Published in final edited form as:

Clin Exp Nephrol. 2013 December ; 17(6): . doi:10.1007/s10157-013-0789-5.

Vasopressin and the Regulation of Aquaporin-2

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Abstract

Water excretion is regulated in large part through the regulation of the osmotic water permeability of the renal collecting duct epithelium. The water permeability is controlled by vasopressin through regulation of the water channel, aquaporin-2 (AQP2). Two processes contribute: 1) regulation of AQP2 trafficking to the apical plasma membrane; and 2) regulation of the total amount of the AQP2 protein in the cells. Regulation of AQP2 abundance is defective in several water balance disorders including many polyuric disorders and the syndrome of inappropriate antidiuresis (SIADH). Here we review vasopressin signaling in the renal collecting duct that is relevant to the two modes of water permeability regulation.

Keywords

Vasopressin; trafficking; transcription; polyuria; hyponatremia; SIADH

I. Introduction

Specialized integral membrane proteins called *aquaporins* mediate water transport across plasma membranes in many cells and tissues [1]. Aquaporins expressed in the kidney are particularly important because they play critical roles in the regulation of water excretion by the kidney. Of the 13 known mammalian aquaporins, eight (Aquaporins 1, 2, 3, 4, 6, 7, 8, and 11) are expressed in the kidney [1] [2]. Aquaporin 1 (AQP1), AQP2, AQP3 and AQP4 are important because they mediate water transport across renal tubule epithelia [1]. AQP1 is expressed at very high levels in the proximal tubule and thin descending limb of Henle [3] [4], correlating with the extremely high water permeability in these segments. In contrast, renal tubule epithelia with very low water permeabilities (thin ascending limb, thick ascending limb and distal convoluted tubules) do not appear to express aquaporins. The terminal part of the renal tubule, the connecting tubules and collecting ducts have variable water permeability that is controlled by the peptide hormone vasopressin [5]. This renal tubule segment expresses three aquaporins: aquaporin 2 (AQP2) in the apical plasma membrane [6] and aquaporin 3 and 4 (AQP3 and AQP4) in the basolateral plasma membrane [7] [8]. Although there is evidence for regulation of the basolateral water channels by vasopressin [7],[9], it is generally accepted that transepithelial water permeability is regulated through effects on the apical water channel, namely AQP2 [1].

Water permeability regulation in the renal collecting duct is regulated by vasopressin in two processes. The first process is 'short-term regulation' occurring over a period of minutes as a result of the regulation of trafficking of AQP2-containing membrane vesicles to and from

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Disclosure: All the authors have declared no competing interest.

the apical plasma membrane in response to vasopressin [10]. The second process is ‘long-term regulation’ occurring over a period of hours to days as a result of regulation of whole cell AQP2 abundance by vasopressin [11] [12] [13]. This short review will focus on the mechanisms involved in vasopressin-mediated regulation of AQP2 in the renal collecting duct by the above two processes. Understanding the mechanisms involved in water transport regulation are critical to the understanding of the pathophysiology of a large number of clinical syndromes characterized by disturbances in water balance. Figure 1 shows a ‘word cloud’ that indicates frequency of word usage in this review and thereby summarizes the major topics in the article.

A. Vasopressin signaling in the renal collecting duct

1. General features of vasopressin signaling—The answer to the question of how vasopressin regulates AQP2 abundance in the renal collecting duct depends on knowledge of vasopressin signaling pathways. The general pathways involved in vasopressin signaling in collecting duct cells are diagrammed in Figure 2. The type 2 vasopressin receptor (V2R) is a G_s-coupled receptor that binds vasopressin and activates two adenylyl cyclases, types III and VI [14], to increase intracellular cyclic AMP (cAMP) levels. Exogenously added cAMP analogs replicate the rapid osmotic water permeability increase seen with vasopressin, leading authors to conclude that the action of vasopressin to increase water permeability in collecting ducts is mediated by cAMP [15];[16]. Downstream effects are believed to be mediated in part by activation of protein kinase A, although other kinases likely play important roles including Akt, Sgk, myosin light chain kinase and calmodulin dependent kinases, and MAP kinases [17],[18],[19],[20]. One substrate for protein kinase A is AQP2 itself at Ser256. However, this site has been shown to be a substrate for other basophilic protein kinases including Akt1 and protein kinase Cδ [21]. In addition, to Ser256, three additional phosphorylation sites in the COOH-terminal tail of AQP2 have been identified, viz. Ser261, Ser264, and Ser269 [22]. Studies with phospho-specific antibodies to these sites have demonstrated that vasopressin regulates phosphorylation of all four COOH-terminal serines, causing increases in phosphorylation of Ser256 [23], Ser264 [24],[25], and Ser269 [24],[26], while decreasing the phosphorylation at Ser261 [27]. Phosphorylation of Ser256 appears to be a prerequisite to phosphorylation at Ser264 and Ser269 [24]. Quantification of Ser269 phosphorylation in rat IMCD showed that in the absence of vasopressin only 3% of total AQP2 was phosphorylated at this site, while 26% was phosphorylated at Ser269 after vasopressin exposure [28]. Similarly, Ser264 phosphorylation increased in response to vasopressin from 1.9 to 3.4 percent of total AQP2. In contrast, Ser261 decreased from 18% to 2% of total AQP2 in response to vasopressin in the same study and Ser256 phosphorylation was constitutively high in these experiments (control, 49% of total; vasopressin, 34% of total), consistent with the idea that Ser256 phosphorylation is a prerequisite to downstream phosphorylation at Ser264 and Ser269, but need not be altered by vasopressin to realize a physiological effect of vasopressin [28]. Results were very similar in cultured mpkCCD collecting duct cells in response to vasopressin exposure.

