Aquaporins in kidney pathophysiology

Yumi Noda, Eisei Sohara, Eriko Ohta and Sei Sasaki

Abstract | Seven aquaporin water channels are expressed in human kidneys, and they have key roles in maintaining body water homeostasis. Impairment of their function can result in nephrogenic diabetes insipidus and other water-balance disorders. A lot of data have increased understanding of the functions and mechanisms of regulation of aquaporins both at the molecular and the clinical level. Research has also focused on aquaporins as therapeutic targets. This Review describes recent progress in uncovering the physiology and pathophysiology of aquaporins in the kidney, with particular attention devoted to AQP2, the most well-studied member of this protein group.

Noda, Y. *et al. Nat. Rev. Nephrol.* **6**, 168–178 (2010); published online 26 January 2010; doi:10.1038/nrneph.2009.231

Introduction

Water makes up two-thirds of the weight of an adult human, and water transport across the cell membrane is essential for cell function and whole-organism survival. The agents that make water movement across the cell membrane possible were, however, elusive until just over 15 years ago, when aquaporin water channels were finally discovered.[1,2] Aquaporins are a family of small integral membrane proteins each comprised of six membrane-spanning helical domains (Figure 1). Both amino and carboxyl termini are located in the cytoplasm. Two widely conserved, membrane-embedded asparagine–proline–alanine (NPA) motifs are positioned in the proximity of the cell membrane's surface; one motif is found close to the membrane–luminal side border, the other to the membrane–cytoplasm border. The two NPA motifs-containing loops protrude into the lipid bilayer from the opposite surfaces, are juxtaposed in the center, and form the water-permeable pore structure. Aquaporins are divided into three families.[3] AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8 are part of Class I and are water-selective, although AQP6 is also permeable to anions and AQP8 to reactive oxygen species. Class II aquaporins, which include AQP3, AQP7, AQP9 and AQP10, are permeable not only to water but also to small neutral solutes, such as glycerol and urea. These aquaporins are also called aquaglyceroporins.[4] Class III aquaporins, which include AQP11 and AQP12, are also called 'superaquaporins' and have two deviated NPA motifs, that is the first NPA motif is replaced in AQP11 by the sequence Asn–Pro–Cys and that of AQP12 by the sequence Asn–Pro–Thr. The second NPA motif is however conserved in both proteins as the sequence Asn–Pro–Ala.[3] Seven aquaporins are expressed in human kidneys, and they have key roles in maintaining body water homeostasis. The importance of these proteins in the mechanisms that regulate urine concentration have been confirmed by animal studies. Aquaporins have also been found to be involved in the regulation of cell migration, cell proliferation, skin hydration, neural activities and of water balance in the brain.[5]

This Review focuses on the evidence of the physiological and pathophysiological role of aquaporins in the kidney, with special attention dedicated to AQP2, the aquaporin for which the most extensive data are available.

Aquaporins in the kidney

The seven aquaporins expressed in the kidney are AQP1, AQP2, AQP3, AQP4, AQP6, AQP7 and AQP11 (Table 1). AQP1 is expressed in the plasma membrane of the proximal tubules, the descending thin limbs of Henle and the descending vasa recta. AQP1 is critical for water reabsorption from urine in these segments and for a functional countercurrent multiplication system (the system that creates the osmotic driving force for passive water reabsorption across the collecting duct). AQP2 is a vasopressin-sensitive water channel expressed in the principal cells of the collecting duct. Secretion of vasopressin in response to dehydration causes AQP2 to translocate from intracellular vesicles to the apical plasma membrane. This reposition enables water reabsorption from the urine into the cell. AQP3 and AQP4 are expressed on the basolateral membrane of the principal cells of the collecting duct, and they constitute potential water exit pathways from these cells. AQP6 is localized in intracellular vesicles of acid-secreting α-intercalated cells of the collecting duct, and this water channel is suggested to be involved in urinary acid secretion. AQP7 is localized in the apical membrane of segment 3 of the proximal tubule, where it facilitates glycerol and water transport. AQP11 is expressed in the proximal tubules and is presumably localized in the endoplasmic reticulum (ER). Knockout of the gene that encodes AQP11 is fatal in mice because of the onset of polycystic kidney disease. This evidence suggests an important role of AQP11 in ER homeostasis.

Department of Nephrology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan (**Y. Noda, E. Sohara, E. Ohta, S. Sasaki**).

Correspondence to: S. Sasaki ssasaki.kid@tmd.ac.jp

Competing interests
The authors declare no competing interests.

AQP2

Physiological role

Water reabsorption in the collecting ducts is the key event for maintenance of body water balance. This process is regulated by AQP2, a vasopressin-sensitive water channel. Under conditions of normal hydration, AQP2 is confined to the cytoplasm of collecting duct cells. When the body is dehydrated and needs to retain water, AQP2 relocates to the apical membrane, thus enabling water reabsorption from the urinary tubule into the cell. AQP2 impairment results in various water balance disorders, including nephrogenic diabetes insipidus (NDI).

Increase in serum osmolality and reduction in effective circulating blood volume stimulate the secretion of vasopressin from the posterior pituitary gland. Vasopressin then binds to the vasopressin V2 receptor (V2R), which is located on the basolateral membrane of the principal cells of the renal collecting duct. V2R activates adenylate cyclase, which catalyzes the conversion of ATP to cyclic AMP. The increase in cyclic AMP level leads to the activation of protein kinase A (PKA), which in turn phosphorylates residue Ser256 of AQP2. As a consequence of AQP2 phosphorylation, subapical storage vesicles that contain AQP2 translocate from the cytoplasm of principal cells to the apical plasma membrane and fuse with it. Relocation of phosphorylated AQP2 to the cell membrane renders the cell water permeable.[6–9] Upon removal of the vasopressin stimulus, AQP2 is shuttled back to the cell cytoplasm, a process that restores the water-impermeability of the cell. This internalization process consists of AQP2 retrieval into early endosomes that express early endosome antigen 1, and subsequent transferral of this water channel to storage vesicles that express Rab-11.[10]

AQP2 phosphorylation by PKA is one of the main signals that trigger AQP2 exocytosis. AQP2 forms homotetramers *in vivo*, and, for successful plasma membrane

Key points

- Aquaporins are a family of membrane proteins that function as water-permeable channels
- Seven different aquaporins are known to be expressed in the human kidney: AQP1, AQP2, AQP3, AQP4, AQP6, AQP7 and AQP11
- AQP2, for which the most data are available, is vasopressin-sensitive and is expressed in the principal cells of the collecting duct; its impairment results in nephrogenic diabetes insipidus and other water balance disorders
- Genetic defects in aquaporins other than AQP2 expressed in the kidney seem very rare and are associated with either mild defects (as for AQP1 and AQP7) or with no obvious deleterious consequence
- AQP3 and AQP4 are expressed in the basolateral membrane of the principal cells of the collecting duct and represent potential exit pathways from these cells for water entering via AQP2
- Besides being water-permeable, AQP6 and AQP7 are also permeable to anions and glycerol, respectively, so that AQP6 is suggested to be involved in acid secretion and AQP7 in glycerol metabolism

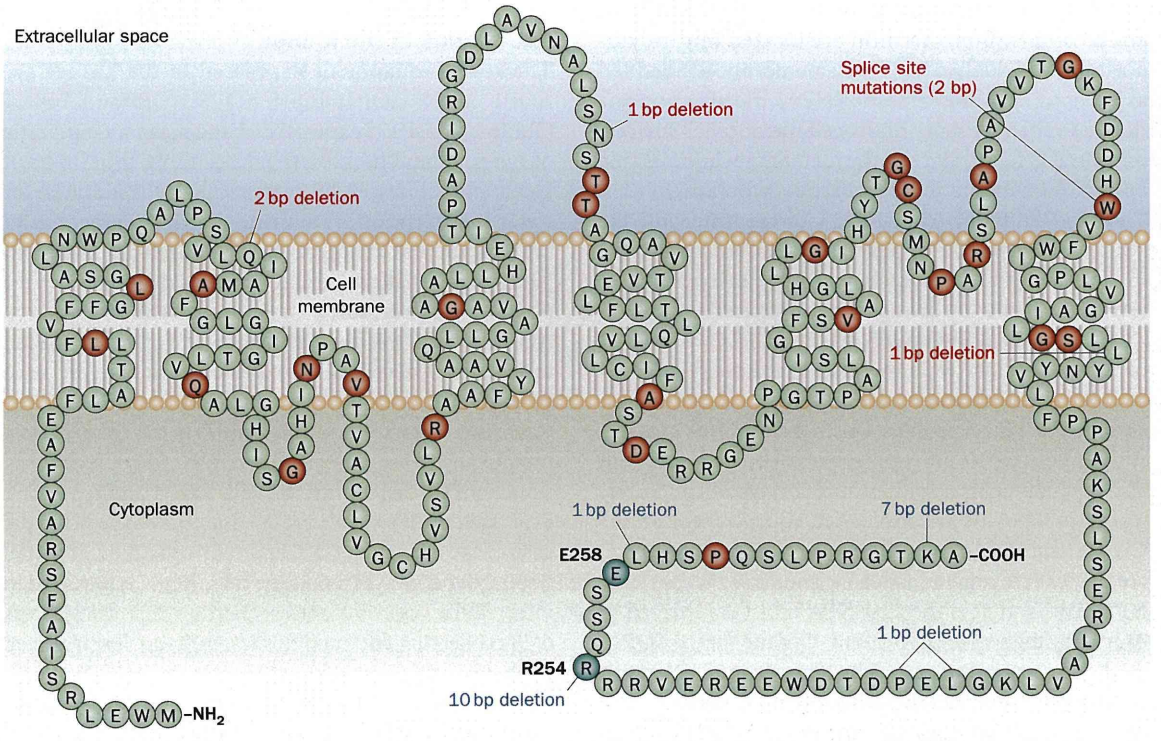


Figure 1 | Structure of aquaporin-2 embedded in the cell membrane reporting the protein mutations that cause nephrogenic diabetes insipidus. Amino acids that when mutated cause the autosomal-recessive form of nephrogenic diabetes insipidus are shown in red. Also in red are shown *AQP2* deletions, and the location of the first amino acid of the protein affected by these nucleotide deletions, that cause autosomal-recessive form of nephrogenic diabetes insipidus. Amino acids when mutated that cause the autosomal-dominant form of nephrogenic diabetes insipidus are shown in blue. Similarly as above, in blue are also reported *AQP2* deletions, and the location of the first amino acid of the protein affected by these nucleotide deletions, that cause the autosomal-dominant form of nephrogenic diabetes insipidus. Abbreviation: bp, base pair.

Table 1 | Aquaporins in the kidney

Aquaporin	Localization	Subcellular distribution	Extrarenal localization	Phenotype of mice with mutations or gene knockout	Phenotype of humans with mutations
AQP1	Proximal tubules, descending thin limbs of Henle, outer medullary descending vasa recta	Apical and basolateral plasma membrane	Erythrocytes, ciliary and lens epithelium choroid plexus, pulmonary vascular endothelium	Urinary concentrating defects, impaired pain sensation	Mild urinary concentrating defects
AQP2	Principal cells of the collecting duct	Apical plasma membrane and subapical vesicles	Epididymis	Diabetes insipidus	Diabetes insipidus
AQP3	Principal cells of the collecting duct	Basolateral plasma membrane	Conjunctiva, pulmonary airway epithelia, colonic epithelia, keratinocytes, erythrocytes	Urinary concentrating defect, reduced skin hydration, impaired wound healing, resistance to the formation of skin tumors	No obvious abnormality
AQP4	Principal cells of the collecting duct	Basolateral plasma membrane	Astroglia, ependyma, retinal glia, muscle fiber cells, keratinocytes pulmonary airway epithelia, stomach parietal cells	Mild urinary concentrating defect, impaired vision, hearing and olfaction	No abnormalities reported
AQP6	Intercalated cells of the collecting duct	Intracellular vesicles	Cerebellum, synaptic vesicles	No report	No abnormalities reported
AQP7	S3 segment of proximal tubules	Apical plasma membrane	Adipose tissue, testis, skeletal muscle, heart, brain, intestine	Glyceroluria, defective glycerol metabolism, obesity, smaller islet cells	Defective glycerol metabolism
AQP11	Proximal tubules	Endoplasmic reticulum	Testis, thymus, intestine, liver	Polycystic kidney disease (fatal)	No abnormalities reported

localization, at least three of four monomers in AQP2 tetramers must be phosphorylated.[11,12] PKA and its substrates are present throughout the cell, therefore, localization of PKA in specific sites is necessary for PKA to effectively phosphorylate its target. Phosphorylation is assisted by protein kinase A anchoring proteins (AKAPs). Tethering of PKA to AKAPs is a prerequisite for AQP2 shuttling to the cell surface.[13] A splice variant of AKAP-18, AKAP-18δ, is specifically involved in AQP2 shuttling,[14] although the involvement of AKAP-220 has also been reported.[15]

Phosphorylation of AQP2 by kinases other than PKA might also contribute to the regulation of AQP2 trafficking. Ser256 in AQP2 is also a substrate for Golgi casein kinase. AQP2 transition through the Golgi apparatus is associated with a PKA-independent increase in AQP2 phosphorylation at Ser256, which suggests that phosphorylation by Golgi casein kinase might be required for Golgi transition.[16] van Balkom *et al.*[17] reported that activation of protein kinase C mediates endocytosis of AQP2, a process that was independent of the phosphorylation state of Ser256. Bouley *et al.*[18] showed the involvement of a cyclic-GMP-dependent pathway in AQP2 exocytosis. In addition, sildenafil citrate, an inhibitor of cyclic GMP phosphodiesterase, induces insertion of AQP2 in the cell membrane.[19]

Besides Ser256, three additional phosphorylation sites are found nearby the AQP2 C-terminus. These modifiable residues are Ser261, Ser264 and Ser269 and have been the focus of research of several groups. Vasopressin induces phosphorylation of AQP2 also at Ser264, and Ser264-phosphorylated AQP2 (pSer264-AQP2) is translocated to the plasma membrane similarly to pSer256-AQP2.[20] On the other hand, the level of pSer261-AQP2 is decreased by vasopressin and pSer261-AQP2 localization is different from that of pSer256-AQP2, which suggests distinct roles for these residues in AQP2 trafficking.[21] Lu *et al.*[22] reported that the phosphorylation state of Ser261 does not affect AQP2 trafficking.[22] Ser269 is reported to be involved in plasma membrane retention of AQP2.[23] Moeller *et al.*[24] reported that phosphorylation of Ser264 and Ser269 depends on prior phosphorylation of Ser256 and that phosphorylation of Ser261 partially depends on the phosphorylation of Ser264 and Ser269.[24] By contrast, Ser256 phosphorylation is not dependent on the state of any of the other phosphorylation sites, which suggests that Ser256 is the most important phorphorylation site of AQP2.

