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Baoxue Yang *Editor*

Aquaporins

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Preface

As described in its preface, the first edition of *Aquaporins*, published in 2017, focused on the gene organization, protein crystal structure, expression localization, physiological functions, and pathophysiological roles in disease development of aquaporins (AQPs). Since the publication of the first edition, this book has been welcomed by readers and downloaded more than 52,000 times and cited 260 times. The second edition of *Aquaporins* represents an extensive revision and a considerable expansion of the first edition. For this second edition, as in the first edition, our approach has been not just to describe what aquaporins (AQPs) are and where they are expressed, but to emphasize their physiological functions and pathophysiological roles in diseases. The contents were redesigned with 15 updated chapters and 9 new chapters. I hope readers like it more than the first edition.

Here I am grateful to the readers who have taken the trouble to write to me with constructive comments and suggestions. I thank all authors and colleagues for their contribution to this book.

Beijing, China — Baoxue Yang

Contents

Classification and Gene Structure of Aquaporins

1

Long Xu, Xiangdong Guo, Weidong Wang, and Chunling Li

Abstract

Aquaporins (AQPs) are a family of membrane water channels that basically function as regulators of intracellular and intercellular water flow. To date, 13 AQPs, distributed widely in specific cell types in various organs and tissues, have been characterized in humans. A pair of NPA boxes forming a pore is highly conserved among all aquaporins and is also key residues for the classification of AQP superfamily into four groups according to primary sequences. AQPs may also be classified based on their transport properties. So far, chromosome localization and gene structure of 13 human AQPs have been identified, which is definitely helpful for studying phenotypes and potential targets in naturally occurring and synthetic mutations in human or cells.

Keywords

Aquaporin · NPA boxes · Gene

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1.1 Classification of Aquaporins

A large number of evidences have shown an unexpected diversity of aquaporins (AQPs) in both prokaryotic and eukaryotic organisms [1, 2] since the discovery of AQP1. More than 300 different aquaporins have been discovered so far in which 13 isoforms have been identified (AQP0–AQP12) in human. AQPs are integral, hydrophobic, transmembrane proteins that primarily facilitate the passive transport of water depending on the osmotic pressure on both sides of membrane. Subsequent studies show that AQPs can transport not only water molecules but also other small, uncharged molecules, i.e., glycerol, urea, down their concentration gradients.

Structural analysis of several AQPs has established that these protein channels share a common structural feature. The functional aquaporin unit is a homotetramer, which comprises six α-helix transmembrane domains with two conserved asparagine–proline–alanine (NPA) motifs embedding into the plasma membrane, a signature sequence of water channels (see Chap. 3). Conformational changes of AQP protein permit other molecules passing through plasma membrane, i.e., urea, glycerol, H_2O_2, NH_3, CO_2, etc.

According to their structural and functional similarities, AQPs are initially subdivided into two subfamilies, classical AQPs (water-selective) and aquaglyceroporins (glycerol channel, Glps) aquaporins. However, further studies revealed

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that both the subfamilies overlap functionally, for examples, some classical AQPs transport water and other small solutes, e.g., glycerol. In addition, a new group of AQPs discovered showed that their structure is highly deviated from the previous AQPs especially around the AQP NPA box [3–5]. This subfamily was later named superaquaporin (also called unorthodox aquaporin) as it has very low homology with the previous two subfamilies [4]. This classification was usually accepted in physiology.

Later, it was found out that several members, e.g., AQP8 and AQP6 in classical AQP family have unique characteristics. Aquaporins are therefore organized into four categories, classical aquaporins, unorthodoxaquaporins, Aqp8-type aquaammoniaporins, and aquaglyceroporns, according to the phylogenetic tree or phylogenetic topology inferred from Bayesian inference (Fig. 1.1) [2, 4, 6]. This classification is identified based on the transport functions and properties of aquaporins.

The first subfamily is that of aquaporins, the water selective or specific water channels, also named as "orthodox," "classical" aquaporins, including AQP0, AQP1, AQP2, AQP4, AQP5, and AQP6. This subfamily of AQPs has been extensively studied, which help us define regulation of AQP expression in the body and their potential roles in physiological and pathophysiological states. Evidence, however, appears to suggest that AQP6 be classified as unorthodox aquaporins, due to low water permeability of AQP6 [7, 8].

The second subfamily of related proteins has low conserved amino acid sequences around the NPA boxes unclassifiable to the first two subfamilies [4]. Mammalian AQP11 and AQP12 are the only two members in this subfamily, which have been called "superaquaporins" or "unorthodox aquaporins." The NPA boxes of these two AQPs are highly deviated from those of other classical AQPs with homology less than 20%, indicating that they belong to a supergene family of AQPs. The signature sequence for these AQPs is the cysteine residue at the nine residues downstream of the C-terminal of the second NPA, which is exposed on the surface of the protein at the periplasmic side of the membrane [9, 10]. The structure and function of AQP11 and AQP12 are currently poorly understood. As this subfamily focuses on deviated NPA itself and unconventional functions, AQP6 and AQP8 are also included previously [11].

The third subfamily is AQP8-type aquaammoniaporins. The structure and function of AQP8 indicate that AQP8 should not be regarded as either a conventional water channel or an aquaglyceroporin. In AQP8, both NPA motifs are conserved (although the first motif is followed by VS, instead of VT). AQP8 has the highest homology to the plant AQP, γTIP, than any mammalian AQPs [11]. AQP8 is characterized as a Hg^{2+}-inhibitable water channel when expressed in Xenopus oocytes [12–14]. AQP8 is unique due to its permeability of NH_3/NH_4^+ [15, 16] in Xenopus oocytes and in AQP8-containing proteoliposomes [17]. While more evidence suggests that AQP8 is not the only aquaporin transporting ammonia, some other classical aquaporins (AQP1, −6) and aquaglyceroporins are also capable of facilitating ammonia transport.

The fourth subfamily is represented by aquaglyceroporins that are permeable to water and other small uncharged molecules (ammonia, urea, in particular glycerol). They also facilitate the diffusion of arsenite and antimonite and play a crucial role in metalloid homeostasis [18]. The aquaglyceroporins, including AQP3, AQP7, AQP9, and AQP10, can be distinguished from aquaporins based on amino acid sequence alignments [19]. The aspartic acid residue in the second NPA box is the signature key for AQP members of this subfamily. This residue is located just the downstream of the arginine forming the aromatic residues/arginine (Ar/R) narrowest filter for the selective water permeation [20]. The aspartic acid residue enlarges this pore constriction and makes more hydrophobic, permeating small molecules larger than water [10]. AQP3 is the first mammalian aquaglyceroporin to be cloned, and it is permeable to glycerol and water [21, 22]. AQP7, AQP9, and AQP10 transport water, glycerol, and urea

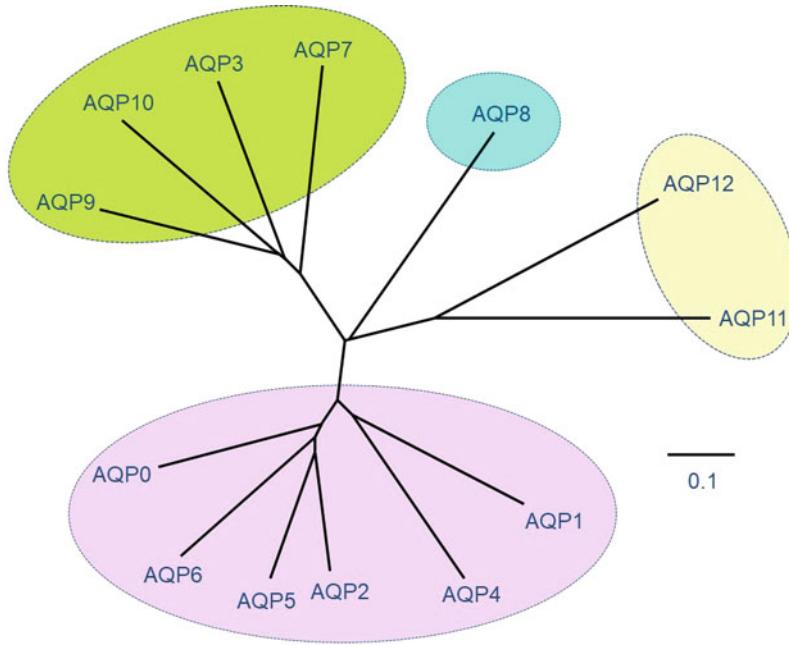

Fig. 1.1 The phylogenetic tree of 13 human AQPs. The tree shows the classical AQPs (AQP0, AQP1, AQP2, AQP4, AQP5, and AQP6) (light pink oval); the aquaammoniaporin AQP8 (light blue oval); the aquaglyceroporins (AQP3, AQP7, AQP9, AQP10, light green oval); and the superaquaporins (AQP11, AQP12, light yellow oval). (Modified from [4])

when expressed in Xenopus oocytes [23–25]. AQP9 is also permeable to a wide range of other solutes in oocytes [25]. Most aquaglyceroporins that transport glycerol and urea are less understood yet.

Additionally, a few isoforms, for example, AQP1, AQP3, AQP8, also facilitate hydrogen peroxide membrane permeation and are called peroxiporins.

As AQPs are present in three domains of life including bacteria, eukaryotes, and archaea, a generally accepted classification will be useful to obtain an overview of widely distributed AQP family in every kingdom of lives. AQP superfamily may therefore be classified based on the primary sequence around highly conserved a pair of NPA boxes, which is critical for the function of AQPs. Four AQP subfamilies are identified: AQP1-like, AQP3-like, AQP8-like, and AQP11-like. Compared to the above, consistency of primary sequence is emphasized in this classification. For example, the presence of Asp (D) in the second NPA box is the key for AQP3-like, while Cys (C) at nine residues downstream of the second NPA box is the key for AQP11-like.

1.2 Isoforms of AQPs

To date, at least 13 isoforms of AQPs have been discovered in humans (Table 1.1). The biological roles of these proteins have been thoroughly investigated in the past 30 years after the discovery of the first water channel AQP1. We have learned substantial base of knowledge on the structure, cellular localization, biological function, and potential pathophysiological significance of these mammalian AQPs, although there are some questions still need to answer.

1.2.1 Classical Aquaporins

1.2.1.1 AQP0

AQP0 is the protein in the fiber cells of the eye lens where it is required for homeostasis and transparency of the lens [26]. AQP0 showed lower water permeability than AQP1, about to 1/40 that of AQP1 [27]. AQP0 in lens also functions as peroxiporins to facilitate membrane transport of hydrogen peroxide [28]. The water transport via AQP0 is regulated by C-terminal

Table 1.1 Genes of human AQPs

Aquaporins	Exon numbers	Location	OMIM
AQP0	8	12q13.3	154,050
AQP1	7	7p14.3	107,776
AQP2	4	12q13.12	107,777
AQP3	6	9p13.3	600,170
AQP4	6	18q11.2-q12.1	600,308
AQP5	5	12q13.12	600,442
AQP6	4	12q13.12	601,383
AQP7	10	9p13.3	602,974
AQP8	6	16q12	603,750
AQP9	6	15q21.3	602,914
AQP10	6	1q21.3	606,578
AQP11	3	11q14.1	609,914
AQP12	4	2q37.3	609,789

References from www.ncbi.nlm.nih.gov/gene/, and omim.org/entry/

cleavage [29]. Deletion of amino acids at the C-terminal end of AQP0 impairs lens fiber organization, integrity, mechanical properties, and lens development [30–32]. AQP0 is also regulated by pH and Ca^{2+}/calmodulin (CaM) [33]. Lowering internal Ca^{2+} concentration or inhibiting calmodulin increased AQP0 water permeability. The molecular dynamics and functional mutation studies reveal that binding to calmodulin inhibits AQP0 water permeability by allosterically closing the cytoplasmic gate of AQP0 [34]. Emerging evidence showed that AQP0 could be a marker of erythroid differentiation and play a critical role of AQP0 in erythropoiesis [35].

1.2.1.2 AQP1

AQP1 is the first water channel discovered [36–38] and the first AQP that was found to function as a gas channel [39, 40]. AQP1 is a widely distributed water channel in the body [41], where it plays a central role in the regulation of water transport through those tissues. Aside of facilitating water movement, studies have revealed that AQP1 could enhance CO_2 and NH_3 permeability [7, 42] and function as a non-selective monovalent cation channel when activated by intracellular cGMP [43]. Phosphorylation of tyrosine Y253 in the C-terminus is involved in the regulation of AQP1 as a cGMP-gated cation channel [44]. Early evidence showed that threonine and serine protein kinase also regulate AQP1 ion channel activity [45]. Recent studies revealed a role of human AQP1 in the facilitated transport of H_2O_2 in smooth muscle [46] and cardio myocytes cell [47] hypertrophy.

1.2.1.3 AQP2

AQP2 is an arginine vasopressin (AVP)-regulated aquaporin which is probably the most thoroughly studied to date. AQP2 displays permeability only to H_2O but not any other small molecules. AQP2 is expressed in principal cells of the collecting ducts and is abundant both in the apical plasma membrane and subapical vesicles [48–50] in the kidney where it deeply involved in urine concentration. Translocation of AQP2 from intracellular compartment to the apical membrane is dependent on the binding of vasopressin to its V2 receptor [49, 50] located in the basolateral plasma membrane, by which vasopressin increases the water permeability.

1.2.1.4 AQP4

AQP4 is a predominant AQP located in central nervous system and is permeable to water [51, 52] and CO_2 [7]. Phosphorylation of AQP4 at cytosolic serine residues (Ser111 and Ser180) is indicated mediating water permeability by gating [53]. AQP4 possesses Ca^{2+}-dependent calmodulin-binding domains at both its cytosolic N- and C-termini. The S276 residue of AQP4 was able to be phosphorylated in vivo and was linked

to Ca^{2+}-CaM-dependent, reversible translocation of AQP4 to the cell surface during extracellular hypotonic challenge of astrocytes [54, 55]. Phosphorylation at AQP4 C-terminus by protein kinase C (PKC) is required for Golgi transition [56].

1.2.1.5 AQP5

AQP5 expression was described in the digestive, renal, respiratory, integumentary, and reproductive systems as well as in sense organs [57]. AQP5 is permeable to water and CO_2 [7, 58]. AQP5 can be directly phosphorylated at Ser156 and Thr259 by protein kinase A (PKA) in the cytoplasmic loop and the C-terminus [59, 60]. However, it increases intracellular Ca^{2+}, but not PKA-induced phosphorylation, that induces AQP5 trafficking to plasma membrane [61, 62].

1.2.1.6 AQP6

AQP6 colocalizes with the H^+-ATPase in intracellular vesicles in the renal collecting duct type-A intercalated cells [8], indicating that AQP6 may functionally interact with H^+-ATPase in the vesicles to regulate intravesicle pH. In response to acid–base changes H^+-ATPase in the intercalated cells is observed translocating from the cytoplasmic vesicles to the apical plasma membrane [63], where no AQP6 is found, indicating that AQP6 lacks intracellular trafficking and functions exclusively at the intracellular sites. The lack in intracellular trafficking of AQP6 is likely due to its intracellular retention [64]. A region within loop C of AQP6 that is responsible for severely hampering plasma membrane expression was recently identified. Serine substitution corroborated that amino acids present within AQP6 194–213 of AQP6 loop C contribute to its intracellular endoplasmic reticulum (ER) retention [64]. This signal may preclude proper plasma membrane trafficking and severely curtail expression of AQP6 in heterologous expression systems [64]. AQP6 appears impermeable to H_2O [7, 65], but in the presence of $HgCl_2$ or at acidic pH (<5.5), the water and anion permeability of AQP6 in oocytes was rapidly increased [8]. Moreover, AQP6 also enables transport of urea, glycerol, and nitrate [66, 67]. The N-terminus of AQP6 seems critical for the trafficking of the protein to the intracellular sites and intracellular vesicles localization [68]. Calcium signals may be involved in internalization of AQP6 as calmodulin can bind AQP6 in a calcium-dependent manner at the N-terminus [69].

1.2.2 Superaquaporins

1.2.2.1 AQP11

AQP11 has a conventional N-terminal Asn-Pro-Ala (NPA) signature motif and an unique amino acid sequence pattern that includes an Asn-Pro-Cys (NPC) motif, which appears essential for full expression of molecular function [3]. Recent evidence strongly suggests that Cys227 of AQP11 plays an important role in the formation of its quaternary structure and molecular function [70]. One reconstruction vesicle study has clearly shown that AQP11 is indeed a water channel that transports water as efficient as AQP1 [71, 72]. Although detailed subcellular localization of AQP11 remains clarified, it has been observed that AQP11 colocalizes with markers of the endoplasmic reticulum [73] and HA-tagged AQP11-transgenic mice [74]. Recent studies showed that AQP11 colocalized to the mitochondrial-associated membrane (MAM) which regulates essential signal transduction [75]. AQP11 facilitates specifically H_2O_2 transport to ER [75] and thus AQP11 constitutes an important regulator of renal and hepatic ER redox homeostasis and signaling. Deficiency or downregulation of AQP11 is associated with endoplasmic reticulum stress and apoptosis in the kidney proximal tubules [73] and in adipocytes [76].

