet to be identified in mammalian cells. In yeast, an increase of environmental osmolality activates putative osmotic sensor proteins (Sln1p, Sho1p, and Msb2p) that activate the MAP kinase Hog1p that in turn activates transcriptional responses involved in glycerol synthesis, leading to an increase of intracellular compatible osmolyte concentration (77). Several mechanisms have been proposed to play the role of an osmosensor in mammalian cells (19). These include cell shrinkage per se, increased intracellular ionic strength, molecular crowding, DNA breaks, cytoskeletal perturbations, cytoskeletal stress, and altered intracellular signaling. Transient receptor potential vanilloid (TRPV) ion channels have additionally been proposed to play an osmosensing role (104). On the other hand, osmotic sensing could by achieved by several elements acting in tandem with each other.

### The AQP2 Water Channel

The susceptibility of renal cells, and especially CD principal cells, to changes in environmental osmolality is very different from that of most nonrenal cells. This is due to the presence of AQP water channels that determine the water permeability properties of membranes in which they are inserted. At least four AQPs actively participate in renal water reabsorption. AQP1 accounts for the transcellular selective water permeability of the proximal tubule and thin descending limb of Henle (tDL) (26, 148) and is expressed in both apical and basolateral membranes of these cells (119). Approximately 75% and 15% of the glomerular filtrate is reabsorbed along these segments, respectively. Although AQP1 water reabsorption is commonly referred to as "constitutive," it should be noted that AQP1 whole cell abundance increases with interstitial tonicity (167). The final urine concentration varies with the body's needs and is determined by reabsorption of remaining water along the CD. This fine regulation is achieved through controlled expression of AQP2 principally expressed in the apical plasma membrane of CD principal cells (50, 128). AQP3 and AQP4 are expressed at the basolateral membrane of CD principal cells and provide an exit for osmotically driven water (81, 164). The clinical importance of AQP2 is illustrated by imbalances of body fluid homeostasis that arise from dysregulated AQP2 expression. Decreased AQP2 expression manifested in nephrogenic diabetes insipidus (NDI) leads to an inability to maximally concentrate urine (95). NDI patients consequently excrete large amounts of hypotonic urine (up to 20 liters per day) that must be compensated by excessive fluid uptake. Conversely, AQP2 overexpression associated with congestive heart failure (130, 178), pregnancy (134) and, under particular circumstances, liver cirrhosis (47), leads to water retention and expanded extracellular fluid. A feature that sets AQP2 apart from the other members of the AQP family is that VP largely mediates its expression. VP secretion from the posterior pituitary gland varies in response to plasma osmolality (170). As discussed below, VP participates in both short-term (minutes) and long-term (hours or days) regulation of CD water permeability by binding to the V<sub>2</sub> receptor located in the basolateral membrane of CD principal cells, which in turn leads to activation of the  $G_s\alpha$ /adenylyl cyclase system, increased concentrations of intracellular cAMP and PKA activation (94). Whereas both AQP2 abundance and cell surface expression are largely under the control of VP, these processes are additionally modulated by numerous other factors that act independently of VP, including extracellular calcium (21, 141), aldosterone (68, 85, 133), and insulin (20, 172).

#### Effects of Hypertonicity on AQP2 Abundance

The year AQP2 was cloned by Fushimi et al. (50) was also the year that water restriction was first found to affect AQP2 whole cell abundance independently of VP (128). This provided one of the first compelling pieces of evidence that factors other than VP control AQP2 expression, and therefore CD water permeability. The remaining part of this review will focus on the role that osmolality plays in regulating AQP2 expression. The influence of other non-VP stimuli on AQP2 expression has been reviewed elsewhere (125, 129).