Intracellular Ca^{2+} mobilization is also involved in vasopressin-mediated AQP2 trafficking,[25] although its precise role remains unclear. In addition to increasing cyclic AMP levels in the cytoplasm of the principal cells of the collecting duct, vasopressin binding to V2R triggers a rapid increase of intracellular Ca^{2+}, which is followed by sustained temporal oscillations of the level of this ion. This process seems important for AQP2 exocytosis. Balasubramanian *et al.*[25] suggest several plausible candidates as downstream effectors of this signaling cascade, such as calmodulin and MLCK. MLCK is a calmodulin-dependent kinase that regulates actin filament organization by phosphorylating the regulatory light chain of myosin II, and thus also activates myosin motor activity. Myosin II and its regulatory light chain are found in the AQP2-binding protein complex (see below),[26] an observation which supports their involvement in AQP2 trafficking. Nevertheless, Lorenz *et al.*[27] demonstrated that cyclic AMP alone is sufficient to induce AQP2 shuttling, without need for an increase

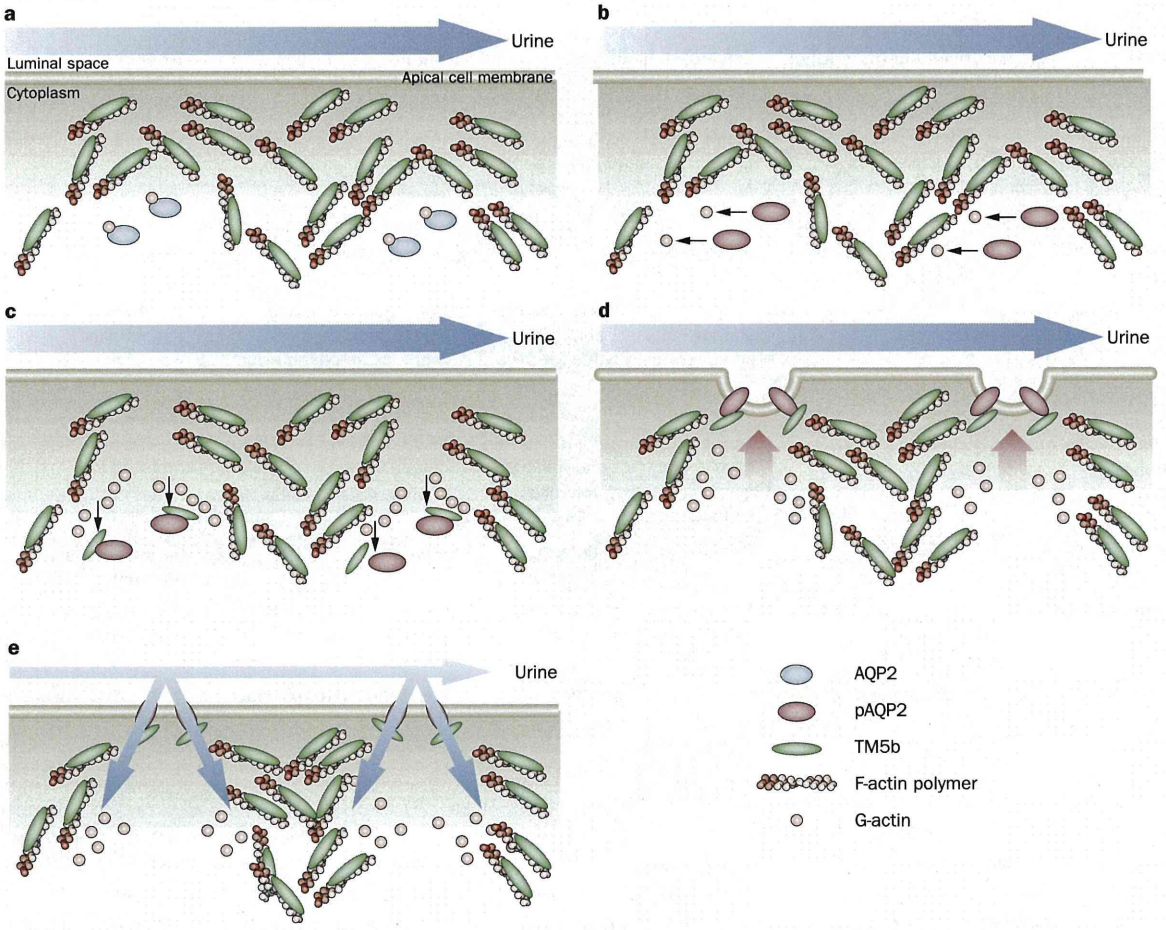


Figure 2 | Mechanism of AQP2 translocation from the cytoplasm of principal cells of the collecting duct to the membrane. **a** | Under physiological conditions, AQP2 binds to G-actin, and F-actin is stabilized by TM5b to form a barrier that inhibits AQP2 translocation to the apical cell membrane. **b** | When the body is dehydrated, vasopressin-triggered signaling of cyclic AMP leads to AQP2 phosphorylation, a modification that prompts G-actin release from AQP2. **c** | AQP2 phosphorylation also promotes competitive binding of TM5b by the modified AQP2, a process that destabilizes the F-actin polymer. **d** | F-actin destabilization enables efficient AQP2 transport to the apical membrane of the cell. **e** | TM5b-bound AQP2 molecules form pores in the cell membrane through which water is reabsorbed into the cell cytoplasm. Abbreviations: AQP2, tetrameric aquaporin-2; pAQP2, tetrameric phosphorylated aquaporin-2; TM5b, tropomyosin 5b.

in cytosolic Ca^{2+} levels in cells of the inner medullary collecting duct.

AQP2 translocation to the apical membrane prompted by forskolin-induced increases in cyclic AMP levels is inhibited by increased levels of extracellular Ca^{2+}. This process is probably mediated by the endogenous calcium-sensing receptor[28] and is associated with an increase in F-actin level.

Several other factors have recently been reported to affect AQP2 trafficking. Nejsum *et al.*[29] reported that in Madin–Darby canine kidney epithelial cells transfected with AQP2 prostaglandin E2 and dopamine induce internalization of AQP2, regardless of AQP2 dephosphorylation.[29] de Seigneux *et al.*[30] reported that aldosterone induces basolateral expression of AQP2, suggesting a role for aldosterone in water metabolism in conditions of increased sodium reabsorption in the collecting ducts.

The actin cytoskeleton is reported to function as a barrier for AQP2 exocytosis. Actin depolymerization is necessary for the cyclic-AMP-dependent translocation of AQP2.[31] In fact, stimulation of prostaglandin E3 receptors has been shown to inhibit vasopressin-induced inactivation of Rho GTPase, vasopressin-induced F-actin depolymerization and AQP2 translocation induced by vasopressin, cyclic AMP or forskolin.[32] Rho GTPase activation by bradykinin stabilizes cortical F-actin and inhibits AQP2 trafficking.[33]

GTPase-activating protein Spa-1 (SPA-1) binds to the C-terminus of AQP2, and this binding is required for AQP2 trafficking.[34] SPA-1 may inhibit Rap1 GTPase activating protein, which triggers F-actin disassembly and may maintain the basal mobility of AQP2.[9,35] SPA-1-deficient mice show impaired AQP2 trafficking and hydronephrosis.[34,36] In humans, mutations in the C-terminus of AQP2, which is the binding region of SPA-1, cause NDI (Figure 1).[9,37,38] Furthermore, AQP2 binds to a multiprotein complex that includes the actin cytoskeleton, and a pattern of competing interactions between AQP2 and G-actin or tropomyosin directs AQP2 trafficking to the apical membrane (Figure 2).[26,39,40]

F-actin assembly might have both inhibitory and facilitatory effects on AQP2 exocytosis.[41,42] Tajika *et al.*[43] showed that actin depolymerization inhibits AQP2 translocation from early endosomes that express early endosome antigen 1 to subapical storage vesicles that express Rab-11.[43] Myosin II and its regulatory light chain are found in an AQP2-binding protein complex,[26] and vasopressin induces myosin light chain phosphorylation, which enhances myosin–actin filament interaction and the formation of actin fibers.[44] Myosin has also been shown to be critical for AQP2 recycling.[45]

In addition to acting as a barrier to prevent AQP2 trafficking, actin fibers may function as 'cables' that promote and direct AQP2 transport. Dynamic actin reorganization may be responsible for the transformation of the actin barrier into a network of actin cables. Myosin and tropomyosin may be involved in this cable formation. Measurement of molecular dynamics with high spatiotemporal resolution may be useful for clarifying the mechanism of this process.

The docking and fusion of AQP2-containing vesicles with the apical membrane involves the action of SNARE proteins including VAMP-2, SNAP-23, syntaxin-3 and syntaxin-4.[6,9] Syntaxin-binding protein 2 (also known as Munc18b) is reported to function as a negative regulator of SNARE complex formation and AQP2-vesicle fusion to the apical membrane.[46]

Following cessation of the vasopressin signal, AQP2 undergoes clathrin-mediated endocytosis.[9] A heat shock protein hsc70, which is important for uncoating clathrin-coated vesicles, binds to the C-terminus of nonphosphorylated AQP2 and is required for AQP2 endocytosis.[47] Kamsteeg *et al.*[48] reported that the myelin and lymphocyte protein (also known as MAL), which is involved in the organization of glycosphingolipid-enriched membrane, interacts with AQP2 and enhances expression of AQP2 at the apical membrane by decreasing the level of internalization of the protein.[48] Ubiquitination at Lys270 of AQP2 is important for AQP2 endocytosis and degradation.[49] Furthermore, LIP5, which is involved in multivesicular body formation, interacts with AQP2 and facilitates its lysosomal degradation.[50]

Collecting duct cells are the site of AQP2-regulated water reabsorption, and they are exposed to great fluctuations in osmotic pressure during transition between antidiuresis and diuresis. The activity of the promoter of the gene that encodes the murine AQP2, is enhanced by hypertonicity and reduced by hypotonicity.[51,52] Acute hypertonicity induces AQP2 accumulation at the cell surface, and chronic hypertonicity induces AQP2 insertion into the basolateral membrane instead of the apical membrane.[53,54] Cell volume regulation in response to changes in the external osmolality is a fundamental property of cells. When cells are exposed to hypotonic extracellular fluid, they swell because of the osmotic water influx. After the swelling, cells start to recover their original volume. This cellular defensive process against hypotonic shock is called regulatory volume decrease.[55] Hypotonicity induces AQP2 internalization, which may contribute to regulatory volume decrease by limiting water entry into collecting duct cells.[56] Furthermore, AQP2 regulates volume decrease by controlling the cytoskeleton.[57]

NDI and AQP2 impairment

Mutations in AQP2 cause congenital NDI, which is characterized by an inability of patients to concentrate urine despite normal or elevated plasma concentrations of vasopressin. This condition results in a massive loss of water through the kidney. In more than 90% of cases of congenital NDI the condition results from mutations in V2R (X-linked NDI). The remaining cases result from mutations in AQP2 (autosomal NDI). *AQP2*, the gene that encodes AQP2, is located in 12q12–q13, and it is comprised of four exons. To date, 40 mutations in *AQP2* have been reported (Figure 1).[58,59] Two inheritance types are possible for the disease; autosomal-recessive NDI is associated with 32 mutations, and autosomal-dominant NDI is associated with eight mutations (Figure 1). Almost all of the mutations in recessive NDI are located in the core region of the protein, and they lead to misfolded proteins that become trapped in the ER and targeted for rapid degradation by the proteasome. On the other hand, AQP2 homotetramers composed only of wildtype protein are properly translocated to the apical membrane. This effect explains the healthy phenotype of the patients' parents.[58]

All mutations in autosomal-dominant NDI locate in the cytosolic C-terminus of AQP2 (Figure 1). This region is important for AQP2 trafficking and these mutations impair trafficking to the apical membrane, although the water channel function of these mutants is preserved. Arg254Leu and Arg254Gln mutations destroy the site for PKA phosphorylation, so that forskolin-induced trafficking to the plasma membrane is impaired.[59,60] The Glu258Lys mutant of AQP2 is missorted to multivesicular bodies and/or lysosomes.[61] AQP2 mutants resulting from three gene deletions, 721delG, 763–772del and 812–818del, have similar extended C-terminal tails, which contain the basolateral-membrane-sorting dileucine (LeuLeu) motif, so these mutated proteins are wrongly translocated to the basolateral membrane.[37,62] In contrast to the AQP2 mutants associated with the recessive form of the disease, AQP2 mutants associated with the dominant form of the disease are not misfolded, so they are able to form heterotetramers with wildtype AQP2. Because of the dominancy of the missorting motif in the mutant proteins, mutant–wildtype tetramers are missorted, which leads to severely decreased amounts of AQP2 on the apical membrane. This effect explains the dominant mode of NDI inheritance in patients with these mutations. We generated gene knockin mice with the heterozygous mutant AQP2 resulting from a gene deletion (763–772del) that produces a mouse model of dominant NDI.[63] The mutant AQP2 is wrongly translocated to the basolateral membrane; it forms a heterotetramer with wildtype AQP2 and shows a dominant-negative effect on the normal apical sorting of wildtype AQP2. The urine concentrating ability of these gene knockin mice is severely

reduced. Furthermore, rolipram, an inhibitor of the phosphodiesterase isozyme 4, is able to increase urine osmolality, which suggests that this drug may be useful in the treatment of dominant NDI.

AQP2 is also involved in acquired NDI. Lithium is widely used to treat bipolar disorder and its most common adverse effect is NDI.[64] In lithium-induced NDI, both AQP2 expression and its trafficking to the apical membrane are inhibited. Lithium enters cells expressing AQP2 via the epithelial sodium channel in the apical membrane and accumulates intracellularly. This accumulation leads to the inhibition of signaling pathways that involve glycogen synthase kinase-3β (GSK3β). Although the mechanism by which AQP2 is dysregulated in this context is not established, the involvement of GSK3β is speculated. Inhibition of GSK3β by lithium increases expression of cyclooxygenase 2 and the local excretion of prostaglandin E2.[65] Prostaglandin E2 is suggested to counteract vasopressin activity by causing endocytic retrieval of AQP2 from the plasma membrane, thus impairing the urinary concentrating ability of the cell. Furthermore, lithium increases the intracellular accumulation of β-catenin,[66] which can serve as an activator of T-cell-factor-dependent transcription. AQP2 downregulation may be achieved via this transcription mechanism.

Hypokalemia and hypercalcemia cause downregulation of AQP2 expression, which results in a vasopressin-resistant urinary concentrating defect. Ureteral obstruction decreases AQP2 expression and impairs urine concentrating capacity.[67] Mouri *et al.*[68] reported that AQP2 translocation to the apical membrane is inhibited by metabolic acidosis, a mechanism that might be responsible for the diuresis in patients with chronic renal failure.

Treatment of NDI due to AQP2 impairment

No cure exists for NDI. Currently, this condition is managed by salt restriction combined with the administration of hydrochlorothiazide diuretics to reduce urine output.[69] Hydrochlorothiazide reduces in fact sodium reabsorption in the distal convoluted tubule, leading to increase in sodium excretion and extracellular fluid volume contraction. As a result, glomerular filtration rate decreases and the proximal tubular sodium and water reabsorption increases. Consequently, less water and sodium are delivered to the collecting ducts, resulting in decrease in urine volume. This antidiuretic effect is enhanced by a low sodium intake. Furthermore, hydrochlorothiazide increases expression of AQP2 and distal renal sodium transporters, which may also contribute to this antidiuretic action.[70] Additional administration of prostaglandin-synthesis inhibitors or the potassium-sparing diuretic amiloride is reported to enhance the effectiveness of NDI management, although long-term use of prostaglandin inhibitors is often complicated by gastrointestinal and hematopoietic adverse effects and renal dysfunction. In any event, current treatment does not completely obviate the excessive water excretion, as adult patients undergoing treatment still void 8–10 l per day. Therefore, extensive efforts to develop therapies continue.

Chemical chaperones that facilitate folding of the mutant protein have been reported to correct the sorting of NDI-causing AQP2 mutants in cell cultures. However, chemical chaperones are not suitable for use *in vivo* because of the high concentrations that would be required to achieve clinically meaningful results.

AQP2 phosphorylation by cyclic GMP kinase is involved in its exocytosis, and the cyclic GMP phosphodiesterase inhibitor sildenafil citrate induces AQP2 membrane insertion.[18,19] Therefore, cyclic GMP phosphodiesterase inhibitors are expected to be effective in the treatment of NDI due to V2R impairment by bypassing the need for cyclic AMP signaling for AQP2 exocytosis. However, in healthy individuals, use of sildenafil citrate has not been associated with either water retention or hyponatremia, which might mean that their effect on AQP2 translocation is, at least in healthy people, negligible.

Li *et al.*[71] generated an animal model for X-linked NDI, in which *AVPR2*, the gene that encodes V2R, was conditionally deleted. These mice show characteristic symptoms of NDI, such as urinary concentrating defects and dilatation of the renal pelvis. An agonist of the EP4 subtype of the prostaglandin E receptor proved highly effective in ameliorating these manifestations of NDI.

We investigated in our mutant AQP2 knockin mice whether phosphodiesterase inhibitors affect the urinary concentration ability of these mice.[63] Among the inhibitors tested, rolipram increased urine osmolality, cyclic AMP content in the papillae, AQP2 phosphorylation, and apical membrane translocation of the mutated AQP2. Interestingly, rolipram also induced the apical translocation of wildtype AQP2 as a consequence of dehydration.

Interaction of phosphorylated AQP2 with tropomyosin 5b is essential for AQP2 trafficking to the apical membrane, suggesting that tropomyosin 5b is a potential therapeutic target for NDI.[40] Knockdown of the gene encoding tropomyosin 5b corrects the trafficking defect of the Ser256Ala AQP2 mutant in Madin–Darby canine kidney cells.[40] Specific inhibition of tropomyosin 5b may be useful for both congenital and acquired NDI because the interaction of tropomyosin 5b with phosphorylated AQP2 is critical for the final step of AQP2 trafficking.

Oiso and colleagues[72] succeeded in developing a method for the targeted expression of AQP2 in collecting ducts using the Sendai virus vector system that did not contain the fusion protein gene (SeV/ΔF). This vector system directs high-level transgene expression and is nontransmissible. Furthermore, in an animal model of NDI, this vector was administered to the animal retrogradely via ureter to renal pelvis, which enabled its efficient expression in the collecting duct in limited area of renal medulla. Viral delivery of *AQP2* in a lithium-induced rat model of NDI led to a reduction in urine output and an increase in urine osmolality for several days. Oiso and colleagues suggest that this strategy may be beneficial in patients with NDI, especially patients

who are unconscious and those in a perioperative situation because this treatment approach can temporarily reverse the polyuria phenotype.