1.2.2.2 AQP12

AQP12 is more closely related to AQP11 than to other aquaporins. With regard to the signature motifs, the first NPA motif of AQP12 is substituted by an Asn-Pro-Thr (NPT) motif and the C-terminal NPA motif is conserved [5, 9]. AQP12 seems to be expressed specifically

in pancreatic acinar cells and retained in intracellular structures [5]. The osmotic water permeability measured by using vesicles from the AQP12 knockout and wild-type mouse pancreas showed only a small nonsignificant difference [77]. One study suggests that AQP12 may function as controlling the proper secretion of pancreatic fluid following rapid and intense stimulation [77].

1.2.3 AQP8-Type Aquaammoniaporins

1.2.3.1 AQP8

So far, AQP8 is the only member in this family. It is a water channel first found in intracellular domains of the proximal tubule and the collecting duct cells [78]. Several studies showed that AQP8 transports water [7, 79] and ammonia [7, 17]. Although AQP8 was shown ultrastructurally localized at inner mitochondrial membrane (IMM) in the liver and functionally permeable to water [79], this was not supported by water permeability study in AQP8-deleted mouse liver cell IMM [80]. In the kidney, AQP8 facilitates transport of NH_3 released from glutamine and glutamate out of the IMM [81] for secretion into the tubule lumen, where the NH_3 buffers acid excreted by epithelial cells, particularly during metabolic acidosis [82]. AQP8 may also facilitate the diffusion of hydrogen peroxide across membranes of mitochondrial in situations when reactive oxygen species is generated, e.g., electron transport chain is highly reduced [75, 83, 84].

1.2.4 Aquaglyceroporins

1.2.4.1 AQP3

AQP3 has a wide tissue distribution. It is permeable to water, glycerol, and urea. Recent studies revealed the pH gating of human AQP3 on both water and glycerol permeabilities using a human red blood cell model and in silico [85]. AQPs also differ in their capacity to transport various substances, such as urea, glycerol, H_2O_2, ions, and gas. Emerging evidence showed that AQP3 is regulated on short-term basis likely via cAMP-PKA pathway [86–88]. In the kidney, the increased basolateral diffusion of AQP3 induced by elevated intracellular cAMP likely altered AQP3 interactions with other proteins or lipids in the plasma membrane, which may be a physiological adaptation to the increased water flow mediated by apical AQP2 [86]. AQP3 was shown to transport H_2O_2 through the plasma membrane [84, 89, 90], which likely plays an important role in initiating intracellular signaling in cell migration [91], inflammation [92], and cancer progression [93, 94].

1.2.4.2 AQP7

AQP7 facilitates transport of water, glycerol, urea, ammonia, arsenite, and NH_3 [7, 23, 95]. Hydropathy analysis predicts six putative transmembrane domains with the N- and C-terminal localized in the cytosol. Six prospective sites of AQP7 for PKA phosphorylation have been identified based on database analysis [96], but the direct regulation by PKA remains to be elucidated, whereas a potential PKC phosphorylation site is found at residue Thr-174 [23]. AQP7 is abundantly expressed in adipose tissue [97] and pancreatic β-cells [98, 99].

1.2.4.3 AQP9

AQP9 is expressed at the sinusoidal plasma membrane of hepatocytes [100], where it serves as a conduit for the uptake of NH_3 and mediates the efflux of newly synthesized urea. AQP9 may also function as a glycerol channel to facilitate glycerol uptake in the liver. AQP9 is also permeable to water, carbamides, CO_2, and NH_3; moreover, AQP9 is suggested playing a crucial role in metalloid homeostasis by transporting antimonite and arsenite [2, 11]. Interestingly, it also transports much larger substrates such as lactate, purine, pyrimidine [2, 25], probably due to a larger pore size disclosed by a 3D structure analysis [101]. AQP9 facilitates the membrane transport of H_2O_2 in mammalian cells and regulates redox-regulated downstream cell signaling [102]. Human AQP9 has a potential N-glycosylation site at Asn142, a potential PKC phosphorylation sites at Ser11 and Ser222, a potential casein kinase II phosphorylation site at

Ser28 [25, 103]. However, little is known about short-term regulation of AQP9.

1.2.4.4 AQP10

AQP10 is an aquaglyceroporin expressed only in the human gastrointestinal tract, but not in the mouse small intestine where it has been demonstrated to be a pseudogene [24, 104]. AQP10 is able to transport water, glycerol, and urea when expressed in *Xenopus* oocytes [24]. AQP10 is also a glycerol channel expressed in the plasma membrane of human adipocytes [105]. Silence of AQP10 in human differentiated adipocytes resulted in a 50% decrease of glycerol and osmotic water permeability, suggesting that AQP10, together with AQP7, is particularly important for the maintenance of normal or low glycerol contents inside the adipocyte, thus protecting humans from obesity [105]. Three potential glycosylated sites for AQP10 were predicted, at least one of them Asn133 in the extracellular loop of AQP10 was confirmed. Glycosylation at Asn133 may increase thermostability of AQP10 when challenged with low temperature, indicating a stabilizing effect of the N-linked glycan [106]. AQP10 mediated increased glycerol flux activated by acidification in human adipocytes [107], likely by a unique gating mechanism combining complex interaction networks between water molecules and protein residues at the loop interface [108].

1.3 Gene Structures of AQPs

Table 1.1 shows chromosome localization and numbers of exons of 13 human AQPs. The gene of AQP0 spans 3.6 kb, contains four exons, and is present in single copy in the haploid human genome. Transcription is initiated from a single site 26 nucleotides downstream from the TATA box [109].

Genomic Southern analysis indicated the existence of a single *AQP1* gene that was localized to human 7p14 by in situ hybridization [110–112]. AQP2 cDNA was cloned as the water channel of the apical membrane of the kidney collecting tubule in the rat [48], which shows 42% identity in amino acid sequence to AQP1. Human AQP2 encodes a deduced protein with 89.7–91% amino acid identity to the rat protein [112–115]. By in situ hybridization, *AQP2* gene was mapped to chromosome 12q13 [113, 115], very close to the site of major intrinsic protein (MIP).

Using a rat AQP3 probe, Ishibashi [116] screened a human kidney cDNA library and isolated a cDNA coding for human AQP3 protein. *AQP3* gene is located at 9p13 and appeared to exist as a single copy with six exons. The initiation site of transcription was identified to be located 64-bp upstream of the first ATG codon. The 5-prime flanking region contained a TATA box, 2 Sp1 sequences, and some consensus sequences including AP-2 sites [117].

Human AQP4 (initially called mercurial-insensitive water channel, MIWC) cDNA cloned from a fatal brain cDNA library showed that the longest open reading frame encoded 301 amino acids with 94% identity to rat AQP4. Analysis of MIWC genomic indicated two distinct but overlapping transcription units from which multiple MIWC mRNAs are transcribed. Later reports revealed that the *AQP4* gene is composed of four exons encoding 127, 55, 27, and 92 amino acids separated by introns of 0.8, 0.3, and 5.2 kb. Genomic Southern blot analysis indicated the presence of a single MIWC gene, localized on chromosome 18q [51, 118].

Human *AQP5* cDNA and gene was isolated and characterized from a human submaxillary gland library, which contained a 795-bp open reading frame encoding a 265-amino acid polypeptide with a transcription initiation site 518 bp upstream of the initiating methionine. *AQP5* gene was mapped to chromosome 12q13 [119].

Ma et al. isolated the cDNA by using degenerate PCR from a human kidney cDNA library that was related to AQP2, having four exons and was organized similarly to AQP0 and AQP2 and later was referred to this gene as *AQP6*, assigned to chromosome 12q13 [120, 121].

Human *AQP7* gene contains 10 exons. An Alu repetitive sequence and binding sites for several different transcription factors within the AQP7 promoter was determined, including a putative

peroxisome proliferator response element and a putative insulin response element, indicating potential involvement of AQP7 in energy metabolism [23, 122, 123].

Like the genes of non-water-selective aquaporins, the *AQP8* gene contains six exons; however, its exon–intron boundaries are different from the boundaries of those other aquaporin genes. *AQP8* gene was mapped to chromosome 16p12 [14, 124].

A partial AQP9 cDNA was isolated by using RT-PCR of leukocyte RNA with primers based on conserved regions of aquaporins [125]. AQP9 shares greater sequence identity with AQP3 and AQP7 than with other members of the family, suggesting that these three proteins belong to a subfamily.

The cDNA encoding AQP10 was isolated from jejunum cDNA library. Sequence analysis predicted that AQP10 is approximately 53% identical to AQP3 and AQP9, Northern blot analysis revealed expression of a 2.3-kb AQP10 transcript in jejunum but not liver [126].

Human *AQP11* gene contains three exons and spans 8 kb and was mapped to chromosome 11q14. Human *AQP12A* gene contains four exons and encodes a 1.5-kb transcript only in pancreas [73, 127].

Genetic variants of AQPs may result in disturbance of molecule selection and transport by AQPs; disruption of the formation of tetramers or arrays; and misfolding, faulty sorting of AQPs, or other dysfunction [81].

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Protein Structure and Modification of Aquaporins

2

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Abstract

Aquaporins (AQPs) allow water molecules and other small, neutral solutes to quickly pass through membrane. The protein structures of AQPs solved by crystallographic methods or cryo-electron microscopy technology show that AQP monomer consists of six membrane-spanning alpha-helices that form the central water-transporting pore. AQP monomers assemble to form tetramers, forming the functional units in the membrane, to transport water or other small molecules. The biological functions of AQPs are regulated by posttranslational modifications, e.g., phosphorylation, ubiquitination, glycosylation, subcellular distribution, degradation and protein interactions. Modifications of AQP combined with structural properties contribute to a better functional mechanism of AQPs. Insight into the molecular mechanisms responsible for AQP modifications as well as gating and transport properties proved to be fundamental to the development of new therapeutic targets or reliable diagnostic and prognostic biomarkers.

Keywords

Aquaporins · Protein structure · X-ray crystallography · Posttranslational modification

2.1 Introduction

Overall aquaporin (AQP) structure is largely conserved among the various AQP classes and species isoforms, despite significant differences in sequence similarities (Table 2.1). Structural studies have provided a relevant insight regarding the determining requirements that enable homotetramer formation and demonstrate the structural basis for transporting water and other small neutral solutes such as ammonia, glycerol, urea, etc. The quaternary structure enables water transport activity in animal AQPs [1], and the "hourglass model" was used to explain the 3D structure of AQPs [2, 3] previously. The secondary structure of AQPs consists of 40% α-helix, 42% – 43% β-sheet and corner structure. The AQPs are tetrameric proteins composed of identical 30 kDa monomers, each of which functions as

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an independent water channel. The monomer has six transmembrane helices (H1–6, tilted at about 30° with respect to the membrane normally), connected by five loops (A-E) in which A, C and E are extracellular, and B and D are intracellular. The hydrophilic terminal amino and carboxyl groups in the monomer are always located in the cytoplasm [1] (Fig. 2.1).

AQP has two conserved asparagine-proline-alanine (Asn-Pro-Ala, NPA) sequences distributed in loop B and loop E, which are embedded in the plasma membrane and correlate with substrate selectivity [1, 4–6]. The position of the NPA motif is stabilized by ion pairs and hydrogen bonds with neighbouring transmembrane helices, which constitute narrow water-permeable pores. This water-permeable pore has electrostatic interactions, where the water molecule in Brownian motion (random movement) in the extracellular environment renders the AQP outer cone walls in a hydrophobic state, causing repulsion [7]. The other conserved structural feature of AQP family is the aromatic/arginine (ar/R) constriction site located at the extracellular side of the channel. The ar/R constriction site contains highly conserved aromatic and arginine residues [6], acting as a selectivity filter (Fig. 2.2). The diameter of AQPs is approximately 3 Å, which is only slightly larger than the 2.8 Å diameter of the water molecule, the pore constriction prevents permeation of all molecules bigger than water [8]. In comparison with aquaporins, aquaglyceroporins present much bigger selectivity size (pore size), which can reach ~3.4 Å in diameter [9].

The latest review reports that, as of December 2020, a total of 31 AQP structures have been revealed in both eukaryotes and prokaryotes, by X-ray crystallography, cryo-electron microscopy (cryo-EM) and NMR [10] (Table 2.2). Understanding of high-resolution structures of aquaporins is helpful to clarify the permeation mechanism of water and other permeable small molecules, it provides novel insights into the regulation of water flow by pH, phosphorylation and mechano-sensitivity [11–15]. The structure of the resolved AQPs will be described in detail below.

Posttranslational modifications (PTMs) are usually highly dynamic processes, which alter the properties and biological function of target protein and increase protein diversity. Proteins can be regulated after translation by the reversible or irreversible addition of functional groups (e.g., phosphorylation, acetylation and methylation), peptides (e.g., ubiquitination, SUMOylation), or other complex molecules (e.g., glycosylation). Through changes in protein conformation, these PTMs have been shown to modulate the localization, stability, activity and interacting partners of their substrate proteins, thus playing pivotal roles in intracellular signalling, protein maturation and folding. The precise effect of PTMs depends on the nature of the covalent modification, the identity of the substrate and the residue that is specifically targeted by the chemical reaction [16]. This section of the review mainly focuses on PTMs of the arginine vasopressin (AVP)-regulated AQP2 that is the best understood and discusses modification of other AQPs at the end of the section.

2.2 Protein Structure of AQPs

2.2.1 Protein Structure the Classical AQPs

2.2.1.1 AQP0

In 2004, successively published the structures of bovine AQP0 (bAQP0) and sheep AQP0 (sAQP0) with resolutions of 2.2 Å and 3.0 Å [17, 18]. The structures obtained by the method of X-ray and cryo-EM are very similar, the only four residues C14F, S20T, M90V and S240T (bAQP0 numbering) are different between bAQP0 and sAQP0 sequences. AQP0 remains a tetramer, and monomer interactions are inseparable from the Proline-Proline motif. Starting from the extracellular side, between residues Asn-115, Thr-120 and His-34, the vestibule narrows to a diameter of ≈10 Å; at residues Phe-48, His-172, Met-176, Ala-181 and Arg-187, the channel narrows a diameter of 1.99 Å, which is the narrowest region of the channel. Gly-180, Ala-181, Gly-182 and Met-183, four backbone carbonyls of successive residues, provide the

Table 2.1 Sequence alignments of human AQPs at the first and second NPA boxes

	NCBI reference sequence	Regions of NPA boxes	Fist NPA boxes	Second NPA boxes
AQP0	NP_036196	68–70; 184–186	NISGAHV**NPA**VTFAFLV	YYTGAGM**NPA**SFAPAI
AQP1	NP_001316801.1	76–78; 192–194	HISGAHL**NPA**VTLGLLL	DYTGCGI**NPA**RSFGSAV
AQP2	NP_000477.1	68–70; 184–186	HISGAHI**NPA**VTVACLV	HYTGCSM**NPA**RSLAPAV
AQP3	NP_004916	83–85; 215–217	QVSGAHL**NPA**VTFAMCF	FNSGYAV**NPA**RDFGPRL
AQP4	NP_001304313	97–99; 213–215	HISGGHI**NPA**VTVAMVC	NYTGASM**NPA**RSGPAV
AQP5	NP_001642	69–71; 185–187	PVSGGHI**NPA**ITLALLV	YFTGCSM**NPA**RSFGPAV
AQP6	NP_001643	82–84; 196–198	KASGAHA**NPA**VTLAFLV	HFTGCSM**NPA**SFGPAI
AQP7	NP_001161	94–96; 226–228	RISGAHM**NAA**VTFANCA	MNTGYAI**NPS**RDLPPRI
AQP8	NP_001160.2	92–94; 210–212	NISGGHF**NPA**VSLAAML	PVSGGCM**NPA**RAFGPAV
AQP9	NP_066190	84–86; 216–218	GVSGGHI**NPA**VSLAMCL	LNSGCAM**NPA**RDLSPRL
AQP10	NP_536354	82–84; 214–216	NVSGAHL**NPA**FSLAMCI	ANCGIPL**NPA**RDLGPRL
AQP11	NP_766627	99–101; 216–218	TLVGTSS**NPC**GVMMQMM	SLTGVF**NPA**LALSLHF
AQP12	NP_945349	81–83; 200–202	TLDGASA**NPT**VSLQEFL	PFTSAFF**NPA**LAASVTF

Highly conserved NPAs (asparagine–proline–alanine) are highlighted and underlined. Sequence was based on NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/)

