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Calcium sensing receptor exerts a negative regulatory action toward vasopressin-induced aquaporin-2 expression and trafficking in renal collecting duct

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Contents

Abstract

Vasopressin (AVP) plays a major role in the regulation of water homeostasis by its antidiuretic action on the kidney, mediated by V2 receptors. An increase in plasma sodium concentration stimulates AVP release, which in turn promotes water reabsorption. Upon binding to the V2 receptors in the renal collecting duct, AVP induces the expression and apical membrane insertion of the aquaporin-2 (AQP2) water channels and subsequent water reabsorption. AVP regulates two independent mechanisms: the short-term regulation of AQP2 trafficking and long-term regulation of the total abundance of the AQP2 protein in the cells. On the other hand, several hormones, acting through specific receptors, have been reported to antagonize AVP-mediated water transport in kidney. In this respect, we previously described that high luminal Ca^{2+} in the renal collecting duct attenuates short-term AVP-induced AQP2 trafficking through activation of the Ca^{2+}-sensing receptor (CaSR). This effect is due to reduction of AVP-dependent cAMP

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generation and possibly hydrolysis. Moreover, CaSR signaling reduces AQP2 abundance both via AQP2-targeting miRNA-137 and the proteasomal degradation pathway.

This chapter summarizes recent data elucidating the molecular mechanisms underlying the physiological role of the CaSR-dependent regulation of AQP2 expression and trafficking.

s0010

1. Introduction

p0010

Regulation of water homeostasis is crucial for all terrestrial organisms. In most mammals, the maintenance of water balance is critically dependent on water intake, the sensation of thirst and the regulation of water excretion in the kidney, which is under the control of the antidiuretic hormone arginine vasopressin (AVP) (Knepper, Kwon, & Nielsen, 2015; Knepper, Nielsen, Chou, & DiGiovanni, 1994). AVP is secreted into the circulation by the posterior pituitary gland, in response to an increase in serum osmolality or a decrease in blood volume. In the kidney, AVP binds to the V2 vasopressin receptor (V2R), which belongs to the superfamily of G protein-coupled receptors (GPCRs) (Birnbaumer, 2000). V2Rs expressed in the basolateral membranes of collecting duct principal cells, in the last portion of the nephron, are functionally coupled to Gs protein causing an increase in intracellular cAMP. The cAMP/PKA signal transduction cascade results in multiple phosphorylating events in the C-terminus of aquaporin-2 (AQP2) representing important signals for triggering AQP2 trafficking toward the apical plasma membrane leading to an increase in luminal water permeability (Hoffert, Pisitkun, Wang, Shen, & Knepper, 2006; Jung & Kwon, 2016; Nedvetsky et al., 2009; Nielsen et al., 1995, 2002; Valenti, 2005). In addition to phosphorylation, AQP2 undergoes different regulated posttranslational modifications, such as ubiquitination and glutathionylation, which are likely to be fundamental for controlling AQP2 cellular localization, stability and function (Moeller, Olesen, & Fenton, 2011; Tamma, Di Mise, et al., 2014). Several proteins participating in the control of cAMP-dependent AQP2 trafficking have been identified including SNAREs, annexin-2, hsc70, A kinase anchoring proteins (AKAPs), small GTPases of the Rho family proteins controlling cytoskeletal dynamics (Centrone et al., 2017; Klussmann et al., 2001; McSorley et al., 2006; Procino, Barbieri, et al., 2008; Schrade et al., 2018; Tamma et al., 2003; Tamma, Procino, Mola, Svelto, & Valenti, 2008; Wang et al., 2010; Wu et al., 2018). In addition to regulate AQP2 trafficking, AVP also regulates the total amount of the water

channel within the cell (Hasler, Leroy, Martin, & Feraille, 2009). Alterations in the AQP2 abundance as well as defects in AVP signaling in the renal collecting can seriously compromise the maintenance of water balance in the body (Noda, Sohara, Ohta, & Sasaki, 2010). Loss-of-function mutations of both V2R and AQP2 are associated with congenital nephrogenic diabetes insipidus (NDI), a rare genetic disorder, which can be quite severe in infants, characterized by a failure to concentrate urine (despite normal or elevated levels of AVP), polyuria, polydipsia and hypernatremia (Bockenhauer & Bichet, 2017).