In addition to a rise in cAMP, vasopressin and its V2R-selective analog dDAVP evoke a rise in intracellular Ca²⁺ concentration in collecting duct cells [29],[30], [31]. The Ca²⁺ mobilization can also be reproduced by addition of exogenous cAMP [31], and a role for one of the isoforms of the cAMP-dependent Rap1GEF, Epac, has been implicated [32]. Both Epac1 and Epac2 are expressed in collecting duct cells and the abundance of Epac1 has been reported to be increased by the V2R-selective vasopressin analog dDAVP [33]. High resolution techniques using Ca²⁺-sensitive fluorescent dyes have demonstrated that the vasopressin-induced rise in intracellular Ca²⁺ consists of aperiodic calcium spikes that are independent of Ca²⁺ changes in neighboring cells [31] [17]. The rise in intracellular Ca²⁺ is associated with an increase in phosphorylation of myosin regulatory light chain [34], via a

Ca^{2+} -calmodulin mediated activation of myosin light chain kinase. Ca^{2+} -calmodulin also enhances cyclic AMP production by stimulating AC3 [14].

Several lines of evidence are consistent with the view that V2R-mediated calcium mobilization is a key element of the mechanism by which vasopressin increases osmotic water permeability in the collecting duct epithelium. Either chelation of intracellular calcium with BAPTA or prevention of Ca^{2+} release with ryanodine inhibited the vasopressin action to increase water permeability in isolated perfused inner medullary collecting ducts [35]. Calmodulin inhibitors strongly inhibited the action of vasopressin to increase osmotic water permeability in isolated perfused IMCD ducts [35]. Downstream targets of Ca^{2+} -calmodulin include both adenylyl cyclase type III [14] and myosin light chain kinase [34], both of which are stimulated. Furthermore, blebbistatin, an inhibitor of conventional non-muscle myosins inhibited the vasopressin-induced morphological changes (cell height increases) in inner medullary collecting duct [36]. Phosphorylation of the myosin regulatory light chain by myosin light chain kinase results in activation of conventional non-muscle myosins II-A and II-B, which are believed to be critical to vasopressin-mediated movement of AQP2-containing vesicles to the apical plasma membrane (*vide infra*).

Vasopressin also causes a PI-3K dependent activation of Akt (protein kinase B) in the IMCD, as well as an inhibition of the ERK1/2, p38, and Jnk MAP kinase pathways [17], [19]. In addition, several other protein kinases have been implicated in collecting duct signaling although not necessarily in vasopressin signaling in the collecting duct. These include Protein kinase C isoforms [37],[38],[39], Rho/CDC42/Rac-dependent kinases [40], G protein-coupled receptor kinase 4 (Grk4) [41], Sgk1 [42],[43], Wnk1 [44],[45], [43], Wnk4 [44],[43] Protein kinase G [46],[47], Casein kinase II [48], mTOR [49], GSK3 β [50] and various tyrosine kinases [51],[52],[53],[54].

2. Phosphoproteomics—Recently, our laboratory has conducted a series of phosphoproteomic studies to identify proteins whose phosphorylation is regulated by vasopressin in collecting duct cells [22],[55],[18], [19],[56] and in renal thick ascending limbs [57]. An in depth presentation of these results is beyond the scope of the current review. All results can be access online at <http://helixweb.nih.gov/ESBL/Database/index.html>. In the following, we mention a few of the vasopressin-regulated phosphoproteins that appear to be most likely to play roles in the regulation of AQP2 by vasopressin.

β -catenin: Among the most consistent phosphoproteomic responses to vasopressin has been a marked increase in β -catenin (*Ctnnb1*) phosphorylation at Ser552, seen in native IMCD cells [55],[58], in cultured mpkCCD cells [18],[59],[56], and in native thick ascending limb cells [57]. In mpkCCD cells, vasopressin the increase in Ser552 phosphorylation was associated with translocation of β -catenin from the cytoplasm to nucleus, consistent with prior observations in A431 tumor cells [60]. In the nucleus, β -catenin functions as a transcriptional coactivator for a number of transcription factors on a number of genes, although possible roles for β -catenin in regulation of AQP2 transcription have not been reported. β -catenin is a key element of the Wnt signaling pathway that is instrumental in collecting duct development in the embryo. Ser552 of β -catenin is known to be phosphorylated either by Akt or by protein kinase A [61].

c-Jun: The transcription factor c-Jun (gene symbol: Jun) dimerizes with c-Fos or one of its homologs to form the AP-1 transcription factor. A decrease in phosphorylation of Ser73 of c-Jun has been observed in both nuclear fractions and whole cell extracts of mouse mpkCCD cells [18],[56]. This is the canonical site for phosphorylation by Jnk and other MAP kinases [62], and its phosphorylation is required for kinase activity [63].