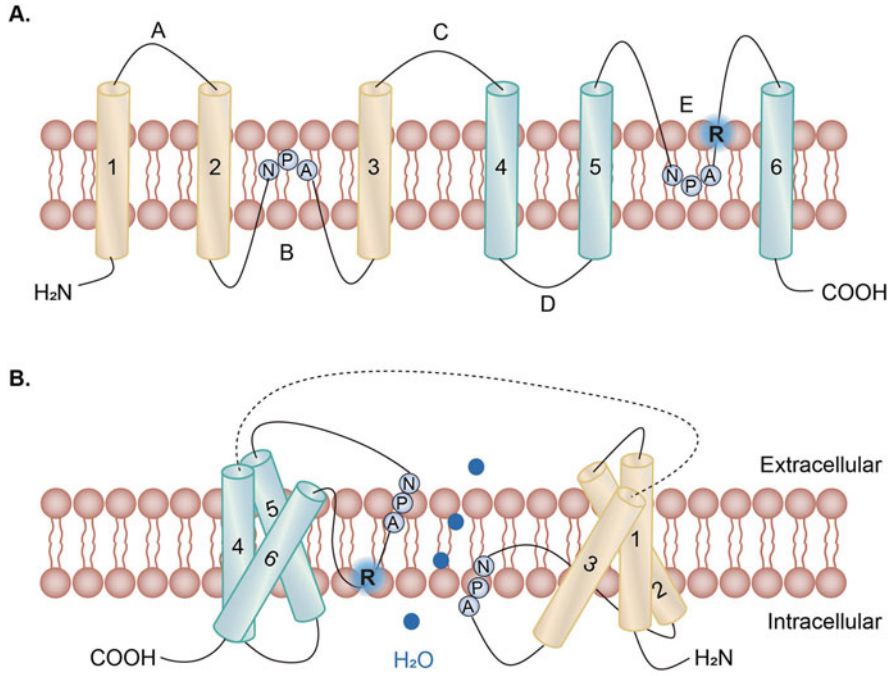

Fig. 2.1 A secondary structure and topology of AQP molecule. (**a**) AQP1 monomer has six membrane-spanning regions (1–6), five loops (A-E) with intracellular amino and carboxy termini as well as internal tandem repeats. (**b**) In the monomer, the hydrophilic loops B and E are bent back into the cavity and meet in the middle to form the putative water-selective gate that contains two consensus NPA motifs. ar/R region is shown close to the entrance of the pore. (Modified from [159])

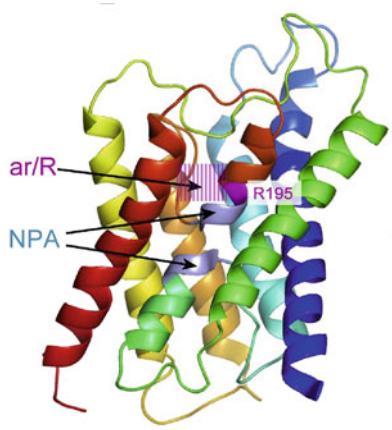

Fig. 2.2 Schematic architecture of AQP1. A ribbon model of AQP1 using a rainbow colour scheme from blue (N-terminal) to red (C-terminal). The narrowest region in the AQP1 pores, previously termed ar/R, is located close to the extracellular entrance of the pore. The Arg195 and NPA motifs are shown in magenta and light blue, respectively. (Reproduced with permission from [44])

canonical AQP hydrogen bond acceptors that align waters through the channel. Farther into the channel, the side chain of Tyr-23 is oriented directly towards the central axis of the channel, with Phe-141, Leu-52 and Leu-168, constricts the channel diameter to 2.5 Å. Asn-68 and Asn-184 in the two NPA motifs orient the key central water molecule, which is responsible for preventing the reorientation necessary for any proton conduction. On the cytoplasmic side of the NPAs, the line of backbone carbonyl oxygens resumes along one wall of the channel from Gly-64, Ala-65, His-66 and Gly-67 and ends at Tyr-149. Together with Val-56, Gly-64, His-66 and Phe-75, Tyr-149 pointing directly into the channel forms another constriction that is the narrowest region of the channel. It accepts a sphere with a maximum diameter of 1.5 Å (Fig. 2.3).

Two mutations with single amino acid substitution in the AQP0 molecule: E134G or T138R will lead to the occurrence of human congenital cataracts [19], and structural analysis of the AQP0 mutation suggests that E134G or T138R would alter the conductance for water, due to removal of orienting factor and distortion in the line of carbonyls [17].

His-40, His-66 and His-172 are hydrogen bond donors, among which His-40 can inhibit the effect of pH or Ca^{2+} on water transport rates. Oocytes expressing mutate AQP0 by changing His-40 to alanine, aspartate, or lysine, no longer displayed the pH-dependent closing as pH was raised to 6.5. The closed-state AQP0 structure was formed at pH 6.0, while the open structure of AQP0 was formed at pH 10.0, which may explain why the AQP0 is regulated by pH [17, 18].

Regulation of water permeability by calmodulin (CaM) is achieved through a Ca^{2+}-dependent interaction between Ca^{2+}–CaM and the cytoplasmic C-terminal domain of AQP0 [20–22]. AQP0 tetramer is in complex with two CaM molecules. Molecular dynamics (MD) simulations showed that CaM binding to the C terminus of AQP0 allosterically modulates the dynamics at the CSII pore constriction site, thus resulting in channel closure [23].

2.2.1.2 AQP1

Models of AQP1 by X-ray crystallography and cryo-EM structural studies at about 4 Å resolution have been successively reported [8, 24], independently confirming the presence of two membrane-inserted non-membrane-spanning helices. The AQP1 monomer contains six tilted membrane-spanning helices forming a right-handed bundle. Beginning at the fourfold axis of the tetramer, the helices are arranged: 2-1-3 (first repeat), 5-4-6 (second repeat) [24]. Each monomer interacts with two neighbouring monomers, likely stabilized by a network of hydrogen bonds between residues Ser-59, Thr-62 and Gln-65 with residues Gln-148, Cys-152 and Thr-156.

The presence of NPA motifs (Asn192-Pro193-Ala194, Asn76-Pro77-Ala78) forms part of the surface of the aqueous pore with the narrowest area of about 3 Å in diameter. Ile-60, Phe-24, Leu-149 and Val-176 form a hydrophobic surface lining the inside of the pore adjacent to Asn-76 and Asn-192 of the NPA motifs. Highly conserved His-74 forms an ion pair with Glu-17. Among the glycerol-permeable homologues, His-80 is usually replaced by glycine [8].

Table 2.2 Determining information related to AQP structure at high resolution

Target	Source	PDB code	Ref
Mammalian			
AQP0	*Bos taurus*	1YMG	Harries et al. [17]
AQP0	*Ovis aries*	1SOR	Gonen et al. [18]
AQP1	*Homo sapiens*	1FQY	Murata et al. [8]
AQP1	*Homo sapiens*	1IH5	Ren et al. [24]
AQP1	*Homo sapiens*	6P0J	Dingwell et al. [27]
AQP1	*Bos taurus*	1J4N	Sui et al. [25]
AQP2	*Homo sapiens*	4NEF	Frick et al. [34]
AQP2	*Homo sapiens*	6QF5	Lieske et al.
AQP4	*Rattus norvegicus*	2D57	Hiroaki et al. [48]
AQP4	*Rattus norvegicus*	2ZZ9	Tani et al. [44]
AQP4	*Homo sapiens*	3GD8	Ho et al. [43]
AQP5	*Homo sapiens*	3D9S	Horsefield et al. [46]
AQP7	*Homo sapiens*	6QZI	de Maré et al. [62]
AQP10	*Homo sapiens*	6F7H	Gotfryd et al. [11]
Plant			
PIP2;1	*Spinacia oleracea*	1Z98	Törnroth-Horsefield et al. [12]
PIP2;4	*Arabidopsis thaliana*	6QIM	Wang et al. [64]
TIP2;1	*Arabidopsis thaliana*	5I32	Kirscht et al. [63]
Eukaryotic microorganism			
AQPy1	*P. pastoris*	2W2E	Fischer et al. [13]
AQPy1	*P. pastoris*	3ZOJ	Kosinska Eriksson et al. [67]
Unicellular protozoan AQP			
PfAQP	*Plasmodium falciparum*	3C02	Newby et al. [45]
Prokaryotic			
AqpM	*M. marburgensis*	2F2B	Lee et al. [68]
AqpZ	*E. coli*	1RC2	Savage et al. [69]
AqpZ	*E. coli*	2ABM	Jiang et al. [47]
AqpZ	*E. coli*	3NK5	Savage et al. [73]
GlpF	*E. coli*	1FX8	Fu et al. [71]
GlpF	*E. coli*	1LD5	Tajkhorshid et al. [35]

Data are based on the PDB protein database. https://www.rcsb.org/

The narrow pore of AQP1 is composed of four amino acid residues His-182, Arg-197, Phe-58 and Cys-191, and a steric limit of ≈2.8 Å is established at the constriction region, which is highly unfavourable for the transport of larger molecules like glycerol. Water molecules can permeate the pore with a minimal energy barrier, whereas the hydrogen-bond isolation generated from bulk water will prevent the transfer of protons (H-bond isolation mechanism). This is probably due to the positive electrostatic field generated by the dipole moments of the pore helices in AQP1 and the availability of water-binding sites which reduce the energy barrier to water. His-182 appears to be critical in determining whether an AQP is specific for water or has additional selectivity for other solutes such as glycerol [25].

Subsequently, the resolution of the AQP1 is further improved to 2.2 Å, some of these molecular details better explain the mechanism by which AQP1 regulates water and solutes [25]. Within the AQP1 selectivity filter, waters are identified at four locations where they form the coordinating hydrogen bonds with residues around, and only the middle two are close enough to each other to form a water-water hydrogen bond [26].

By using solid-state nuclear magnetic resonance to determine multiple interatomic distances, it is found that a conformation of loop

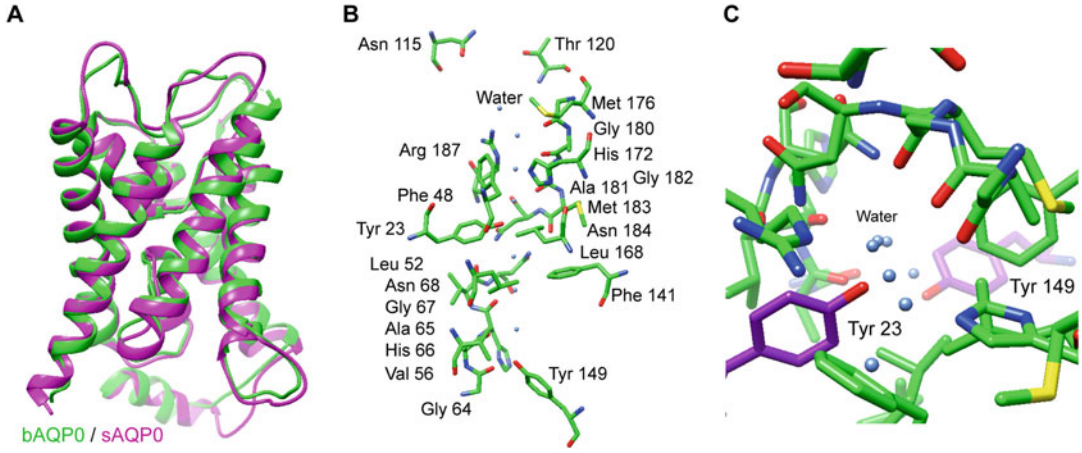

Fig. 2.3 Monomer channel views of bAQP0. (**a**) The overall comparison of bAQP0 and sAQP0 monomer channel structures, with bAQP0 structure in green and sAQP0 structure in magenta. (**b**) Key residues surrounding the water molecules in the channel. (**c**) Stereo view from the extracellular side of the channel. Tyr-23 and Tyr-149 point directly into the channel. (Modified from [17])

C is stabilized both by interactions within the loop and with other regions on the extracellular surface of hAQP1. Such as Ala130-Val133, Asn134-Gln137, as well as Ala130-Tyr186, Asn49-Asp185 and possible interactions between Asn-127 and Asn-205 [27, 28]. Cys-189 and Ala-73 reside at comparable locations on the extracellular/cytoplasmic side of the narrowing, the structure can explain the inhibition of the double mutant C189S, A73C by mercury [29–31].

2.2.1.3 AQP2

Mutation or defective trafficking of AQP2 leads to nephrogenic diabetes insipidus (NDI), a water balance disorder characterized by large amounts of hypoosmotic urine output, hypoosmotic urine volumes, leading to dehydration. The structure of human AQP2 provides insight into the mechanism of how AQP2 mutations induce NDI [32]. AQP2 displays the conserved AQP fold like other water channels [33–35]. In the X-ray structure, the striking difference between AQP2 and all other mammalian AQPs is the highly variable position of the short C-terminal helix. Specifically, four hydrophobic leucine residues (Leu-230, Leu-234, Leu-247 and Leu-240) that align on the same side of the C-terminal helix of protomer C and insert into the protomer D of another AQP2 tetramer. Also, the unusual flexibility of the C-terminus of AQP2 may arise from two consecutive prolines (Pro-225 and Pro-226) that form a hinge region. The N terminus adopts a conformation that allows to form hydrogen bond interactions between Glu-3, Ser-82 and Arg-85, which is important in the gating mechanism (Fig. 2.4).

It is worth noting that there are two Cd^{2+} ions (Cd1 and Cd2) built per tetramer of AQP2 at the cytoplasmic side of the membrane during crystallization, Cd1 binds at the membrane interface between protomers a and d and is ligated by Glu-155(a) of loop D and Gln-57(d) of TM helix 2, as well as two water molecules. Cd2 is located between loop B and the C-terminal tail in protomer c and is ligated by His-80(c), Glu-232 (c) and one water molecule. Mutations near Cd1 can cause ER retention of AQP2 [34, 36]. Besides, a radioactive calcium-binding assay strongly suggests that the observed Cd^{2+}-binding sites represent Ca^{2+}-binding sites in vivo. The transient changes in the intracellular Ca^{2+} levels trigger alteration in the structure of AQP2 [37]. Thirty-one NDI-causing mutations are identified in the AQP2 crystal structure, most of which are located within the transmembrane region [32, 38].

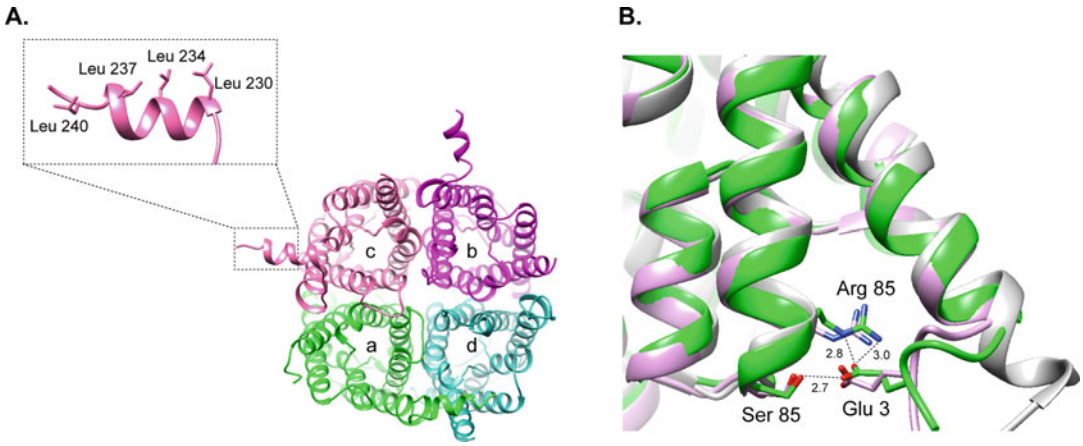

Fig. 2.4 Structure of the C and N termini of AQP2. (**a**) Overview of the AQP2 tetramer from the intracellular side. There is an interaction between the C-terminal helix of monomer c (pink) and the symmetry-related protomer d, with the four leucines on the helix highlighted. (**b**) Overlay of the N termini of protomer a and d with HsAQP5 (light pink) and BtAQP1 (grey). For protomer a (green), Glu-3 interacts with Ser-82 and Arg-85, similar to the structural arrangement seen in AQP5. In contrast, the TM1 of BtAQP1 extends into the cytoplasm. (Modified from [34])

Ubiquitination of Lys-270 at the C-terminus causes AQP2 internalization, which enhances endocytosis and targets AQP2 to multivesicular bodies (MVBs) for subsequent lysosomal degradation [39]. Lysosomal trafficking regulator-interacting protein 5 (LIP5) has been shown to facilitate AQP2 lysosomal degradation by interacting with AQP2 [40], which involved in MVB formation. LIP5-binding sites to AQP2 are Leu-230, Leu-234, Leu-247 and Leu-240, all of which locate within the AQP2 C-terminal helix [40]. This proves that the observed interaction arises from the propensity of the C-terminal helix to participate in protein–protein interactions. It has been reported that alanine mutations in the sixth transmembrane domain of AQP2 (Leu-217, Leu-218 and Leu-222, Leu-223) inhibit vasopressin-induced translocation [41] (Fig. 2.5).

2.2.1.4 AQP4

AQP4 is the predominant water channel in the mammalian brain, its role in cerebral water balance has been implicated in neuropathological disorders [42]. Two studies on crystal structures with resolutions up to 1.8 Å and 2.8 Å reveal the molecular basis for the water selectivity of the human AQP4 channel [43, 44]. The X-ray structure of hAQP4 at 1.8 Å resolution shows that each monomer is surrounded by 6 and 2 half-length alpha-helices (M1–M8) and tetramerizes along the central axis. In the mid-membrane section of AQP4, Phe-195, Leu-191 and Leu-75 create a hydrophobic block, which can be blocked by four aliphatic chains of phospholipids or fatty acids in plasmodium falciparum aquaglyceroporin (PfAQP) [45], and in the human AQP5 where a single lipid molecule is found [46]. Unlike the structures of water-selective AQPs such as AQPZ and AQP1, the asparagines of the two NPA motifs bond to two different water molecules in the centre of the channel, which is supported by molecular dynamics simulation studies [25, 47]. Similar to the water-selective bAQP1, the arginine (Arg-216) environment in hAQP4 provides NεH and NηH as donors to the waters in transit, whereas in the glycerol conducting GlpF, the selectivity filter arginine (Arg-206) has only one of the NηH, which determines its low water conductivity [35] (Fig. 2.6).