Other pathologies associated with AQP2

AQP2 has a critical role in the pathophysiology of many diseases associated with water balance disorders. The best-known example is congestive heart failure (CHF). Water retention and hyponatremia are common and clinically important complications of CHF. Plasma vasopressin levels are suppressed by hyponatremia in healthy individuals; however, these levels are not suppressed in patients with hyponatremia who have CHF.[73,74] Upregulation of AQP2 expression and increased AQP2 trafficking to the apical membrane of principal cells of the collecting duct have been shown in rat models of cardiac failure.[75,76] Futhermore, water retention and hyponatremia in these rats are reversed by a V2R antagonist.[76] These findings indicate that hyponatremia is caused by nonosmotic stimulation of vasopressin, which promotes the expression and trafficking of AQP2. In patients with heart failure, V2R antagonists promote electrolyte-free water excretion and elevate serum sodium concentration.[77–79] Tolvaptan, a vasopressin antagonist, has been shown to improve several symptoms of heart failure, such as dyspnea, in these patients.[80]

Water retention and hyponatremia are observed in patients with hepatic cirrhosis. In these patients, nonosmotic secretion of vasopressin occurs secondary to splanchnic arterial vasodilation and relative arterial underfilling.[74] In cirrhotic rats, AQP2 expression was increased and correlated with the volume of ascites.[81] In patients with hyponatremic cirrhosis, V2R antagonists are effective at inducing free water diuresis and raising plasma sodium levels.[78,82]

During pregnancy, arterial underfilling secondary to systemic arterial vasodilation with nonosmotic vasopressin secretion and upregulation of AQP2 is observed.[83,84] Administration of a V2R antagonist increases electrolyte-free water excretion in pregnant rats.[83]

A urinary concentrating defect is observed in patients with the nephrotic syndrome.[85,86] AQP2 expression is decreased in nephrotic rats.[85,87] However, changes in AQP2 expression levels have not yet been confirmed in patients with the nephrotic syndrome.

Syndrome of inappropriate antidiuretic hormone secretion (SIADH) is a condition in which plasma vasopressin levels are not appropriately suppressed despite hypo-osmolality. SIADH is the predominant cause of euvolemic hyponatremia and a commonly encountered disorder.[88] SIADH occurs frequently in association with vascular, infectious or neoplastic abnormalities in the lung or central nervous system. In patients with SIADH, the V2R antagonist OPC-31260 was shown to be effective in increasing urine volume and plasma sodium levels.[89] However, the long-term effects of its administration are limited in rats with SIADH.[90] Although AQP2 protein expression is reduced shortly after administration of the V2R antagonist to rats with SIADH, it increases again in parallel with the decline of the therapeutic effects.

Urinary AQP2 excretion level is associated with vasopressin activity in the kidney and is, therefore, a clinically useful biomarker.[91] AQP2 is excreted into the urine through the secretion of exosomes originating from internal vesicles of multivesicular bodies.[92] During this process, the outer membrane multivesicular bodies fuses with the apical plasma membrane. Urinary AQP2 excretion is increased by dehydration or vasopressin and decreased by hydration. Urinary AQP2 excretion is also increased in patients with CHF and hepatic cirrhosis and in pregnant women.[93–95] In patients with CHF, administration of a V2R antagonist produced a significant increase in urine flow and solute-free water excretion, accompanied with a dramatic decrease in urinary AQP2 excretion.[93] Augmentation of urinary excretion of AQP2 is also found in SIADH.[96] Urinary excretion of AQP2 is a sensitive marker of the antidiuretic activity of vasopressin.

Other AQPs

AQP1

AQP1 is located on the apical and basolateral membrane of the proximal tubules, the descending thin limbs of Henle and the outer medullary descending vasa recta. In these segments, AQP1 is constitutively active as a water-selective pore.[2]

Deletion of *Aqp1* in mice results in marked polyuria. Urinary osmolality in *Aqp1*-null mice is low and unresponsive to vasopressin secretion or water deprivation.[97] This phenotype was explained by two distinct mechanisms: impaired near-iso-osmolar water re-absorption by the proximal tubule and reduced medullary hypertonicity resulting from impaired countercurrent exchange.[98] Precise analysis of *Aqp1*-null mice clarified that AQP1 is the principal water channel in the proximal tubules and the descending thin limbs of Henle and provides the major route for transepithelial water permeability in these segments.[98]

In 2001, two unrelated individuals with a deficiency in AQP1 expression were reported.[99] One of these individuals was homozygous for a deletion of exon 1 of *AQP1*. The second individual was homozygous for a frame-shift mutation in exon 1 of *AQP1*. Although both patients seemed asymptomatic under normal conditions, they showed an impaired urinary concentrating ability following 24 h water restriction as compared with healthy controls (450 versus 1,000 mmol/kg H_2O). Such a defect can become clinically meaningful in circumstances that require maximal urinary concentration, for instance during vomiting and diarrhea.[100]

A role of AQP1 in cell migration has also been suggested, after defective cell migration was demonstrated in *AQP1*-null endothelial cells.[101] Migration of proximal tubules from *Aqp1*-null mice was reduced by more than 50% compared to that of wildtype cell.[102] In addition, the kidneys of *Aqp1*-null mice showed greater tubular injury than kidneys of wildtype mice after ischemia–reperfusion-induced and endotoxemia-induced acute kidney injury.[102,103] These results suggest an important role for AQP1 in the migration of proximal tubular cells and in the response of the proximal tubule to injury.

AQP3

AQP3 is expressed in the principal cells of the connecting tubule and collecting duct, and it enables water entry into the interstitium. AQP3 is constitutively localized in the basolateral plasma membrane. AQP3, together with AQP4, represents potential exit pathways from these cells for water entering the cell via AQP2. AQP3 expression is increased by thirst and by vasopressin or aldosterone secretion.[104] *Aqp3* knockout mice exhibit a NDI-like phenotype, which indicates that AQP3 has an important role in urine concentration.[5] AQP3 deficiency was reported in humans.[105] This defect is caused by homozygous mutation affecting the 5' donor splice site of intron 5 of *AQP3*. This mutation causes the skipping of exon 5 and generates a frameshift change and premature stop codon. However, phenotypes associated with this defect were not reported possibly because the patients seemed normal.

AQP4

AQP4 is expressed in the principal cells of the collecting duct. AQP4 is more abundant in the inner medullary collecting duct cells than AQP3. Organization of AQP4 into orthogonal arrays of particles might enhance AQP4 water permeability.[106] Similarly to AQP3, AQP4 is constitutively localized in the basolateral plasma membrane. *Aqp4* knockout mice have a mild urinary concentrating defect.[5] In contrast to AQP3, AQP4 expression is not regulated by thirst or vasopressin secretion. AQP4 water permeability is decreased by high levels of protein kinase C and dopamine. This effect is mediated by phosphorylation at Ser180 of AQP4.[107] Evidence from a study published in 2009 indicates that AQP4 expression and its organization into orthogonal arrays of particles are regulated by vasopressin.[108]

The molecular structure of AQP4 has been largely elucidated. Hiroaki *et al.*[109] proposed the structural basis for an orthogonal array formation of AQP4 and AQP4-mediated cell adhesion by electron crystallography of double-layered, two-dimensional crystals. The 1.8 Å crystal structure of human AQP4 published in 2009 reveals the molecular basis for the water selectivity of this channel.[110]

AQP6

AQP6 is localized in the intracellular vesicles in intercalated cells in the collecting duct in the kidney.[111] The immunolabeling pattern of AQP6 strongly suggests preferential localization of this water channel in type-A intercalated cells.[112–114] AQP6 has low water permeability and acts primarily as an anion transporter.[115] The anion permeability of AQP6 is increased by exposure to low pH. Furthermore, ion conductance across the membrane is increased by the mercuric ion, Hg^{2+}, which is a well-known water channel inhibitor. AQP6 is expressed in acid-secreting type-A intercalated cells and co-localizes with V-type H^+-ATPase, therefore, AQP6 is suggested to function to promote urinary acid secretion.

Intracellular vesicles are acidified by V-type H^+-ATPase activity in acid-secreting intercalated cells, and anion conductance must be ensured in acidic vesicles to maintain electroneutrality within them. Chloride channel protein 5 (ClC-5) is co-localized with V-type H^+-ATPase,[116] however, ClC-5 is inhibited at pH <6.5.[117] AQP6 conductance seems to be switched on at low pH, when ClC-5 is switched off, which suggests that ClC-5 might function at an early stage of acidification and AQP6 at a later stage.[112]

Liu *et al.*[118] showed that a single amino acid substitution at Asn60 for Gly60 switches the function of AQP6 from that of an anion channel to that of a water-selective channel. This evidence provides a critical advance toward understanding the structural basis of AQP6 anion permeability.

AQP7

AQP7 is classified as an aquaglyceroporin, because it effects the transport of glycerol as well as water.[4] *Aqp7*-null mice show adipocyte hypertrophy, which resulted from defective glycerol exit and consequent accumulation of glycerol and triglycerides inside the cells.[119] In the kidney, AQP7 is localized in the brush border of proximal straight tubules (S3 segment).[120]

We reported that *Aqp7* knockout mice showed marked glyceroluria,[121] which indicates that glycerol can be reabsorbed through AQP7 in the proximal straight tubule, and that there might be no other glycerol reabsorbing system to compensate for this defect in the distal nephron segment.

The amount of glycerol re-absorbed via AQP7 is small compared with glycerol dietary intake.[4] However, in fasting *Aqp7* knockout mice, blood glucose levels are decreased, which indicates that glycerol loss via urine excretion might affect the plasma glucose levels.[4] One explanation for this observation is that reabsorbed glycerol might be converted to glucose in the proximal tubule or that glycerol transport to the liver may be important for glucose metabolism.

In *Aqp7* knockout mice, water permeability of the brush border membrane vesicles was slightly but significantly lower than that in wildtype mice,[121] which suggests that AQP7 makes a small contribution to the water permeability of the proximal straight tubules. This contribution has been estimated to be one-eighth that of AQP1.[122] As expected from the small decrease in water permeability, *Aqp7* knockout mice did not show a urine concentrating defect.[121]

The case of a man with a point mutation in AQP7 has been reported. However, no renal symptoms were mentioned, although he did have defective glycerol metabolism.[123]

AQP11

AQP11 and AQP12 are the most recently identified members of the AQP family.[124,125] In rats, they share a similar genomic structure as they have three exons. This arrangement makes them distinct from other AQPs—AQP0, AQP1, AQP2, AQP4, AQP5 and AQP6 have four exons and AQP3, AQP7, AQP8, AQP9 and AQP10 have six exons. Furthermore, AQP11 and AQP12 have atypical NPA boxes (see above),[126] which suggests that

the structure and function of their pores are unique. These two water channels have, therefore, been classified as superaquaporins.

Northern blot analysis of rat tissues revealed that AQP11 is widely expressed in various tissues. The highest expression is found in the testis; this protein is also moderately expressed in the kidney, liver, intestine, and brain.[124,127] AQP12 is expressed only in the pancreas.[125] Interestingly, neither protein is localized in the plasma membrane, but both are located in the intracellular space,[125,127] presumably in the ER.

Aqp11 knockout mice are born healthy but grow poorly and die before weaning because of uremia caused by polycystic kidney disease.[127] The renal cysts originate in the proximal tubule. Interestingly, proximal tubular cells of *Aqp11* knockout mice were unusually swollen and contained multiple giant vacuoles 1 week after birth and before the cysts formed. Ribosomes were attached to some of these vacuoles, suggesting that the vacuoles originated from the ER. In addition, immunofluorescence microscopy also showed that the vacuoles probably originated from the ER, confirming the hypothesis that AQP11 might be localized at the ER. Similar vacuoles were also observed in other organs outside the kidney, such as hepatocytes around the portal area and intestinal epithelial cells at the tip of villi, although they neither developed cysts nor other abnormalities.

Conclusions

In the past several years, considerable progress has been achieved in understanding the regulation and pathophysiological roles of aquaporins, although the precise mechanisms by which these water channels are regulated have not been thoroughly clarified. Understanding of these mechanisms is, however, necessary to develop therapies for conditions resulting from aquaporin dysfunction. Several investigative approaches with high spatiotemporal resolution have become available to cell biology investigation and enable to assess molecular dynamics in a small area of live cells. The molecular mechanisms of aquaporin regulation will be uncovered by application of these approaches. At the same time, animal models, such as aquaporin knockout mice, can be used to confirm the pathophysiological mechanisms hypothesized on the basis of cell biology research and to develop therapeutics to treat conditions arising from the dysregulation of aquaporin activity. Continued studies at both the molecular level and the whole organism level will enable to gain molecular insight in the pathophysiology of water balance homeostasis and will lead to the development of therapeutic approaches that have the potential to treat water balance disorders including NDI, heart failure, hepatic cirrhosis and hypertension.

Review criteria

PubMed was searched for full-length articles in English published up to 31 July 2009, with no set earliest date of publication, using the keywords "aquaporin", "AQP" and "nephrogenic diabetes insipidus".

1. Preston, G. M., Carroll, T. P, Guggino, W. B. & Agre, P. Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. *Science* **256**, 385–387 (1992).
2. Nielsen, S. *et al.* Aquaporins in the kidney: from molecules to medicine. *Physiol. Rev.* **82**, 205–244 (2002).
3. Ishibashi, K., Hara, S. & Kondo, S. Aquaporin water channels in mammals. *Clin. Exp. Nephrol.* **13**, 107–117 (2009).
4. Rojek, A., Praetorius, J., Frøkiaer, J., Nielsen, S. & Fenton, R. A. A current view of the mammalian aquaglyceroporins. *Annu. Rev. Physiol.* **70**, 301–327 (2008).
5. Verkman, A. S. Aquaporins: translating bench research to human disease. *J. Exp. Biol.* **212**, 1707–1715 (2009).
6. Brown, D., Breton, S., Ausiello, D. A. & Marshansky, V. Sensing, signaling and sorting events in kidney epithelial cell physiology. *Traffic* **10**, 275–284 (2009).
7. Hoffert, J. D., Chou, C. L. & Knepper, M. A. Aquaporin-2 in the "-omics" era. *J. Biol. Chem.* **284**, 14683–14687 (2009).
8. Boone, M. & Deen, P. M. Physiology and pathophysiology of the vasopressin-regulated renal water reabsorption. *Pflugers Arch.* **456**, 1005–1024 (2008).
9. Noda, Y. & Sasaki, S. Regulation of aquaporin-2 trafficking and its binding protein complex. *Biochim. Biophys. Acta* **1758**, 1117–1125 (2006).
10. Takata, K., Matsuzaki, T., Tajika, Y., Ablimit. A. & Hasegawa, T. Localization and trafficking of aquaporin 2 in the kidney. *Histochem. Cell Biol.* **130**, 197–209 (2008).
11. Kamsteeg, E. J., Heijnen, I., van Os, C. H. & Deen, P. M. The subcellular localization of an aquaporin-2 tetramer depends on the stoichiometry of phosphorylated and nonphosphorylated monomers. *J. Cell Biol.* **151**, 919–930 (2000).
12. Schenk, A. D. *et al.* The 4.5 A structure of human AQP2. *J. Mol. Biol.* **350**, 278–289 (2005).
13. Klussmann, E., Maric, K., Wiesner, B., Beyermann, M. & Rosenthal, W. Protein kinase A anchoring proteins are required for vasopressin-mediated translocation of aquaporin-2 into cell membranes of renal principal cells. *J. Biol. Chem.* **274**, 4934–4938 (1999).
14. Henn, V. *et al.* Identification of a novel A-kinase anchoring protein 18 isoform and evidence for its role in the vasopressin-induced aquaporin-2 shuttle in renal principal cells. *J. Biol. Chem.* **279**, 26654–26665 (2004).
15. Okutsu, R. *et al.* AKAP220 colocalizes with AQP2 in the inner medullary collecting ducts. *Kidney Int.* **74**, 1429–1433 (2008).
16. Procino, G. *et al.* Ser-256 phosphorylation dynamics of aquaporin 2 during maturation from the endoplasmic reticulum to the vesicular compartment in renal cells. *FASEB J.* **17**, 1886–1888 (2003).
17. van Balkom, B. W. *et al.* The role of putative phosphorylation sites in the targeting and shuttling of the aquaporin-2 water channel. *J. Biol. Chem.* **277**, 41473–41479 (2002).
18. Bouley, R. *et al.* Nitric oxide and atrial natriuretic factor stimulate cGMP-dependent membrane insertion of aquaporin 2 in renal epithelial cells. *J. Clin. Invest.* **106**, 1115–1126 (2000).
19. Bouley, R. *et al.* Stimulation of AQP2 membrane insertion in renal epithelial cells *in vitro* and *in vivo* by the cGMP phosphodiesterase inhibitor sildenafil citrate (Viagra). *Am. J. Physiol. Renal Physiol.* **288**, F1103–F1112 (2005).
20. Fenton, R. A. *et al.* Acute regulation of aquaporin-2 phosphorylation at Ser-264 by vasopressin. *Proc. Natl Acad. Sci. USA* **105**, 3134–3139 (2008).
21. Hoffert, J. D. *et al.* Dynamics of aquaporin-2 serine-261 phosphorylation in response to short-term vasopressin treatment in collecting duct. *Am. J. Physiol. Renal Physiol.* **292**, F691–F700 (2007).
22. Lu, H. J. *et al.* The phosphorylation state of serine 256 is dominant over that of serine 261 in the regulation of AQP2 trafficking in renal epithelial cells. *Am. J. Physiol. Renal Physiol.* **295**, F290–F294 (2008).
23. Moeller, H. B., Knepper, M. A. & Fenton, R. A. Serine 269 phosphorylated aquaporin-2 is targeted to the apical membrane of collecting duct principal cells. *Kidney Int.* **75**, 295–303 (2009).
24. Moeller, H. B., MacAulay, N., Knepper, M. A. & Fenton, R. A. Role of multiple phosphorylation sites in the COOH-terminal tail of aquaporin-2 for water transport: evidence against channel gating. *Am. J. Physiol. Renal Physiol.* **296**, F649–F657 (2009).