Phosphorylation of c-Jun at Ser73 has been shown to enhance transcriptional activation [62], [64]. Transcriptomic analysis has demonstrated that c-Jun, JunB, and c-Fos are expressed in both native rat IMCD [43] and cultured mpkCCD (clone 11) cells [65] at levels well above the median for all transcripts (see databases accessible at <http://helixweb.nih.gov/ESBL/Database/index.html>). AP-1 has been implicated in regulation of Aqp2 transcription by Yasui et al. [66]. The Ser73 phosphorylation site of c-Jun (LAS*PELER) exhibits a “proline-directed” motif consistent with a role for either MAP kinases in the response to vasopressin. Additional phosphoproteomic data indicates that vasopressin treatment in native collecting duct cells decreases the phosphorylation of Jnk2 (Mapk9) and p38 α (Mapk14) at their canonical activation sites [19].

Myosin light chain kinase: Vasopressin strongly decreases myosin light chain kinase (Mylk) phosphorylation at Ser364 in nuclear fractions of cultured mpkCCD cells Bolger. As noted above Mylk phosphorylates two myosin light chain isoforms in native inner medullary collecting duct cells and is an important downstream component of vasopressin signaling in inner medullary collecting duct cells via its effect on AQP2 trafficking [34]. Its role in the nucleus is, however, unclear but it may be involved in the organization of chromatin or the mechanism of splicing of transcripts [56]. In a previous studies, Schenk et al. [59] identified Mylk’s target myosin regulatory light chain B as well as the corresponding conventional non-muscle myosin heavy chains coded by the Myh9 and Myh10 genes in nuclear fractions of mpkCCD (clone 11) cells (<http://helixweb.nih.gov/ESBL/Database/mNPD/index.html>). The Ser364 phosphorylation site of Mylk is in a region of the protein associated with binding of Mylk to actin microfilaments (stress fibers) [67].

3. V1a receptor—In addition to the V2 receptor, there is evidence that a second vasopressin receptor, the V1a receptor, is expressed in collecting ducts in the renal cortex [68],[69],[70],[71]. Recent evidence suggests that the V1a receptor resides in intercalated cells where it interacts with the mineralocorticoid receptor (*Nr3c2*) to regulate acid-base transport [72].

B. Short-term regulation by vasopressin: AQP2 trafficking

1. General features of short-term regulation—Classic studies in isolated perfused cortical collecting ducts showed that vasopressin produces a rapid increase in the osmotic water permeability [15]. In isolated perfused rat renal IMCDs, a half-maximal increase is seen 8 min after vasopressin addition [16].

The increase in osmotic water permeability of the collecting duct epithelium occurs as a result of recruitment of AQP2 to the apical plasma membrane of the collecting duct cells [10]. In the absence of vasopressin, AQP2 is present chiefly in intracellular vesicles believed to be recycling endosomes [73],[74]. Water permeability measurements as a function of time coupled to mathematical modeling studies [75],[76] have demonstrated that the vasopressin-induced redistribution of AQP2 to the apical plasma membrane takes place through two general processes: 1) *speeding up* of the rate of exocytic insertion of AQP2 *into* the plasma membrane and 2) *slowing down* of the endocytic removal of AQP2 *from* the apical plasma membrane. Brown *et al.* [77],[78] have provided evidence that the amount of AQP2 in the plasma membrane is a result of a balance between continuing endocytosis and exocytosis of AQP2, both in the presence and absence of vasopressin. They showed, for example, that vasopressin-induced redistribution of AQP2 to the plasma membrane can be mimicked simply by inhibiting endocytosis, e.g. by expression of a dominant-negative form of dynamin [79]. Based on these observations, the mechanism of vasopressin action in regulation of AQP2 trafficking must focus on separate effects on exo- and endocytosis of AQP2-containing vesicles.