p0015 Hypercalcemia–induced hypercalciuria, can also causes acquired NDI, but the mechanisms underlying hypercalcemia-induced NDI are not well understood. Several studies point to a critical role of CaSR activation in reducing coupling efficiency between the V2R and adenylyl cyclase reducing AQP2 transcription and trafficking (Bustamante et al., 2008; Procino, Mastrofrancesco, et al., 2008;Procino et al., 2004; Ranieri et al., 2015, 2018; Riccardi & Valenti, 2016). An opposite rare genetic disorder, the Nephrogenic Syndrome of Inappropriate Antidiuresis (NSIAD), has been identified, caused by gain-of-function mutations of the V2R and characterized by hyponatremia despite undetectable AVP levels (Erdélyi et al., 2015; Feldman et al., 2005). On the other hand, inappropriate antidiuresis is the most common cause of hyponatremia and it is characterized by inability to excrete a free water load, inappropriately concentrated urine, hypoosmolality and natriuresis. The most common clinical entity is the idiopathic syndrome of inappropriate antidiuretic hormone secretion (SIADH), which is linked to hypersecretion of AVP (Cuesta & Thompson, 2016).

p0020 This chapter summarizes some of new understanding in the regulation of AQP2 trafficking and AQP2 protein abundance with a special focus on the negative feedback of CaSR signaling on AVP-induced AQP2 expression and trafficking in renal collecting duct.

s0015 2. Regulation renal collecting duct water permeability by the vasopressin—aquaporin-2 system

p0025 The renal collecting duct water permeability is controlled by AVP through the regulation of the water channel AQP2 in two processes: short- and long-term regulation. The short-term regulation occurs over a period of minutes resulting in an increased trafficking of AQP2-bearing vesicles from an intracellular pool to the apical plasma membrane in response to AVP stimulus. The complex molecular signals controlling, at short-term,

AQP2 trafficking in response to AVP, include rapid changes of the activity of kinases, phosphatases, AKAPs, E3-ligases and regulation of cytoskeleton remodeling all important steps for exocytosis and endocytosis of AQP2.

p0030 The long-term regulation, instead, occurs over a period of hours to days due to AVP regulation of cellular AQP2 total abundance.

s0020 2.1 Short-term regulation of AQP2 by vasopressin

p0035 The expression of AQP2 at the apical membrane is tightly regulated by AVP. Binding of AVP to its specific V2 receptor (V2R) localized to the basolateral membrane of collecting duct principal cells, increases the intracellular concentration of cAMP via adenylyl cyclase type III and VI, resulting in the activation of protein kinase A (PKA) (Hoffert, Chou, Fenton, & Knepper, 2005), although other kinases, such as Akt, Sgk, p38-MAPK, may play a role in response to AVP stimulation (Nedvetsky et al., 2010; Pisitkun et al., 2008; Rinschen et al., 2010). One target for PKA is AQP2 at serine-256, although AQP2 may be a substrate for other basophilic protein kinases such as Akt1 and protein kinase Cδ (Douglass et al., 2012). PKA-dependent phosphorylation of the water channel AQP2, at S256, has been considered essential to promote the translocation of AQP2 from an intracellular vesicles pool to the apical plasma membrane (Katsura, Gustafson, Ausiello, & Brown, 1997; Nielsen et al., 1995) although this concept has been recently questioned. Several studies clearly indicate that cAMP can also stimulate exocytosis independently of PKA action (Ozaki et al., 2000; Seino & Shibasaki, 2005). cAMP activates Epac (exchange protein activated by cAMP) and PKA similarly (Christensen et al., 2003). Epac is a cAMP downstream effector expressed in different tissues (Kawasaki et al., 1998) and functions as a guanine-nucleotide-exchange factor for Rap1 and Ras (de Rooij et al., 1998; Kawasaki et al., 1998). Stimulation of isolated renal tubules with a selective agonist of Epac, mimics AVP-induced intracellular calcium release and AQP2 targeting at the apical plasma membrane independently of PKA signaling activation (Yip, 2006). Recently, Cheung and coworkers (Cheung, Terlouw, Janssen, Brown, & Bouley, 2019) reported that Src kinase inhibition causes serine 256-independent AQP2 membrane trafficking and induces phosphorylation of serine 269 independently of serine 256 phosphorylation. In this line, previous study by Ando et al. (2016) demonstrated that Wnt5a induces accumulation of AQP2 to the apical membrane inducing phosphorylation of serine 269 independently of serine 256.Together these novel findings support the

concept that AVP-mediated AQP2 trafficking may occur independently from cAMP (Lei et al., 2018).