Based on the cryo-EM structure of AQP4, it is proposed that a short helix in the loop C (residues

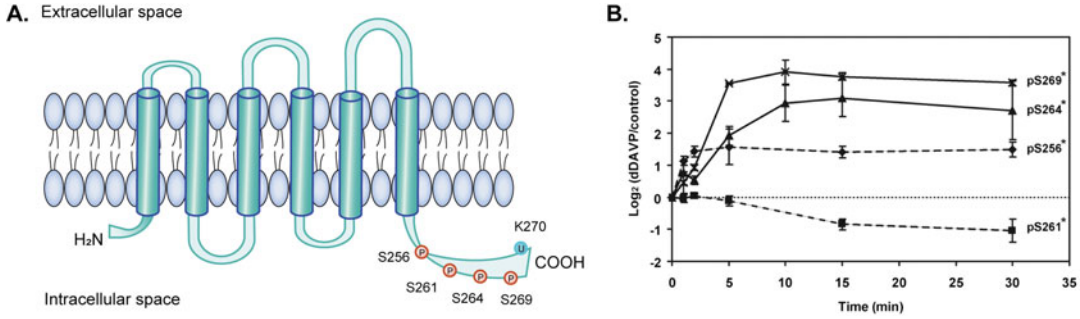

Fig. 2.5 Illustration of the topology of AQP2 and time course studies of AQP2 C-terminal phosphorylation. (**a**) Schematic illustration of the topology of AQP2 and the C-terminal phosphorylation (S256, S261, S264, S269) and ubiquitination sites (K270) of AQP2. (**b**) Time course of changes in AQP2 phosphorylation at S256, S261, S264 and S269 in response to 1 nm dDAVP (a V2R agonist) in rat inner medullary collecting duct (IMCD) tubule suspensions. Note that maximal phosphorylation at S256 occurs quickly, whereas it takes longer for maximal phosphorylation to occur at the other S264 and S269. Phosphorylation at the S256, S264 and S269 sites remains high as long as the agonist is present. In contrast, dDAVP stimulation results in decreased phosphorylation at S261. (Reproduced with permission from [85])

139–142) is the main site providing for AQP4-mediated cell-cell adhesion [48].

The reason why AQP4 is insensitive to mercury inhibition is probably a lack of reactive cysteine residues in the channel lumen corresponding to Cys-191 in AQP1 [30]. AQP4 conductance is reduced ~50% when Ser-180 is phosphorylated by protein kinase C [49]. However, the structure of the S180D mutant generated to mimic phosphorylated Ser180 showed no significant differences from the structure of the wild-type channel. The observed differences are likely due to crystallization conditions that induce non-physiological conformations [50]. AQP4 conductance is increased ~40% when phosphorylated by protein kinase G at Ser-111 [51].

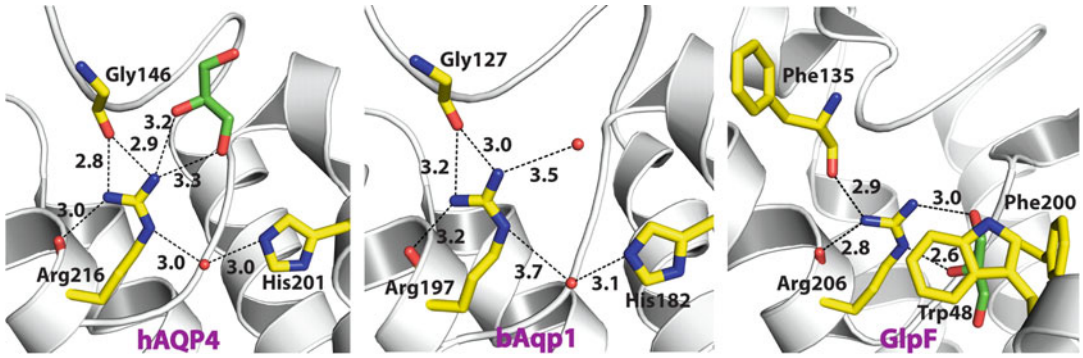

Fig. 2.6 Comparison of the hydrogen bond network of the selectivity filter arginine of hAQP4, bAQP1 and GlpF. Residues of the selectivity filter and glycerol molecules are shown as sticks. Water molecules are shown as blue spheres. Similar to Arg-197 in water-selective bAQP1, the NεH, Nη1H, Nη2H of Arg-216 in the selectivity filter of hAQP4 are all hydrogen bonded to other acceptor oxygens of the protein, leaving NεH and NηH as donors to the waters. While in the glycerol conducting GlpF, Arg-206 has only one of the NηH satisfied. (Reproduced with permission from [43])

2.2.1.5 AQP5

HAQP5 has 63% amino acid sequence identity with the paralog hAQP2, AQP5 is able to traffick from intracellular membrane to the apical membrane of epithelial cells after C-terminal modifications [52], in response to cAMP [53] or cevimeline [54]. In the structure of AQP5 at 2.0 Å resolution [46], five and seven water molecules are unambiguously observed along the water transport channel in each of the protomers, six of which sit at conserved positions, which has water-mediated hydrogen bond interactions. The water channel narrows to an average radius of 1.02 Å near the ar/R constriction region. A lipid occludes the putative central pore, preventing the passage of gas or ions through the centre of the tetramer. In contrast, both AQP0 and AQP1 do not contain lipids in the central pores, probably because the large Phe residues at this site preventing lipid insertion, whereas in this position of AQP5 is a Leu [17, 25, 55]. AQP5 also contains a mercury-sensitive cysteine residue at position 182, locating just upstream of the NPA sequence in loop E [56].

HAQP5 contains several consensus phosphorylation sites including Ser-152, Ser-156, Ser-231 and Ser-233. Ser-152 and Ser-156 located in intracellular loop D form a close association with the C-terminus of AQP5, and it is therefore hypothesized that phosphorylation at Ser-156 may lead to structural changes in loop D, which would break its interaction with the C-terminus, thereby flagging the protein for translocation to the plasma membrane [46]. The phosphomimetic mutation of Ser-156 to glutamate (S156E) increased constitutive membrane expression of AQP5, but studies on crystal structure of mutant show that such a mutation does not cause any significant structural changes to the protein [57].

2.2.2 Protein Structure of Aquaglyceroporins

In addition to transporting water across cell membranes, AQPs also transport other solutes (such as urea or glycerol) and play an important role in osmotic pressure regulation [58]; some AQPs also facilitate the transport of volatile substances (e.g., carbon dioxide (CO_2) and ammonia (NH_3)) across membranes [59]. The structural properties of the channel centre determine the selective permeation mechanism to the substrate.

2.2.2.1 AQP7

AQP7 facilitates permeation of glycerol through cell membranes and plays a crucial role in lipid metabolism [60]. In particular, Xenopus oocytes expressing human AQP7 exhibited higher osmotic water permeability and ^{3}H-glycerol uptake than oocytes expressing GlpF [61]. In 2020, two high-resolution X-ray structures of AQP7 were identified [62]; the structures show that AQP7 is an open channel with well-ordered glycerol and water molecule lining the pore. The conserved ar/R selectivity filter, the NPA signature motifs and the glycerol molecules form a hydrogen bond network characterized by partly turning the hydroxyl groups towards the more hydrophobic side of the pore. Interestingly, the human AQP7 has unusual NPA motifs, where both the asparagines are conserved, but rare substitutions create NAA (Asn-94, Ala-95 and Ala-96) and NPS (Asn-226, Pro-227 and Ser-228) motifs. The selectivity filter is known to be wider in the aquaglyceroporins compared with the orthodox AQPs, for facilitating the transport of the larger glycerol molecule, where the glycerol molecule binds to Arg-229. MD simulations, performed on glycerol passing through the pores of AQP7, show a significantly reduced osmotic permeability coefficient for water in the presence of glycerol, which suggests that glycerol prevent an unrestricted flux of water through human AQP7. And studies have shown that residue Phe-74 of AQP7 in selectivity filter prevents the passage of small solutes [61].

2.2.2.2 AQP10

The crystal structure of human AQP10 determined at 2.3 Å resolution unveils the molecular basis for pH modulation—an exceptionally wide selectivity (ar/R) filter and a unique cytoplasmic

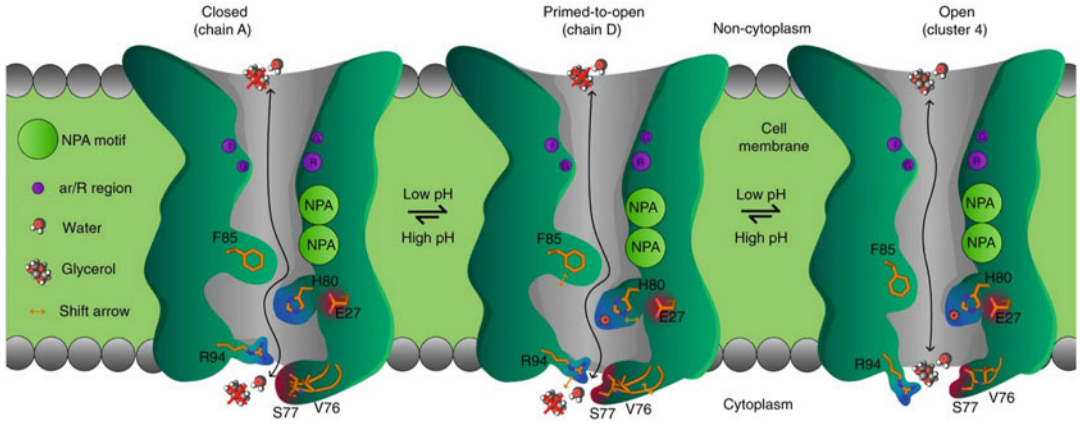

Fig. 2.7 Human AQP10 pH-gated glycerol flux mechanism. Proposed hAQP10 pH-gated glycerol flux mechanism in adipocytes and likely other cell types. Glycerol, but not water, permeation is decreased at pH 7.4. AQP10 glycerol-specific opening is stimulated by pH reduction, triggering H80 protonation that renders the residue to interact with E27. Concerted structural changes of the nearby F85 and the cytoplasmic V76–S77 loop thereby allow glycerol passage. (Reproduced with permission from [11])

gate, both unique to AQP10. It is proposed that the pH-dependent gating mechanism of hAQP10 was triggered by the protonation of His-80, through the high-resolution crystal structure. The permeation of glycerol is decreased at pH 7.4. AQP10 glycerol-specific opening is stimulated by decreased pH, triggering His-80 protonation that renders the residue to interact with Glu-27. Concerted structural changes of the nearby Phe-85 and the cytoplasmic Val-76–Ser-77 loop thereby allow glycerol passage [11] (Fig. 2.7).

AQP7 and AQP10 also have significant differences when permeating glycerol. The AQP10 has a narrowing on the intracellular side of the pore that permits water flux but does not allow the passage of glycerol, in contrast, the narrowest part of the pore coincides with the selectivity filter in AQP7. AQP7 is not regulated in a pH-dependent manner like AQP10, even this histidine (His-92) is sequentially conserved among the human AQPs. Another difference is that the Val-97 in AQP7 replaces the Phe-85 at the same position in AQP10, leading to a wider diameter of the pore in this part of the channel [11, 62].

2.2.3 Structure of Other AQPs

2.2.3.1 Plant AQPs

Land plants have evolved an ability to cope with rapid changes in the availability of water by regulating all AQPs located on the plasma membrane. The X-ray structure of spinach plasma membrane aquaporin SoPIP2;1 was revealed in a closed conformation at 2.1 Å resolution and an open conformation at 3.9 Å resolution [12]. SoPIP2;1 crystallized as a tetramer displaying extended hydrophobic interactions between monomers, as all other AQP crystals do, and seven water molecules are observed within the SoPIP2;1 channel. Molecular dynamics simulations have identified that loop D caps the channel from the cytoplasm and thereby occludes the pore in the closed conformation, while in the open conformation, loop D is displaced up to 16 Å. This movement opens a hydrophobic gate blocking the channel entrance from the cytoplasm.

The structure of *Arabidopsis thaliana* aquaporin AtTIP2;1 reveals the relatively wide pore and the polar nature of the selectivity filter, which may clarify the ammonia permeability

[63]. An extended selectivity filter with the conserved arginine (Arg-200) adopts a unique unpredicted position. Mutational studies show that the four allelic substitutions (F56H, N127H, H180I, C189G) of AtTIP2;1 in HsAQP1 can make it have the same ammonia permeability.

AtPIP2;4 can mediate H_2O_2 transport in addition to water, and its 3D structure is consistent with the SoPIP2;1 [64], the difference is that Cd^{2+} cations do not need to maintain a closed conformation. AtPIP2;4, SoPIP2;1 and hAQP1 are all transporters of both H_2O and H_2O_2, but with different efficiencies, the former is more efficient.

2.2.3.2 Eukaryotic Microorganism AQPs

The presence of AQPs in yeast *Saccharomyces cerevisiae* enhances the host's tolerance to rapid freezing [65] and osmotic shock as aquaglyceroporins control the cellular osmolyte content [66]. Pichia pastoris encodes a single AQP, Aqy1, which has 34 additional N-terminal residues compared with its closest hAQP1. The X-ray structure of the Aqy1 shows a novel gate-like function of Aqy1 amino terminus by folding to form a cytoplasmic helical bundle with a tyrosine (Tyr31), which enters the water channel and occludes the cytoplasmic entrance [13]. In contrast, the water channel in spinach is closed by a unique conformation of loop D, folding over the cytoplasmic entrance and blocking the channel [12]. Molecular dynamics simulations and site-directed mutagenesis in combination with functional studies suggest that water flow is regulated through a combination of mechano-sensitive gating and posttranslational modifications (such as phosphorylation) of Aqy1.

A work published in *Science* further improved the crystal structure resolution of Aqy1 to 0.88 Å resolution [67], revealing the H-bond interactions between asparagine residues in the dual NPA motif and water molecules, and observing a polarized water-water H-bond configuration within the channel. Additionally, the tautomeric states of histidine and arginine residues in the selectivity filter (SF) were also assigned and where the four water positions at SF were observed to be too close together to occupy simultaneously.

2.2.3.3 Unicellular Protozoan AQPs

The aquaglyceroporin in the malaria parasite *Plasmodium falciparum* (PfAQP) conducts glycerol and water at a very high rate, surpassing its closest known structural homologue GlpF. Its 2.05 Å crystal structure indicates that the reason may be that the generally conserved arginine in the selective filter forms two hydrogen bonds in GlpF, while there are three hydrogen bonds in all water-selective AQPs and PfAQP. In addition, the two NPA regions of PfAQP (NLA and NPS) are involved in maintaining the orientation of asparagine in the centre of the channel [45].

2.2.3.4 Prokaryotic AQPs

The crystal structure of the transmembrane channel protein AQPM from the archaea *Methanothermobacter marburgensis* shows that the isoleucine (Ile-187) replaces the key histidine residue found in the lumen of the water channel. This becomes a glycine residue in aquaglyceroporins. The change in AQPM generates a selectivity filter, which is wider and more hydrophobic (2.54 Å) than that of AQP1 (1.86 Å) [68].

The *Escherichia coli* genome encodes two AQPs, GlpF and AQPZ. AQPZ is a homotetramer of four water-conducting channels, which can regulate the selective passage of water molecules, and does not allow other unfamiliar, small molecules to pass [69]. A 3.2 Å crystal structure of the tetrameric AQPZ reveals two distinct Arg-189 confirmations associated with water permeation through the channel constrictions. In one of the four monomers, the guanidino group of Arg-189 points towards the periplasmic vestibule, opening up the constriction to accommodate the binding of a water molecule through a tridentate H-bond. In the other three monomers, the Arg-189 guanidino group bends over to form a H-bond with carbonyl oxygen of the Thr-183, thus occluding the channel [47, 70].

The structure of GlpF was solved during 2000–2001 [35, 71]. The constriction region of the channel is composed of Gly-191, Arg-206, Trp-48 and Phe-200 corresponding to the His-182, Arg-197, Phe-58 and Cys-191 in AQP1. This change makes the pore size of GlpF

slightly larger than that of AQP1 and reduces its hydrophilicity, which determines its permeability to glycerol.

AQPZ's preference for water transport and GlpF's preference for glycerol were demonstrated by in vitro and in vivo functional experiments [72]. AQPs have a smaller pore size than aquaglyceroporins, and the selectivity filter is the narrowest point in GlpF, AQPZ and AQP1. In AQPZ, this selectivity filter is formed by the sidechains of Phe-43, His-174 and Arg-189 and the carbonyl of Thr-183. The GlpF selectivity filter, which is larger and more hydrophobic than in AQPZ, contains the typical aquaglyceroporin substitutions of F43W, H199G and T200F, and the GlpF wild-type structure contains both a water and a glycerol molecule bound at the selectivity filter [69]. By replacing the three AQP-specific SF residues of AQPZ into their AQGP-specific counterparts in GlpF (F43W/H174G/T183F), it is shown that the polarity and size of the channel dominate water and glycerol conduction energetics by functional analysis and X-ray crystallography [73].