Together with the demonstration that enhanced CD water permeability by VP was due to AQP2 accumulation at the cell surface (115, 127, 145, 180), VP was shown to increase AQP2 protein abundance (37, 72). It was demonstrated soon after that VP increases AQP2 mRNA content (182) and that a cAMP responsive element (CRE) and an activator protein 1 (AP-1) element located in the AQP2 promoter, which respectively bind cAMP-responsive element protein (CREP) and c-Fos/ c-Jun, mediate VP-induced AQP2 transcription (78, 118, 183). Numerous observations from animal studies led to the suspicion that environmental osmolality might also participate in regulating AQP2 abundance by acting independently of VP. One of the first clues was provided by a study that showed that reduced AQP2 abundance following lithium treatment could be partly restored in water-restricted animals, and to a greater extent than that achieved by VP treatment (113). Water restriction was later shown to return AQP2 abundance to normal levels in animals treated with the V<sub>2</sub> receptor antagonist OPC-31260 (112). By performing the exact opposite experiment, Ecelbarger et al. (39) showed that water loading decreased whole cell AQP2 content despite ongoing V2 receptor stimulation. The possibility that environmental osmolality regulates AQP2 abundance independently of cAMP/PKA was further evidenced by the finding that AOP2 expression decreases with medullary osmolality in senescent animals despite unchanged papillary cAMP levels (140). AQP2 expression could be restored in these animals by VP administration, but the authors concluded that this event is most likely mediated by an increase of papillary osmolality rather than a direct effect of VP itself (29). On the other hand, medullary osmolyte washout following long-term (4-5 days) furosemide treatment had no effect on AQP2 abundance shedding some doubt on the enhancing effect of increased environmental osmolality on AOP2 abundance (114, 165). Using cultured outer medullary collecting ducts (OMCD) cells, Furuno et al. (48) showed, for the first time, that hypertonic challenge alone (24 h) increased AQP2 mRNA abundance. The effect of extracellular osmolality on AOP2 abundance was further tested by Storm et al. (156) who measured AQP2 mRNA and protein abundance in primary cultured IMCD cells exposed to a hypertonic medium. The authors found that both AQP2 mRNA and protein expression levels increased in a dose-dependent manner after long-term (days) hypertonic stimulation. The finding that osmolality did

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not influence cytomegalovirus (CMV) promoter-driven AQP2 expression provided evidence that osmolality influences AQP2 abundance by increasing its transcription. Using a luciferase reporter plasmid driven by various fragments of the murine AQP2 promoter, Kasano et al. (87) confirmed the VP-independent enhancing effects of hypertonicity on AQP2 transcription in Madin-Darby canine kidney cells. Enhanced AQP2 mRNA and protein expression following 24 h of hypertonic challenge was also observed in cultured CCD mpkCCD<sub>cl4</sub> cells (70), a cell line that exhibits many major characteristics of CD principal cells including VP-inducible expression of endogenous AQP2 (67). Interestingly, AQP2 expression levels decreased shortly ( $\leq 3$  h) following hypertonic challenge implying a time-dependent biphasic effect of hypertonicity on AQP2 expression (70). What are the mechanisms underlining the effects of hypertonicity on AQP2 transcription? Hypertonicity does not increase cAMP concentration (69) or cAMP response element-binding protein (CREB) phosphorylation (156) indicating that its effects are not mediated by cAMP. This is further supported by the observation made in mpkCCD<sub>cl4</sub> cells that the extent of both time-dependent effects of hypertonicity on AQP2 expression was similar in the absence or presence of VP. Moreover, the extent of stimulatory effect mediated by hypertonicity was not altered by PKA inhibition (70). Together, these observations indicate that the effects of hypertonicity do not rely on the "classical" cAMP/PKA pathway.