p0040 Several studies have shown that cytoskeleton remodeling plays a key role in modulating AQP2 trafficking. Chemical disruption of microtubules inhibited the AVP-induced osmotic water permeability in the mammalian collecting duct (Brown & Stow, 1996; Sabolic, Katsura, Verbavatz, & Brown, 1995). Dynamics of actin cytoskeleton are also known to be involved in controlling the cellular distribution of AQP2 (Valenti, 2005).

p0045 Specifically, exposure of IMCD and renal CD8 cells to Clostridium toxin B, which inhibits proteins of Rho family (Rho, Rac and Cdc42) or Clostridium toxin C3, specifically inhibiting Rho, caused a partial depolymerization of actin filaments associated with an increase of the cells surface expression of AQP2 resulting in a significant increase of the osmotic water permeability, even in the absence of AVP stimulation (Klussmann et al., 2001; Tamma et al., 2001). Interestingly, immunoaffinity chromatography studies showed that the C-terminus of AQP2 strongly binds β- and γ-isoforms of actin (Noda, Horikawa, Katayama, & Sasaki, 2004).

p0050 Cell surface expression of AQP2 is the results of a balance between the exocytosis and the endocytosis. Dennis Brown group has convincingly showed that AVP exerts its action by stimulation of the exocytic pathway (Brown, Hasler, Nunes, Bouley, & Lu, 2008).

p0055 Docking and fusion of AQP2 vesicles to the apical membrane is a complex mechanism displaying several similarities with synaptic vesicles fusion.

p0060 Annexin-2 was found to be functionally involved in the fusion of highly purified AQP2 vesicles with plasma membrane (Tamma et al., 2008). Moreover, similarly to the crucial role played in synaptic vesicle fusion, SNARE proteins (where NSF stands for N-ethyl-maleimide-sensitive fusion protein) have been demonstrated to be essential for AQP2 vesicle fusion (Gouraud et al., 2002). Selective protein silencing of VAMP2, VAMP3 and SNAP23 inhibited AQP2 insertion at the apical plasma membrane (Procino, Barbieri, et al., 2008; Procino, Mastrofrancesco, et al., 2008). Moreover, knockout mice for VAMP8 develop hydronephrosis (Wang et al., 2010).

p0065 In a very recent study, we have provided evidence that the fusion of AQP2 vesicles to the plasma membrane requires Gi protein modulation of the potassium channel TASK-2 resulting vesicle osmotic swelling, a process that facilitates the fusion process (Centrone et al., 2018).

p0070 Conversely, vesicle-mediated internalization of plasma membrane, involves the coordinate activity of a dynamic complex of proteins including, clathrin, dynamin, hsc70, endophilin, anphiphysin, synaptojanin, epsin,

adaptor protein-2 (AP-2) and flotillin (Grant & Donaldson, 2009; Mettlen et al., 2009). The involvement of clathrin in AQP2 internalization is well described. AQP2 is accumulated in clathrin-enriched membrane domains during AVP stimulation and washout (Sun, Wu, & Wu, 2002).

p0075 AQP2 undergoes different regulated posttranslational modifications, such as phosphorylation, ubiquitination and glutathionylation, which are likely to be fundamental for controlling AQP2 cellular localization, stability and function (Kamsteeg et al., 2006; Medvar, Raghuram, Pisitkun, Sarkar, & Knepper, 2016; Moeller et al., 2011; Nedvetsky et al., 2010; Tamma, Ranieri, et al., 2014).

p0080 *AQP2 phosphorylation*. AVP-induced translocation of AQP2 vesicles to the plasma membrane requires PKA-dependent phosphorylation of AQP2 at S256. The crucial role of cAMP-dependent phosphorylation of AQP2 was first investigated by Kuwahara et al. (1995) in 1995. Subsequently, phosphoproteomic studies revealed a more complex action of AVP on AQP2, showing that AVP stimulation increases S256, S264 and T269 while decreases S261 phosphorylation in the C-terminus (Hoffert et al., 2006). A time course study reveals that phosphorylation at S256 rapidly increases within few seconds followed by phosphorylation at S269, S264 and S261 dephosphorylation (Hoffert et al., 2008). Ser261 phosphorylation follows mono-ubiquitination at K270, endocytosis and stabilizes AQP2 ubiquitination and intracellular localization (Tamma, Robben, Trimpert, Boone, & Deen, 2011). Short-term AVP stimulation (30 min) causes a relocalization of AQP2-pS264from predominantly intracellular vesicles, to both the basolateral and apical plasma membranes. After 60 min, part of the AQP2-pS264 was observed in clathrin-coated vesicles, early endosomal compartments, and recycling compartments, but not lysosomes (Fenton et al., 2008). Quantitative mass spectrometry and immunoblotting analysis with phosphospecific antibodies showed that AVP increases the phosphorylation of AQP2 at Ser269 which is mainly located in the apical plasma membrane (Hoffert et al., 2008). This observation is confirmed analyzing the cellular localization of the mutant protein mimicking the constitutive phosphorylated AQP2 at S269 (AQP2-S269D) indicating that S269 phosphorylation might be a strong apical retention signal (Hoffert et al., 2008; Moeller, Knepper, & Fenton, 2009).