2. Actin dynamics and regulation of AQP2 exocytosis—An important process regulating exocytosis is vasopressin-induced depolymerization of F-actin in the subapical cell cortex in collecting duct cells, first demonstrated by Richard Hayes and his colleagues [80] and subsequently confirmed by multiple research groups. The dense cortical network of actin filaments is viewed as a barrier to movement of AQP2-containing vesicles to the apical plasma membrane, and actin depolymerization is therefore expected to facilitate exocytic insertion of AQP2-laden vesicles. The state of actin polymerization in the collecting duct is regulated by Rho family kinases [81]. In collecting duct, Rho activation appears to be associated with redistribution of AQP2 from the plasma membrane to intracellular compartments, presumably by inhibition of AQP2 exocytosis. In natural killer lymphocytes, RhoA has been shown to be phosphorylated by protein kinase A at Ser188, near the COOH-terminus of the protein, an effect that causes dissociation of RhoA from membrane-associated effectors [82], chiefly Rho/Rac/Cdc42-activated kinases. Vasopressin increases Ser188 phosphorylation of RhoA in collecting duct cells leading to actin depolymerization [83]. Exposure of CD8 collecting duct cells to *Clostridium* toxin C3, which specifically inhibits RhoA, also caused a partial depolymerization of F-actin associated with increased plasma membrane localization of AQP2 [84]. The COOH-terminus of AQP2 strongly binds actin [85] through a multiprotein complex discovered via proteomics [86]. These authors found that phosphorylation of AQP2 at Ser256 was associated with an increase in the affinity of AQP2 to tropomyosin-5b (also known as tropomyosin-1), decreasing its interaction with G-actin, thereby contributing to F-actin depolymerization [87]. Studies by Tamma et al [88] also show that FERM domain-containing proteins (four-point-one, ezrin, radixin, and moesin), known to bridge between integral membrane proteins and the actin cytoskeleton, participate in Rho signaling and in regulation of AQP2 exocytosis.

3. Aquaporin-2 phosphorylation and regulation of AQP2 endocytosis—As noted above, vasopressin regulates the amount of AQP2 in the apical plasma membrane of collecting duct cells in part by regulating the rate of AQP2 endocytosis [75],[76],[77]. Of the four COOH-terminal phosphorylation sites that are regulated by vasopressin, two have been implicated in the regulation of endocytosis, viz. Ser261 and Ser269.

Ser261: Studies using quantitative LC-MS/MS and immunoblotting with phospho-specific antibodies have demonstrated that vasopressin strongly decreases phosphorylation of AQP2 at Ser261 in rat IMCD cell suspensions [22], [27], [89]. The group of Deen has argued that Ser261 phosphorylation may play a role in regulation of endocytosis of AQP2 by speeding the degradation of AQP2 in association with ubiquitylation at K279 [90]. They also report that internalization of AQP2 with ATP and dopamine (agents that counteract the action of vasopressin to increase water permeability) coincides with an increase in S261 phosphorylation in AQP2 [91]. However, studies in which LLC-PK1 cells were transfected with S261A and S261D mutant forms of AQP2 led the authors to conclude that the phosphorylation state of AQP2 at Ser261 does not appreciably affect trafficking of AQP2 [92]. More studies will be needed to identify the physiological role of AQP2 phosphorylation at Ser261.

Ser269: Studies using quantitative mass spectrometry and immunoblotting with phospho-specific antibodies have demonstrated that vasopressin strongly increases phosphorylation of AQP2 at Ser269 in rat IMCD cell suspensions [24]. The vasopressin-mediated increase in AQP2 phosphorylation at Ser269 occurred significantly more slowly ($t_{1/2} = 3.2$ min) than the increase in Ser256 phosphorylation ($t_{1/2} = 41$ s) [24]. The response time correlates closely with the time course of the water permeability response to vasopressin in isolated perfused IMCD segments (initial increase at 35 s, half-maximal response at 8 min) [16], indicating a plausible role of phosphorylation at Ser256 and Ser269 in the regulation of AQP2

trafficking. Using phosphospecific antibodies to AQP2, Hoffert et al [24] and Moeller et al. [26] showed that in rat renal IMCDs that AQP2 phosphorylated at Ser269 was present only in the apical plasma membrane, whereas AQP2 phosphorylated at any of the other three COOH-terminal sites was found throughout the cell in intracellular vesicles as well as in the plasma membrane. Mutation of the Ser269 site to Asp, mimicking the charge state of a phosphorylated serine, resulted in constitutive localization of AQP2 in the plasma membrane [24], suggesting that AVP-mediated phosphorylation of AQP2 at Ser269 inhibits the endocytosis of AQP2. This conclusion was bolstered by biotin internalization assays in MDCK cells showing that the internalization of S269D mutated form of AQP2 from the plasma membrane was slower than that of wild type AQP2 [93]. The slower internalization corresponded with reduced interaction of S269D-mutated AQP2 with several proteins involved in endocytosis, including Hsp70, Hsc70, dynamin, and clathrin heavy chain. Additional experiments in LLC-PK1 cells transfected with S269A and S269D mutant forms of AQP2 further supported the critical role of Ser269 phosphorylation in maintenance of AQP2 at the cell surface [94].

B. Long-term regulation of AQP2 protein abundance

It is well established that long-term administration of vasopressin markedly increases AQP2 abundance in the renal collecting duct [11] [12] [13]. It is useful to begin by identifying possible mechanisms by which this regulation could occur (Figure 3). The amount of AQP2 in a given collecting duct cell at steady state represents a balance between production of AQP2 by translation and removal from the cell by either degradation or exosomal secretion, processes that are reviewed in the following paragraphs.