2.3 Protein Modification of AQPs

Through altering protein conformation, post-translational modifications (PTMs) have been shown to modulate the localization, stability, activity and interacting partners of their substrate proteins, thus playing critical roles in intracellular signalling, protein maturation and folding. Some AQPs are subject to post-translational modifications by the reversible or irreversible addition of functional groups (e.g., phosphorylation, acetylation and methylation), peptides (e.g., ubiquitination, SUMOylation) or other complex molecules (e.g., glycosylation). As PTMs of AQP2 are well studied, the following will mainly focus on AQP2 modification and its physiological significance.

2.3.1 Protein Modification of AQP2

2.3.1.1 Phosphorylation

The modulation of protein abundance in plasma membrane requires a delicately regulated translocation (trafficking) from intracellular compartment to the membrane, which is achieved through multiple sorting signals and PTMs. Phosphorylation is one of the most well-studied PTMs, which is often involved in regulation of protein function and cellular distribution. AQP2 phosphorylation is one of the best characterized examples.

AQP2 is expressed in the principal cells of the kidney collecting ducts [74–76], its intracellular distribution is finely regulated by AVP. Stimulation with AVP results in a predominant translocation of AQP2 to apical membrane localization from subapical compartments. This renders the apical membrane highly permeable to water, and it is a key event in formation of concentrated urine and thus in regulation of body water balance. This intracellular re-distribution of AQP2 induced by AVP is closely associated with phosphorylation/dephosphorylation (and/or ubiquitination) of AQP2 [77, 78].

Phosphorylation of AQP2 at multiple sites in the C-terminus governs its translocation to the apical membrane from intracellular vesicles [79, 80]. AQP2 contains numerous putative phosphorylation sites for various protein kinases, e.g., PKA, PKG, PKC and casein kinase II [81] based upon bioinformatic analysis [82]. Phosphorylation of the serine at position 256 (S256) of AQP2 in the C-terminal tail of AQP2 was the first to be identified and the best characterized phosphorylation site of AQP2 [80, 83, 84]. Large-scale phospho-proteomic analysis later demonstrated that beside S256 phosphorylation site, the polyphosphorylated region of AQP2 contains S261, S264 and S269 (Fig. 2.5a). S256-AQP2 is a target for PKA-induced phosphorylation, which was evidenced by in vitro phosphorylation assays of AQP2 C-terminal peptides [85]. Kinases other than PKA may also be involved in AQP2 C-terminal phosphorylation. PKG is proposed to modulate AQP2 trafficking. The agonist of PKG, cGMP, has been shown to mediate translocation of AQP2 to the plasma membrane in AQP2-transfected LLC-PK1 cells and in isolated kidney slices [86]. Activators of the cGMP pathway, such as atrial natriuretic peptide (ANP), L-arginine, cGMP phosphodiesterase type 5 (PDE5) inhibitors sildenafil citrate, elevated

intracellular cGMP levels, resulting accumulation of AQP2 in plasma membrane [86–88]. These data suggest a positive role of PKG on AQP2 trafficking. However, one study showed that ANP and NO (nitric oxide) signalling deceased S256-AQP2 phosphorylation, reduced AQP2 in the plasma membrane, antagonizing vasopressin-mediated water permeability in inner medullary collecting duct cells [89]. This may be due to the systemic counterregulatory effects acting on AQP2 regulation. In addition, activation of PKC pathway mediates endocytosis of AQP2 that was independent of the phosphorylation state of AQP2 at serine 256 [80]. AKT (also known as PKB) was also shown to mediate vasopressin-stimulated AQP2 membrane accumulation [90]. The protein kinases responsible for S261, S264 and S269 phosphorylation appear more complex [79, 85, 91, 92].

Phosphorylated AQP2 at S256 (pS256) is detected in both intracellular vesicles and the apical plasma membrane in the collecting duct principal cells, where its abundance is increased in response to AVP treatment [93]. The expression of phosphorylated AQP2 at S264 was found in plasma membrane-associated compartments and early endocytic pathways. This phosphoform of AQP2 was found to increase in abundance in both the apical and basolateral plasma membrane of principal cells after acute dDAVP (1-deamino-8-D-arginine vasopressin, a vasopressin V2 receptor agonist) treatment [94]. Similar to pS256 of AQP2, pS269 was associated with membrane accumulation of AQP2, indicating a role in AQP2 plasma membrane targeting [85, 95, 96]. AQP2 in early endosomes is dephosphorylated at S269 during Rab5-mediated endocytosis, and in recycling endosomes, AQP2 can be phosphorylated at S269 in response to vasopressin prior to apical trafficking [97]. pS261-AQP2 is predominantly localized within the cell in compartments different from the endoplasmic reticulum, Golgi apparatus, and lysosomes [91].

In IMCD tubule suspensions, the specific V2R agonist dDAVP or exogenous cAMP increased phosphorylation of AQP2 at S256, S264, and S269, which remains high as long as the agonist is present [85]. Phosphorylation of S256 increases initially and maximal phosphorylation at S256 occurs rapidly, whereas maximal phosphorylation at the other sites (S264 and S269) occur relatively slow. In contrast, dDAVP stimulation results in decreased phosphorylation at S261 [85] (Fig. 2.5b).

S256 phosphorylation appears required and strongly facilitates phosphorylation of S264 and S269 [85], as the S264 and S269 phosphoforms of AQP2 are not observed in cells expressing the S256 mutated form of AQP2 or in kidney sections from a mouse model with a mutation of S256 to leucine [85, 98]. Recent evidence demonstrated that S256 phosphorylation alone is necessary and sufficient for regulated membrane accumulation of AQP2 induced by AVP (or cAMP), independently of the phosphorylation state of any other sites in the C terminus, e.g., S264 or S269 [99]. These observations strongly suggest that S256 phosphorylation is a priming event for phosphorylation of S264 and S269 and plays a critical role in intracellular translocation of AQP2. Both S256 and S269 phosphorylation are involved in the insertion of AQP2 into the apical plasma membrane [100], although the phosphoform of AQP2-pS269 has a distinct cellular localization in the apical plasma membrane [98]. The role of AQP2 phosphorylation at S264 in subcellular distribution of AQP2 in the cell remains unclear [94]. Earlier studies revealed that the increased monophosphorylation of AQP2 at S256 with vasopressin stimulation of rat IMCD coincided with decreased phosphorylation of AQP2 at S261, which was associated with intracellular vesicle distribution, suggesting that phosphorylation of S256 and S261 may inversely regulate AQP2 trafficking [79, 91, 94, 101].

2.3.1.2 Ubiquitination

It is known that two major protein degradative pathways to function in mammalian cells, the ubiquitin proteasome pathway and lysosomal proteolysis pathway. Ubiquitin (Ub), a 76-amino acid peptide, plays a key role in proteasome-mediated protein degradation. Ubiquitin labels protein through a conjugation system comprising E1 activation, E2 conjugation, and E3 ligation

enzymes. Following conjugation to proteins, ubiquitin serves to target them for degradation by cytosolic proteasome complex. Ubiquitination of certain plasma membrane proteins can promote their internalization via endocytotic pathway, followed by their degradation in lysosomes [102]. Protein ubiquitination is reversed by deubiquitinating enzymes (DUBs), which is essential for cellular homeostasis [103].

The first example of ubiquitination of an AQP was reported more than 20 years ago. The studies demonstrated that AQP1 was able to be ubiquitinated and degraded by the proteasome. Exposure to hypertonic medium induced decrease of AQP1 ubiquitination and markedly increased stability of AQP1 protein, thereby contributing to overall protein induction [104].

There are three putative potential attachment sites (cytosolic lysine residues) for AQP2 ubiquitination at positions 228, 238, and 270, but site mutation study revealed that K270 is the only substrate for ubiquitination, with one to three ubiquitins added in a K63-linked chain [39]. The ubiquitination of AQP2 at the plasma membrane results in the internalization of AQP2, transport to intracellular multivesicular bodies and subsequent proteasomal degradation [39]. Transcriptome analysis and liquid chromatography-tandem mass spectrometry proteomic analysis identified that five common isoforms of E3 ligases (UBR4, UHRF1, NEDD 4, BRE1B and Cullin-5) are putatively associated with dDAVP-induced AQP2 regulation [105]. For example, a vasopressin-activated calcium-mobilizing receptor Cullin-5, a member of the cullin gene family of scaffold proteins of the E3 complex [106], was observed to be upregulated during dDAVP withdrawal, which was associated with increased prevalence of AQP2 among the ubiquitinated proteins in intracellular vesicles fractions. This finding suggests that CUL5 may play a role in the attachment of Ub to AQP2, resulting in an ubiquitination of AQP2, internalization of AQP2 and reduction of AQP2 abundance after dDAVP withdrawal, presumably via lysosomal and/or proteosomal degradation [105]. The E3 ubiquitin ligase CHIP, which is highly expressed throughout the collecting duct, can also interact with AQP2, Hsp70 and Hsc70 and is modulated in abundance by vasopressin. CHIP knockout mice or CRISPR/Cas9 mice without CHIP E3 ligase activity showed higher AQP2 abundance and altered renal water-handling capacity, as seen in reduced water intake and urine output, and increased urine osmolality [107]. Integrated information from multiple large-scale proteomic and transcriptomic datasets showed that NEDD 4 and NEDD 4L have the highest probability of interacting with AQP2 [108], and the ubiquitination regulation of AQP2 differs in different subcellular types [109].

Phosphorylation of AQP2 at S256 and dephosphorylation at S261 cause its translocation from intracellular vesicles to the apical membrane, whereas ubiquitination of AQP2 at K270 induces its internalization and lysosomal degradation, or released in exosomes into the urine via exocytosis. Phosphorylation and ubiquitination are highly dynamic and a cross-talk between two has been proposed [110]. Phosphorylation and ubiquitination likely act in concert and finely regulate AQP2 protein function. Together with the plasma membrane targeting signal of S256, S264 and S269 phosphorylation and intracellular S261 phosphorylation, K270 ubiquitination fine tunes the subcellular distribution of AQP2 (Fig. 2.8).

Two studies examined the potential interplay between polyubiquitylation and polyphosphorylation of AQP2 [101, 111]. Stimulation with dDAVP or forskolin induces pS256 on AQP2 monomers, followed by increased S269 and S264 phosphorylation and reduced S261 phosphorylation, resulting in steady-state redistribution of AQP2 from vesicles to the apical membrane, whereas increased AQP2 ubiquitination induced endocytosis and steady-state redistribution of AQP2 to intracellular vesicles. Interestingly, phosphorylation of S261 on AQP2 occurs after ubiquitin-mediated endocytosis, suggesting that phosphorylation of S261 does not induce AQP2 ubiquitination itself, but likely stabilize ubiquitinated AQP2 (Fig. 2.8).

Phosphorylation often occurs as a priming event for ubiquitination, and ubiquitination can regulate protein phosphorylation by regulation of

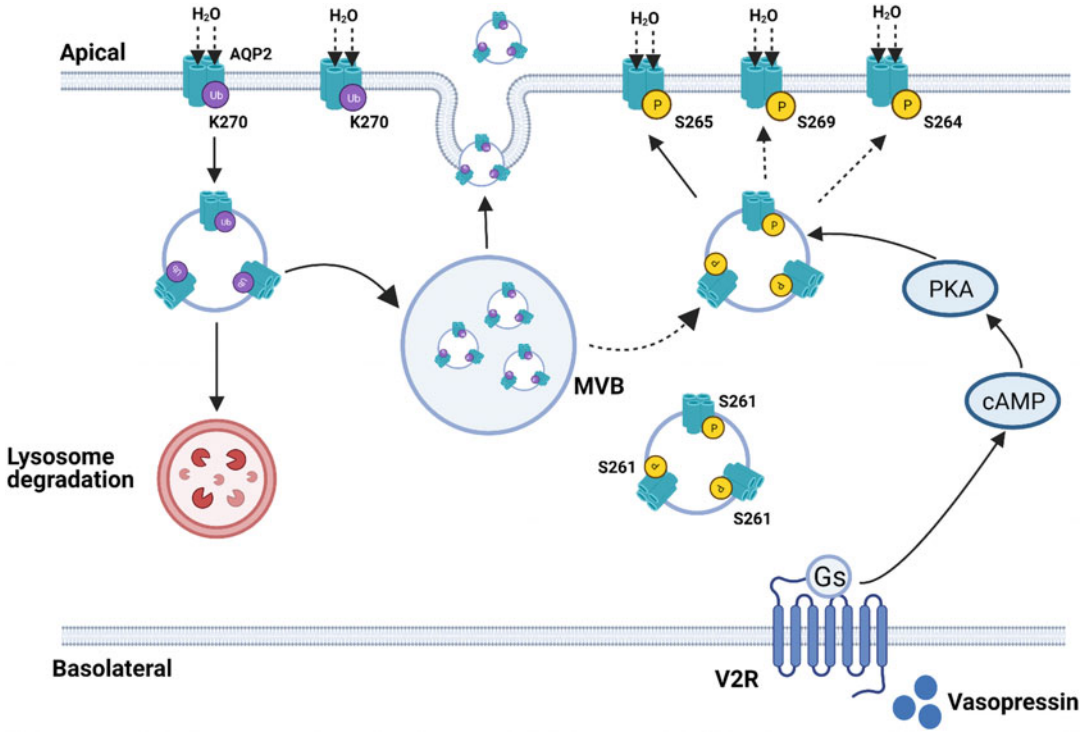

Fig. 2.8 Phosphorylation and ubiquitination of AQP2 determines the intracellular localization. Arginine-vasopressin (AVP)-induced phosphorylation at S256 on AQP2 monomers, followed by increased S269 and S264 phosphorylation and reduced S261 phosphorylation, resulting in steady redistribution of AQP2 from intracellular vesicles to the apical plasma membrane. AQP2 is ubiquitinated with one or more ubiquitin proteins at K270. Ubiquitination occurs in the membrane after removal of AVP stimulation and mediates steady redistribution of AQP2 to intracellular vesicles. Ubiquitination of AQP2 may be sorted to the multivesicular body (MVB), where AQP2 is either degraded in lysosomes or released in exosomes into the urine via exocytosis

kinase activity [110]. AQP2 phosphorylation was demonstrated to be able to override dominant endocytic signal of K63-linked polyubiquitylation. In polarized epithelial cells and kidney tissue, distribution of AQP2 on the plasma membrane is regulated by phosphorylation at S256 and S269. The rate of AQP2 endocytosis was reduced by prolonging phosphorylation specifically at S269. AQP2 phosphorylation at S269 and ubiquitylation at K270 can occur in parallel, with increased S269 phosphorylation and decreased AQP2 endocytosis occurring when K270 polyubiquitylation levels are maximal [111]. The study suggests that site-specific phosphorylation can counteract polyubiquitylation to determine its final localization.

2.3.1.3 SUMOylation

Besides ubiquitin, the best-studied ubiquitin-like protein is Small Ubiquitin-like MOdifier (SUMO). SUMOylation is a reversible PTM where SUMOs are covalently attached to lysine residues in the target proteins, similar to ubiquitination. SUMOylation has been found to be involved in multiple nuclear processes, such as chromatin organization, transcription and DNA repair. SUMOylated proteins also play important roles in the regulation of channel activity, receptor function, G-protein signalling, cytoskeletal organization, exocytosis, and autophagy [112]. So far there is no evidence showing involvement of SUMOylation in regulation of AQP expression.

2.3.1.4 Glutathionylation

As an important PTM, S-Glutathionylation is an important regulatory reversible protein modification, which exerts protection of cysteine residues against irreversible oxidation during redox imbalance [113]. The relationship between AQP2 and S-glutathionylation is of potential interest because reactive oxygen species (ROS) may influence the expression and the activity of different transporters and channels, including AQPs. Evidence suggested that in mpkCCD cells, vasopressin stimulated translation of seven glutathione S-transferase (GST) proteins functioning to conjugate the tripeptide glutathione to substrates, e.g., cysteine, likely indicating the involvement of redox into vasopressin-activated signal transduction pathway [114]. Glutathione is one of the major cellular antioxidant molecules that are continuously converted into the reduced form of GSH. Topological analysis of AQP2 suggests that Cys75 and Cys79 on cytosolic B-loop might be target of S-glutathionylation [115]. Subsequently, the study later demonstrated that AQP2 is subjected to S-glutathionylation both in kidney tissue and in HEK cells stably expressing AQP2 [115]. The S-glutathionylation of AQP2 is tightly modulated by changes in cellular ROS content both in renal tissue and in HEK cells stably expressing AQP2, specifically an oxidant inducer caused a significant increase in AQP2 S-glutathionylation secondary to increases in ROS content, indicating that this redox-sensitive PTM is linked to the redox condition of the tissue; however, whether S-glutathionylation affects the localization and the activity of AQP2 is not reported [115].