p0085 *AQP2 ubiquitination*. It has been reported that at short-term, the cAMP elevating agent forskolin, promoted K63-linked short chain ubiquitination of AQP2 at the apical plasma membrane (Kamsteeg et al., 2006). Interestingly, short chain ubiquitination increases during forskolin washout

corresponding to a higher rate of AQP2 retrieval from the plasma membrane to cellular vesicles (Kamsteeg et al., 2006). Short chain ubiquitination of AQP2 has become recognized as a key signal for intracellular trafficking, endocytosis and degradation in lysosomes (Woelk et al., 2006). In situ mutagenesis experiments revealed that AQP2 is ubiquitinated, within the C-terminus, at lysine 270 (K270). On the other hand forskolin decreased AQP2 polyubiquitination resulting in a significant decrease of S261 phosphorylation (Nedvetsky et al., 2010).

p0090 *AQP2 glutathionylation.* We recently demonstrated for the first time that AQP2 is subjected to S-glutathionylation in native mammalian kidney and in renal cell culture and that this posttranslational modification is modulated by the oxidative stress (Tamma, Ranieri, et al., 2014). Topological analysis of AQP2 suggests that Cys75 and Cys79, on cytosolic B-loop, might be target of S-glutathionylation. Indeed, previous data have shown that AVP stimulation increased S-glutathionylation of different proteins in mpkccd cells, indicating the involvement of ROS in AVP activated signal transduction pathway (Sandoval et al., 2013). The physiological role of this posttranslational modification in AQP2 is, however, not clarified and additional studies will be needed to better elucidate the physiological relevance.

s0025 2.2 Aquaporin-2 protein abundance: Long-term regulation

p0095 The total cellular AQP2 abundance is the resulted balance of protein synthesis and protein removal via degradation or exosomal secretion. Prolonged exposure to AVP increases the total abundance of cellular AQP2 (Ecelbarger et al., 1997; Nielsen, DiGiovanni, Christensen, Knepper, & Harris, 1993).

p0100 AQP2 abundance depends on production of AQP2 mRNA or by direct regulation of translation. In the past few years it has become clear that alternative mechanisms controlling AQP2 abundance exist and AQP2 mRNA available for translation does not reflect the total AQP2 mRNA due to regulated sequestration of mRNAs and mRNA degradation via micro-RNA (miRNAs) (Alsaleh & Gottenberg, 2014).

p0105 As an example, it has been reported that expression of AQP2 is regulated by external osmolality and solute composition, via the tonicity-responsive enhancer binding protein (TonEBP), which plays a key role in protecting renal cells from hypertonic stress by stimulating transcription of specific gene (Storm, Klussmann, Geelhaar, Rosenthal, & Maric, 2003). In the mouse mpkCCD cell line the expression of AQP2 mRNA, AVP increased

endogenous AQP2 mRNA and protein levels (Hasler et al., 2002; Hasler, Vinciguerra, Vandewalle, Martin, & Feraille, 2005). Conversely, AQP2 mRNA as well as AQP2 protein abundance decreased in cells deficient for TonEBP (Hasler et al., 2006).

p0110 *Urinary exosome excretion of AQP2.* Urinary excretion of intact AQP2 was firstly described in 1995 by Kanno et al. (1995). The molecular mechanism responsible of AQP2 delivery into the urine is not fully clarified yet, although it is well accepted that it occurs via exosome secretion (Pisitkun, Shen, & Knepper, 2004). Under steady-state conditions, an increase in urinary AQP2 excretion could be due to an increased AQP2 synthesis or to a decrease in degradation. Both mechanisms depend on an increase in cellular abundance of AQP2, which could be reflective of AVP stimulation. AQP2 excretion with exosomes may represent a further mechanism of regulation of AQP2 abundance within the cell.