The factors that mediate increased AQP2 expression in response to sustained hypertonic challenge have been investigated and are schematically illustrated in Fig. 1. Hypertonicity may accentuate AQP2 transcription by affecting transcription factor(s) that act on the AQP2 promoter. Observations made in mutant mice that lack TonEBP activity suggest that TonEBP could be such a factor. Indeed, in surviving mice harboring a functionally inactive TonEBP gene, kidney protein expression levels of genes targeted by TonEBP (AR, BGT1, SMIT, and TauT) together with AQP2 were reduced compared with wildtype kidney (107). In another study, mice expressing dominantnegative TonEBP (DN-TonEBP), restricted to the kidney CD, displayed decreased levels of both UT-A1, used as a control (124), and AQP2 mRNA compared with nontransgenic mice (102). Because the differentiation states of transgenic CD cells might be altered as a consequence of chronic hypertonic stress that cannot be counterbalanced by TonEBP, the role that TonEBP may play on AQP2 transcription was examined in cultured mpkCCD<sub>cl4</sub> cells that retain an intact phenotype (65). These cells are a good model for investigation of TonEBPmediated events since they display high levels of TonEBP expression and activity (65). Decreased TonEBP activity in



AQP2

cells transfected with either RNA interference (RNAi) against TonEBP or cDNA encoding DN-TonEBP was associated with reduced basal and hypertonicity-induced expression RNA levels of both AR and AQP2 genes. The extent of decreased AQP2 expression was similar in the presence and absence of VP, indicating that TonEBP acts independently of VP-mediated events. As initially pointed out by Storm et al. (156), a highly conserved TonE sequence (TGGAA) is located upstream of the CRE and AP-1 cis elements of the AQP2 promoter. Hypertonicity-induced luciferase activity in cells transfected with a reporter construct 5'-flanked by the first 517 bp of the AQP2 promoter containing the TonE sequence was abolished in cells transfected with RNAi against TonEBP and in cells harboring a mutant TonE sequence (65). This provided the first evidence that enhanced AQP2 transcription by hypertonicity is mediated by TonEBP via its binding to a TonE element of the AQP2 promoter. Interestingly, a more recent study has shown that hypotonicity decreases AQP2 transcription and that this event that may be mediated by TonE (146). This would be expected since TonEBP activity increases with extracellular tonicity (176) and that consequently TonEBP activity is low under conditions of hypotonicity. It is worthwhile stressing here the various roles that TonEBP plays in the corticomedullary osmolality gradient: not only does it help protect cells from hypertonicity by promoting HSP70 activity and accumulating small organic osmolytes, but it also helps establish the very gradient that cells need protection from by promoting UT-A expression (84). AQP2 uses this gradient as part of the water reabsorption process, an event that depends on TonEBP-mediated AQP2 transcription. Recently, hypertonicityinduced activation of calcineurin-sensitive nuclear factor of activated T cells (NFATc), which belongs to the same family of transcription factors as TonEBP, was found to increase AQP2 transcription (103). In this pathway, a hypertonicity-nduced increase of intracellular Ca<sup>2+</sup> increases activity of the serine/threonine phosphatase calcineurin, which in turn dephosphorylates NFATc. This promotes nuclear translocation of NFATc, which increases AQP2 transcription by binding to the AQP2 promoter at several sites, including that found to bind TonEBP.

