CaSR-Regulated microRNAs

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The calcium sensing receptor (CaSR) is a unique G protein-coupled receptor (GPCR) activated by extracellular Ca2+ and by other physiological cations, aminoacids, and polyamines. CaSR is the main controller of the extracellular Ca2+ homeostatic system by regulating parathyroid hormone (PTH) secretion and, in turn, Ca2+ absorption and resorption. Recent advances highlight novel signaling pathways activated by CaSR signaling involving the regulation of microRNAs (miRNAs).

Keywords: calcium sensing receptor (CaSR) ; calcium ; microRNAs

1. CaSR and AQP2 Interplay

A postulated mechanism for the process occurring in the collecting duct is that, during the vasopressin antidiuretic action promoting water reabsorption from the lumen, urinary Ca^{2+} concentration increases secondary to urine concentration. Increased Ca^{2+} levels, in turn, activate the CaSR located on the apical membrane of the principal cells. CaSR activation reduces the vasopressin-stimulated insertion of AQP2 into the plasma membrane and the rate of water reabsorption, consequently reducing the risk of Ca^{2+} supersaturation [1][2][3][4]. Maintenance and regulation of water balance is essential for all physiological processes and is critically dependent on water intake and water output in the kidney under the control of the antidiuretic hormone vasopressin. Dysregulation associated with water balance is responsible of several disorders, such as congenital nephrogenic diabetes insipidus (NDI), idiopathic syndrome of inappropriate antidiuretic hormone secretion (SIADH), nephrogenic syndrome of inappropriate antidiuresis (NSIAD), and autosomal dominant polycystic kidney disease (ADPKD) (revised in Ranieri et al., 2019) [5].

Already in the 1997, Sands and coworkers reported evidence of the presence of an apical *“Calcium/polycation receptor proteins (CaRs)”* in rat kidney terminal inner medullary collecting duct (tIMCD) that specifically reduces vasopressin-elicited osmotic water permeability when luminal calcium rises. This evidence provides support for a unique and new tIMCD apical membrane signaling mechanism linking calcium and water metabolism [6].

However, clinical evidence for an effect of luminal calcium on AQP2-mediated water reabsorption was provided for the first time, in humans (enuretic children), in a study of Valenti and collaborators, demonstrating that urinary AQP2 and calciuria correlate with the severity of enuresis [7]. 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 [7]. Further evidence has been provided, more recently, 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 an increase in blood hematocrit (reflecting water loss) which coincided with a reduction of urinary AQP2 likely paralleled by an increase in urinary calcium due to bone demineralization [8].

All these results strongly support the indication that urinary calcium can modulate the vasopressin-dependent urine concentration through a down-regulation of AQP2 trafficking.

In a previous study, we demonstrated that in cultured renal cells and microdissected collecting ducts, the inhibitory effect of CaSR signaling on AQP2 trafficking to the plasma membrane is associated with a significant decrease in cAMP-induced AQP2 phosphorylation at serine 256 (pS256) and AQP2 trafficking, resulting in a reduced osmotic water permeability response [9]. Specifically, calcimimetics activation of CaSR reduced AQP2 translocation to the plasma membrane in response to the cAMP elevation forskolin-induced. 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, exploited to mimic “tonic” activation of CaSR [10]. The physiological consequence of the negative feedback on cAMP-induced AQP2-pS256 phosphorylation and trafficking stimulated by CaSR signaling is lowering the osmotic water permeability response both in cells and in isolated mouse collecting duct [9].

This theory that elevated concentration of calcium in urine counteract vasopressin action via the activation of CaSR expressed at luminal membrane of principal cells has been further validated in a mouse model double-knockout (dKO) for Pendrin/NaCl Cotransporter (NCC) [11], which display significant calcium wasting and severe volume depletion, despite high circulating vasopressin levels [12].

Due to 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 of AQP2-pS261 and ubiquitinated AQP2. In addition, in dKO mice, exposure of inner medulla kidney slices to the proteasome inhibitor MG132 increased total AQP2 by 50%, indicating that the rate of AQP2 degradation via proteasome is significantly higher. It has been recently suggested that CaSR expressed at the apical membrane of collecting duct principal cells could mediate the effects of hypercalciuria in reducing vasopressin-elicited osmotic water permeability and urinary concentrating ability by the activation of autophagic degradation of AQP2. Indeed, 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 [13].

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 mitogen-activated protein kinase (p38-MAPK), an enzyme activated by CaSR signaling and known to phosphorylate AQP2 at Ser261 [14][15]. Of note, CaSR inhibition with the calcilytic NPS2143 reduced AQP2-pS261 levels in dKO mice, demonstrating that CaSR acts upstream of p38-MAPK and mediates the upregulation of AQP2-pS261. Moreover, inhibition of p38-MAPK caused a drastic decrease in AQP2-pS261, along with a nearly five-fold increase in total AQP2. Furthermore, in dKO mice, p38-MAPK inhibition results in a drastic reduction in ubiquitinated AQP2 that is paralleled by a strong increase in total AQP2 [11].

In addition to 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 [16][17]. Moreover, vitamin D-elicited hypercalcemia/hypercalciuria is associated with polyuria in humans. At the end, dihydrotachysterol (DHT) induces AQP2 water channel downregulation despite unaltered AQP2 mRNA expression in rats, suggesting a higher rate of AQP2 degradation attributed to activation of the calcium-sensitive protease calpain [18].

Ultimately, 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.

Of note, regulation events of post-transcriptional gene expression can occur and be involved in several diseases, under the direct control of the small non-coding RNAs, the microRNAs (miRNAs) [19].