25. Balasubramanian, L., Sham, J. S. & Yip, K. P. Calcium signaling in vasopressin-induced aquaporin-2 trafficking. *Pflugers Arch.* **456**, 747–754 (2008).
26. Noda, Y., Horikawa, S., Katayama, Y. & Sasaki, S. Identification of a multiprotein "motor" complex binding to water channel aquaporin-2. *Biochem. Biophys. Res. Commun.* **330**, 1041–1047 (2005).
27. Lorenz, D. *et al.* Cyclic AMP is sufficient for triggering the exocytic recruitment of aquaporin-2 in renal epithelial cells. *EMBO Rep.* **4**, 88–93 (2003).
28. Procino, G. *et al.* Extracellular calcium antagonizes forskolin-induced aquaporin 2 trafficking in collecting duct cells. *Kidney Int.* **66**, 2245–2255 (2004).
29. Nejsum, L. N., Zelenina, M., Aperia, A., Frøkiaer, J. & Nielsen, S. Bidirectional regulation of AQP2 trafficking and recycling: involvement of AQP2–S256 phosphorylation. *Am. J. Physiol. Renal Physiol.* **288**, F930–F938 (2005).
30. de Seigneux, S. *et al.* Long-term aldosterone treatment induces decreased apical but increased basolateral expression of AQP2 in CCD of rat kidney. *Am. J. Physiol. Renal Physiol.* **293**, F87–F99 (2007).
31. Tamma, G. *et al.* Rho inhibits cAMP-induced translocation of aquaporin-2 into the apical membrane of renal cells. *Am. J. Physiol. Renal Physiol.* **281**, F1092–F1101 (2001).
32. Tamma, G. *et al.* The prostaglandin E2 analogue sulprostone antagonizes vasopressin-induced antidiuresis through activation of Rho. *J. Cell Sci.* **116**, 3285–3294 (2003).
33. Tamma, G., Carmosino, M., Svelto, M. & Valenti, G. Bradykinin signaling counteracts cAMP-elicited aquaporin 2 translocation in renal cells. *J. Am. Soc. Nephrol.* **16**, 2881–2889 (2005).
34. Noda, Y. *et al.* Aquaporin-2 trafficking is regulated by PDZ-domain containing protein SPA-1. *FEBS Lett.* **568**, 139–145 (2004).
35. Harazaki, M. *et al.* Specific recruitment of SPA-1 to the immunological synapse: involvement of actin-bundling protein actinin. *Immunol. Lett.* **92**, 221–226 (2004).
36. Kometani, K. *et al.* Role of SPA-1 in phenotypes of chronic myelogenous leukemia induced by BCR-ABL-expressing hematopoietic progenitors in a mouse model. *Cancer Res.* **66**, 9967–9976 (2006).
37. Kuwahara, M. *et al.* Three families with autosomal dominant nephrogenic diabetes insipidus caused by aquaporin-2 mutations in the C-terminus. *Am. J. Hum. Genet.* **69**, 738–748 (2001).
38. Kuwahara, M., Asai, T., Terada, Y. & Sasaki, S. The C-terminal tail of aquaporin-2 determines apical trafficking. *Kidney Int.* **68**, 1999–2009 (2005).
39. Noda, Y., Horikawa, S., Katayama, Y. & Sasaki, S. Water channel aquaporin-2 directly binds to actin. *Biochem. Biophys. Res. Commun.* **322**, 740–745 (2004).
40. Noda, Y. *et al.* Reciprocal interaction with G-actin and tropomyosin is essential for aquaporin-2 trafficking. *J. Cell Biol.* **182**, 587–601 (2008).
41. Noda, Y. & Sasaki, S. The role of actin remodeling in the trafficking of intracellular vesicles, transporters, and channels: focusing on aquaporin-2. *Pflugers Arch.* **456**, 737–745 (2008).
42. Noda, Y. & Sasaki, S. Actin-binding channels. *Prog. Brain Res.* **170**, 551–557 (2008).
43. Tajika, Y. *et al.* Differential regulation of AQP2 trafficking in endosomes by microtubules and actin filaments. *Histochem. Cell Biol.* **124**, 1–12 (2005).
44. Chou, C. L. *et al.* Non-muscle myosin II and myosin light chain kinase are downstream targets for vasopressin signaling in the renal collecting duct. *J. Biol. Chem.* **279**, 49026–49035 (2004).
45. Nedvetsky, P. I. *et al.* A role of myosin Vb and Rab11-FIP2 in the aquaporin-2 shuttle. *Traffic* **8**, 110–123 (2007).
46. Procino, G. *et al.* AQP2 exocytosis in the renal collecting duct – involvement of SNARE isoforms and the regulatory role of Munc18b. *J. Cell Sci.* **121**, 2097–2106 (2008).
47. Lu, H. A. *et al.* Heat shock protein 70 interacts with aquaporin-2 and regulates its trafficking. *J. Biol. Chem.* **282**, 28721–28732 (2007).
48. Kamsteeg, E. J. *et al.* MAL decreases the internalization of the aquaporin-2 water channel. *Proc. Natl Acad. Sci. USA* **104**, 16696–16701 (2007).
49. Klumperman, J. & Deen, P. M. Short-chain ubiquitination mediates the regulated endocytosis of the aquaporin-2 water channel. *Proc. Natl Acad. Sci. USA* **103**, 18344–18349 (2006).
50. van Balkom, B. W. *et al.* LIP5 Interacts with aquaporin 2 and facilitates its lysosomal degradation. *J. Am. Soc. Nephrol.* **20**, 990–1001 (2009).
51. Kasono, K. *et al.* Hypertonicity regulates the aquaporin-2 promoter independently of arginine vasopressin. *Nephrol. Dial. Transplant.* **20**, 509–515 (2005).
52. Saito, T. *et al.* Hypotonicity reduces the activity of murine aquaporin-2 promoter induced by dibutyryl cAMP. *Exp. Physiol.* **93**, 1147–1156 (2008).
53. Hasler, U. *et al.* Acute hypertonicity alters aquaporin-2 trafficking and induces a MAPK-dependent accumulation at the plasma membrane of renal epithelial cells. *J. Biol. Chem.* **283**, 26643–26661 (2008).
54. van Balkom, B. W. *et al.* Hypertonicity is involved in redirecting the aquaporin-2 water channel into the basolateral, instead of the apical, plasma membrane of renal epithelial cells. *J. Biol. Chem.* **278**, 1101–1107 (2003).
55. Okada, Y. *et al.* Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). *J. Physiol.* **532**, 3–16 (2001).
56. Tamma, G. *et al.* Hypotonicity induces aquaporin-2 internalization and cytosol-to-membrane translocation of ICln in renal cells. *Endocrinology* **148**, 1118–1130 (2007).
57. Li, Y. H. *et al.* Aquaporin-2 regulates cell volume recovery via tropomyosin. *Int. J. Biochem. Cell Biol.* **41**, 2466–2476 (2009).
58. Loonen, A. J., Knoers, N. V., van Os, C. H. & Deen, P. M. Aquaporin 2 mutations in nephrogenic diabetes insipidus. *Semin. Nephrol.* **28**, 252–265 (2008).
59. Savelkoul, P. J. *et al.* p.R254Q mutation in the aquaporin-2 water channel causing dominant nephrogenic diabetes insipidus is due to a lack of arginine vasopressin-induced phosphorylation. *Hum. Mutat.* **30**, E891–E903 (2009).
60. de Mattia, F. *et al.* Lack of arginine vasopressin-induced phosphorylation of aquaporin-2 mutant AQP2–R254L explains dominant nephrogenic diabetes insipidus. *J. Am. Soc. Nephrol.* **16**, 2872–2880 (2005).
61. Kamsteeg, E. J. *et al.* Missorting of the Aquaporin-2 mutant E258K to multivesicular bodies/lysosomes in dominant NDI is associated with its monoubiquitination and increased phosphorylation by PKC but is due to the loss of E258. *Pflugers Arch.* **455**, 1041–1054 (2008).
62. Asai, T. *et al.* Pathogenesis of nephrogenic diabetes insipidus by aquaporin-2 C-terminus mutations. *Kidney Int.* **64**, 2–10 (2003).
63. Sohara, E. *et al.* Pathogenesis and treatment of autosomal-dominant nephrogenic diabetes insipidus caused by an aquaporin 2 mutation. *Proc. Natl Acad. Sci. USA* **103**, 14217–14222 (2006).
64. Grünfeld, J. P. & Rossier, B. C. Lithium nephrotoxicity revisited. *Nat. Rev. Nephrol.* **5**, 270–276 (2009).
65. Rao, R. *et al.* Lithium treatment inhibits renal GSK-3 activity and promotes cyclooxygenase 2-dependent polyuria. *Am. J. Physiol. Renal Physiol.* **288**, F642–F649 (2005).
66. Nielsen, J. *et al.* Proteomic analysis of lithium-induced nephrogenic diabetes insipidus: mechanisms for aquaporin 2 down-regulation and cellular proliferation. *Proc. Natl Acad. Sci. USA* **105**, 3634–3639 (2008).
67. Li, C., Wang, W., Knepper, M. A., Nielsen, S. & Frøkiaer, J. Downregulation of renal aquaporins in response to unilateral ureteral obstruction. *Am. J. Physiol. Renal Physiol.* **284**, F1066–F1079 (2003).
68. Mouri, T. *et al.* Acute and chronic metabolic acidosis interferes with aquaporin-2 translocation in the rat kidney collecting ducts. *Hypertens. Res.* **32**, 358–363 (2009).
69. Robben, J. H., Knoers, N. V. & Deen, P. M. Cell biological aspects of the vasopressin type-2 receptor and aquaporin 2 water channel in nephrogenic diabetes insipidus. *Am. J. Physiol. Renal Physiol.* **291**, F257–F270 (2006).
70. Kim, G. H. *et al.* Antidiuretic effect of hydrochlorothiazide in lithium-induced nephrogenic diabetes insipidus is associated with upregulation of aquaporin-2, Na-Cl co-transporter, and epithelial sodium channel. *J. Am. Soc. Nephrol.* **15**, 2836–2843 (2004).
71. Li, J. H. *et al.* A selective EP4 PGE2 receptor agonist alleviates disease in a new mouse model of X-linked nephrogenic diabetes insipidus. *J. Clin. Invest.* **119**, 3115–3126 (2009).
72. Suga, H. *et al.* Novel treatment for lithium-induced nephrogenic diabetes insipidus rat model using the Sendai-virus vector carrying aquaporin 2 gene. *Endocrinology* **149**, 5803–5810 (2008).
73. Szatalowicz, V. L. *et al.* Radioimmunoassay of plasma arginine vasopressin in hyponatremic patients with congestive heart failure. *N. Engl. J. Med.* **305**, 263–266 (1981).
74. Schrier, R. W. Vasopressin and aquaporin 2 in clinical disorders of water homeostasis. *Semin. Nephrol.* **28**, 289–296 (2008).
75. Nielsen, S. *et al.* Congestive heart failure in rats is associated with increased expression and targeting of aquaporin-2 water channel in collecting duct. *Proc. Natl Acad. Sci. USA* **94**, 5450–5455 (1997).
76. Xu, D. L. *et al.* Upregulation of aquaporin-2 water channel expression in chronic heart failure. *J. Clin. Invest.* **99**, 1500–1505 (1997).
77. Gheorghiade, M. *et al.* Effects of tolvaptan, a vasopressin antagonist, in patients hospitalized with worsening heart failure: a randomized controlled trial. *JAMA* **291**, 1963–1971 (2004).
78. Schrier, R. W. *et al.*; SALT Investigators. Tolvaptan, a selective oral vasopressin V2-receptor antagonist, for hyponatremia. *N. Engl. J. Med.* **355**, 2099–2112 (2006).
79. Abraham, W. T., Shamshirsaz, A. A., McFann, K., Oren, R. M. & Schrier, R. W. Aquaretic effect of lixivaptan, an oral non-peptide, selective V2