What factors mediate decreased AQP2 mRNA expression shortly following hypertonic challenge (Fig. 1)? Contrary to its stimulatory effect in the course of sustained hypertonicity, TonEBP does not play a role in decreased AQP2 expression at the onset of hypertonic challenge. Indeed, the extent of decreased AQP2 expression was not affected by downregulated TonEBP activity in RNAi and DN-TonEBP-transfected cells, in either the presence or absence of VP (65). On the other hand, several observations indicate that increased NF-KB activity induced by hypertonicity may reduce AQP2 expression. First, polyuria arising in various models of tubulo-interstitial inflammatory diseases displaying increased NF-kB activity is associated with decreased levels of AQP2 expression (2, 41, 46, 60). Second, hypertonicity alters NF-κB activity in numerous cell types including skin fibroblasts, monocytic cells, gastric epithelial cells, peripheral blood mononuclear cells, and vascular smooth muscle cells (1, 71, 91, 137, 149). However, depending on the cell type, hypertonicity either promotes or represses NF-KB activity. Several pieces of evidence indicate that hypertonicty increases NF-kB activity in the renal medulla. Transgenic mice with a luciferase reporter gene driven by a NF-KB response element displayed increased luciferase activity following 24 h of water deprivation, an effect that was reproduced in renal medullary interstitial cells (RMIC) after 20 h of exposure to hypertonic NaCl medium (64). Hypertonicity was additionally found to increase binding of proteins from RMIC nuclear extracts to a NF-kB consensus-containing DNA probe (64). Events mediating downregulated AQP2 expression by LPS-induced NF-kB activation have been specifically addressed (60). On the basis of downregulated V<sub>2</sub> receptor expression occurring in animals several hours following intraperitoneal LPS injection, it was concluded that LPS reduces AQP2 expression as a consequence of decreased V<sub>2</sub> receptor activity (60). However, this cannot explain decreased AQP2 expression at the onset of hypertonic challenge since hypertonicity, assuming that it does increase NF-KB activity in CD principal cells, does not affect cAMP levels and does not decrease PKA activity (69, 70). The AQP2 promoter contains two highly conserved kB elements located upstream of the TonE sequence suggesting that NF- $\kappa$ B may modulate AQP2 expression by binding to its promoter. This possibility was addressed in a study performed on mpkCCD<sub>cl4</sub> cells that investigated in tandem the effects of hypertonicity on NF-kB activity and the role of NF-kB on AQP2 transcription (66). Hypertonicity was indeed found to increase NF-kB activity in these cells to a similar extent to that induced by LPS, as shown by increased NF-kB-driven luciferase activity and by increased expression levels of several NF- $\kappa B$  genes under the control of NF- $\kappa$ B transcriptional activity (TNF $\alpha$ , MCP-1, and I $\kappa$ B $\alpha$ ). Increased expression of these genes induced by LPS was accompanied by decreased AQP2 mRNA and protein expression. The extent of decreased AOP2 expression was similar in cells bathed in isotonic or hypertonic medium and in cells treated or not with VP. This same study showed decreased AQP2 mRNA expression occurring in rat kidney slices in response to LPS. This occurred in the presence of either the  $V_2$ receptor antagonist SR121463B or 8Br-cAMP further indicating that the effect of LPS on AQP2 expression arises from events occurring downstream of the V2 receptor. Mutation of either or both KB elements of the AQP2 promoter abolished the LPS-induced decrease of AQP2-driven luciferase activity. Moreover, these mutations had no effect on increased luciferase activity following sustained (12h) hypertonic challenge but did abolish the effect of LPS that occurred in a hypertonic environment. What members of the NF-kB family mediate AQP2 transcriptional control? Combined chromatin immunoprecipitation and RNAi assays indicate that an increase of NF-kB activity, induced by endotoxins or an increase of environmental tonicity, lead to p65 release from and increased p50 and p52 binding to kB elements of the AQP2 promoter (66). Since NF-KB dimers containing p65 classically activate transcriptional activity while those containing p50/p52 mono and dimers act as repressors, reduced AQP2 transcription in response to an increase of NF-kB activity may arise from both occurrences. The biological importance of time-dependent changes in AQP2 transcriptional activity is discussed below.

# Effects of Hypertonicity on AQP2 Trafficking

The vast majority of studies aimed at investigating mechanisms that govern short-term control of AQP2 expression describe the effects of VP on AQP2 plasma membrane accumulation (10, 12, 16, 18, 168). Here, the effects of hyperto2012

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nicity on AQP2 trafficking are described and compared with those induced by VP.