s0030 3. Aquaporin-2 and calcium-sensing receptor (CaSR) interplay

p0115 The kidney is a key organ for calcium homeostasis, and its ability to sense extracellular calcium levels in the urinary filtrate and the interstitial fluid is owing to the extracellular calcium-sensing receptor (CaSR), which is expressed in multiple sites along the nephron (Brown & MacLeod, 2001; Riccardi & Brown, 2010; Riccardi & Valenti, 2016; Ward & Riccardi, 2002).

p0120 The extracellular calcium-sensing receptor (CaSR) is a G-protein–coupled seven-transmembrane spanning receptor that resides on the cell surface as a dimer. It is highly expressed in the parathyroid glands, where it regulates the production and secretion of parathyroid hormone (PTH) in negative feedback fashion. It is also expressed in numerous other tissues, where it has diverse but less well defined functions (Riccardi & Kemp, 2012).

p0125 In the kidney, CaSR is expressed across the entire length of the nephron, with the highest expression on the basolateral membrane of thick ascending limb epithelial cells where it plays a crucial role in the regulation of divalent mineral cation transport by inhibiting calcium reabsorption in response to a stimulation by an increase in plasma calcium levels (Brown & MacLeod, 2001; Hebert, Brown, & Harris, 1997; Hofer & Brown, 2003; Pearce & Thakker, 1997; Ward & Riccardi, 2002).

p0130 In the proximal tubule, the CaSR is expressed apically where can directly and rapidly blunt the phosphaturic action of PTH, thereby modulating the inhibitory effect of PTH on Pi absorption (Ba, Brown, & Friedman, 2003).

p0135 In the collecting duct, CaSR is localized to the apical membrane of the principal cells and co-expressed with the aquaporin-2 (AQP2) water channel in intracellular vesicles (Brown, 1999; Riccardi & Brown, 2010; Sands et al., 1997). Several in vitro, in vivo, and human observational studies have suggested that CaSR signaling inhibits AVP-induced trafficking and expression of AQP2 (Bustamante et al., 2008; Earm et al., 1998; Procino et al., 2004; Procino et al., 2012; Ranieri et al., 2015, 2018; Renkema et al., 2009; Valenti et al., 2000, 2002).

p0140 A postulated mechanism for this process is that, during antidiuresis, the actions of AVP promoting water reabsorption from the lumen, causes an increase in urinary Ca2+ concentration secondary to urine concentration, which in turn activates the CaSR located on the apical membrane of the principal cells. The activation of the CaSR reduces the AVP-stimulated insertion of AQP2 into the plasma membrane and the rate of water reabsorption and consequently reduces the risk of Ca2+ supersaturation (Procino et al., 2004, 2012; Procino, Barbieri, et al., 2008; Procino, Mastrofrancesco, et al., 2008). Clinical evidence for an effect of luminal calcium on AQP2-mediated water reabsorption was provided for the first time in a previous study from our research group in enuretic children demonstrating that urinary AQP2 and calciuria correlate with the severity of enuresis in children (Valenti et al., 2000). Interestingly, hypercalciuric enuretic children receiving a low calcium diet to reduce hypercalciuria, had decreased overnight urine output (reduced nocturnal enuresis) paralleled by an increase in nighttime AQP2 excretion and osmolality (Valenti et al., 2002). More recently, we provided further evidence for the inverse relationship between calciuria and water metabolism in a bed rest study. Immobilization results in alterations of renal function, fluid redistribution and bone loss, which couples to a rise of urinary calcium excretion. Under these conditions it was observed that bed rest induced increase in blood hematocrit (reflecting water loss) which coincided to a reduction of urinary AQP2 likely paralleled by an increase in urinary calcium due to bone demineralization (Tamma, Di Mise, et al., 2014). These results further support the view that urinary calcium can modulate the AVP-dependent urine concentration through a down-regulation of AQP2 expression/trafficking.

p0145 Besides the effect on AQP2 trafficking, previous findings demonstrated that high external calcium reduces AQP2 expression both in the collecting

duct cell line mpkCCD and in hypercalciuric rats (Bustamante et al., 2008; Sands, et al., 1998). In humans, vitamin D-elicited hypercalcemia/hypercalciuria is associated with polyuria. In rats dihydrotachysterol (DHT) induces AQP2 water channel down-regulation despite unaltered AQP2 mRNA expression suggesting higher rate of AQP2 degradation attributed to activation of the calcium-sensitive protease calpain (Puliyanda, Ward, Baum, Hammond, & Harris, 2003). Altogether, these data support a direct effect of luminal calcium on AQP2 expression in collecting duct principal cells and point to a role of calcium in regulating both AQP2 trafficking and expression.