receptor vasopressin antagonist, in the New York Heart Association functional class II and III chronic heart failure patients. *J. Am. Coll. Cardiol.* **47**, 1615–1621 (2006).
80. Gheorghiade, M. *et al.* Short-term clinical effects of tolvaptan, an oral vasopressin antagonist, in patients hospitalized for heart failure: the EVEREST Clinical Status Trials. *JAMA* **297**, 1332–1343 (2007).
81. Asahina, Y. *et al.* Increased gene expression of water channel in cirrhotic rat kidneys. *Hepatology* **21**, 169–173 (1995).
82. Gerbes, A. L. *et al.* Therapy of hyponatremia in cirrhosis with a vasopressin receptor antagonist: a randomized double-blind multicenter trial. *Gastroenterology* **124**, 933–939 (2003).
83. Ohara, M. *et al.* Upregulation of aquaporin 2 water channel expression in pregnant rats. *J. Clin. Invest.* **101**, 1076–1083 (1998).
84. Chapman, A. B. *et al.* Temporal relationships between hormonal and hemodynamic changes in early human pregnancy. *Kidney Int.* **54**, 2056–2063 (1998).
85. Apostol, E. *et al.* Reduced renal medullary water channel expression in puromycin aminonucleoside–induced nephrotic syndrome. *J. Am. Soc. Nephrol.* **8**, 15–24 (1997).
86. Bohlin, A. B. & Berg, U. Renal water handling in minimal change nephrotic syndrome. *Int. J. Pediat. Nephrol.* **5**, 93–98 (1984).
87. Fernández-Llama, P. *et al.* Concentrating defect in experimental nephrotic syndrome: altered expression of aquaporins and thick ascending limb Na+ transporters. *Kidney Int.* **54**, 170–179 (1998).
88. Ishikawa, S. E., Saito, T., Saito, T., Kasono, K. & Funayama, H. Pathophysiological role of aquaporin-2 in impaired water excretion. *Prog. Brain Res.* **170**, 581–588 (2008).
89. Saito, T. *et al.* Acute aquaresis by the nonpeptide arginine vasopressin (AVP) antagonist OPC-31260 improves hyponatremia in patients with syndrome of inappropriate secretion of antidiuretic hormone (SIADH). *J. Clin. Endocrinol. Metab.* **82**, 1054–1057 (1997).
90. Kazama, I. *et al.* BSC1 inhibition complements effects of vasopressin V2 receptor antagonist on hyponatremia in SIADH rats. *Kidney Int.* **67**, 1855–1867 (2005).
91. Kanno, K. *et al.* Urinary excretion of aquaporin-2 in patients with diabetes insipidus. *N. Engl. J. Med.* **332**, 1540–1545 (1995).
92. Pisitkun, T., Shen, R. F. & Knepper, M. A. Identification and proteomic profiling of exosomes in human urine. *Proc. Natl Acad. Sci. USA* **101**, 13368–13373 (2004).
93. Martin, P. Y. *et al.* Selective V2-receptor vasopressin antagonism decreases urinary aquaporin-2 excretion in patients with chronic heart failure. *J. Am. Soc. Nephrol.* **10**, 2165–2170 (1999).
94. Ivarsen, P. *et al.* Increased urinary excretion of aquaporin 2 in patients with liver cirrhosis. *Gut* **52**, 1194–1199 (2003).
95. Buemi, M. *et al.* Urinary excretion of aquaporin-2 water channel during pregnancy. *Cell. Physiol. Biochem.* **11**, 203–208 (2001).
96. Ishikawa, S. *et al.* Close association of urinary excretion of aquaporin-2 with appropriate and inappropriate arginine vasopressin-dependent antidiuresis in hyponatremia in elderly subjects. *J. Clin. Endocrinol. Metab.* **86**, 1665–1671 (2001).
97. Verkman, A. S. Dissecting the roles of aquaporins in renal pathophysiology using transgenic mice. *Semin. Nephrol.* **28**, 217–226 (2008).
98. Pallone, T. L., Edwards, A., Ma, T., Silldorff, E. P. & Verkman, A. S. Requirement of aquaporin-1 for NaCl-driven water transport across descending vasa recta. *J. Clin. Invest.* **105**, 215–222 (2000).
99. King, L. S., Choi, M., Fernandez, P. C., Cartron, J. P. & Agre, P. Defective urinary-concentrating ability due to a complete deficiency of aquaporin-1. *N. Engl. J. Med.* **345**, 175–179 (2001).
100. Schrier, R. W. & Cadnapaphornchai, M. A. Renal aquaporin water channels: from molecules to human disease. *Prog. Biophys. Mol. Biol.* **81**, 117–131 (2003).
101. Saadoun, S., Papadopoulos, M. C., Hara-Chikuma, M. & Verkman, A. S. Impairment of angiogenesis and cell migration by targeted aquaporin-1 gene disruption. *Nature* **434**, 786–792 (2005).
102. Hara-Chikuma, M. & Verkman, A. S. Aquaporin-1 facilitates epithelial cell migration in kidney proximal tubule. *J. Am. Soc. Nephrol.* **17**, 39–45 (2006).
103. Wang, W. *et al.* Role of AQP1 in endotoxemia-induced acute kidney injury. *Am. J. Physiol. Renal Physiol.* **294**, F1473–F1480 (2008).
104. Kwon, T. H. *et al.* Regulation of collecting duct AQP3 expression: response to mineralocorticoid. *Am. J. Physiol. Renal Physiol.* **283**, F1403–F1421 (2002).
105. Roudier, N. *et al.* AQP3 deficiency in humans and the molecular basis of a novel blood group system, GIL. *J. Biol. Chem.* **277**, 45854–45859 (2002).
106. Yang, B., van Hoek, A. N. & Verkman, A. S. Very high single channel water permeability of aquaporin-4 in baculovirus-infected insect cells and liposomes reconstituted with purified aquaporin-4. *Biochemistry* **36**, 7625–7632 (1997).
107. Zelenina, M., Zelenin, S., Bondar, A. A., Brismar, H. & Aperia, A. Water permeability of aquaporin-4 is decreased by protein kinase C and dopamine. *Am. J. Physiol. Renal Physiol.* **283**, F309–F318 (2002).
108. van Hoek, A. N. *et al.* Vasopressin-induced differential stimulation of AQP4 splice variants regulates the in-membrane assembly of orthogonal arrays. *Am. J. Physiol. Renal Physiol.* **296**, F1396–F1404 (2009).
109. Hiroaki, Y. *et al.* Implications of the aquaporin-4 structure on array formation and cell adhesion. *J. Mol. Biol.* **355**, 628–639 (2006).
110. Ho, J. D. *et al.* Crystal structure of human aquaporin 4 at 1.8 A and its mechanism of conductance. *Proc. Natl Acad. Sci. USA* **106**, 7437–7442 (2009).
111. Yasui, M. pH regulated anion permeability of aquaporin-6. *Handb. Exp. Pharmacol.* **190**, 299–308 (2009).
112. Yasui, M. *et al.* Rapid gating and anion permeability of an intracellular aquaporin. *Nature* **402**, 184–187 (1999).
113. Yasui, M., Kwon, T. H., Knepper, M. A., Nielsen, S. & Agre, P. Aquaporin-6: an intracellular vesicle water channel protein in renal epithelia. *Proc. Natl Acad. Sci. USA* **96**, 5808–5813 (1999).
114. Ohshiro, K. *et al.* Expression and immunolocalization of AQP6 in intercalated cells of the rat kidney collecting duct. *Arch. Histol. Cytol.* **64**, 329–338 (2001).
115. Ikeda, M. *et al.* Characterization of aquaporin-6 as a nitrate channel in mammalian cells. Requirement of pore-lining residue threonine 63. *J. Biol. Chem.* **277**, 39873–39879 (2002).
116. Günther, W., Lüchow, A., Cluzeaud, F., Vandewalle, A. & Jentsch, T. J. CIC-5, the chloride channel mutated in Dent's disease, colocalizes with the proton pump in endocytotically active kidney cells. *Proc. Natl Acad. Sci. USA* **95**, 8075–8080 (1998).
117. Friedrich, T., Breiderhoff, T. & Jentsch, T. J. Mutational analysis demonstrates that CIC-4 and CIC-5 directly mediate plasma membrane currents. *J. Biol. Chem.* **274**, 896–902 (1999).
118. Liu, K. *et al.* Conversion of aquaporin 6 from an anion channel to a water-selective channel by a single amino acid substitution. *Proc. Natl Acad. Sci. USA* **102**, 2192–2197 (2005).
119. Hara-Chikuma, M. *et al.* Progressive adipocyte hypertrophy in aquaporin-7-deficient mice: adipocyte glycerol permeability as a novel regulator of fat accumulation. *J. Biol. Chem.* **280**, 15493–15496 (2005).
120. Nejsum, L. N. *et al.* Localization of aquaporin-7 in rat and mouse kidney using RT-PCR, immunoblotting, and immunocytochemistry. *Biochem. Biophys. Res. Commun.* **277**, 164–170 (2000).
121. Sohara, E. *et al.* Defective water and glycerol transport in the proximal tubules of AQP7 knockout mice. *Am. J. Physiol. Renal Physiol.* **289**, F1195–F1200 (2005).
122. Sohara, E., Rai, T., Sasaki, S. & Uchida, S. Physiological roles of AQP7 in the kidney: lessons from AQP7 knockout mice. *Biochim. Biophys. Acta* **1758**, 1106–1110 (2006).
123. Kondo, H. *et al.* Human aquaporin adipose (AQPap) gene. Genomic structure, promoter analysis and functional mutation. *Eur. J. Biochem.* **269**, 1814–1826 (2002).
124. Morishita, Y., Sakube, Y., Sasaki, S. & Ishibashi, K. Molecular mechanisms and drug development in aquaporin water channel diseases: aquaporin superfamily (superaquaporins): expansion of aquaporins restricted to multicellular organisms. *J. Pharmacol. Sci.* **96**, 276–279 (2004).
125. Itoh, T. *et al.* Identification of a novel aquaporin, AQP12, expressed in pancreatic acinar cells. *Biochem. Biophys. Res. Commun.* **330**, 832–838 (2005).
126. Ishibashi, K. Aquaporin subfamily with unusual NPA boxes. *Biochim. Biophys. Acta* **1758**, 989–993 (2006).
127. Morishita, Y. *et al.* Disruption of aquaporin-11 produces polycystic kidneys following vacuolization of the proximal tubule. *Mol. Cell. Biol.* **25**, 7770–7779 (2005).

Acknowledgments
Our research work is supported in part by Grant-in-Aid for Creative Scientific Research from the Japan Society for the Promotion of Science (17GS0312).

皮膚臨床 52（11）特：50；1552～1555，2010

2 掌蹠多汗症

3）ボツリヌス毒素注射

伊東慶子＊　玉田康彦＊　大嶋雄一郎＊　柳下武士＊　渡辺大輔＊

1 はじめに

掌蹠多汗症の主な治療法として，塩化アルミニウム溶液の外用療法，抗コリン剤などの内服療法，多汗部位を水道水に浸し，直流ないし交流電流を流すイオントフォレーシス，A型ボツリヌス毒素（BT-A）の局所注射が皮膚科領域では一般的である。胸腔鏡下胸部交感神経遮断術（endoscopic thoracic sympathectomy，以下 ETS）はほぼ完全に手掌の発汗を止めることができ，その効果の持続性や永続性が利点であることから上記の治療に満足できない重症例に適応となる。

2 有用性の報告

ボツリヌス菌毒素は，グラム陽性菌の *Clostridium botulinum* が産生する神経毒素で A～G 型の7種がある。このなかで A 型ボツリヌス毒素（BT-A）は精製度が高く，効力や作用時間が最も優れており，コリン作動性神経の接合膜からのアセチルコリン放出を抑制する作用がある[1]。BT-A は Botox®（Allergan Inc.）と Dysport®（Ipsen）が日本では主に使われており，両者の力価について，Botox® は Dysport® の 1.5～4 倍とされている[2)3)]。

BT-A は以前から眼瞼痙攣や斜視の治療に使用されているが，1996 年 Bushara らは BT-A が腋窩多汗症に有効であることをはじめて報告し[4]，その後，掌蹠多汗症へも応用されるようになった。

今までの報告によると，Schnider らは 11 例の片手に Dysport® 120 U，もう一方に生理食塩水を投与した比較試験で，3 カ月後も Dysport® 群で発汗低下を観察している[5]（レベルⅡ）。また 19 例に対して片手に Botox® 100 U，反対の手には生理食塩水を注射したところ，28 日後 Botox® を投与した手掌はすべての症例で発汗低下が認められ，両群とも握力低下などはみられていない[6]。また Solomon と Hayman は，20 例の難治性の手掌多汗症に Botox® を投与したところ 19 例に著しい発汗低下がみられ，4～9 カ月も効果が持続したと記載し[7]，Bodokh は片方の手掌に Botox® を注射し，もう片方をコントロールにして Minor 法で発汗量を観察したところ，15/20（75%）の患者で注射部位の発汗量が低下したとしている[8]。

本邦でも，村上ら[9]が BT-A を補助療法として用いることにより，5 例の局所多汗症患者で QOL が改善したとし，BT-A は手掌多汗症に有用と推察される。

3 適応

塩化アルミニウム溶液の外用療法，抗コリン剤などの内服療法は簡便であるが重症例には対処できないこともある。水道水イオントフォレーシスは効果的であるが，頻回施行の不便さ，治療時に枚か 20～30 分を要することや刺激感，水疱形成などの副作用がみられることもあり，また通電するため心疾患患者には禁忌であるなどの制約がある。これらの治療法に抵抗性の場合に BT-A の局所注射が行われているが，保存的治療と外科的治療の中間的な位置づけとされており，その利点は

＊ Keiko ITO, Yasuhiko TAMADA, Yuichiro OHSHIMA, Takeshi YANAGISHITA & Daisuke WATANABE, 愛知医科大学，皮膚科学教室（玉田康彦教授，主任：渡辺大輔教授）
［別刷請求先］伊東慶子：愛知医科大学皮膚科（〒480-1195 愛知県愛知郡長久手町岩作雁又 21）
キーワード：掌蹠多汗症，A 型ボツリヌス毒素，ボトックス，発汗量，BT-A 90 単位

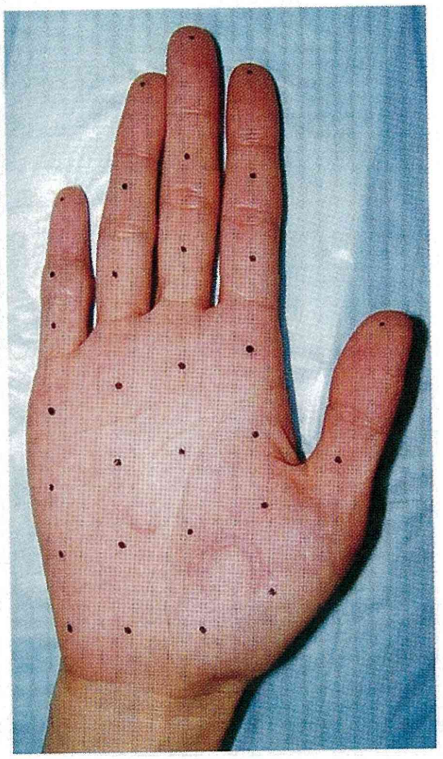


図1　BT-A の注射部位：2 単位ずつ 30 カ所に計 60 単位を局注する。（文献 10）より引用）

従来の保存的治療に比べ，効果の持続時間が長く，ETS で時々みられる代償性発汗がほとんどないことであろう。

4 治療の実際・治療成績

我々は，手掌多汗症患者 27 名の片側にのみ Botox® を 2 単位ずつ 30 カ所に計 60 単位を皮内注射し（**図 1**），もう一方をコントロールとして換気カプセル法およびヨードデンプンひまし油法にて経時的に発汗量を計測した。Botox® を投与した手掌では発汗量の低下がみられ（**図 2-a**，**b**），約 6 カ月間にわたって持続した。換気カプセル法で安静時の発汗量を測定し 1 mg/cm^2/min 以上群，それ未満の群に分けて比較検討したところ，1 mg/cm^2/min 以上群では Botox® 治療後も発汗量の減少が少なく推移していた（**図 3**）[10]。

その後，発汗量が 2 mg/cm^2/min 以上群では Botox® 60 単位の投与では発汗量の減少が少なく満足のいく効果を得られないため，90 単位に増量して約 7 カ月間にわたって観察したところ，観察期間中発汗量の低下が持続していた。さらに，発汗量 2.0～2.5 mg/cm^2/min の群と 2.5 mg/cm^2/

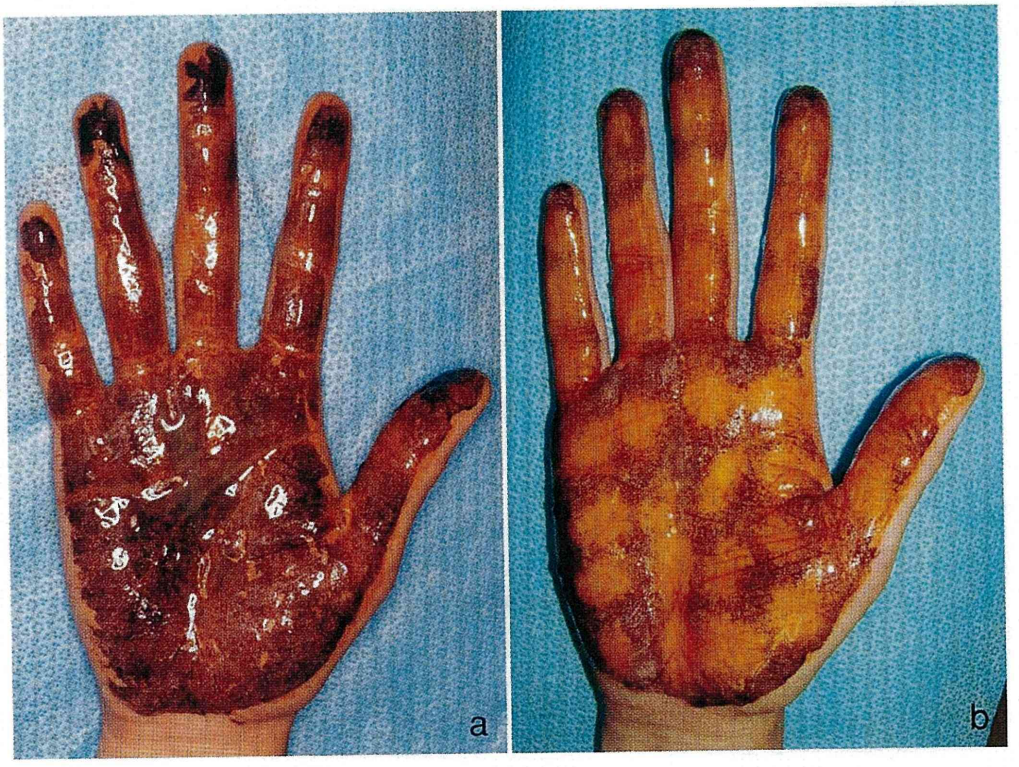
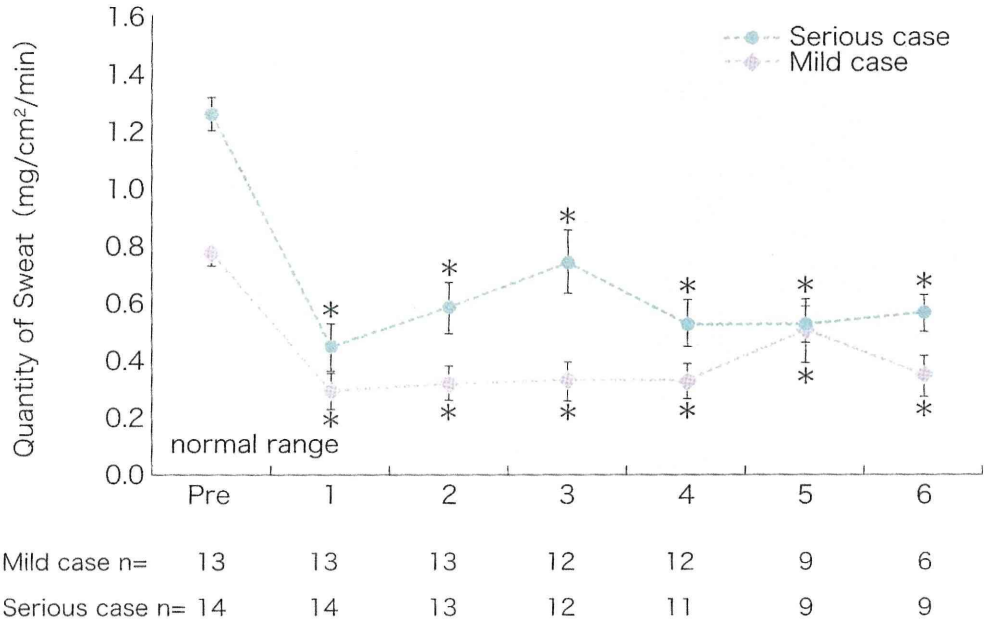
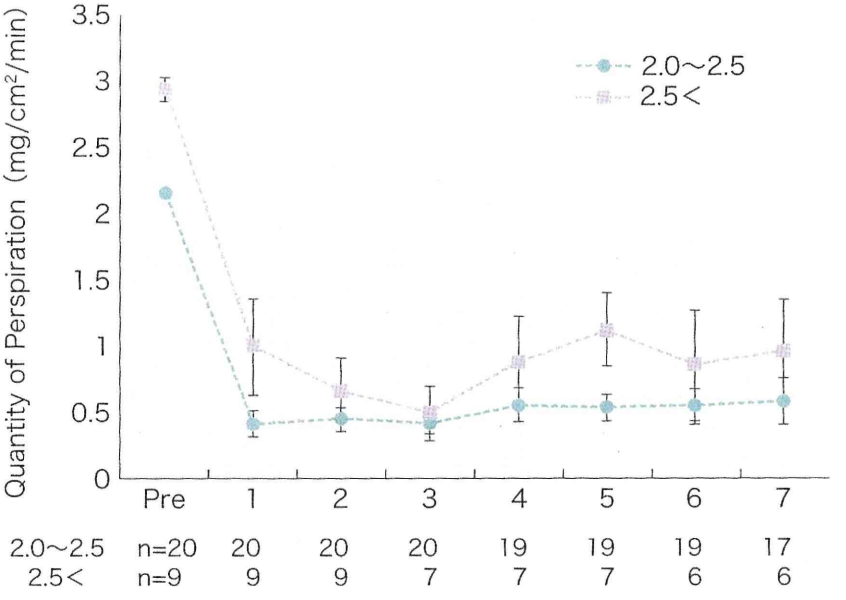