AQP2 has long been known to accumulate in both intracellular vesicles and at the cell surface, suggesting regulated trafficking activity of AQP2-containing vesicles to and from the plasma membrane (27, 128). Mechanisms that regulate docking and fusion of AQP2-bearing vesicles to the plasma membrane, together with vesicle budding and internalization from the cell surface, have been extensively investigated and are the subject of numerous fine reviews (10, 16–18, 125, 168). It is now well understood that AQP2 recycles constitutively between an intracellular pool and the cell surface but that numerous factors modulate this activity. Well before the discovery of aquaporins, enhanced water permeability elicited by hypertonic stimulation was associated with particle clustering at the apical surface of frog bladder epithelium (6, 14). This is in line with a recent study that showed the occurrence of decreased AQP2 expression at the plasma membrane of cultured renal CD8 cells at the onset of hypotonic challenge (10 min) (163). PKA-dependent phosphorylation of AQP2 at Ser256 is imperative for its accumulation at the cell surface following VP stimulation (17). Similar to VP, the effect of hypotonicity appears to rely on Ser256 phosphorylation since reduced AQP2 cell surface expression was associated with decreased AQP2 phosporylation (163). The effects of acute hypertonicity on AQP2 trafficking were investigated and these findings are summarized in Fig. 2. In contrast to acute hypotonicity, an increase of environmental tonicity from 300 to  $\geq$  350 mosmol/kgH<sub>2</sub>O was shown to induce an accumulation of AQP2 at the plasma membrane within minutes (<30 min) of stimulation (69) in kidney slices and in cultured renal cell lines [mCCDcl1, LLC-PK1, and MDCK cell lines (51, 69, 89)]. As observed in kidney slices but also in cultured cells, acute hypertonic challenge directed AQP2 mostly toward the apical pole. The amount of AQP2 expressed at the cell surface was dependent on the extent of hypertonic challenge, with a maximal effect comparable to that achieved by VP and was reversed by placing cells back in isotonic medium. Hypertonic stimulation not only induced AQP2 to accumulate at the cell surface but in the trans-Golgi network (TGN) as well. Despite low levels of cAMP concentration, PKA activity is increased on the onset of hypertonic challenge (42, 70), possibly resulting from PKAc release from a NF-kB-IkB-PKAc complex (185). Both PKA activity and AQP2 phosphorylation at Ser256 are required for hypertonicity-induced AQP2 accumulation at the cell surface but not the TGN. Two major signaling pathways, one cAMP/PKA dependent the other cGMP dependent, have so far been described to increase AQP2 expression at the cell surface (11, 101, 131). A novel aspect of AQP2 trafficking at the onset of hypertonic challenge is that AQP2 accumulation at the cell surface, but not TGN, is dependent on p38, ERK1/2, and JNK1/2 MAP kinase activity. In fact, AQP2 cell surface accumulation by acute hypertonicity, but not VP, was greatly reduced by pharmacological inhibition of just one of these MAP kinases (69). It seems that the effects of environmental tonicity on AQP2 cell surface expression are not restricted to acute tonicity alone. Indeed, AQP2 is expressed in both apical and basolateral membranes of CD principal cells of the inner medulla (128) and sustained hypertonic stimulation was shown to redirect AQP2 expression from the apical to the basolateral membrane (169). Although the physiological relevance of basolateral AQP2 expression can only be speculated on, it is possible that it reinforces AQP3- and AQP4-mediated water exit out of the cell. In any case, the observation that

Fig. 2. Hypertonicity profoundly affects AQP2 trafficking. On the onset of hypertonic challenge, both AQP2 endocytotic and exocytotic activity are reduced leading to an accumulation of AQP2 at both the apical plasma membrane (PM) and the trans-Golgi network (TGN), as illustrated by solid red arrows. AQP2 accumulation at the apical PM is dependent on a hypertonicity-induced increase of p38, ERK1/2, and JNK1/2 activity, on a hypertonicity-induced increase of PKA activity that occurs in the absence of an increase of intracellular cAMP concentration, and on AQP2 phosphorylation at S256 (69). Sustained hypertonic challenge leads to an accumulation of AQP2 at the basolateral PM (169), as illustrated by a dashed red arrow. +, Events that are induced by hypertonicity; -, events that are repressed by hypertonicity.