Urinary exosome excretion—The secretion of intact AQP2 into the urine was first identified by Kanno et al [95]. Later, the mechanism of AQP2 delivery into the urine was identified to be via exosome secretion based on work by Pisitkun and colleagues [96]. Exosomes are the internal vesicles of multivesicular bodies (late endosomes) that are delivered to the lumen of the renal tubule by every cell type facing the urinary space [97]. It has been shown that a large fraction of AQP2 endocytosed from the cell surface is delivered to multivesicular bodies in collecting duct cells. The proteome of urinary exosomes has been elucidated [96],[98] and is publically available as a publicly accessible resource (<http://dir.nhlbi.nih.gov/papers/lkem/exosome/>). Multiple publications have reported measurement of urinary AQP2 excretion as an indicator of the state of the kidney with regard to AQP2 regulation [95] [99] [100] [101] [102] [103]. However, such measurements are difficult to interpret unless long term steady state timed urine collections are made [97], [104], [105]. Specifically, under steady state conditions, an increase in urinary AQP2 excretion could reflect either an increase in synthesis or a decrease in degradation. Both of these processes would be expected to be associated with increased cellular abundance of AQP2, which could be reflective of an increase in vasopressin action. However, if steady-state conditions are not established, an increase in urinary AQP2 excretion could merely be a reflection of a transient increase in AQP2 endocytosis, which would indicate a decrease in vasopressin action in the collecting duct.

AQP2 degradation—The rate of AQP2 protein degradation can be measured in cultured cells by pulse-chase methodologies [106]. However, such measurements are impractical in intact animals. AQP2 degradation can presumably occur via incorporation into lysosomes or via proteasomal degradation. Proteins are marked for both via ubiquitination. Mono- or oligo-ubiquitination targets proteins for lysosomal degradation, while multi-ubiquitination targets proteins for proteasomal degradation. Recently, Deen et al demonstrated that AQP2 can be oligo-ubiquitinated at lysine-270, which is the second from last amino acid at the COOH-terminus [107]. Recently, data from two laboratories [20],[108] have provided

evidence that vasopressin may increase the half life of the AQP2 protein, but the magnitude of the increase was too small to account for the observed long term increase in AQP2 expression [13],[109].

AQP2 production—The rate of AQP2 production in collecting duct cells can theoretically be due to changes in the abundance of AQP2 mRNA or by direct regulation of translation, e.g. as seen in the endoplasmic reticulum stress response [110]. Vasopressin produces a clear increase in AQP2 mRNA abundance in rat collecting ducts [111],[9], suggesting that increased AQP2 translation, if it occurs, is due at least in part to increased AQP2 mRNA levels. The reader should be aware that AQP2 mRNA levels may not necessarily reflect amount of AQP2 mRNA available for translation because of regulated sequestration of mRNAs [112], a process that has not been investigated with regard to long-term regulation of AQP2 abundance. The total abundance of AQP2 mRNA can theoretically change as a result of changes in the transcription rate or changes in the mRNA degradation rate. There has been recent progress in mechanisms involved in regulation of mRNA degradation via micro-RNAs (miRNAs) [113], products of non-protein-coding genes. miRNAs can bind to specialized motifs in the 3'-untranslated regions of mRNA transcripts and target them for degradation via the RISC (RNA-induced silencing complex) mechanism. Although several potential miRNA binding motifs can be identified in the 3'-untranslated region of the AQP2 transcript (unpublished observations) there is as yet no evidence that AQP2 mRNA abundance is regulated by specific miRNAs.

Despite the possibilities described above for regulation of AQP2 protein abundance by vasopressin, most workers believe that the regulation of AQP2 protein abundance is largely due to regulation of AQP2 gene transcription. Progress in AQP2 transcriptional regulation was further advanced through the development of the mpkCCD cell line from micro-dissected cortical collecting duct cells of SV40 large T antigen transgenic mouse [114]. When grown on membrane supports that allow cell polarization, this cell line has barely detectable AQP2 mRNA and protein levels in the absence of vasopressin, but addition of vasopressin to the basolateral medium markedly increases endogenous AQP2 mRNA and protein levels in dose- and time-dependent manner [115]. The increases in AQP2 mRNA and protein levels induced by vasopressin are abolished by actinomycin D, a drug that inhibits transcription, indicating transcriptional regulation of *AQP2* gene expression by vasopressin. The overall response to vasopressin in the mpkCCD cells is reminiscent of Brattleboro rats that have minimal AQP2 mRNA and protein due to the lack of endogenous vasopressin [13;116]. When infused with vasopressin, the Brattleboro rats respond with an increase in AQP2 mRNA and protein levels [13]. Thus, vasopressin is necessary for the long-term maintenance of *AQP2* gene expression in the collecting ducts.

One strategy to investigate transcriptional regulation is to analyze 5'-flanking regions of a gene for *cis*-element motifs to which transcription factors bind and regulate gene transcription. The isolation of the AQP2 gene provides a starting point for this analysis [117]. Analysis of the 5'-flanking regions of the *Aqp2* gene from several species has identified several conserved binding motifs that play putative roles [65] [118] [119] including a cAMP-response element (CRE), a GATA site, an Sp1 site, several Ets sites, a Hox site, a Forkhead box, an NFAT site and an RXR site. In addition, an AP1 binding site has been reported Yasui. Furthermore, a site for Kruppel-like factor (Klf) binding has been identified in the first intron of the *Aqp2* gene [118]. The latter is a class of zinc finger transcription factors, usually involved in gene repression.