図 2　BT-A の注射前後の比較（Minor 法）（文献 10）より引用）
a：注射前，手掌全体に発汗がみられる。
b：注射 1 カ月後，注射部に一致して発汗が抑制されている。



	Pre	1	2	3	4	5	6
Mild case n=	13	13	13	12	12	9	6
Serious case n=	14	14	13	12	11	9	9

図3 Serious case and Mild case 発汗量の推移：BT-A を 60 単位投与し，投与後 1〜6 カ月間発汗量の低下を認めた。Serious case では BT-A 治療後も発汗量の減少が少なく推移していた。（文献 10）より引用）
統計学的有意差 Repeated measures ANOVA と Dunnett 検定を行った。
*$p<0.01$



		Pre	1	2	3	4	5	6	7
2.0〜2.5	n=20	20	20	20	19	19	19	17
2.5<	n=9	9	9	7	7	7	6	6

図4 2.0〜2.5 mg/cm²/min 群と 2.5 mg/cm²/min 以上群の発汗量の推移：BT-A を 90 単位投与し，投与後 1〜7 カ月間発汗量の低下を認めた。2.5 mg/cm²/min 以上群では BT-A 治療後も発汗量の減少が少なく推移していた。

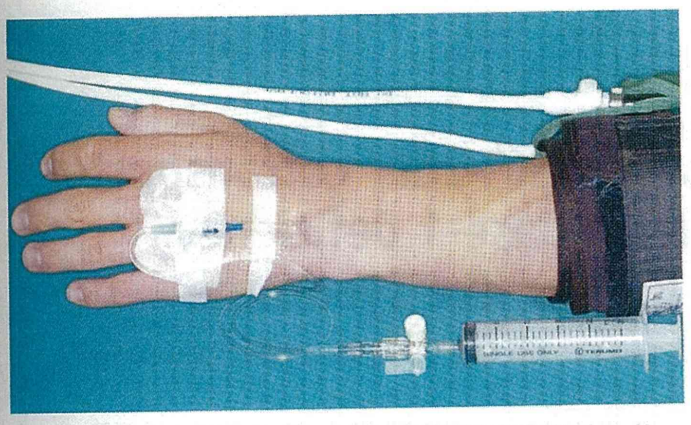


図5　Bier's Block を用いた局所静脈内麻酔法：片方の手背より 22 G でルートを確保しダブルターニケットを前腕末梢側に巻く。同腕を垂直に高く上げ脱血し 230～250 mmHg で加圧後腕を水平に下ろし静脈用 Xylocaine2％を 100 mg ゆっくりと投与する。

min 以上の群で分けて比較検討したところ，2.5 mg/cm^2/min 以上の群では有効期間が少し短縮していた（図 4）。

5 利点

手掌多汗症に対しては，BT-A 1 回の局所投与で 6～7 カ月の発汗量の減少を期待できる。しかし重症度に応じた投与量の調整が必要であろう。

6 欠点

BT-A 療法の問題点として注射時の疼痛，手の筋力低下，BT-A に対する中和抗体の産生などが挙げられる。注射時の痛みのコントロールのため注射前に局所麻酔薬の外用，アイスパックでの冷却，麻酔薬の静脈注射（Bier's block）[11]（図 5），末梢神経ブロック[12]が行われている。手指の筋力低下は投与量に比例するが，重症化することはなく一過性で経過観察のみで軽快することが多い[6]。中和抗体の産生されることはまれであるが，注射間隔を 2～3 カ月以上あけることで回避できる。

文　献

1）Rusciani L et al：J Drugs Dermatol, **1**：147-151, 2002
2）Brin MF：Muscle Nerve suppl, **6**：s146-168, 1997
3）Shimonetta M M et al：Br J Dermatol, **149**：1041-1045, 2003
4）Bushara KO et al：Clin Exp Derm, **21**：276-278, 1996
5）Schnider P et al：Br J Derm, **136**：548-552, 1997
6）Lowe NJ et al：Dermatol Surg, **28**：822-827, 2002
7）Solomon BA, Hayman R：J Am Acad Dermatol, **42**：1026-1029, 2000
8）Bodokh I：Ann Dermatol Venereol, **130**：561-564, 2003
9）村上義之，高梨真教：西日皮膚，**66**：53-59，2004
10）Yamashita N et al：J Dermatol, **35**：325-329, 2008
11）Blaheta JH et al：Dermatol Surg, **28**：666-672, 2002
12）Hayton M et al：Br J Dermatol, **149**：447-451, 2003

Impaired phosphorylation of Na^+-K^+-$2Cl^-$ cotransporter by oxidative stress-responsive kinase-1 deficiency manifests hypotension and Bartter-like syndrome

Shih-Hua Lin[a,b], I-Shing Yu[c], Si-Tse Jiang[d], Shu-Wha Lin[c], Pauling Chu[a], Ann Chen[b], Huey-Kang Sytwu[e], Eisei Sohara[f], Shinichi Uchida[f], Sei Sasaki[f], and Sung-Sen Yang[a,b,1]

[a]Division of Nephrology, Department of Medicine, Tri-Service General Hospital, [b]Graduate Institute of Medical Sciences, and [e]Graduate Institute of Microbiology and Immunology, National Defense Medical Center, Taipei 114, Taiwan; [c]Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University, Taipei 100, Taiwan; [d]National Laboratory Animal Centre, National Applied Research Laboratories, Taipei 115, Taiwan; and [f]Department of Nephrology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo 113-8519, Japan

Edited by Maurice B. Burg, National Heart, Lung, and Blood Institute, Bethesda, MD, and approved September 12, 2011 (received for review May 11, 2011)

Na^+-K^+-$2Cl^-$ cotransporters (NKCCs), including NKCC1 and renal-specific NKCC2, and the Na^+-Cl^- cotransporter (NCC) play pivotal roles in the regulation of blood pressure (BP) and renal NaCl reabsorption. Oxidative stress-responsive kinase-1 (OSR1) is a known upstream regulator of N(K)CCs. We generated and analyzed global and kidney tubule-specific (KSP) OSR1 KO mice to elucidate the physiological role of OSR1 in vivo, particularly on BP and kidney function. Although global $OSR1^{-/-}$ mice were embryonically lethal, $OSR1^{+/-}$ mice had low BP associated with reduced phosphorylated (p) STE20 (sterile 20)/SPS1-related proline/alanine-rich kinase (SPAK) and p-NKCC1 abundance in aortic tissue and attenuated p-NKCC2 abundance with increased total and p-NCC expression in the kidney. KSP-$OSR1^{-/-}$ mice had normal BP and hypercalciuria and maintained significant hypokalemia on a low-K^+ diet. KSP-$OSR1^{-/-}$ mice exhibited impaired Na^+ reabsorption in the thick ascending loop on a low-Na^+ diet accompanied by remarkably decreased expression of p-NKCC2 and a blunted response to furosemide, an NKCC2 inhibitor. The expression of total SPAK and p-SPAK was significantly increased in parallel to that of total NCC and p-NCC despite unchanged total NKCC2 expression. These results suggest that, globally, OSR1 is involved in the regulation of BP and renal tubular Na^+ reabsorption mainly via the activation of NKCC1 and NKCC2. In the kidneys, NKCC2 but not NCC is the main target of OSR1 and the reduced p-NKCC2 in KSP-$OSR1^{-/-}$ mice may lead to a Bartter-like syndrome.

Bartter syndrome | electrolytes | hormone | knockout mice | volume

Recent studies have shown that Na^+-K^+-$2Cl^-$ cotransporters (NKCCs) and the Na^+-Cl^- cotransporter (NCC) play very important roles in the regulation of blood pressure (BP) and extracellular volume. NKCCs consist of ubiquitous NKCC1 and renal-specific NKCC2. NKCC1 can modulate BP through vascular and renal effects (1–4). NKCC2 and NCC are two renal Na^+ cotransporters expressed in the thick ascending limbs (TALs) and distal convoluted tubules (DCTs) of the kidney, respectively, accounting for 20% and 10% of filtered Na^+ reabsorption (5). In human essential hypertension and salt-sensitive or spontaneously hypertensive animal models, activation of NKCC1 and NKCC2 has also been reported to play a pivotal role in the pathogenesis of hypertension (6, 7). In addition, activation of NCC by gene mutations in *WNK1* and *WNK4* leads to an autosomal dominant salt-sensitive hypertension known as pseudohypoaldosteronism type II (PAHII) (8). On the other hand, loss-of-function mutations in the *SLC12A1* and *SLC12A3* genes encoding NKCC2 and NCC can lead to renal salt-wasting hypotension with hypokalemic metabolic alkalosis, known as Bartter syndrome (BS) (9) and Gitelman syndrome (GS) (10), respectively.

In vitro studies have shown that posttranscriptional phosphorylation of NKCC1/2 and NCC plays a crucial role in the regulation of normal transport activity. Oxidative stress-responsive kinase-1 (OSR1) (11) and STE20 (sterile 20)/SPS1-related proline/alanine-rich kinase (SPAK) (12), two downstream substrates of With-No-Lysine kinase (WNK) 1/4, are the upstream phosphorylators of NKCC1/2 and NCC. Threonine or serine residues in their N-terminal conserved domains (T206/96, T211/101, and T224/114 in mouse NKCC1/2; T53, T58 and S71 in mouse NCC) are the phosphorylation sites of OSR1 and SPAK. The docking site on the conserved C-terminal domains of OSR1/SPAK interacts with the RFXV/I motif on the N terminus of NKCC/NCC and then increases NKCC/NCC phosphorylation and function (13–16). We have also reported that increased phosphorylated (p) OSR1/SPAK abundance can enhance p-NCC expression in the PHAII-causing *Wnk4* D561A knock-in mice (17), whereas the reverse is true in the *Wnk4* hypomorphic knockout (KO) mice (18). These findings support that OSR1 and SPAK are important regulators of NKCC and NCC in vivo.

Because OSR1 and SPAK share high homology in their catalytical and regulatory domains and their expression in tissues often overlaps, the creation and analysis of distinct OSR1 or SPAK KO mice is warranted to tease apart the role of each kinase in vivo. For this purpose, we first generated SPAK KO mice and found that $SPAK^{+/-}$ mice exhibited hypotension with decreased p-NKCC1 abundance in aortic tissues and $SPAK^{-/-}$ mice presented a GS phenotype caused by reduced total and p-NCC expression (19). In the present study, we generated global and kidney tubule-specific (KSP) OSR1 KO mice to elucidate the physiological role of OSR1 in vivo (*SI Text* and Figs. S1 and S2). Results to be reported indicate that global $OSR1^{-/-}$ mice were embryonically lethal and $OSR1^{+/-}$ mice had low BP associated with reduced p-SPAK expression and p-NKCC1 abundance in aortic tissue and attenuated p-NKCC2 abundance with increased total and p-NCC expression in the kidney. KSP-$OSR1^{-/-}$ mice manifested Bartter-like syndrome because of impaired NKCC2 phosphorylation and function in the TAL with a compensatory increase in NCC phosphorylation and expression. This study provides in vivo evidence that OSR1 is primarily involved in the regulation of BP and renal tubular Na^+ reabsorption via the phosphorylation of NKCC1 and NKCC2 but not NCC.

Results

Phenotype in Global $OSR1^{+/-}$ and KSP-$OSR1^{-/-}$ Mice. First, we examined BP and electrolyte homeostasis in the global $OSR1^{+/-}$ and KSP-$OSR1^{-/-}$ mice on a normal diet (0.4% Na^+ wt/wt, 1% K^+ wt/wt). Compared with WT littermates, the global $OSR1^{+/-}$ mice had relative hypotension ($P < 0.05$) without serum and urine

Author contributions: S.-H.L. and S.-S.Y. designed research; I.-S.Y. and S.-S.Y. performed research; I.-S.Y., S.-T.J., and S.-W.L. contributed new reagents/analytic tools; S.-H.L., P.C., A.C., H.-K.S., E.S., S.U., S.S., and S.-S.Y. analyzed data; and S.-H.L. and S.-S.Y. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: sungsenyang@hotmail.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1107452108/-/DCSupplemental.

Table 1. BP and blood and urine biochemistry values in global OSR1$^{+/-}$ mice

Diet	Normal		Low Na^+	
Genotype (*n*)	WT (*n* = 12)	$OSR^{+/-}$ (*n* = 12)	WT (*n* = 8)	$OSR^{+/-}$ (*n* = 8)
BP, mmHg				
Systolic	111 ± 6	99 ± 5*	108 ± 7	95 ± 6*
Diastolic	58 ± 4	49 ± 8*	54 ± 4	51 ± 11
Mean	74 ± 4	64 ± 7*	71 ± 5	62 ± 7*
Weight, g	22.8 ± 2.2	21.6 ± 2.3	23.6 ± 3.5	22.1 ± 4.3
Plasma				
PAC, pg/mL	932 ± 413	835 ± 212	1,325 ± 313	1,138 ± 323
PRA, ng·mL·h	6.4 ± 3.0	6.5 ± 2.9	12.2 ± 2.5	10.3 ± 3.5
Na^+, mmol/L	157 ± 3	156 ± 2	153 ± 6	154 ± 5
K^+, mmol/L	4.3 ± 0.3	4.1 ± 0.3	4.2 ± 0.4	4.3 ± 0.4
Cl^-, mmol/L	115 ± 2	113 ± 4	115 ± 2	113 ± 4
Ca^{2+}, mg/dL	9.5 ± 0.2	9.6 ± 0.4	9.3 ± 0.5	9.4 ± 0.6
Mg^{2+}, mg/dL	2.9 ± 0.1	3.1 ± 0.2	3.0 ± 0.3	3.1 ± 0.4
Cr, mg/dL	0.13 ± 0.05	0.12 ± 0.04	0.11 ± 0.08	0.11 ± 0.06
Urine, mL/d	1.77 ± 0.82	1.86 ± 0.73	1.25 ± 0.63	1.23 ± 0.65
Na^+, μmol/d	220 ± 56	262 ± 65	53 ± 8	50 ± 10
K^+, μmol/d	435 ± 133	520 ± 108	514 ± 155	539 ± 178
Cl^-, μmol/d	256 ± 85	306 ± 85	80 ± 22	84 ± 14
Mg^{2+}, mg/d	0.47 ± 0.11	0.50 ± 0.13	0.53 ± 0.21	0.48 ± 0.22
FE_{Na}, %	0.65 ± 0.24	0.68 ± 0.37	0.25 ± 0.09	0.23 ± 0.11
FE_K, %	18.3 ± 6.1	20.2 ± 8.6	20.6 ± 8.2	22.1 ± 9.4
FE_{Cl}, %	0.57 ± 0.12	0.61 ± 0.19	0.21 ± 0.04	0.16 ± 0.05
FE_{Mg}, %	10.5 ± 2.3	10.6 ± 2.5	10.5 ± 2.3	10.6 ± 2.5
Ca/Cr, mg/mg	0.17 ± 0.05	0.19 ± 0.08	0.19 ± 0.10	0.18 ± 0.07

Cr, creatinine; FE_{Na}, FE_K, FE_{Cl}, and FE_{Mg} represent the fractional excretion of Na^+, K^+, Cl^-, and Mg^{2+}, respectively.
*$P < 0.05$ vs. WT on the same diet.