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sustained hypertonicity can redirect AQP2 trafficking is of special interest to cell biology as it implies that hypertonicity not only induces protein trafficking via elements already in place but can reprogram the trafficking machinery itself to better adjust cell function to environmental changes.

The intracellular elements that mediate the effects of acute hypertonicity on AQP2 trafficking are presently unknown. Whereas VP- and hypertonicity-induced accumulation of AQP2 at the cell surface most certainly share common mediators, the numerous differences between the two stimuli indicate that their effects are governed by different sets of elements. Indeed, VP stimulation has previously been shown to increase AQP2 cell surface expression by inducing an accumulation of AQP2 in "endocytosis-resistant" membrane domains (13) in addition to enhancing AQP2 exocytosis (108, 132, 184). Acute hypertonicity, on the other hand, reduces both endocytotic and exocytotic activity in cultured renal cells (69). Hypertonicity, unlike VP, does not increase cAMP concentration indicating that PKA activation by each stimulus is achieved through distinct mechanisms (42). MAP kinase activation is essential for AQP2 cell surface accumulation by hypertonicity but not VP. Finally, AQP2 accumulation at the TGN was dissipated by VP (69). Numerous proteins that play various roles in vesicle trafficking have been proposed to influence AQP2 cell surface accumulation. Candidate proteins that may play various roles in altered AQP2 trafficking by hypertonicity include those implicated in cytoskeletal remodeling and those involved in vesicle transport and fusion/budding processes. Actin is an obvious candidate. RhoA-mediated depolymerization of actin stress fibers has been shown to increase AQP2 cell surface expression (93). Trafficking processes require actin remodeling and hypertonicity profoundly alters actin cytoskeletal organization. F-actin is rapidly polymerized in response to hypertonicity (22, 35) under the combined action of the small GTPases Rac and Cdc42, cortactin and Arp2/3 (a regulator of F-actin nucleation), which all form a complex with actin (35). In the TAL, RVI efficacy following hypertonic challenge is increased by the simultaneous depolymerization of the F-actin cortical ring and polymerization of a dense diffuse F-actin network (22). These observations indicate that actin, together with proteins that mediate its polymerization state, play an essential role in altered AQP2 trafficking in the hypertonicity-challenged cell. It is possible that hypertonicity additionally affects microtubule organization. Interestingly, microtubule depolymerization by microtubule-disrupting agents was found to induce a transient (10- to 30-min long) accumulation of AQP2 at the apical membrane of MDCK cells (161). Longer periods of microtubule disruption led to a scattering of AQP2-containing vesicles that was confined to subapical domains of MDCK cells (161) but that was apparent throughout the entire cytoplasm in cells of the whole kidney (145). The presence of the microtubule-associated motor protein dynein and the associated dynactin complex in AQP2containg vesicles (116) may impart a role for these proteins in the effects of hypertonicity on AQP2 trafficking. In addition to actin and microtubules, any protein shown to colocalize in the same vesicles as AQP2, such as TRPC3, SNAP-23, syntaxin-4, and Hrs-2 (4, 57, 79, 111, 151), could potentially play a role in altered AQP2 trafficking by hypertonicity. Other candidate proteins include those identified by functional studies such as nonmuscle myosin II and myosin light chain kinase, vesicle-associated membrane protein (VAMP)/synaptobrevin-2, myelin and lymphocyte-associated protein (MAL), and heat shock protein (HSP)70 (25, 59, 86, 109). AQP2 has been shown to be located in clathrin-coated pits (158). Since hypertonicity is known to effectively impair clathrin-mediated endocytosis (73), proteins involved in this process, including dynamin (158), provide attractive candidates. Proteins involved in AQP2 phosphorylation at S256 and at other phosphorylation sites (S261, S264, and S269) shown to be regulated by VP (17, 49, 74–76, 88) may play key roles in orchestrating increased AQP2 cell surface expression by hypertonicity. Finally, the identification of molecules that mediate MAP kinase-dependent trafficking (61, 123, 142, 147, 186) will undoubtedly provide new insight for processes governing AQP2 trafficking under hypertonic conditions.