2.3.1.5 Glycosylation

In their extracellular loops, AQPs contain N-linked glycosylation consensus sites, some of which are not efficiently recognized during protein synthesis by oligosaccharyltransferase, generating a mixture of glycosylated and nonglycosylated species. N-glycosylation is not believed to be important in the transport function of AQPs. In AQP1, the site of N-glycosylation is Asn42, which lies in a potential N-glycosylation consensus sequence. The early study using site-directed mutant of Asn42 showed that the non-glycosylation of AQP1 failed to affect water permeability in oocytes [116]. In AQP2, the glycosylated form has a shorter half-life than the non-glycosylated form [114], indicating that N-linked glycosylation is not necessary for the stability of AQP2. The glycosylation seems important for cell surface expression of AQP2 [117] but is not essential for routing, evidenced by that inhibition of glycosylation does not prevent delivery of AQP2 to the plasma membrane in response to increased cAMP [118]. In addition, glycosylation appears not essential for tetramerization of AQP2 in the endoplasmic reticulum, as part of tetrameric complexes with one or more nonglycosylated AQP2 molecules [117]. However, early data indeed suggested that addition of a single *N*-linked oligosaccharide moiety can partially compensate for ER folding defects induced by disease-related mutations [119]. The functionality of glycosylated hAQP10 is unaffected compared to the non-glycosylated protein, but its thermal stability is increased by 3–6 °C, suggesting a stabilizing effect of the N-linked glycan [120].

2.3.1.6 Other PTMs

N-terminal acetylation has been proposed to be a determinant of protein stability [121]. AQP2 was one of the proteins identified with N-terminal acetylation [114]. N-linked acetylation, carbamylation, and oleoylation have been discovered on AQP0 at the N-terminal amino acid residues by using direct tissue profiling method designed for membrane protein analysis [122, 123]. Although biological and physiological significance of these PTMs is still undetermined, it might play potential roles in protein–protein interactions and thus regulation of water permeability in the eye. Another study revealed that the N-terminal cysteines of AQP4 are post-translationally modified with palmitic acid, and this palmitoylation likely inhibited the formation of AQP4 square arrays in Chinese hamster ovary cells transfected with AQP4 [124].

2.3.2 PTM of Other AQPs

In addition to the typical AQP2 undergoing phosphorylation regulation, other AQPs also undergo extensive phosphorylation modification as to controls cellular water balance. Here are a few examples about phosphorylation regulation of several AQPs, in particular, AQP0, AQP1, AQP4, AQP5 and AQP8, which have been implicated to trigger membrane-specific trafficking.

2.3.2.1 AQP0

Phosphorylation of the C-terminus of AQP0 is up-regulated in the cortex in a normal lens, and the phosphorylation prediction data base flags the serines at positions 229, 235 and 231 as consensus PKA and PKC phosphorylation sites [125]. Pathological conditions of inappropriate phosphorylation or calcium/CaM regulation of AQP0 contributes to the development of a cataract [126].

2.3.2.2 AQP1

AQP1 water permeability has been shown to be dynamically regulated by several hormones. In *Xenopus* oocyte expression system, water permeability of AQP1 was increased by vasopressin (AVP) and decreased by ANP [127, 128]. Early data from in vitro and in vivo studies suggest that phosphorylation by PKA results in trafficking of AQP1 from an intracellular compartment to the apical membrane [129–132]. In addition, PKC positively regulates both water permeability and ionic conductance of AQP1 channels by phosphorylating Thr157 and Thr239 [133]. A study demonstrated that the signalling molecules cAMP and cGMP promote trafficking of AQP1 into the brush border membrane of proximal tubular cells from intact endosomal compartment [134]. In the same study, cAMP and cGMP have both reduced the ubiquitination of AQP1 and increased AQP1 protein stability, as two potential ubiquitination sites (Lys-243 and Lys-267) were indicated in the AQP1 amino acid sequence [102, 104]. Previous data have shown that a hypotonicity-induced translocation of AQP1 occurs rapidly, which is Ca^{2+}/calmodulin, PKC and microtubule dependent [135–137]. On the other hand, interestingly, exposure to hypertonicity also increases AQP1 expression in cultured renal proximal and inner medullary cells [138, 139]. The effect of hypertonicity may be mediated by promoter-mediated activation of AQP1 synthesis [140] and by inhibition of AQP1 protein degradation [104].

2.3.2.3 AQP4

It is well established that AQP4 water permeability can be regulated by reversible protein phosphorylation. There are several potential phosphorylation sites of AQP4 for PKA, PKC, PKG, casein kinase (CK) and calcium/calmodulin-dependent protein kinases (CaMK).

The Ser111 residue of AQP4 is a potential site for both PKA phosphorylation and calcium-dependent CaMKII phosphorylation. The phosphorylation of Ser111 by PKA increases water permeability of AQP4 [49, 51, 102]. Agents that stimulate cAMP production including forskolin, AVP and V2 receptor agonist were reported to increase the water permeability in a renal cell line transfected with AQP4 [102]. The increased membrane water permeability of an astrocyte cell line transfected with AQP4 cDNA induced by Ser111 phosphorylation was able to be reversed by a Ca^{2+}/CaMKII inhibitor, suggesting that phosphorylation of Ser111 via CaMKII increases the water permeability of AQP4 [141]. Calmodulin binds directly at the carboxy terminus of AQP4, causing specific conformational changes and driving AQP4 to localize on the cell membrane [142]. It is therefore reasonable to speculate that Ser111 is phosphorylated by PKA in kidney cells and by CaMKII in astrocytes, both phosphorylation leads to increased permeability of AQP4. Early studies have suggested that Ser111 could also be phosphorylated by PKG via CaMKII-NO-cGMP-PKG signalling [51]. In contrast to phosphorylation of Ser111, phosphorylation of Ser180 by PKC downregulates AQP4 water permeability both in *Xenopus* oocyte expression system and in cultured kidney epithelial cells [49, 129], which is

previously considered due to a gating effect, since expression of AQP4 in the cytosolic compartment is negligible both under basal conditions and in hormone-stimulated cells [49]. Recent research has shown that addition of laminin to astrocytes in culture can affect the membrane localization and function of AQP4 through the PKC pathway [143].

However, evidence from crystal structure, functional studies and molecular dynamics simulations seems not support phosphorylation dependent gating of AQP4 via Ser111 and Ser180 [7, 144, 145]. Mass spectrometry data demonstrated that AQP4 plasma membrane trafficking or channel gating is not significantly modulated by phosphorylation at COOH-terminal serine residues [144].

2.3.2.4 AQP5

AQP5 membrane trafficking has been shown to be affected by cAMP in a PKA-dependent manner [52, 53]. Elevated intracellular cAMP appears to have distinct acute and chronic effects, which cause a decrease in AQP5 membrane abundance in short-term (minutes) and increased total AQP5 protein in long-term (hours) [146]. Two consensus PKA sites in AQP5 that are able to be phosphorylated have been identified, Ser156 in cytoplasmic loop D [147] and Thr259 [52, 148] in the carboxy-terminus. However, mutation of these phosphorylation sites resulted in constructs with the same membrane abundance as wild-type AQP5, indicating that phosphorylation may not occur under basal conditions. In contrast, AQP5 phosphorylation at Thr259 by cAMP-PKA was shown to be associated with lateral diffusion of AQP5, potentially regulating water flow in glandular secretions [149]. Previous data demonstrated that membrane expression of AQP5 is affected by Ser156 phosphorylation, by increased targeting or decreased internalization or both [57]. Recent data have shown that AQP5 requires the C-terminal domain to pass protein quality control and be transported to the plasma membrane, where Leu262 was shown to be critical for the plasma membrane localization of AQP5 [150]. In a phosphorylation-dependent manner, AQP5 can be gated by extracellular pH, with higher activity at physiological pH 7.4 [151].

2.3.2.5 AQP8

AQP8 is primarily located within the liver cell in a vesicular compartment [152, 153] and in mitochondria [154]. The expression of AQP8 on cell surface is very low under basal conditions [153, 155]; however, hormone glucagon or its second messenger cAMP strongly induced redistribution of AQP8 to the plasma membrane from intracellular compartment [155, 156]. Thereby, the water permeability of plasma membrane is increased, facilitating osmotic water transport and canalicular bile formation. These studies suggest that both PKA and PI3K pathways are involved in glucagon-induced trafficking of AQP8 [155, 157]. Recent data have shown that extracellular hypertonicity can induce increased AQP8 gene expression, and use of inhibitors of the PI3K signalling pathway reduces AQP8 expression [158].

2.4 Summary and Prospect

As the structures of AQPs have been reported one after another, not only the selective transport mechanism of AQPs for substrates, including water, glycerol, ions, etc., is revealed at the molecular level, but also a better understanding of the functional mechanisms of AQPs and how it is regulated by PTM and protein–protein interactions can be achieved. Most importantly, AQPs serve as important drug targets, and their specific inhibitors also have druggable potential. Based on the structure of AQPs, it will better guide the design of drugs.

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Expression Regulation and Trafficking of Aquaporins

3

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Abstract

Aquaporins (AQPs) mediate the bidirectional water flow driven by an osmotic gradient. Either gating or trafficking allows for rapid and specific AQP regulation in a tissue-dependent manner. The regulatory mechanisms of AQP2 are discussed mainly in this chapter, as the mechanisms controlling the regulation and trafficking of AQP2 have been very well studied. The targeting of AQP2 to the apical plasma membrane of collecting duct principal cells is mainly regulated by the action of arginine vasopressin (AVP) on the type 2 AVP receptor (V2R), which cause increased intracellular cAMP or elevated intracellular calcium levels. Activation of these intracellular signaling pathways results in vesicles bearing AQP2 transport, docking and fusion with the apical membrane, which increase density of AQP2 on the membrane. The removal of AQP2 from the membrane requires dynamic cytoskeletal remodeling. AQP2 is degraded through the ubiquitin proteasome pathway and lysosomal proteolysis pathway. Finally, we review updated findings in transcriptional and epigenetic regulation of AQP2.

Keywords

Aquaporin2 · Arginine vasopressin · cAMP · Trafficking · V2R

3.1 Introduction

Aquaporins (AQPs) are expressed in a wide range of tissues and usually spatially located within a certain region of the cell. AQPs mediate the bidirectional water flow driven by an osmotic gradient. The transport of water mediating by AQPs is regulated either by gating, a conformational change, or by altering the AQP density in particular membrane. The trafficking of AQPs is regulated at the transcriptional and/or translational level and also involves shuttles of AQPs between intracellular storage vesicles and the target membrane. Posttranslational modification (PTM), especially phosphorylation, are one of the important mechanisms regulating redistribution of AQPs in the cell. The regulation of AQPs, either through gating or trafficking, allows for rapid and specific water regulation in a tissue-dependent manner. There is another relatively long-term regulation by which increased/decreased protein abundance of AQPs is affected by systemic hormones (e.g., vasopressin, insulin,

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angiotensin II), local molecules, and other common microenvironment signals including pH, divalent cation concentrations, and osmolality. These regulations of AQPs are often associated with certain physiological or pathophysiological conditions.

In this section, regulatory mechanisms of AQP2 are discussed mainly. The mechanisms controlling the regulation and trafficking of AQP2 and the critical role of it in maintenance of body water balance in mammals have been thoroughly studied during the past 30 years. As highlighted by Fenton RA et al. in his review, in some respect AQP2 has become a "model protein" for understanding protein trafficking in epithelia, the role of posttranslational modifications, and the complex hormone-regulated signaling mechanisms that control exocytic and endocytic transport events [1].

3.2 Gating of AQPs

In plants and yeast, the plasma membrane-localized AQPs are gated in response to environmental stress [2]. Gating regulation of aquaporin has been reviewed recently [3]. In mammals, gating regulates the water permeability of AQP0, in a pH-dependent and Ca^{2+}-calmodulin dependent manner [4, 5].

AQP1 is shown to function as an ion channel upon cGMP activation. Phosphorylation of tyrosine Tyr253 in the carboxyl terminal domain of AQP1 acts as a master switch regulating responsiveness of AQP1 to cGMP, and the tetrameric central pore is the ion permeation pathway [6]. In an early study on mechanism of gating and ion conductivity of a possible tetrameric pore in aquaporin1, cGMP is found to interact with an unusually arginine-rich, cytoplasmic loop facilitating its outward motion, which is hypothesized to trigger the opening of a cytoplasmic gate [6].

Gating of AQP4 via phosphorylation has also been suggested [7]. It is well established that eukaryotic AQPs can be gated by phosphorylation, but the trigger for AQP5 gating is still unclear [8]. AQP5 channel can switch between different conformations characterized by distinct rates of water flux, thus changing between open and closed, and between wide and narrow conformations, respectively [9].

Data on gating of AQP2 via phosphorylation is still debatable. Studies from different research groups by using similar systems or different systems have failed to unanimously agree [10–12]. Recently molecular dynamics stimulations are used to investigate the gating mechanism of AQP2. The aromatic/Arg (ar/R) selectivity filter region is a gating site of AQP2, depending on the side-chain conformation of His172. The H172G mutant of aromatic residue His172 is very important in AQP2 gating mechanism, due to its ring orientation and approaching Arg187, resulting to the narrower pore of AQP2 channel and decreased transport of water molecules [13]. Mutation of V168M and G64R on the AQP2 structure impede the permeation of water molecules, indicating that mutant of V168M and G64R also involve in the gating of AQP2 [14]. The electric field direction also plays an important role in the gating of AQP2. The same direction of external electric field and intrinsic electric field along the +Z direction of the AQP2 channels induces selectivity filter regions remaining in the wide conformation, increasing the water permeability [15].

3.3 Trafficking of AQP2 to the Membrane

Facilitated transport processes across epithelia require an apically to basally polarized distribution of transmembranous transport proteins like AQP. AQPs must be transported in vesicles specifically to the apical or basolateral plasma membrane domain, which requires trafficking machineries, including exocytosis, endocytosis, sorting, clustering, and the maintenance of integral membrane proteins at the plasma membranes [16].

Following translation, AQP2 is folded into its monomeric conformation, and subsequently a tetrameric complex, in the endoplasmic reticulum. These tetramers are later transported to the Golgi apparatus where two monomers are N-glycosylated before they are transported

through the trans-Golgi network to different subcellular compartments [17]. A large proportion of AQP2 that exits the trans-Golgi network stored in some form of endosomal vesicles and upon relevant stimulus (e.g., arginine vasopressin, AVP) is transported to the apical plasma membrane [17, 18]. Trafficking of intracellular vesicles containing AQP2 to the membrane, docking and fusion of AQP2 vesicles with the apical plasma membrane (exocytosis), and removal of AQP2 from the membrane (endocytosis) are likely attributed to total plasma membrane abundance of AQP2 [17].

3.3.1 The cAMP-Mediated Effect of Vasopressin on AQP2 Trafficking

AQP2 is present in the principal cells of the renal collecting ducts. AQP2 abundance and intracellular localization in response to the AVP determine water reabsorption in this segments [17–19]. In the absence of AVP, AQP2 is localized in subapical vesicles. Upon stimulation of AVP, a predominantly apical membrane localization of AQP2 is induced. Classically, AVP binds to the basolaterally located vasopressin V2 receptor (V2R), which is coupled to adenylate cyclase (AC) by the heterotrimeric G-protein, Gs. The binding of vasopressin to its receptor causes α-subunit of G-protein to release GDP, bind to GTP, and dissociate from the β- and γ-subunits. This G-αGTP complex, in turn, activates adenylate cyclase to synthesize cAMP which activates protein kinase A (PKA). PKA in turn directly or indirectly phosphorylates AQP2 at the carboxyl terminus. AQP2 can be phosphorylated at four sites—Ser256, Ser261, Ser264, and Ser269 (in humans, Ser269 is conserved as Thr269). Ser256 and Ser269 seem to be the most important to AQP2 targeting and accumulation in the plasma membrane, and thereby they appear to be keystones for the regulation of both endocytosis and exocytosis of AQP2. In contrast, AVP decreases phosphorylation at Ser261, which may increase the stability of the AQP2 protein, without affecting its trafficking [18–23]. The phosphorylation of AQP2 then increases transport or trafficking via the cytoskeleton from the storing cytoplasmic vesicles to the apical membrane (Fig. 3.1).

As PKA has many cellular targets, localization of PKA to specific sites of targets is necessary for a timely and spatially effective phosphorylation of target protein. This is mediated by PKA-anchoring proteins (AKAP). AKAPs bind to both PKA R-subunits and other signaling molecules, thereby allowing the phosphorylation of PKA substrates specifically [24]. For the phosphorylation of AQP2, anchoring of PKA by AKAP in close proximity to AQP2 is a prerequisite [16, 25]. Several splice variants of AKAP18, AKAP18 delta [26], and AKAP220 [27] have been reported to be involved in the shuttling of AQP2 [28–30]. Recent omics data indicate several AKAPs expressed in renal collecting ducts [31], which probably coordinate PKA activity to regulate AQP2 phosphorylation in the vasopressin signaling pathway.

3.3.2 The Role of Calcium in Vasopressin-Induced AQP2 Trafficking

Several studies have demonstrated a role of intracellular Ca^{2+} mobilization in vasopressin-mediated AQP2 trafficking. By binding to V2 receptors, vasopressin causes a transient increase in intracellular Ca^{2+} concentration and calcium oscillations in IMCD cells [32–34]. However, calcium mobilization induced by vasopressin appears not due to coupling of the V2 receptor to the G-protein alpha-subunit Gq/11, as inositol 1,4,5-trisphosphate (IP3) levels are not increased or protein kinase C is not activated in collecting duct cells [35]. It was suggested that the calcium mobilization appears to result from the effects of PKA-mediated phosphorylation of IP3 receptors [36].