Table 2. BP and blood and urine biochemistry values in KSP-OSR1$^{-/-}$ mice

Diet	Normal		Low Na^+	
Genotype (*n*)	WT (*n* = 10)	KSP-$OSR^{-/-}$ (*n* = 10)	WT (*n* = 8)	KSP-$OSR^{-/-}$ (*n* = 8)
BP, mmHg				
Systolic	110 ± 3	107 ± 4	109 ± 2	101 ± 6*
Diastolic	57 ± 4	60 ± 7	51 ± 4	46 ± 9
Mean	75 ± 2.3	74 ± 6	69 ± 3	62 ± 7
Weight, g	23.6 ± 3.5	22.8 ± 4.3	22.1 ± 4.5	23.2 ± 5.3
Plasma				
PAC, pg/mL	999 ± 329	867 ± 216	1,433 ± 265	1,289 ± 332
PRA, ng·mL·h	8.7 ± 3.5	7.9 ± 4.2	11.4 ± 3.5	12.3 ± 4.2
Na^+, mmol/L	154 ± 3	153 ± 2	152 ± 6	150 ± 5
K^+, mmol/L	4.2 ± 0.2	3.7 ± 0.2*	4.3 ± 0.3	3.6 ± 0.3*
Cl^-, mmol/L	114 ± 3	112 ± 4	114 ± 3	112 ± 4
Ca^{2+}, mg/dL	9.8 ± 0.3	9.7 ± 0.2	9.8 ± 0.3	9.7 ± 0.2
Mg^{2+}, mg/dL	2.9 ± 0.2	3.0 ± 0.1	2.8 ± 0.3	3.0 ± 0.3
Cr, mg/dL	0.14 ± 0.06	0.13 ± 0.05	0.13 ± 0.05	0.12 ± 0.08
Urine, mL/d	1.92 ± 0.80	2.68 ± 0.21*	1.54 ± 0.80	2.13 ± 0.68*
Na^+, μmol/d	232 ± 72	250 ± 55	93 ± 17	103 ± 26
K^+, μmol/d	311 ± 122	375 ± 110	489 ± 222	518 ± 110
Cl^-, μmol/d	203 ± 84	247 ± 78	65 ± 34	72 ± 24
Mg^{2+}, mg/d	0.60 ± 0.29	0.56 ± 0.14	0.58 ± 0.21	0.55 ± 0.15
FE_{Na}, %	0.55 ± 0.14	0.58 ± 0.27	0.22 ± 0.06	0.24 ± 0.12
FE_K, %	20.1 ± 4.1	29.5 ± 4.5*	22.1 ± 6.2	32.8 ± 6.5*
FE_{Cl}, %	0.52 ± 0.14	0.54 ± 0.16	0.20 ± 0.04	0.22 ± 0.05
FE_{Mg}, %	11.4 ± 2.1	10.1 ± 1.1	10.4 ± 3.1	9.1 ± 2.5
Ca/Cr, mg/mg	0.16 ± 0.03	0.21 ± 0.04*	0.18 ± 0.02	0.23 ± 0.05*

Cr, creatinine; FE_{Na}, FE_K, FE_{Cl}, and FE_{Mg} represent the fractional excretion of Na^+, K^+, Cl^-, and Mg^{2+}, respectively.
*$P < 0.05$ vs. WT on the same diet

electrolyte abnormalities (Table 1). The KSP-OSR1$^{-/-}$ mice had normal BP; however, unlike the global OSR1$^{+/-}$ mice, they showed significant hypokalemia with an increased fractional excretion of K^+ (FE_K) ($P < 0.05$) and hypercalciuria ($P < 0.05$) (Table 2). In addition, the ambient osmolarity of spot urine was significantly reduced in KSP-OSR1$^{-/-}$ mice (1,805 ± 389 vs. 2,414 ± 525 mOsm/L in WT, $n = 10$; $P < 0.05$). When the global OSR1$^{+/-}$ and KSP-OSR1$^{-/-}$ mice were fed a low-Na^+ diet (0.05% Na^+ wt/wt), the

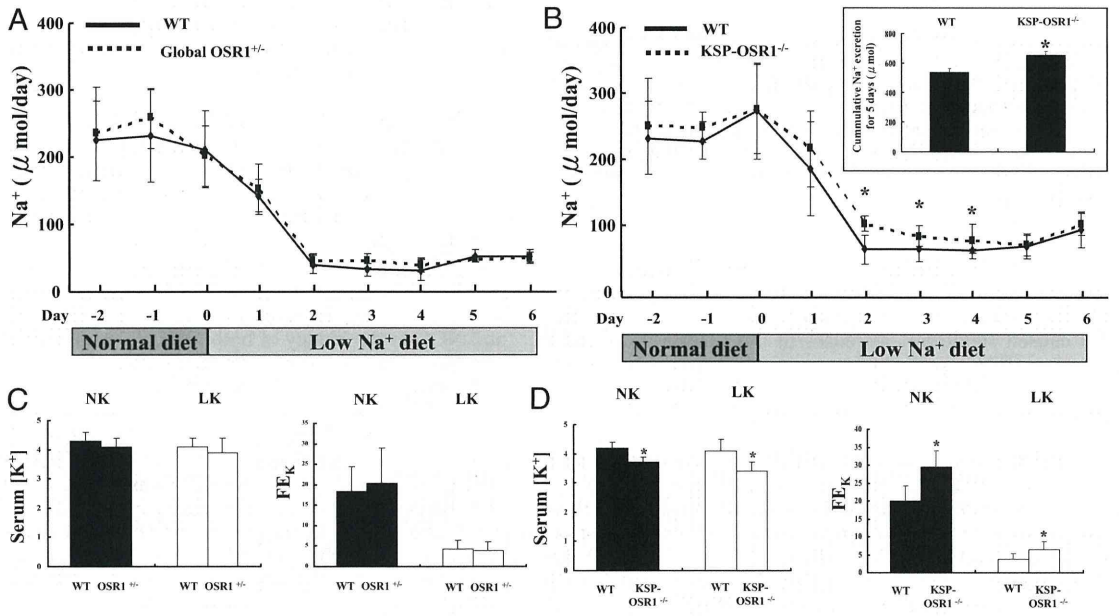


Fig. 1. Na^+ balance and renal K^+ handling in OSR1$^{+/-}$ and KSP-OSR1$^{-/-}$ mice. The daily urine Na^+ excretion rate in WT and global OSR1$^{+/-}$ (*A*) or KSP-OSR1$^{-/-}$ (*B*) mice on a normal-Na^+ diet for 3 d and then on a low-Na^+ diet for 6 d was determined. (*B*, *Inset*) Cumulative Na^+ excretion from day 1 to day 5 of the low-Na^+ diet between WT and KSP-OSR1$^{-/-}$ mice. *$P < 0.05$ vs. WT ($n = 6$ per group). Serum K^+ concentration and urinary FE_K in WT, OSR1$^{+/-}$ (*C*), and KSP-OSR1$^{-/-}$ (*D*) mice on a normal-K^+ (NK) diet and a low-K^+ (LK) diet. *$P < 0.05$ vs. WT ($n = 8$ per group).

PHYSIOLOGY

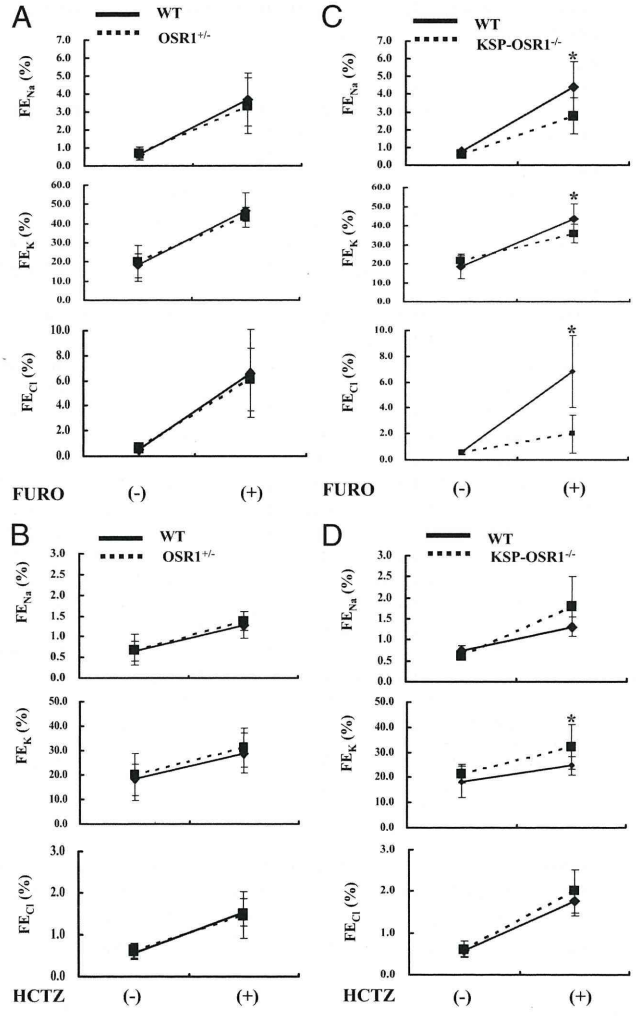


Fig. 2. Effect of furosemide (FURO) and HCTZ administration on Na^+, K^+, and Cl^- excretion. FE_{Na}, FE_K, and FE_{Cl} represent the fractional excretion of Na^+, K^+, and Cl^-, respectively. Preserved response of FE_{Na}, FE_K, and FE_{Cl} in OSR1$^{+/-}$ mice after FURO (*A*) and HCTZ (*B*) treatment is shown. *$P < 0.05$ vs. WT ($n = 6$ per group). There was a blunted response of FE_{Na}, FE_K, and FE_{Cl} in KSP-OSR1$^{-/-}$ mice after FURO treatment (*C*) but a similar response of FE_{Na}, FE_K, and FE_{Cl} between WT and KSP-OSR1$^{-/-}$ mice after HCTZ (*D*) treatment. *$P <$ 0.05 vs. WT ($n = 6$ per group).

severity of hypotension did not increase in OSR1$^{+/-}$ mice, whereas the previously normotensive KSP-OSR1$^{-/-}$ mice developed relative systolic hypotension ($P < 0.05$ for systolic BP only). A low-Na^+ diet also caused significant increases in the plasma aldosterone concentration (PAC) and plasma renin activity (PRA) in both global OSR1$^{+/-}$ and KSP-OSR1$^{-/-}$ mice, which were not significantly different from their WT littermates (Tables 1 and 2).

Renal Na^+ and K^+ Handling in Global OSR1$^{+/-}$ and KSP-OSR1$^{-/-}$ Mice. We further evaluated renal Na^+ and K^+ handling in the global OSR1$^{+/-}$ and KSP-OSR1$^{-/-}$ mice. On normal Na^+ diets, all these different groups of mice had similar urine Na^+ excretion rates and fractional excretion of Na^+ (FE_{Na}) (Tables 1 and 2). On paired-fed low-Na^+ diets, the urinary Na^+ excretion quickly dropped in all three groups (WT, global OSR1$^{+/-}$, and KSP-OSR1$^{-/-}$) of mice. There was no significant difference in the degree of reduced urine Na^+ excretion between the global OSR1$^{+/-}$ and WT littermates (Fig. 1*A*). However, KSP-OSR1$^{-/-}$ mice clearly demonstrated more urine Na^+ excretion than WT controls on the second, third, and fourth days (Fig. 1*B*), supporting the presence of a renal tubular defect in KSP-OSR1$^{-/-}$ mice. Compared with WT controls, the average increased Na^+ excretion was 115 ± 7 μmol per mouse for the 6 d of the low-Na^+ diet in the KSP-OSR1$^{-/-}$ mice (Fig. 1*B*).

We then evaluated K^+ handling in the mice on a low-K^+ diet (0.3% K^+ wt/wt) for 6 d. WT and OSR1$^{+/-}$ mice showed no significant difference in serum K^+ concentration [3.9 ± 0.5 mmol/L in OSR1$^{+/-}$ ($n = 8$) vs. 4.1 ± 0.3 mmol/L in WT ($n = 8$); $P = 0.48$] and urinary FE_K [$4.0 \pm 2.1\%$ in OSR1$^{+/-}$ ($n = 8$) vs. $4.3 \pm 2.1\%$ in WT ($n = 8$); $P = 0.61$] (Fig. 1*C*). The KSP-OSR1$^{-/-}$ mice, which already had hypokalemia on a regular diet, showed more pronounced hypokalemia [3.5 ± 0.3 mmol/L in KSP-OSR1$^{-/-}$ ($n = 8$) vs. 4.1 ± 0.4 mmol/L in WT ($n = 8$); $P < 0.05$] and urinary FE_K [$6.5 \pm 2.2\%$ in KSP-OSR1$^{-/-}$ ($n = 8$) vs. $3.8 \pm 1.5\%$ in WT ($n = 8$); $P < 0.05$] (Fig. 1*D*) after a low-K^+ diet.

Diuretic Response in Global OSR1$^{+/-}$ and KSP-OSR1$^{-/-}$ Mice. To determine the function of NKCC2 and NCC, two substrates of OSR1, in these global OSR1$^{+/-}$ and KSP-OSR1$^{-/-}$ mice, we administered the NKCC2 inhibitor furosemide and NCC inhibitor hydrochlorothiazide (HCTZ), respectively. Like WT mice, global OSR1$^{+/-}$ mice showed a dramatic and similar increase in the FE_{Na}, FE_K, and FE_{Cl} in response to furosemide (Fig. 2*A*) and HCTZ (Fig. 2*B*), suggesting that their NKCC2 and NCC functions were not obviously affected. Compared with WT and global OSR1$^{+/-}$ mice, KSP-OSR1$^{-/-}$ mice exhibited a blunted response to furosemide (Fig. 2*C*) but a normal response to HCTZ (Fig. 2*D*), indicating that their NKCC2 function was reduced and NCC function was well preserved (Fig. 2*D*).

Expression of OSR1, SPAK, and NKCC1 in Aortic Tissues of Global OSR1$^{+/-}$ Mice. Because OSR1, SPAK, and NKCC1 are coexpressed in vascular smooth muscle and NKCC1 activity is known to play an important role in the regulation of aortic contractility and BP (1, 2), we examined whether the OSR1/SPAK-NKCC1 pathway could be involved in the hypotension of global OSR1$^{+/-}$ mice. Relative protein expression of total OSR1 ($59 \pm 22\%$; $P < 0.01$), p-OSR1 ($44 \pm 12\%$; $P < 0.01$), and p-SPAK ($54.6 \pm 2.6\%$; $P < 0.01$) was significantly reduced, along with dramatically reduced p-NKCC1 expression (T206) ($53 \pm 22\%$; $P < 0.01$) despite unchanged total SPAK and NKCC1 abundance in aortic tissue (Fig. 3). These findings suggested that defective phosphorylation of OSR1 may cause decreased NKCC1 phosphorylation in blood vessels, which led to the lower BP.

Renal OSR1, SPAK, NKCC2, and NCC in Global OSR1$^{+/-}$ and KSP-OSR1$^{-/-}$ Mice. In the kidneys, we had previously reported that OSR1 is mainly distributed in the TAL to downstream renal tubules and is dominantly expressed in the medulla, colocalizing with NKCC2 (19). Because KSP-OSR1$^{-/-}$ mice displayed a BS-like phenotype with hypercalciuria and normal magnesemia and a blunted response to furosemide corresponding to a TAL lesion rather than GS physiology (a DCT lesion with hypocalciuria and hypomagnesemia) (20), the expression of total and p-OSR1, SPAK, NKCC2, and NCC in the kidney of both OSR1$^{+/-}$ and KSP-OSR1$^{-/-}$ mice was further evaluated by semiquantitative immunoblotting (IB).