2. CaSR-Regulated miRNAs

MiRNAs are ubiquitous endogenous, short non-coding, most frequently of 19–25 nucleotides in length, single-stranded (ss)RNA transcripts that act as post-transcriptional regulators of gene expression by blocking protein translation and/or inducing messenger RNA (mRNA) degradation. miRNAs may act as transcriptional or splicing regulators within the nucleus [19], and be involved in genetic exchange with adjacent cells, through exosomes [20]. Many miRNAs display tissue-specific expression patterns and are involved in the development and maintenance of organ function. Approximately 60% of protein-coding genes are influenced by miRNAs [21] 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 [22]. Since their discovery in 1993 [23], numerous miRNAs have been identified in humans and other eukaryotic organisms, and their role as key regulators of gene expression is still being elucidated.

Only since 2012 have the miRNA activated by CaSR been indicated as key regulators of diverse proteins involved in different pathophysiological circumstances. Indeed, Hou and co-workers described the physiological function of claudins in the paracellular transport mechanisms with a focus on renal Ca^{2+} handling [24] (revised also in 2016 in [25]). In the thick ascending limb of Henle, paracellular Ca^{2+} reabsorption involves the functional interplay of three important claudin genes: claudin-14, -16, and -19, associated with human kidney diseases with hypercalciuria, nephrolithiasis, and bone mineral loss. A novel microRNA-based signaling pathway downstream of CaSR that directly regulates claudin-14 gene expression has been described indicating that claudin-14 is a key regulator for renal Ca^{2+} homeostasis. Through physical interaction, claudin-14 blocks the paracellular cation channel made of claudin-16 and -19, critical for Ca^{2+} reabsorption in the tick ascending limb. The molecular cascade of CaSR-microRNAs-claudins forms a regulatory loop to maintain proper Ca^{2+} homeostasis in the kidney [24][26]. Under normal dietary condition, claudin-14 proteins are suppressed by two microRNA

molecules, miR-9 and miR-374. Both microRNAs directly target the 3′-UTR of claudin-14 mRNA; induce its mRNA decay and translational repression in a synergistic manner, causing claudin-14 to decline, leading to decreases in cation permeation [26][27][28][29]. These data indicate that the regulation of miRNA by CaSR signaling may occur on several layers within the kidney.

Moreover, the silencing of the CaSR has been demonstrated to induce tumors in colorectal cancer, associated with increased expression of miR135b and miR-146b, which are considered to be oncogenic [30]. In colon cancer cell lines other miRNAs—miR21, miR-145, and miR-135a—are inversely correlated with CaSR expression [31][32].

Furthermore, altered expression of miRNAs have been implicated in parathyroid function and may have an important role in the development of parathyroid tumors [33][34].

Our recent studies suggest that CaSR may regulate AQP2 expression also via miRNA [11] (**Table 1**).

Table 1. MicroRNA expression downstream CaSR signaling.

miRNA		Target mRNA	Target Protein	Target Organ	References
miR-9 miR-374	↓	*CLDN14*	Claudin-14	Thick Ascending Limb cell, kidney	Hou J, Organogenesis 2012 [24]; Gong Y, Hou J, JASN 2014 [27]; Gong Y et al., JASN 2015 [28]; Hou J, Curr Opin Nephrol Hypert 2016 [25]
miR-21 miR-135a miR-135b	↓	Tumor suppressors	Tumor suppressor proteins	Human colon carcinoma cell lines, colon	Singh N et al., Int J Cancer, 2013 [31][32]
miR-145	↑	Oncogenes	Oncoproteins	Human colon carcinoma cell lines, colon	Singh N et al., Int J Cancer, 2013 [31][32]
miR-375 miR-429 miR-361	↑	*PTH*	Parathormone	Parathyroid	Shilo V et al., FASEB J 2015 [33]
miR-137	↑	*AQP2*	Aquaporin-2	Collecting duct, kidney	Ranieri M et al., FASEB J 2018 [11]

However, despite several studies having demonstrated that transcriptional and post-transcriptional regulation of AQP2 play crucial roles in AQP2 expression levels within the cell, along with a profound impact on water homeostasis [35][36], little is known about the role of miRNA in the regulation of AQP2 expression.

Several studies highlight an emerging role of miRNAs in AQP regulation (reviewed in [37]). Specifically, miRNAs have been identified as endogenous modulators of the expression of several AQPs [38][39][40][41][42][43][44][45][46][47][48]. Two AQP2-targeting miRNAs, miR-32 and miR-137, were reported to decrease AQP2 expression in kidney collecting duct cells independently of vasopressin regulation [41]. The authors demonstrated a significant decrease of AQP2 translation in mpkCCDc14 cells transfected with miR-32 or miR-137 providing novel insights into the regulation of AQP2 by RNA interference.

Specifically, in dKO mice, miR-137 was found to be about 1.7-fold higher compared to WT mice, which was in line with the reduced translation of AQP2 mRNA. Noteworthy, miR-137 transcript levels were increased by the calcimimetic NPS-R-568 in WT mice; furthermore, in dKO mice, miR-137 transcript levels were drastically reduced in response to CaSR or p38-MAPK inhibition with the calcilytic NPS2143 or SB203580, respectively, providing the first evidence that CaSR signaling directly acts upstream of the miR-137-AQP2 axis [11].

These findings represent the first demonstration that CaSR can regulate AQP2 expression via AQP2-targeting miRNA.

The discovery of miRNAs as endogenous modulators of AQPs offers a potential therapeutic approach for the regulation of AQP-related disorders [37].

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