Ryanodine inhibitors, calmodulin inhibitors, or intracellular Ca^{2+} chelators were shown to block vasopressin-stimulated translocation of AQP2 to the plasma membrane and increase of osmotic water permeability in primary cultured

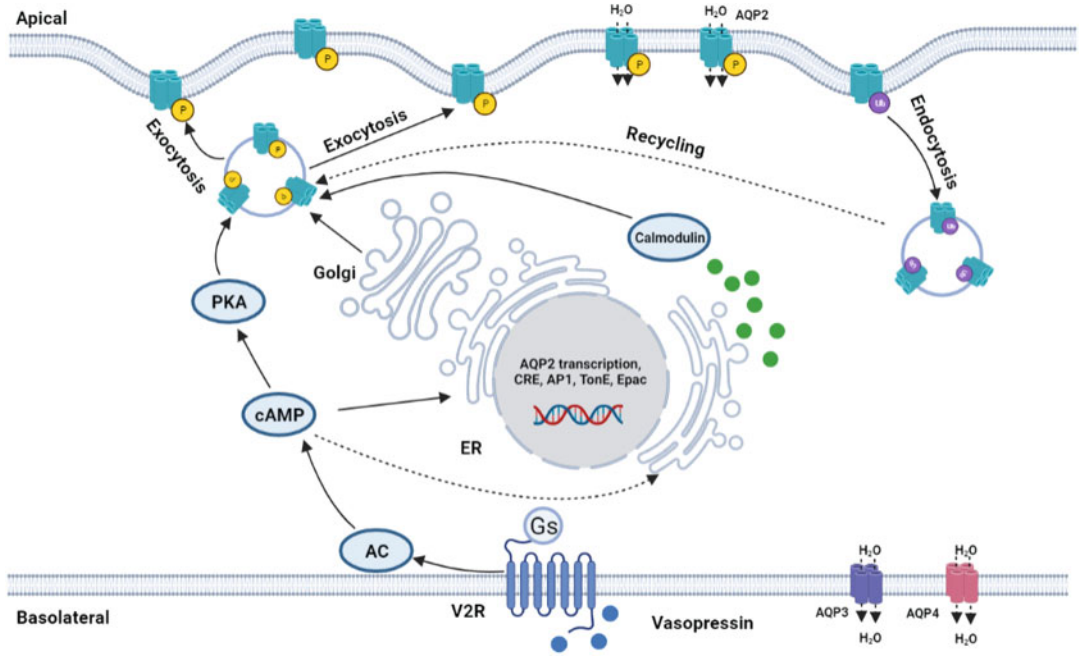

Fig. 3.1 Protein regulation of AQP2 by AVP. AVP binds to the vasopressin type-2 receptor (V2R), present on the basolateral membrane of renal collecting duct principal cells. This induces a signaling cascade, involving Gs protein-mediated activation of adenylate cyclase (AC), a rise in intracellular cAMP, activation of protein kinase A (PKA), and subsequent phosphorylation of AQP2. This results in the redistribution of AQP2 from intracellular vesicles to the apical membrane. AVP stimulation also results in increased intracellular Ca^{2+} levels via Ca^{2+} release from calmodulin-dependent ryanodine-sensitive intracellular stores, which induces apical membrane expression of AQP2. On the long term, vasopressin increases AQP2 expression via activating transcriptional factors, which stimulates transcription of AQP2 at the AQP2 promoter. Once the water balance is restored, AVP levels drop and AQP2 is internalized via ubiquitination. Driven by the transcellular osmotic gradient, water enters principal cells through AQP2 and pass through basolateral plasma membrane via AQP3 and AQP4 to the blood. *ER,* endoplasmic reticulum

IMCD cells [32–34]. These observations suggest that vasopressin-induced intracellular increase of Ca^{2+} is important for AQP2 translocation to the apical membrane (Fig. 3.1). This involves intracellular Ca^{2+} released from ryanodine-sensitive stores and the influx of extracellular Ca^{2+}. However, Ca^{2+} release from endoplasmic reticulum cannot maintain a prolonged intracellular Ca^{2+} mobilization required for an adequate response of collecting duct cells to vasopressin. The Ca^{2+} necessary to sustain cellular response to vasopressin is provided by means of store-operated calcium entry via ORAI1 channel [37]. In contrast, data from other studies in primary cultured epithelial cells from renal inner medulla showed that cAMP is sufficient for triggering the exocytic recruitment of AQP2, which is not evoked by vasopressin-induced intracellular calcium increases [38]. Interestingly, Wnt5a, a ligand for frizzled receptors increasing intracellular calcium [39], is recently shown to induce AQP2 protein expression, phosphorylation and trafficking via Wnt5a/calcium/calmodulin/calcineurin signaling pathway [39, 40].

3.3.3 Vesicles Bearing AQP2 Transport to the Membrane

For the coordinated delivery of vesicles to specific sites, their transport along the cytoskeleton is needed [16]. How the cytoskeleton precisely

modulates AQP2 trafficking is unclear. The AQP2 C-terminus interacts directly with actin monomers and phosphorylation of AQP2 enhances its interaction with tropomyosin-5b, causing F-actin destabilization and promoting AQP2 exocytosis [41], thereby the reorganization of microtubules and actin cytoskeleton are essential in AQP2 trafficking. The actin cytoskeleton provides a cage anchoring AQP2 in unstimulated cells, preventing their exocytosis. The binding of AVP to V2R causes the depolymerization of F-actin in collecting duct cells, which is critical in promoting the trafficking and fusion of AQP2-bearing vesicles with the apical membrane [42, 43]. Interestingly, evidence shows that at the same time when vasopressin induces F-actin depolymerization facilitating AQP2 apical membrane insertion, vasopressin also frees α-actinin 4 to enter the nucleus where it binds glucocorticoid receptor to enhance *AQP2* gene expression [44]. Indeed, AQP2 itself can directly modulate the local actin cytoskeleton depolymerization and subsequent exocytosis. PKA-induced phosphorylation of AQP2 at Ser256 reduced the direct binding of AQP2 to G-actin, but increased the affinity of AQP2 to myosin-Vb, one of the central regulators in apical trafficking. This interaction results in a reduced quantity of myosin-Vb that bounds to F-actin, resulting in F-actin destabilization that allows translocation of AQP2 vesicles to the plasma membrane [41, 45, 46] (Fig. 3.2). AQP2 also interacts with ERM (Ezrin, Radixin and Moesin) family proteins [47], key in cross-linking actin filaments with the plasma membrane. The A-kinase anchoring protein 220 (AKAP220) is a ubiquitously expressed vesicular and membrane-associated anchoring protein that positively regulate actin polymerization and microtubule stability during membrane protrusion [48]. Early studies showed that AKAP220 is physically associated with AQP2 in the principal cells of the kidney collecting ducts [27]. This study supports the role of actin-barrier dynamics in the subcellular localization of AQP2 in the kidney.

There are some binding proteins at C-terminus of AQP2 (or in AQP2-bearing vesicles) that mediate AQP2 sorting and the destination of AQP2. A large-scale proteomic analysis showed that more than 180 proteins were identified, including SNARE proteins, trans-Golgi network markers, motor proteins, etc. These proteins interact with AQP2 via direct binding, indirect linkage, forming a protein complex, or colocalization in the same vesicles [49, 50], which is actively involved in regulation of AQP2 dynamics. For example, immunoisolated AQP2-bearing intracellular vesicles are associated with the presence of a large variety of actin-related cytoskeletal proteins such as actin-related protein (Arp)2/3, b-actin and c-actin, myosin isoforms, tubulin, Rab GTPases [49], suggesting a complex network of proteins that interact with actin during AQP2 vesicular trafficking. For example, Rho GTPase activation stabilizes cortical F-actin and inhibits AQP2 trafficking [51]. The GTPase-activating protein Spa-1 (SPA-1) inhibits Rap1 GTPase-activating protein, which triggers F-actin disassembly and may maintain the basal mobility of AQP2 [52]. Recently, an actin-related protein Arp2/3 was found essential for AQP2 trafficking, specifically for its delivery into the post-trans-Golgi network exocytotic pathway to the plasma membrane [52]. Myosin II and its regulatory light chain are present in an AQP2-binding protein complex [53], and it is critical for AQP2 recycling [53]. Myosin light chain kinase, which regulates actin filament organization by phosphorylating the regulatory light chain of myosin, was recently showed to be required for vasopressin-induced actin depolymerization and AQP2 transition from early to late endosomes [54] (Fig. 3.2).

3.3.4 Docking and Fusion of Vesicles Bearing AQP2 with the Apical Membrane

Fusion of AQP2-bearing vesicles with the plasma membrane is a key terminal step in vasopressin-regulated water transport. The docking and fusing of AQP2-bearing vesicles is mediated by SNARE (Soluble *N*-ethylmaleimide sensitive factor attachment protein receptors) mechanisms [17] which involves vesicle (v) SNAREs (soluble NSF attachment protein receptors) and target

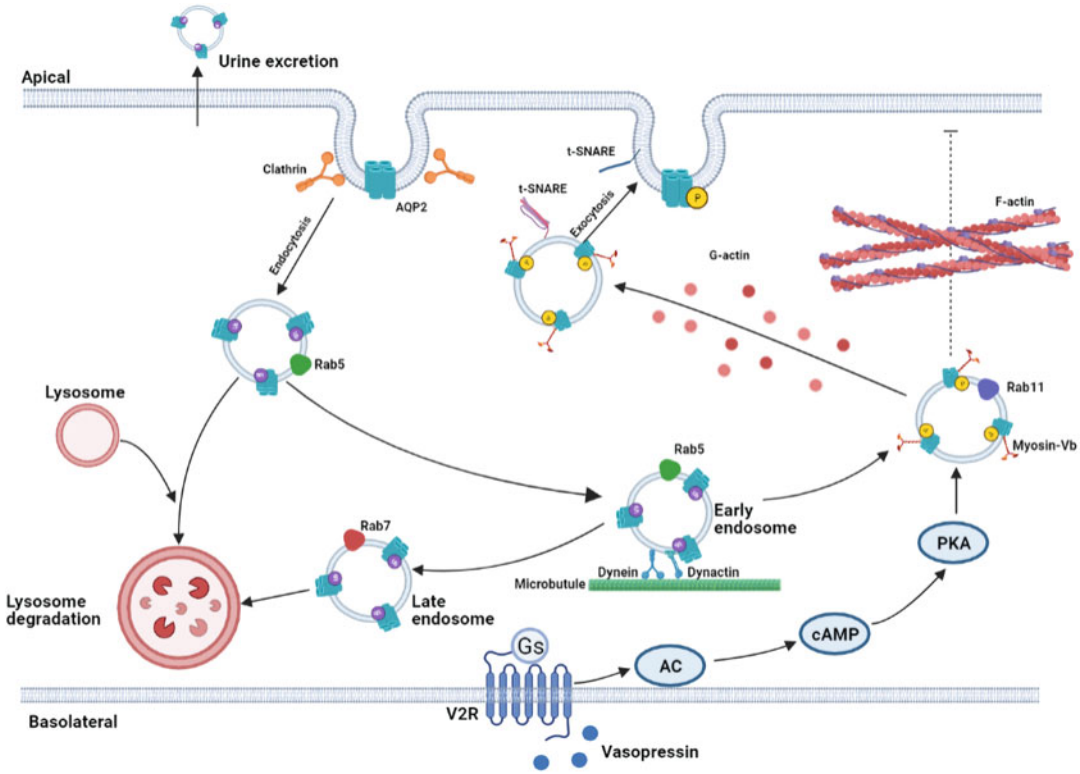

Fig. 3.2 Exocytosis and endocytosis of AQP2. AVP triggers cAMP signaling and induces phosphorylation of AQP2 which dissociates G-actin from AQP2 and promotes AQP2 interaction with myocin-Vb. This releases myocin-Vb from F-actin and induces destabilization and depolymerization of the F-actin network, allowing vesicles bearing-AQP2 transport to the membrane. AQP2-bearing vesicles contain specific v-SNAREs that bind to specific t-SNAREs on the apical plasma membrane; AQP2 is thus fused with the apical plasma membrane. After AVP washout, AQP2 localizes to clathrin-coated pits and undergoes clathrin-mediated endocytosis. Internalized AQP2 can be targeted either to recycling pathways or to degradation via lysosomes. Internalized Rab5-mediated AQP2 vesicles are transported to early (Rab5), late endosomes (Rab7), and multivesicular bodies (MVBs) transporting along microtubules for storage. From MVBs, they can then either be lysosomally degraded or recycled via the Rab-11-dependent slow recycling pathway

membrane (t) SNAREs. Multiple components of the SNARE system are found in the collecting duct principal cell. The v-SNARE proteins vesicle-associated membrane protein (VAMP)-2 and VAMP-3 are found in AQP2-containing vesicles [18, 27], and t-SNARES (syntaxin-4, syntaxin 3, SNAP23, and SNAP25) are observed in the apical membrane of principal cells [55, 56]. Snapin, an intermediate scaffolding molecule, was found to serve as a linker between AQP2 and the t-SNARE complex and can aid AQP2 trafficking from storage vesicles to the apical plasma membrane [55, 56]. The cleavage of VAMP-2 by tetanus toxin blocked the AVP-mediated AQP2 translocation to the plasma membrane, suggesting a role of v-SNARS in AQP2 docking [57]. Knockdown of Munc18, a protein-inhibiting SNARE-mediated membrane fusion, increased AQP2 membrane accumulation, whereas knockdown of VAMP-2, VAMP-3, syntaxin 3, and SNAP23 inhibited AQP2 fusion at the apical membrane [58]. These studies strongly suggest involvement of SNARE in AQP2 docking and fusion to the membrane. It is noted that many other proteins (e.g., annexin-2, GTPase, AKT substrate) are also involved in

AQP2 trafficking and exocytosis, although their precise roles and how they interact with AQP2 (or AQP2-bearing vesicles) remains to be fully established [17] (Fig. 3.2).

3.3.5 Removal of AQP2 from the Membrane and Degradation

Regulated endocytosis of AQP2 contribute significantly to final plasma membrane levels of AQP2. Inhibition of endocytosis can increase the amount of AQP2 at the apical membrane [17, 59–61], indicating another way to increase the water permeability of collecting ducts. Endocytosis of AQP2 is shown to require dynamic actin cytoskeletal remodeling, and actin is involved in multiple steps in the endocytosis pathway, including coated pit formation, constriction, internalization, splitting, and merging of clathrin-coated vesicles, and lateral mobility on the cell surface [62].

In the endocytotic process, AQP2 accumulates in clathrin-coated pits and is internalized via a clathrin-mediated process in a dynamin-dependent manner [63–65]. The role of dynamin in AQP2 endocytosis is confirmed by the finding that GTPase-deficient dynamin mutants exhibit arrested endocytosis and accumulation of AQP2 in the apical membrane independently of vasopressin stimulation [65]. The microtubule-associated motor protein dynein and the associated dynactin complex are associated with intracellular vesicles bearing AQP2 [66], indicating a role of microtubule complex in AQP2 trafficking.

The AQP2 C-terminus interacts directly with the actin cytoskeleton linker Ezrin, an actin-binding protein facilitating endocytosis of AQP2 [66]. Direct binding of Ezrin to AQP2 promotes AQP2 endocytosis, linking AQP2 trafficking to the dynamic actin cytoskeletal network [66]. While, knockdown of Ezrin was associated with elevated AQP2 membrane accumulation and decreased AQP2 endocytosis.

Hsc70, a heat shock protein, which is important for uncoating clathrin-coated vesicles, may bind to the C-terminus of non-phosphorylated AQP2 and is reported to be required for AQP2 endocytosis [67]. Mimicking phosphorylation of AQP2 at S256 and S269 decreased their interaction with clathrin, hsp/hsc70, and dynamin alongside a decreased rate of endocytosis [12, 67]; thus, phosphorylation of AQP2 may alter the efficiency of pit maturation and clathrin-coated vesicle and modulate quantity of AQP2 in clathrin-coated pits and internalization [68].

AQP2 was shown to interact with caveolin-1, a principal component of caveolae membranes that are involved in receptor-independent endocytosis [69]. Both AQP2 and caveolin-1 were internalized in response to forskolin removal [69], indicating that AQP2 is internalized through caveolae/caveolin-1 dependent mechanisms. In addition, evidences support the role of membrane rafts in regulation of AQP2 endocytosis. Reagents depleting membrane cholesterol induces plasma membrane accumulation of AQP2 in vivo and in vitro, likely a result of decreased AQP2 internalization [60, 61, 70, 71].

When endocytosis starts, AQP2 is internalized into early, late endosomes and multivesicle bodies (MVBs) for sorting or storage. MVBs can be either lysosomally degraded or recycled via the Rab11-dependent recycling pathway. By these ways, AQP2 could be recycled either via the trans-Golgi network or directly to the plasma membrane, leading to AQP2 expression in plasma membrane. MVBs degradation could lead to downregulation of AQP2 in the plasma membrane and the cells [72, 73].