Proteomic studies of nuclei isolated from inner medullary collecting duct cells have identified transcription factors that can potentially bind to some of these sites: CRE (*Creb1* and *Crebl1*), GATA (*Trps1*), Ets (*Etv5*), Hox (*Hoxb7* and *Hoxa2*), NFAT (*Nf45*), RXR

(*Nr1h2*) and Klf (*Klf15*) [118]. Additional proteomics studies were conducted in mouse mpkCCD cells to identify transcription factors and transcriptional coregulators that undergo nuclear translocation from the cytosol in response to vasopressin [59]. Three transcription factors underwent significant increases in nuclear abundance in response to the V2 receptor selective analog dDAVP, namely JunD, Elf3, and Gatad2b, which potentially bind to the AP1, the Ets and the GATA sites of the *Aqp2* gene, respectively. In addition, there were marked vasopressin-induced increases in the nuclear abundances of transcriptional coactivators, β -catenin (*Ctnnb1*) and CREB binding protein (*Crebbp*). Follow up studies were carried out using phosphoproteomic techniques to identify nuclear proteins in mouse mpkCCD cells that undergo changes in phosphorylation in response to vasopressin [56]. Of the 1,251 phosphorylation sites quantified, 39 changed significantly in response to dDAVP. Phosphorylation of the AP1 transcription factor c-Jun at was markedly decreased at Ser73, the canonical target for the MAP kinase, c-Jun N-terminal kinase 2, which is downregulated in response to vasopressin in the inner medullary collecting duct [19]. Phosphorylation of β -catenin in nuclei of mpkCCD cells was markedly increased at Ser552 [59], a site that is known to be targeted by either protein kinase A or Akt, both of which are activated in response to vasopressin [17]. β -catenin serves a dual role in the collecting duct cell as part of adherens junctions (complexed to cadherins) and as a nuclear transcriptional co-regulator as part of the canonical Wnt signaling pathway. Its role in regulation of *Aqp2* gene transcription has not been reported, although it has been implicated in the downregulation of *Aqp2* gene expression in lithium-induced nephrogenic diabetes insipidus [120]. In the following, we review functional studies providing evidence for regulatory roles of some of these proteins.

CRE-dependent transcriptional regulation—Given the role of cyclic AMP in vasopressin signaling, it has long been assumed that a cyclic-AMP response element (CRE) may be responsible for regulation of *AQP2* gene transcription via one of the CREB proteins. Studies using deletion or site-specific mutagenesis of the *AQP2* CRE confirmed the importance of this *cis* element in vasopressin-stimulated *AQP2* transcription [121] [122] [66].

GATA-dependent transcriptional regulation—In addition to the CRE, Rai et al found, using DNase footprint analysis, a protected GATA element downstream of the CRE site in the *AQP2* 5'-flanking region [119]. They further showed that deletion of this *cis*-element abolished protein-DNA binding and increased promoter activity, suggesting a negative regulatory role of GATA element. They also found that deletion of a 5' flanking region containing the GATA element leads to an increased reporter activity in the mouse outer medullary collecting duct cells [123]. Subsequently, however, using mouse outer medullary collecting duct cells, these authors showed that over-expressing GATA-3 transcription factor increases GATA element activity [124], pointing to the conclusion that the GATA element may be part of a complex non-linear regulatory network.

NFAT-dependent transcriptional regulation—Using mpkCCD cells, the nuclear factor of activated T cells (NFAT) family of transcription factors was found to be involved in osmolality-regulated *AQP2* gene expression [125]. There are five members in the NFAT family including NFATc1-4 and NFAT5 also known as tonicity-responsive enhancer binding protein (TonEBP, which share amino acid similarity mainly in the DNA binding domain [126]. The NFATc transcription factors share a serine-proline rich NFATc homology region that confers their regulation via de-phosphorylation by calcineurin, a calcium-dependent serine/threonine phosphatase. Both NFATc and TonEBP were found to regulate *AQP2* gene expression in mpkCCD cells. When grown on filter membranes, the mpkCCD cells responded to hypertonic medium with increases in *AQP2* mRNA and protein

levels [125]. These increases in AQP2 mRNA and protein abundance were largely reduced when a small interfering RNA reduced TonEBP expression, or when a dominant negative TonEBP mutant was expressed, demonstrating a role of TonEBP in osmolality-regulated *AQP2* gene expression. NFATc in particular NFATc1 was found to enhance AQP2 promoter activity [127]. When ionomycin plus phorbol 12-myristate 13-acetate were used to activate calcineurin activity, an increase in AQP2 mRNA and protein levels were seen in mpkCCD cells, consistent with NFATc activation in *AQP2* gene expression regulation. Hypertonicity promotes NFATc1 nuclear translocation and over-expression of NFATc1 enhances AQP2 promoter activity in mpkCCD-c14 cells. Thus, the NFAT transcription factors may have regulatory roles in *AQP2* gene expression in response to tonicity. However, some skepticism regarding the role of tonicity changes in the regulation of *AQP2* gene expression is warranted since AQP2 protein abundance was not altered by measures that change the osmolality of the renal medullary interstitium in rats [109]. Furthermore in the terminal IMCD, increases in medullary tonicity produced by water restriction in rats did not result in translocation of TonEBP into the nucleus despite clear nuclear translocation in neighboring loop of Henle cells [128].