s0035 3.1 Role of CaSR in the regulation of AQP2 trafficking/abundance

p0150 Studies performed in cultured renal cells and microdissected collecting ducts have demonstrated that the inhibitory effect of CaSR signaling on AQP2 trafficking to the plasma membrane is associated to a significant reduction in cAMP-induced AQP2 phosphorylation at serine 256 and AQP2 trafficking, resulting in inhibition of osmotic water-permeability response (Ranieri et al., 2015).

p0155 Specifically, acute CaSR activation by selective agonists reduced AQP2 translocation to the plasma membrane in response to the cAMP elevating agent forskolin (FK). Moreover, in hypercalciuric patients, evaluation of renal response to dDAVP demonstrated a moderate urinary concentrating defect in these subjects paralleled by reduced urinary excretion of AQP2 (Procino et al., 2012). These findings support the hypothesis that the CaSR–AQP2 interplay represents an internal renal defense to mitigate the effect of hypercalciuria on the risk of calcium saturation during antidiuresis as initially suggested by Sands and coworkers (1997).

p0160 In our recent work we demonstrated that CaSR activation in renal cells modulates AQP2 trafficking/expression by alteration of its phosphorylation state (Ranieri et al., 2015). Interestingly, CaSR activation *per sé* induced a significant reduction of the basal AQP2-pS256 levels in HEK-293 cells thus having an opposite effect with respect to AVP action. These data were also confirmed in HEK-293 cells transfected with two gain-of-function variants of CaSR, the CaSR-N124K mutation and the CaSR-R990G polymorphism, used to mimic "tonic" activation of CaSR (Ranieri et al., 2013). Since, under basal conditions, the activation of CaSR by its positive allosteric modulator, NPS-R568, did not significantly alter the cAMP-dependent PKA activity, the hypothesis is that CaSR activation reduces basal

AQP2-pS256 levels as a consequence of the activation of the calcium-regulated protein phosphatase 2A (PP2A), known to dephosphorylate AQP2-pS256 (Tamma, Lasorsa, et al., 2014).

p0165 The physiological consequence of the negative feedback on cAMP-induced AQP2-pS256 phosphorylation and trafficking elicited by CaSR signaling is blunting the osmotic water-permeability response both in cells and in microdissected mouse collecting duct (Ranieri et al., 2015). Since a strong reduction in PKA activation in response to FK was observed upon CaSR activation, it is likely that the reduced cAMP accumulation is a consequence of inhibition of the calcium-sensitive adenylate cyclase (AC6) as demonstrated in HEK-293 cells (Ranieri et al., 2015).

p0170 The concept that high concentrations of urinary calcium counteract AVP action via the activation of the calcium-sensing receptor (CaSR) expressed in the luminal membrane of collecting duct cells, has been further validated in a mouse model double-knockout for Pendrin/NaCl cotransporter (Ranieri et al., 2018). These mice display significant calcium wasting and develop severe volume depletion, despite increased circulating AVP levels (Soleimani et al., 2012). The hypothesis that the CaSR-mediated impairment of AQP2 expression/trafficking underlies this AVP resistance in dKO mice found substantial confirmation in this study. Due to the severe hypercalciuria, a tonic activation of the luminal CaSR in the collecting duct is expected in this dKO mice model, and quite interestingly, those mice had a strong reduction in total AQP2 expression associated with a significantly higher expression ofAQP2-pS261 and ubiquitinated AQP2. In addition, in dKO mice, exposure of inner medulla kidney slices to the proteasome inhibitor MG132 increased total AQP2 by50% indicating that the rate of AQP2 degradation via proteasome is significantly higher. Interestingly, the functional link between CaSR and AQP2 degradation was supported by the observation that the reduced total AQP2 and higher levels of AQP2-pS261 found in dKO mice are paralleled by higher levels of p38-MAPK, an enzyme activated by CaSR signaling and known to phosphorylate AQP2 at ser261 (Nedvetsky et al., 2010; Trepiccione et al., 2014). Of note, CaSR inhibition with the calcilytic NPS2143 reduced AQP2-pS261 levels in dKO, demonstrating that CaSR acts upstream of p38-MAPK and mediates the upregulation of AQP2-pS261.