Unlike VP, acute hypertonicity leads to an accumulation of AQP2 at the TGN (69). Classically, newly synthesized proteins are sorted at the TGN where they are selectively targeted to apical and basolateral domains. The importance of the TGN in protein sorting is especially pertinent to AQP2 since different stimuli promote its expression at apical (VP and acute hypertonicity) and basolateral (sustained hypertonicity) plasma membranes. AQP2 could reach the plasma membrane via various routes. AQP2 could hypothetically traffic between the TGN and recycling endosomes from where it reaches the plasma membrane. Alternatively, AQP2 could reach the plasma membrane directly from the TGN via secretory vesicles. A third possibility, demonstrated for the apical delivery of glycosylphosphatidylinositol-anchored proteins in MDCK cells (138), consists of newly synthesized AQP2 to exit the TGN in membrane-bound carriers that fuse to the basolateral plasma membrane. From there, AQP2 would be rapidly internalized into clathrin-free transport intermediates and redirected to the apical membrane. Possibly, AQP2 trafficking to the plasma membrane involves all three pathways. Why does AQP2 accumulate at the TGN at the onset of hypertonic challenge and could this observation help elucidate how AQP2 reaches the cell surface? Two observations provide us with clues of the origin of AQP2 residing in the TGN at the onset of hypertonic challenge (69). First, recycling AQP2, rather than newly synthesized protein, was found to be the major contributor to the formation of the TGN patch following hypertonic challenge. This conclusion was drawn from the observation that AQP2 accumulation at the TGN at the onset of hypertonic challenge was maintained in the presence of cycloheximide, a protein synthesis inhibitor, or brefeldin A, which blocks translocation of proteins the endoplasmic reticulum to the Golgi apparatus (69). AQP2 accumulation at a structure identified as the TGN was also previously found to be induced by low temperature or treatment with the H<sup>+</sup>-ATPase inhibitor bafilomycin (62). In that study, at least part of the AQP2 pool residing in the TGN was found to consist of internalized AQP2 initially expressed at the cell surface. Second, VP drastically reduced AQP2 accumulation at the TGN suggesting that VP overrides the effect of hypertonicity by inducing AQP2 to leave the TGN, possibly for its immediate recruitment to the plasma membrane. These observations indicate that AQP2 accumulation at the cell surface following acute hypertonic challenge could result from its delivery to the apical plasma membrane by recycling endosomes and secretory vesicles where it would be trapped as a consequence of reduced clathrin-dependent, endocytotic activity. The latter event may also explain AQP2

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accumulation at the TGN. In any case, AQP2 accumulation at the apical cell surface and TGN by acute hypertonicity confirms the tight functional correlation between these two structures in AQP2 trafficking.