Ets-dependent transcriptional regulation—Another group of transcription factors that have been implicated in regulation of AQP2 transcription are the Ets family transcription factors. Yu et al has investigated the potential role of the Ets *cis*-elements in the vasopressin response [65]. Specifically, vasopressin was shown to increase AQP2 transactivation by three Ets-family transcription factors (Elf3, Elf5 and Ehf) in promoter-reporter studies.

C. Dysregulation of AQP2 in Disease

Several clinically important water balance disorders have been shown to be associated with dysregulation of AQP2, according to results from animal models of various disease states. Such disease states can be divided into 1) polyuric syndromes, 2) ECF volume-expanded states, and 3) the syndrome of inappropriate antidiuresis (SIADH) [1].

1. Aquaporin-2 in polyuric syndromes—The amount of AQP2 in collecting duct cells has been shown to be decreased in a variety of polyuric disorders [1]. One such disorder is central diabetes insipidus (CDI). CDI is associated with low or absent circulating vasopressin due to a defect in the production or secretion of the vasopressin in the hypothalamus and neurohypophysis. The Brattleboro rat has provided us with an animal model for CDI. These rats lack circulating vasopressin owing to a mutation in the vasopressin-neurophysin gene [129]. Owing to the absence of vasopressin, these rats have extremely low levels of AQP2 in their kidneys [13]. Infusion of vasopressin can restore kidney AQP2 protein levels to normal [13]. However, vasopressin infusions as long as 7 days may be required to fully restore AQP2 abundance to levels seen in wild-type rats [130]. The slow response complicates approaches to diagnosis of CDI in humans, since a single dose of vasopressin is unlikely to fully restore concentrating capacity. In effect, animals and humans with CDI always have a component of nephrogenic diabetes insipidus (NDI) due to their low renal AQP2 levels.

Compulsive water drinking is another cause of polyuria due to CNS abnormalities as is sometimes seen in psychiatric patients. Here, excessive water drinking maintains circulating vasopressin levels very low, thereby suppressing AQP2 levels in the kidney [131].

Another form of overdrinking which is common in modern society is “cultural overhydration”, exemplified by the recommendation to drink eight 8-ounce glasses of water per day, independent of thirst [132]. Excessive coffee or soft drinking intake can also result

in chronic suppression of circulating vasopressin levels and consequently in renal AQP2 levels. Overdrinking can also lead to impairment of athletic performance [133].

Nephrogenic diabetes insipidus (NDI) is a syndrome of varied etiology that is associated with polyuria. Heritable NDIs are relatively rare genetic disorders occurring as a result of mutations in specific genes associated with collecting duct function. Most common is X-linked NDI due to mutations in the vasopressin V2 receptor [134]. Interestingly, most cases of non-X-linked NDI appear to be associated with mutations in the AQP2 gene [135]. The latter can either be autosomal dominant or recessive, depending in part on the site of the AQP2 mutation.

In humans, the most common forms of NDI are the ‘acquired’ NDI syndromes. Acquired NDI is most often seen in association with sustained ureteral obstruction [136],[137],[138], sustained hypokalemia [139],[140], hypercalcemia [141] or sustained lithium intake [142],[143],[144], [50],[145],[120]. Each of these four syndromes is associated with depletion of AQP2 protein from the collecting ducts and connecting tubules as a result of an impairment of the long-term regulatory process that determines the abundance of AQP2 in the renal collecting ducts. Only one of the four, hypercalcemia-induced polyuria seems to be associated with a defect in AQP2 trafficking, i.e. short-term regulation of AQP2 [141]. In general, further studies at a molecular level are needed to understand the mechanism of AQP2 depletion from the kidney in these syndromes.

2. Aquaporin-2 in volume-expanded states—Here we briefly review the roles of vasopressin and AQP2 in volume expanded states, viz. congestive heart failure, hepatic cirrhosis, and nephritic syndrome. For more information, an excellent review on the role of AQP2 in ECF volume-expanded states has been published by Schrier and colleagues [146].