p0175 Conversely, inhibition of p38-MAPK, caused a drastic decrease in AQP2-pS261, along with a nearly 5-fold increase in total AQP2. In addition, in dKO mice, p38-MAPK inhibition results in a drastic reduction in ubiquitinated AQP2 that is paralleled by a strong increase in total AQP2.

p0180 Previous work in a kidney proximal epithelial cell model, in airway smooth muscle cells, and in H-500 Leydig cancer cells also provided evidence that CaSR activation stimulates p38-MAPK phosphorylation (Maiti, Hait, & Beckman, 2008; Tfelt-Hansen & Brown, 2005; Yarova et al., 2015), demonstrating that CaSR p38-MAPK is a downstream effector of CaSR. In line with these data, specific CaSR inhibition caused a strong reduction in p38-MAPK paralleled by increased total AQP2 abundance and reduced p261-AQP2 and ubiquinated AQP2 levels pointing to CaSR as the upstream receptor that orchestrates the modulation of AQP2 expression in dKO mice (Ranieri et al., 2018).

p0185 The concept that CaSR expressed at the apical membrane of collecting duct principal cells could mediate effects of hypercalciuria in reducing AVP-elicited osmotic water permeability and urinary concentrating ability by activation of autophagic degradation of AQP2 has been recently suggested (Khositseth et al., 2017). Proteomic analysis of inner medullary collecting ducts isolated from parathyroid hormone–treated rats revealed increased autophagic degradation of a specific set of proteins including AQP2. Interestingly, PTH withdrawal was associated with termination of AQP2 degradation autophagic degradation and normalization of total AQP2 levels and renal concentrating ability (Khositseth et al., 2017). Since CaSR expressed in parathyroid glands is the major player regulating the release of PTH these data further strength the link between CaSR signaling and regulation of AQP2 expression.

s0040 3.2 AQP2 regulation via microRNA pathway

p0190 MicroRNAs (miRNAs) are naturally occurring small non-coding RNAs that regulate posttranscriptional gene expression and are involved in several diseases. miRNAs may act as transcriptional or splicing regulators within the nucleus (Hwang, Wentzel, & Mendell, 2007), and be involved in genetic exchange with adjacent cells, through exosomes (Valadi et al., 2007). Approximately 60% of protein-coding genes are influenced by miRNAs (Friedman, Farh, Burge, & Bartel, 2009) that play crucial roles in several biological processes, including control of cell cycle and differentiation, proliferation and metabolism. As such, miRNA deregulation is being increasingly associated with several human pathologies.

p0195 Several studies highlight an emerging role of miRNAs in AQP regulation (reviewed in Gomes, da Silva, Rodrigues, Castro, & Soveral, 2018). Specifically, miRNAs have been identified as endogenous modulators of the expression of several AQPs (Chao et al., 2017; Chen, Shi, Liu, &

Sun, 2018; Huebert et al., 2011; Kim, Jung, Lee, & Kwon, 2015; Li, Shi, Gao, Ma, & Sun, 2018; Luo et al., 2018; Sepramaniam et al., 2010; Tang, Pei, Bai, & Wang, 2016; Wang et al., 2015; Xiong et al., 2018; Zheng et al., 2017). Regarding AQP2, two AQP2-targeting miRNAs, miR-32 and miR-137, were reported to decrease AQP2 expression in kidney collecting duct cells independently of AVP regulation (Kim et al., 2015). The authors demonstrated a significant decrease of AQP2 translation is in mpkCCDc14 cells transfected with miR-32 or miR-137 providing novel insights into the regulation of AQP2 by RNA interference.

p0200 The concept that CaSR may regulate AQP2 expression also via miRNA, is an intriguing hypothesis. In a previous seminal work, Gong and coworkers demonstrated that in the thick ascending limb, high external calcium—via activation of CaSR—regulates the expression levels of 2 miRNAs, miR-9 and miR-374, which, in turn, reduces the expression of Claudin-14, a protein that blocks the paracellular cation channel, leading to decreases in cation permeation (Gong et al., 2012). These data indicate that the regulation of miRNA by CaSR signaling may occur on several layers within the kidney.

p0205 On the other hand, despite several studies that have demonstrated that transcriptional and posttranscriptional regulation of AQP2 play a crucial role in AQP2 expression levels within the cell, along with a profound impact on water homeostasis (Hasler et al., 2009; Hanne B Moeller et al., 2011), little is known about the role of miRNA in the regulation of AQP2 expression.