Although renal total OSR1 ($75 \pm 11\%$; $P < 0.05$) and p-OSR1 ($69 \pm 21\%$; $P < 0.05$) expression was reduced in OSR1$^{+/-}$ mice (Fig. 4*A*), p-SPAK ($128 \pm 11\%$; $P < 0.05$) was mildly increased (Fig. 4*B*). The expression of total NKCC2 was not affected, but p-NKCC2 (T96) ($72 \pm 15\%$; $P < 0.05$) was significantly reduced (Fig. 4*C*). Total NCC ($135 \pm 7\%$; $P < 0.05$), p-NCC (T58) $121 \pm 6\%$; $P <$ 0.05), and p-NCC (S71) ($156 \pm 8\%$; $P < 0.05$) were also significantly increased (Fig. 4*D* and Fig. S3*A*). As expected, total OSR1 and p-OSR1 were virtually absent from the kidney tissue of KSP-OSR1$^{-/-}$ mice (Fig. 5*A*). However, total SPAK ($130 \pm 13\%$; $P <$ 0.05) and p-SPAK ($138 \pm 13\%$; $P < 0.05$) (Fig. 5*B*) were markedly increased. As in OSR1$^{+/-}$ mice, expression of total NKCC2 was not affected but p-NKCC2 (T96) was more dramatically reduced ($32 \pm 14\%$; $P < 0.01$) (Fig. 5*C*). Total NCC ($158 \pm 9\%$; $P < 0.01$),

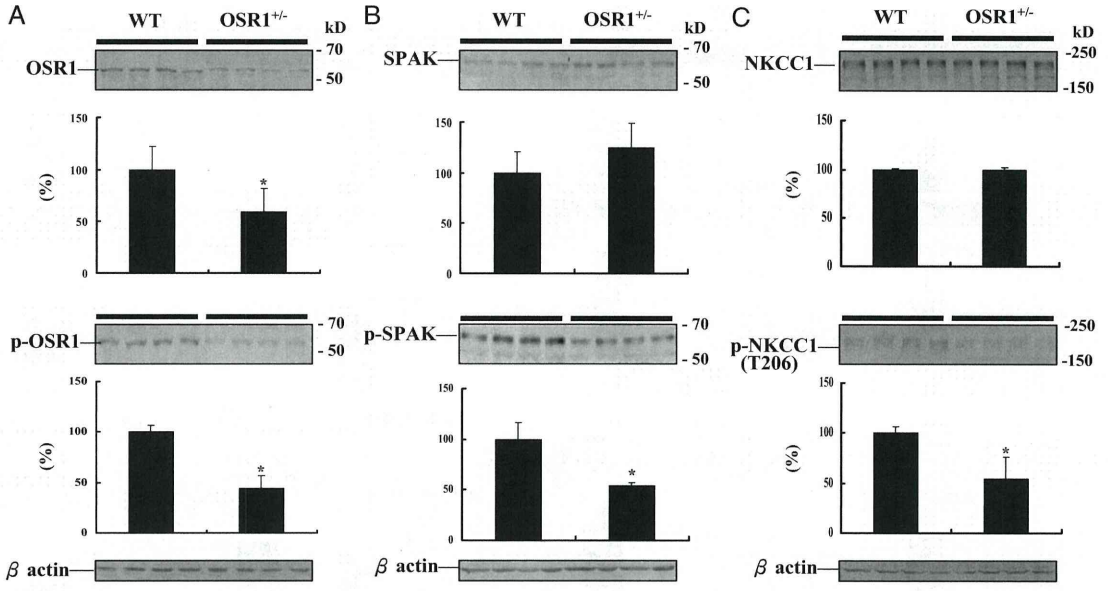


Fig. 3. Expression of OSR1, SPAK, and NKCC1 in aortic tissues of OSR1$^{+/-}$ mice. Semiquantitative IB (*Upper*) and densitometry (*Lower*) of total and p-OSR1 (*A*), total and p-SPAK (*B*), and total and p-NKCC1 (T206) (*C*) in aortic tissues of WT and OSR1$^{+/-}$ mice (n = 4 per group). *P < 0.05 vs. WT.

p-NCC (T53) (138 ± 11%; $P < 0.05$), p-NCC (T58) (127 ± 9%; $P < 0.05$), and p-NCC (S71) (145 ± 15%; $P < 0.05$) (Fig. 5*D* and Fig. S3*B*) were significantly increased in KSP-OSR1$^{-/-}$ mice.

We also examined the cellular localization of NKCC2 and NCC in the kidney tissue of OSR1$^{+/-}$ and KSP-OSR1$^{-/-}$ mice. The cellular distribution of total NKCC2 and p-NKCC2 (T96) was still luminally condensed, albeit less so for p-NKCC2 (T96) (Fig. S4 *A* and *B*). Likewise, total NCC and p-NCC were still luminally condensed (Fig. S4 *C* and *D*).

Discussion

In this study, we generated and analyzed global and KSP-OSR1 KO mice to elucidate the physiological role of OSR1 in vivo in the regulation of the NKCC1 and kidney-specific NKCC2 and NCC, focusing on BP and renal tubular Na^+ reabsorption. As previously reported in OSR1 gene-trapped or kinase-dead knock-in mice (21, 22), global homozygous OSR1$^{-/-}$ mice were embryonically lethal. Heterozygous OSR1$^{+/-}$ mice exhibited hypotension, markedly reduced p-NKCC1 abundance in aortic tissue, and attenuated p-NKCC2 in kidney. KSP-OSR1$^{-/-}$ mice recapitulated the reduced renal Na^+ reabsorption on low-Na^+ diets and remarkably decreased expression of p-NKCC2. They also had a blunted response to furosemide and a parallel increase in NCC expression and phosphorylation, supporting the notion that TAL function was defective. These results indicate that OSR1 is crucial not only in the regulation of BP but in renal tubular Na^+ reabsorption, primarily in the TAL rather than the DCT.

In the global OSR1$^{+/-}$ mice, markedly decreased p-NKCC1 in aortic tissue and kidneys may contribute to obvious hypotension. NKCC1, as a downstream target of OSR1, has been known to play a pivotal role in BP control through vascular and renal effects, as shown in NKCC1 KO mice (1–4). On the one hand, inactivation of NKCC1 in blood vessels causes reduced intracellular Cl^- concentration and, consequently, decreased Ca^{2+} influx through L-type Ca^{2+} channels, which may lead to vessel relaxation and hypotension (23). On the other hand, defective NKCC1 expression in the basolateral membrane of inner medullary collecting ducts and renin-producing juxtaglomerular (JG) cells (24) may cause the impairment of renal Na^+ reabsorption. However, hyperreninemia and hyperaldosteronism with increased renal Na^+ transporters, including NKCC2 and NCC observed in NKCC1 KO mice, could help minimize hypotension (3, 4).

Reminiscent of the reduced NKCC1 phosphorylation with reduced aortic contractility in SPAK$^{+/-}$ mice featuring hypotension but normal serum and urine electrolytes (19), the hypotension observed in global OSR1$^{+/-}$ mice reiterates the importance of OSR1/SPAK-NKCC1 phosphorylation signaling in the vascular tissue on BP control. Because both OSR1$^{+/-}$ and SPAK$^{+/-}$ mice had normal total NKCC1 but reduced p-NKCC1, it appeared that intact expression of both OSR1 and SPAK was required for adequate NKCC1 phosphorylation in the aortic tissue. Based on the attenuated rather than increased p-SPAK expression in the aortic tissue of OSR1$^{+/-}$ mice, SPAK phosphorylation may be dependent on the OSR1 activity in the vessels.

In addition to vascular NKCC1, kidney-specific NKCC2 and NCC, two other OSR1 substrates in the TAL and DCT, respectively (5), were also examined in global OSR1$^{+/-}$ mice. A significant decrease in p-NKCC2 and a parallel increase in both p-SPAK and p-NCC in the OSR1$^{+/-}$ mice strongly suggested that a salt-wasting phenotype was present. Based on the Guyton type renal function curve (a plot between mean arterial pressure and urinary Na^+ intake and excretion) (25), OSR1$^{+/-}$ mice showed a shift to the left, supporting a defect in renal Na^+ transport. Their renal tubule Na^+ defect was mild, however, because they did not exhibit negative renal Na^+ balance even on low-salt diets. Furthermore, their responses to furosemide and thiazide challenges were also normal. Nevertheless, hypotension in OSR1$^{+/-}$ mice might help dampen the tendency of impaired renal Na^+ reabsorption. In response to vascular hypotension or renal Na^+ wasting, one should expect an increase in PRA and PAC. However, PRA and PAC were similar between the WT and OSR1$^{+/-}$ mice, suggesting that PRA and PAC were inappropriately low in OSR1$^{+/-}$ mice. Perhaps the reduced p-NKCC2 observed in OSR1$^{+/-}$ mice may have blunted tubuloglomerular feedback, leading to the impaired release of renin from JG cells as shown in NKCC2 isoform KO mice (26, 27). However, we could not exclude the direct regulation of aldosterone secretion by OSR1, which was also abundantly expressed in adrenal tissues.

To focus on the specific role of OSR1 in the regulation of NKCC2 and NCC in the kidney, we further created the KSP-OSR1 KO mice. NKCC2 has three different full-length splice variants called A, B, and F isoforms (5). Inactivating mutations in NKCC2 can cause antenatal BS, an autosomal recessive renal Na^+-losing nephropathy with chronic hypokalemia and hypercalciuria with

PHYSIOLOGY

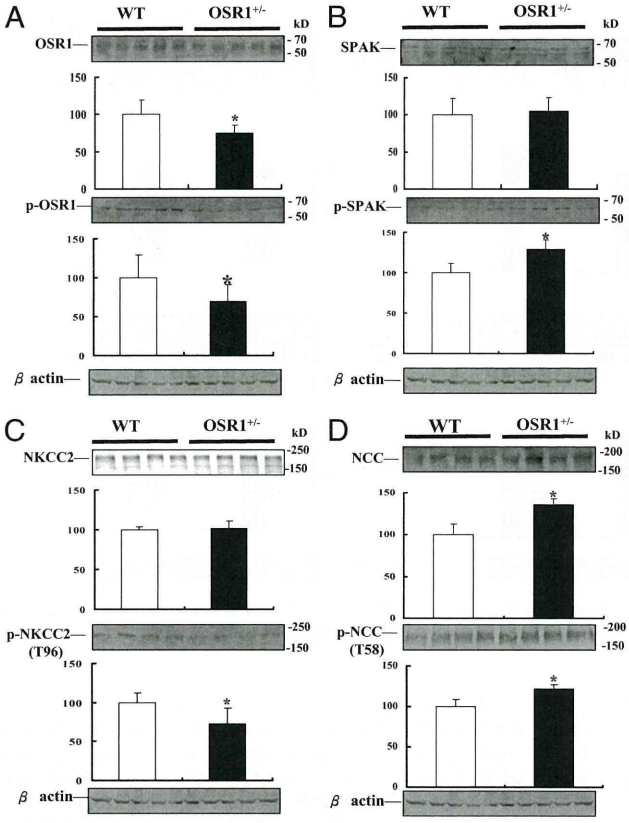


Fig. 4. Expression of OSR1, SPAK, NKCC2, and NCC in kidney tissues of OSR1$^{+/-}$ mice. Semiquantitative IB (*Upper*) and densitometry (*Lower*) of total and p-OSR1 (*A*), total and p-SPAK (*B*), total and p-NKCC2 (T96) (*C*), and total and p-NCC (T58) (*D*) in kidney tissues of WT and OSR1$^{+/-}$ mice (n = 4 per group). *P < 0.05 vs. WT.

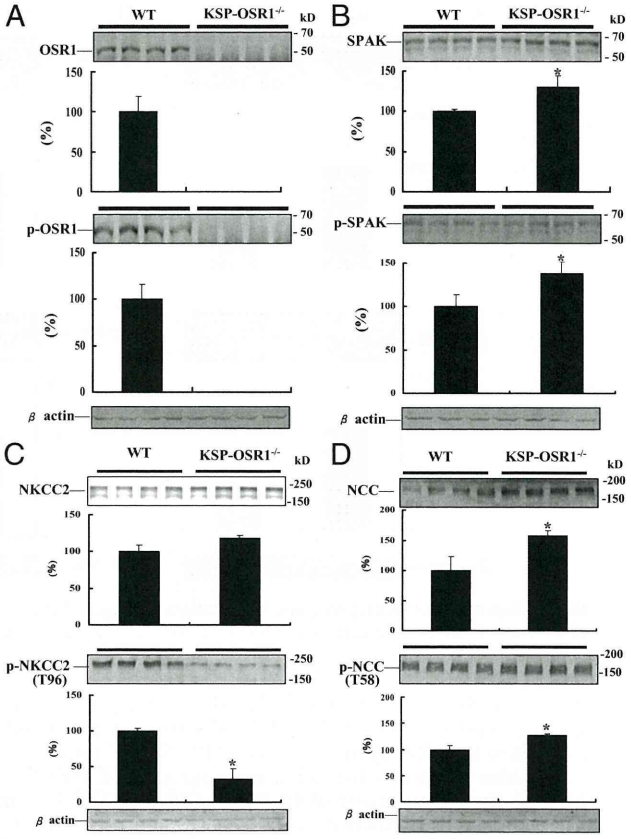


Fig. 5. Expression of OSR1, SPAK, NKCC2, and NCC in kidney tissues of KSP-OSR1$^{-/-}$ mice. Semiquantitative IB (*Upper*) and densitometry (*Lower*) of total and p-OSR1 (*A*), total and p-SPAK (*B*), total and p-NKCC2(T96) (*C*), and total and p-NCC (T58) (*D*) in kidney tissues of WT and KSP-OSR1$^{-/-}$ mice (n = 4 per group). *P < 0.05 vs. WT.

nephrocalcinosis (9). Growth retardation and impaired urine concentration with polyuria are also prominent features in patients who have BS with NKCC2 mutations (28, 29) and in total NKCC2-null mice (30). Unlike total NKCC2-null mice, NKCC2 A- or B-isoform null mice exhibit normal growth but a mild defect in the TAL, with slightly reduced urine osmolality and no polyuria (26, 27). Our KSP-OSR1$^{-/-}$ mice had marked diminution in p-NKCC2 expression and a partially blunted response to furosemide, indicating a moderate defective function of the TAL. Because mice have very high Na^+ intake, a defect in renal tubular Na^+ absorption can be concealed without modification of dietary Na^+. On a low-Na^+ diet, KSP-OSR1$^{-/-}$ mice showed more urine Na^+ excretion than WT mice, supporting the presence of a renal tubular defect. The salt phenotype in KSP-OSR1$^{-/-}$ mice resembled that in NKCC2 A or B KO mice because they all had residual NKCC2 function. Furthermore, the enhanced total and p-SPAK and total p-NCC expression seen in these mice may be compensatory responses to the Na^+ reabsorption defect upstream.

In the kidneys, we have reported that OSR1 and SPAK are expressed in both TAL and DCT. On the one hand, SPAK is mainly expressed in the cortex, especially in the DCT, and SPAK$^{-/-}$ mice with reduced total and p-NCC expression manifest a GS-like syndrome. On the other hand, OSR1 is predominantly expressed in the medulla, especially in the TAL (19). It is reasonable to expect that NKCC2 but not NCC might be the major substrate of OSR1 in renal tubules. Consistent with this notion, we observed reduced p-NKCC2 but increased total and p-NCC abundance in KSP-OSR1$^{-/-}$ mice. However, unlike the decreased NCC phosphorylation in the setting of decreased total NCC abundance in SPAK$^{-/-}$ mice (19), the reduced NKCC2 phosphorylation with unchanged total NKCC2 abundance in KSP-OSR1$^{-/-}$ mice suggests a different mechanism at work. A recent elegant study has shown that adaptor protein (AP)-3, a lysosomal protein, is involved in the sorting process of NCC from the trans-Golgi network to lysosomes and from endosomes to lysosomes (31). Of interest, the most important SPAK/OSR1 phosphoacceptor site on NCC, the T58 residue in the N terminus, is embedded in one of the putative canonical AP-3 binding motifs (YXXθ) and participates in the sorting mechanism of NCC. However, none of the putative canonical AP-3 binding motifs are found in the NKCC2 N terminus. Furthermore, NKCC2 phosphorylation at S130, another important phosphoacceptor, has been shown to be activated by SPAK/OSR1-independent pathways, perhaps via AMP-activated protein kinase, and may also be involved in the regulation of NKCC2 apical sorting (32). KSP-OSR1$^{-/-}$ and SPAK$^{-/-}$ mice may be used further to explore the mechanisms of NKCC2 and NCC phosphorylation on their protein trafficking.

In addition to Na^+ wasting, KSP-OSR1$^{-/-}$ mice had other electrolyte disturbances, such as hypercalciuria and hypokalemia with renal K^+ wasting. The TAL also accounts for 20–25% of filtrated K^+ and Ca^{2+} reabsorption in parallel with Na^+ reabsorption. Although K^+ is directly reabsorbed by NKCC2, the reabsorption of Na^+ provides the main driving force for Ca^{2+} reabsorption via paracellular routes (5). Reduced NKCC2 function would thus impede normal K^+ and Ca^{2+} reabsorption in the TAL, leading to increased urine K^+ and Ca^{2+} excretion. When these