Congestive heart failure (CHF): Severe heart failure is characterized by defects in renal handling of water and sodium resulting in extracellular fluid expansion and hyponatremia. Studies by Nielsen et al [147] and by Xu et al. [148] have investigated changes in renal aquaporin expression in rats with CHF induced by ligation of the left coronary artery. Both studies demonstrated that the renal water retention and hyponatremia in severe CHF in rats is associated with a marked increase in the abundance of AQP2 protein. In parallel to the increase of AQP2 protein, there was an increase in AQP2 mRNA levels in kidney inner medulla and cortex [148]. Furthermore, there was a marked increase in the abundance of AQP2 in the apical plasma membrane [147], consistent with an increase in apical water permeability. The rats manifested significantly increased plasma vasopressin levels, presumably owing to non-osmotic factors related to reduced arterial filling [148]. Furthermore, in this study, administration of the V₂ receptor antagonist OPC31260 (a vaptan) was associated with a significant increase in urine output and a rise in plasma osmolality, associated with a significant reduction in AQP2 expression. Similar results were seen when tolvaptan was administered to rats with CHF due to an induced autoimmune cardiomyopathy [149]. Thus the water retention and hyponatremia seen in CHF appears to be attributable to the nonosmotic release of vasopressin. Based on this model, V2 receptor antagonists (vaptans) are now being used clinically to ameliorate the hyponatremia seen in severe congestive heart failure in patients [150].

Hepatic cirrhosis: Hepatic cirrhosis is another clinically important syndrome associated with water retention. However, unlike CHF, the changes in expression of AQP2 protein levels vary between different animal models of hepatic cirrhosis. With cirrhosis induced by common bile duct ligation (CBDL), despite evidence of marked salt and water retention, there was a significant decrease in AQP2 abundance [103], [151]. Despite a lack of a rise in circulating vasopressin, there was a distinct aquaretic effect of the vaptan OPC31260 [151].

Thus, increased expression of AQP2 is not a uniform finding in rat models of volume-expanded states. In the study by Fernandez-Lama et al. [103], CBDL was associated with hyponatremia in response to water loading, showing that excessive water retention can occur in the absence of increased AQP2 levels.

In another model of hepatic cirrhosis induced by intraperitoneal administration of carbon tetrachloride, Fujita et al. [152] found a significant increase in both AQP2 protein levels and AQP2 mRNA. In a different model of carbon tetrachloride-induced cirrhosis, using carbon tetrachloride inhalation, AQP2 abundance was not increased, however, despite significant salt and water retention [153]. There was, however, evidence for increased trafficking of AQP2 to the plasma membrane consistent with the presence of elevated levels of vasopressin in the plasma. Interestingly, there was also a marked increase in the level of the basolateral water channel AQP3, presumably due to increased circulating vasopressin levels. Although the explanation for the differences between cirrhosis induced by CBDL and carbon tetrachloride remains to be determined, it seems clear that the level of AQP2 expression is not the only, or even the chief, determinant of water retention in cirrhosis.

Nephrotic syndrome: Nephrotic syndrome (NS) is characterized by extracellular volume expansion with excessive renal water and sodium reabsorption that is ultimately the consequence of excessive excretion of plasma proteins resulting in low oncotic pressure in the plasma. In contrast to the hyponatremia seen in CHF and cirrhosis, rats with experimental NS do not develop hyponatremia despite extensive extracellular fluid volume expansion. This absence of hyponatremia may reflect an absence of upregulation of AQP2 expression in the collecting duct. Rather, a marked downregulation of AQP2 protein levels were seen in the kidneys of rats with NS induced either by puromycin aminonucleoside (PAN) [154] or adriamycin [155]. This reduced expression of collecting water channels could represent a physiologically appropriate response to extracellular volume expansion as seen with the vasopressin-escape phenomenon (vide infra). This, as seen with hepatic cirrhosis, there are factors other than than vasopressin than can affect AQP2 expression levels.

3. Aquaporin-2 in the syndrome of inappropriate antidiuresis (SIADH)—In SIADH, inappropriately high levels of circulating vasopressin result in water retention and hyponatremia. In human patients, this is most often seen with malignancies, especially certain forms of lung cancer. In rat models of SIADH, a number of studies have documented increased AQP2 protein and mRNA levels [152] [156] [9]. The serum sodium concentration typically does not inexorably fall, but instead the degree of hyponatremia appears to be limited by a process countering the water-retaining action of vasopressin, viz., the process of ‘vasopressin escape’. Work by Ecelbarger et al [9] in rats has revealed that the escape is a result of suppression of AQP2 mRNA and protein levels through effects on AQP2 gene transcription rate. The process is associated with a fall in intracellular cyclic AMP levels in collecting duct cells, which in turn is due to a decrease in coupling between the vasopressin V2 receptor and the two adenylyl cyclases that it activates [157]. There is also an associated downregulation of vasopressin V2 receptors, presumably due to receptor endocytosis [156].

A nephrogenic form of SIADH has been recently identified that is not due to increased circulating vasopressin levels but instead appears to be due to constitutive V2 receptor signaling in collecting duct cells [158]. At least a subset of these patients experience water retention because of activating mutations of the V2 receptor.

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Figure 1. Word cloud indicating the most frequency of word use by size of representation.

Figure 2. Vasopressin signaling network

Designators represent official gene symbols. The annotations for these proteins may be obtained at <http://www.ncbi.nlm.nih.gov/protein/> From Pisitkun et al [17].

Figure 3. Processes involved in regulation of AQP2 protein abundance
See text for explanation.