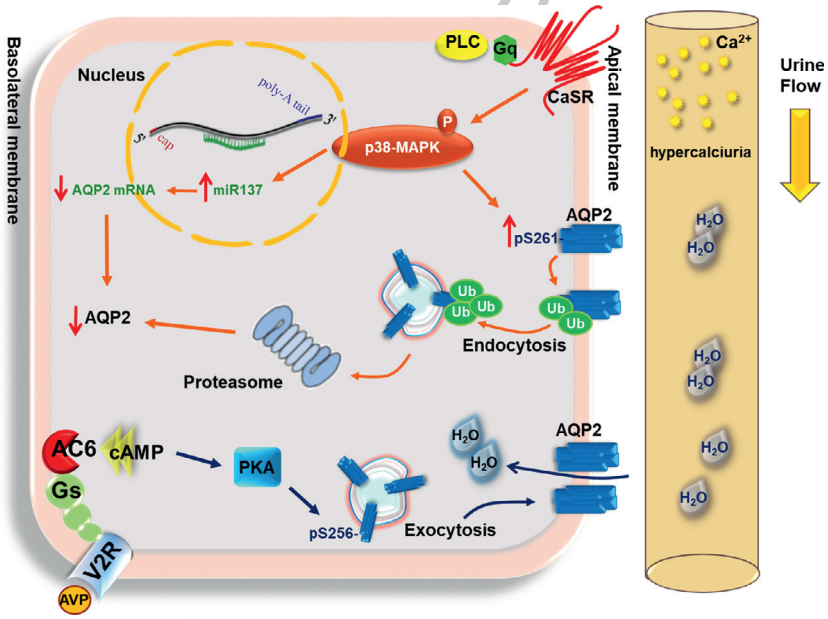
p0210 Of interest, we have recently correlated the AQP2-targeting miR-137 with the reduced expression of AQP2 in Pendrin/NaCl cotransporter dKO mice model, a mechanism found to be mediated by CaSR signaling (Ranieri et al., 2018). Therefore, in addition to the enhanced rate of AQP2 degradation, low AQP2 levels observed in dKO mice are also a result of reduced AQP2 mRNA translation via a novel miRNA pathway modulated by CaSR signaling.

p0215 Specifically, in dKO mice, miR-137 was found about 1.7-fold higher compared to WT mice, which was in line with the reduced translation of AQP2 mRNA. Worthy of attention, miR-137 transcript levels were increased by the calcimimetic NPS-R-568 providing the first evidence that the CaSR signaling directly acts upstream of the miR-137-AQP2 axis. These findings represent the first demonstration that CaSR can regulate AQP2 expression via AQP2-targeting miRNA.

p0220 The discovery of miRNAs as endogenous modulators of AQPs offers a potential therapeutic approach for the regulation AQP-related disorders (Gomes et al., 2018).

s0045 ## 4. Conclusions

p0225 The identification of a novel physiological mechanisms that, in the inner medulla, may link the activation of CaSR to the regulation of the AQP2 expression and trafficking has opened new avenues for understanding AQP2 expression regulation. A bimodal down-regulation of AQP2 expression appears to be involved in this process: CaSR signaling (i) increases AQP2-pS261 and AQP2 ubiquitination resulting in AQP2 protein degradation, (ii) down-regulates AQP2 expression via AQP2-targeting miR-137 pathway. Both pathways contribute to reduce renal concentrating ability and may explain the AVP resistance observed in some pathological setting characterized by hypercalciuria expected to cause sustained stimulation of CaSR in the inner medulla (see proposed model in Fig. 1). Q1



f0010 **Fig. 1** Schematic model. The proposed model shows that high urinary calcium levels in the renal inner medulla activate CaSR, resulting in the activation and phosphorylation of p38-MAPK that, in turn, phosphorylates AQP2 at Ser261, causing AQP2 internalization, ubiquitination (Ub), and proteasomal degradation. In parallel, CaSR signaling promotes the synthesis of miRNA-137 via the activation of p38-MAPK, which results in reduced AQP2 mRNA translation. *Adapted from Ranieri, M., Zahedi, K., Tamma, G., Centrone, M., Di Mise, A., Soleimani, M., & Valenti, G. (2018). CaSR signaling down-regulates AQP2 expression via a novel microRNA pathway in pendrin and NaCl cotransporter knockout mice.* FASEB Journal 32, *2148–2159 doi:10.1096/fj.201700412RR.*

st0060 ## Acknowledgments

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Non-Print Items

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