#### *Physiological Implications of Altered AQP2 Expression by Hypertonicity*

What is the biological relevance of enhanced AQP2 apical expression at the onset of hypertonic challenge together with the time-dependent decrease, then increase of AQP2 transcriptional activity? Targeting of existing AQP2 protein to the apical cell surface may actually help protect cells from excessive water loss immediately following hypertonic challenge. Indeed, increased water efflux out of the cell via basolateral AQP3 and AQP4 but also AQP2 following an increase of interstitial osmolality may be partially counterbalanced by compensatory water entry from the nephron lumen via the apical membrane. A similar protective role during hypotonicityinduced cell swelling has been previously been proposed (163). In that study, hypotonicity was found to decrease AQP2 expression at the apical cell surface of cultured CD8 cells, an event that was accompanied by increased translocation of nucleotide-sensitive chloride current protein to the plasma membrane and increased Cl- efflux. Decreased AQP2 expression at the apical surface of hypotonicity-challenged cells could protect cells by limiting apical water entry thereby reducing cell swelling. Increased and decreased AQP2 expression at the cell surface immediately following (i.e., within minutes) an increase or decrease, respectively, of environmental osmolality, strongly suggests a role for AQP2 in cell volume regulation in osmotically challenged CD principal cells. The presence of AQP2 at the cell surface may additionally be of critical importance for the rapid activation of RVD mechanisms following hypotonicity-induced cell swelling. Activation of RVD mechanisms, linked to fibrosis transmembrane conductance regulator (CFTR) and K<sup>+</sup> channel activation and to an AQP2-facilitated increase of intracellular Ca<sup>2+</sup> concentration, was shown to occur more rapidly in CCD cells transfected with AQP2 (43, 52). Similar to AQP2, other AQPs may help control cell volume in nonrenal cells. Cell swelling in cultured corneal endothelial cells in response to hypotonic challenge was slower in the absence of AQP1 (99). Salivary secretion is a dynamic process and acinar cells are repeatedly subjected to cell shrinkage and swelling. AQP5 expressed in acinar cells was shown to be involved in controlling cell volume since both hypertonicity-induced cell shrinkage and hypotonicity-induced cell swelling were reduced as a consequence of deficient AQP5 function (98). TRPV4, whose activation may depend on AQP5 (105), may directly participate in regulating cell volume by mediating reduced AQP5 abundance under hypotonic conditions (152). A similar functional relationship between AQP2 and TRPC3 may exist in principal CD cells since TRPC3 was found to physically associate with AQP2 (57). AQP2 accumulation at the apical plasma membrane at the onset of hypertonic challenge was found to occur simultaneously with a decrease of AQP2 transcriptional activity. A decrease of AQP2 abundance may help reduce AQP2 expression at the basolateral membrane of CD cells, thereby reducing water loss via the basal side of cells. Reduced AQP2 synthesis may consequently reinforce the effects of enhanced apical AQP2 expression and protect cells from excessive water loss. Among numerous cellular events, the protective role of NF- $\kappa$ B against proapoptotic stimuli is particularly well characterized. Its role in reducing AQP2 abundance would bestow NF- $\kappa$ B with a novel means of suppressing apoptosis in CD principal cells. At a transcriptional level, hypertonicity increases TonEBP activity more slowly than NF- $\kappa$ B activity (65, 66) allowing NF- $\kappa$ B to act first on the AQP2 promoter and then be supplanted by an increase of TonEBP/NFATc activity after longer periods of time. Once the cell has adapted to its new hypertonic environment, increased AQP2 expression by TonEBP would enhance water reabsorption, helping to secure whole body water homeostasis thereby allowing the organism to better adapt to water restriction.

#### Conclusion

Cells of the renal CD are exposed to recurrent variations of extracellular osmolality as a consequence of transitions between diuretic and antidiuretic states that follow physiological and behavioral change. These cells must first adapt to their changing environment and then assume appropriate functional roles to meet the organism's needs. AQP2 plays a critical role in adjusting water homeostasis critical for mammalian survival. Through changes of AQP2 transcription and cell surface expression, AQP2 may play important roles in both cell survival and adjusted function in response to hypertonicity. Increased AQP2 expression at the apical surface together with decreased AQP2 transcription may together help protect cells from the devastating effects of hypertonicity at the onset of hypertonic challenge by decreasing cell shrinkage. After longer periods of time, once the adaptation process to the new hypertonic environment is complete, an increase of AQP2 transcription and whole cell abundance together with increased AQP2 expression at the basolateral surface would enhance transcellular water transport, helping the organism to adapt to water restriction. A comparison of these effects with those induced by hypotonicity helps us understand processes mediating each event. Such events are not restricted to AQP2 but concern other AQPs located in other osmotically challenged tissues as well. Further research dedicated to each of these AQPs will undoubtedly expand our knowledge in the role that AOPs play during aniosmotic challenge and will allow us to further identify their functional mediators.

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