A number of Rab GTPases: Rab4, Rab5, Rab18, and Rab21 (associated with early endosomes), Rab7 (associated with late endosomes), and Rab11 and Rab25 (associated with recycling endosomes) have been identified in immuno-isolated AQP2-containing intracellular vesicles [66], suggesting roles of these Rab GTPases in AQP2 trafficking [74]. For example, after endocytosis, AQP2 is retrieved to early endosomes through a PI3K-dependent mechanism and then is transferred to Rab11-positive storage vesicles [50, 75, 76] (Fig. 3.2). A heterotrimeric retromer complex made up of three vacuolar sorting proteins (Vps): Vps26,

Vps29, and Vps35 is found to interact with Rabs mediating cargo (e.g., AQP2) sorting to plasma membrane, transport from endosomes to the trans-Golgi network or degradation [77].

Ubiquitination works as a signal for endocytosis and subsequent degradation by multivesicular body or proteasome. Ubiquitination of lysine 270 of AQP2 is important for AQP2 endocytosis and degradation [78]. The E3 ubiquitin ligase CHIP ubiquitinylates AQP2 through interacting with AQP2, Hsp70, and Hsc70. CHIP knockdown increases AQP2 expression in the plasma membrane, indicating its involvement in AQP2 endocytosis and degradation [78]. Some AQP2 transferred to multivesicular body is excreted into the urine as exosomes [79–81].

A database reports 139 AQP2-interacting proteins identified by mass spectrometry in rat inner medullary collecting duct [78]. This interactome delineates an overall picture of a dynamic biological process in which AQP2 is synthesized in the rough ER, matures via the Golgi apparatus, transported to endosomes that move into or out of the plasma membrane, and regulated in the plasma membrane [78].

3.4 Transcriptional Regulation of AQP2

3.4.1 Transcription Factors Responsible for the Expression Regulation of *Aqp2*

Aside from intracellular trafficking and PTM, the protein levels of AQP2 are also regulated transcriptionally. Several different transcription factors (TF), such as CREB, the AP1 [82], NFAT family (TonEBP and NFATc) [83, 84], and NF-γB [84] have been involved in this regulation. Vasopressin treatment or dehydration for a certain time results in increased water permeability of the collecting ducts, a response called "long-term regulation". This response is mainly attributed to an increased abundance of AQP2 protein due to stimulated transcription of the *Aqp2* gene [85], which is mediated by the vasopressin-V2R signaling cascade [86]. Sequencing of the 5-flanking region of the *Aqp2* gene revealed several putative cis-binding element motifs including a cAMP-response element (CRE) and an SP-1 site [87, 88]. CRE present in the *Aqp2* gene regulates transcription of the gene [83, 89]. While the PKA-CRE pathway is shown to be involved in the initial rise in AQP2 levels after dDAVP stimulation, long-term regulation of AQP2 may involve the activation of Epac [90]. Hypertonicity affects transcription of many genes through the interaction between the tonicity-responsive enhancer (TonE) and its transcription factor TonEBP. TonEBP knockout mice show downregulated protein expression of AQP2, confirming the role of TonE/TonEBP in AQP2 transcription [83]. A system-level analysis of cell-specific *Aqp2* gene expression in renal collecting duct revealed many transcriptional regulators and transcriptional regulators binding elements that were involved in the transcription of the *Aqp2* gene. The transcriptional regulators that bind to ETS, HOX, RXR (retinoid X receptor family), CREB, and GATA (GATA-binding factors) of the *Aqp2* gene are likely to be involved in cell-specific regulation of *Aqp2* gene expression [91], providing further insight into the transcription regulation of the *Aqp2* gene. Recently, a Bayes' theorem was used to integrate several omics data sets to stratify the 1344 TFs present in the mouse genome with regard to probabilities of regulating *Aqp2* gene transcription [92]. The analysis identified 17 of 1344 TFs that are most likely to be involved in the regulation of *Aqp2* gene transcription, including Cebpb, Elf1, Elf3, Ets1, Jun, Junb, Nfkb1, Sp1. Atf1, Irf3, Klf5, Klf6, Mef2d, Nfyb, Nr2f6, Stat3, and Nr4a1. Surprisingly, among these TFs, CREB (Creb1) is absent [92]. A recent genome-wide mapping of DNA accessibility and binding sites for CREB and C/EBPβ showed that in vasopressin-sensitive collecting duct cells, C/EBPβ (but not CREB), a pioneer transcription factor critical to cell-specific gene expression, bound strongly at the identified enhancer downstream from *Aqp2* [92]. Thus, any role for CREB in the regulation of A*qp2* gene transcription is unlikely to be direct.

3.4.2 AQP2-Targeting miRNAs in the Kidney

miRNAs, important modulators of gene expression that act via regulation of the mRNA translation, provide novel insights in the intricate regulation of protein expression and function [93]. Integrated bioinformatic analysis of the miRNAome and proteome suggested that AQP2 expression was regulated by epigenetic machinery and various transcription factors [93]. About 19 major miRNAs (miR-1193, miR-3549, miR-181d, miR-92b, miR-463, miR-342, miR-93, miR-3573, miR-127, miR-324, miR-411, miR-1, miR-873, miR-16, miR-3074, miR-206, miR-678, miR-496, and miR-298) were found to be responsive to vasopressin in rat kidney inner medullary collecting duct (IMCD) cells using microarray chip assay. miR-127, miR-1, miR873, miR-16, miR-206, miR-678, miR-496, miR-298, and miR-463 exhibited 1.3-fold increase in changes in expression after vasopressin stimulation [93].

Among them four miRNAs (miR-32, miR-137, miR-216a, and miR-216b) are shown to target the 3′-untranslated region of rat AQP2 mRNA. Target seed regions of miR-32 and miR-137 were also conserved in the 3-′-untranslated region of mouse AQP2 mRNA. Overexpression of miR-32 or miR-137, which was not identified as vasopressin-responsive microRNAs, decreased dDAVP-regulated AQP2 mRNA and protein levels in mpkCCDc14 cells, indicating that the interaction of miRNAs with the AQP2 regulatory pathway is likely vasopressin-independent [90]. Consistent with this, a downregulation of AQP2 expression induced by stimulation of the calcium sensing receptor signaling is likely attributed to the miR-137 generation [93].

Dicer is a critical regulator of the biogenesis of miRNA, which helps to process pre-miRNAs to mature miRNAs in the cytoplasm. Dicer$^{AQP2Cre1+}$ mice (selectively suppressed Dicer expression in AQP2-positive cells of the mouse kidney collecting ducts) have severely reduced AQP2 in the kidney. Among 56 differentially expressed miRNAs, 31 had at least a twofold difference in expression (14 upregulated and 17 downregulated) between Dicer$^{AQP2Cre1+}$ and control mice. Only three miRNAs (miR-7688-5p, miR-8114, and miR-409-3p) altered in the renal inner medulla of Dicer$^{AQP2Cre1+}$ mice, which could be putative regulators of AQP2 expression. However, Luciferase assays failed to demonstrate a direct interaction of AQP2 or the three potential transcription factors with miR-7688-5p, miR-8114, and miR-409–3p. In fact, these miRNAs were found involved in epigenetic control (Phf2, Kdm5c, and Kdm4a) or transcriptional regulation (GATA3, GATA2, and ELF3) of AQP2 [93].

3.5 Epigenetic Regulation of AQP2

Histone H3 lysine 27 (H3K27) is a histone marker associated with open chromatin and increased transcription. H3K27 acetylation mapping is widely used to identify open regions of DNA overlapping both promoters and enhancers [93]. Vasopressin or dDAVP induced a marked increase in histone H3K27 acetylation (H3K27ac) across the body of the AQP2 gene, and in the promoter, as well as in upstream of the AQP2 transcriptional start site in mpkCCDc14 cells [90]. A very recent study demonstrated that in hypokalemia-induced nephrogenic diabetes insipidus, the level of acetylated H3K27 (H3K27ac) was decreased in the Aqp2 promoter region, which was associated with reduced Aqp2 mRNA levels. Histone deacetylases inhibitors prevented the downregulation of AQP2 mRNA and protein, likely by enhancing H3K27 acetylation [93]. This study indicates a key role of H3K27ac in AQP2 regulation during pathophysiological conditions. Epigenetic regulation of other AQPs including DNA acetylation or methylation is seen in other reviews [94].

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Transport Characteristics of Aquaporins

4

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Abstract

Aquaporins (AQP) are a class of the integral membrane proteins. The main physiological function of AQPs is to facilitate the water transport across plasma membrane of cells. However, the transport of various kinds of small molecules by AQPs is an interesting topic. Studies using in vitro cell models have found that AQPs mediated transport of small molecules, including glycerol, urea, carbamides, polyols, purines, pyrimidines and monocarboxylates, and gases such as CO_2, NO, NH_3, H_2O_2 and O_2, although the high intrinsic membrane permeabilities for these gases make aquaporin-facilitated transport not dominant in physiological mechanism. AQPs are also considered to transport silicon, antimonite, arsenite and some ions; however, most data about transport characteristics of AQPs are derived from in vitro experiments. The physiological significance of AQPs that are permeable to various small molecules is necessary to be determined by in vivo experiments. This chapter will provide information about the transport characteristics of AQPs.

Keywords

Aquaporins · AQPs · Aquaglyceroporins · Water channel

4.1 Water Transport Mediated by Aquaporins

The main physiological function of aquaporins (AQPs) is to facilitate the water transport across plasma membrane of cells (Fig. 4.1) [1, 2]. Water transporting property of AQPs was first confirmed via biophysical function studies of AQP1 that possesses extremely high water permeability reaching $2 \sim 3 \times 10^9$ water molecules per subunit per second [3].

Almost all of rat AQPs are permeable to water with various single-channel water permeability including: AQP0 (0.25×10^{-14} cm^3/s) [4], AQP1 (6.0×10^{-14} cm^3/s) [4], AQP2 (3.3×10^{-14} cm^3/s) [4], AQP3 (2.1×10^{-14} cm^3/s) [4], AQP4 (24×10^{-14} cm^3/s) [4], AQP5 (5.0×10^{-14} cm^3/s) [4]. The water permeability of other AQPs was measured by various groups: human AQP6 was reported to be inhibited by $HgCl_2$ [5], nevertheless, when the rat AQP6 expressed in oocytes, it was activated by

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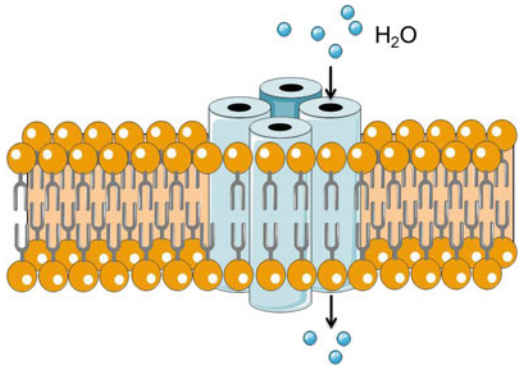

Fig. 4.1 AQP1 mediates transmembrane water permeability. AQP1 is organized as a tetrameric assembly of four identical polypeptide subunits. Water molecules pass through a pore of each AQP1 monomer

Hg^{2+} to dramatically increase the osmotic water permeability ($P_f = 93.0 \times 10^{-4}$ cm/s) [6]. The mouse AQP6 was identified to have low water permeability activated by Hg^{2+} [7]. Another group confirmed that rat AQP6 lacks water permeability [8]. AQP7 was initially found and cloned from rat testis, and water permeability coefficient of *Xenopus* oocytes injected with rat AQP7-cRNA reached 186 μm/s [9]. The cloning and water transport measurement of mouse AQP8 were performed using *Xenopus* oocytes, and the single channel water permeability of AQP8 was up to 8.2×10^{-14} cm^3/s [10]. Rat AQP9 cRNA-injected *Xenopus* oocytes expressed ~fourfold increase of coefficients of osmotic water permeability (P_f) [11]. The function of AQP10 was also examined in Xenopus oocyte expression system, in which the osmotic water permeability increased up to sixfold with AQP10 expression [12]. Using CHO cells transfected with GFP-AQP11 to measure the water permeability of AQP11, the osmotic water permeability P_f value enhanced up to 8.0×10^{-4} cm/s [13]. Another group confirmed that mouse AQP11 was water permeable using stopped-flow analysis of vesicles containing mouse AQP11 [14]. Whether AQP12 could transport water has not been determined yet.

With selective pore for the rapid movement of water across cell membranes, AQPs are crucial for the transport of water and regulation of water homeostasis. In body, there are two trans-tissue water flow routes: transcellular water flow mediated by AQPs and paracellular flow. AQPs are thought to be the specific channels for rapid water transport in response to osmotic gradient, making a critical contribution to the regulation of transcellular water flow [15].

When expressed in *X. laevis* oocytes, AQP1 exhibited significantly high osmotic water permeability that was 20-fold higher than that of the control oocytes [16, 17]. AQP1 protein reconstituted into membrane proteoliposomes causes the 50-fold raise in water permeability [18, 19]. This process occurs with low Arrhenius activation energy and is inhibited by $HgCl_2$ or other mercurial and is reversed by a reducing agent. Water permeability mediated by most AQPs can be inhibited by mercurial reagents such as $HgCl_2$ whose mechanism was elucidated by molecular dynamics simulations [20]. Mercury inhibits water and glycerol transport by mammalian AQPs through binding to cysteine residues [21]. However, AQP4 and AQP6 are not inhibited by $HgCl_2$ [22, 23].

To explain how a simple pore without moving parts could allow rapid transit of water without movement of protons, the groups of Robert Stroud and Bing Jap solved the atomic structures of AQP1 from bovine red blood cells at 2.2 Å resolution [24]. Later, several groups performed molecular dynamics simulations on the basis of this solved structure [25, 26]. Now, the essence of how AQPs facilitate the movement of water but not protons has been revealed. Peter Agre explained how AQP1 could selectively accommodate water molecules transporting in his Nobel lecture [27]: water maintains the bulk solution condition at the extracellular vestibule and an internal vestibule both have the hourglass structure of the AQP1 molecule. In a single file, water could pass through a 20 Å channel that separates vestibules, the water molecules could interact with pore-lining residues to prevent the formation of hydrogen bonds between the water molecules. Especially near the top of the bridged site, the channel reaches its narrowest constriction of 2.8 Å, thus the pore is so narrow that it just accommodates a single water molecule. The mechanism of repealing proton from its

permeation of AQP includes [24, 27–29]: (1) The side chain in loop E forms a fixed positive charge and a conserved histidine residue of another wall forms a partial positive charge, these two positive charges collectively repel protons. (2) Moreover, a single water molecule could form hydrogen bonds simultaneously when it transiently undergoes a transient dipole reorientation, which also serves to be the barrier to protons.

AQPs mediate the bidirectional water flow driven by an osmotic gradient, which can be adjusted either by gating, conformational change, or altering the AQPs density in a particular membrane. Among them, the protein abundance of AQPs can be affected by systemic hormones (e.g., vasopressin, insulin, angiotensin II), local molecules (e.g., purine, prostaglandins, bradykinin, dopamine) and other common microenvironment signals, including pH, divalent cation concentrations and osmolality. The functions of aquaporins are regulated by posttranslational modifications, such as phosphorylation, ubiquitination, glycosylation, subcellular distribution, degradation, and protein interactions [30].

The structure and mechanism of AQP0 gating have always been controversial. Gonen et al. observed two contraction sites along the pores of AQP0, suggesting that the structure of AQP0 in sheep may be closed. In addition, AQP0 also exhibits unusually low water conductance compared to other AQPs, and pH gating can only regulate water conductance threefold, so the structural changes between the open and closed conformations may be unclear yet [31]. Due to tight contacts between extracellular regions, differences in crystal packing may lead to a gating effect of AQP0.

In previous studies, "capping" and "pinching" gating mechanisms were proposed based on channel analysis of AQPs crystal structure. AqpZ is a typical representative of "pinching" gating mechanism. In the crystal structure model of AqpZ homologous tetramer, the R189 side chain has two different conformations of "up" and "down" Further molecular dynamics simulations showed that the R189 side chain could swing up and down rapidly. Therefore, R189 residue is considered to be the switch of AqpZ gating, which swings up and down the side chain to change the diameter of the nearby channel to control the channel switch. Recently, Yang et al. investigated the structure, dynamics and water proximity of key "gated" residues of AqpZ in a functionally active state, revealing that the water molecular channel of AqpZ is in a "permanently open" state. Therefore, it is now widely accepted that most AQPs function as the permanently open channels for water permeation without a gate while a permanently open conformation of the R189 side chain of AqpZ exists in native or native-like membrane environments [32].

4.2 Glycerol Transport Mediated by AQPs

In addition to the primary function of AQPs to facilitate water transport, glycerol transport could be another significant function of AQPs. Aquaglyceroporins, including AQP3, AQP7, AQP9 and AQP10, are a subset of aquaporin family and the exclusive mammalian proteins with the ability to permeate glycerol with their relatively broad solute specificity and sequence homology. One of the physiological functions of aquaglyceroporins is to facilitate the transport of glycerol across the cell membrane. Such glycerol-transporting function of aquaglyceroporins is involved in the movement of glycerol and energy metabolism process.

AQP3 (originally called glycerol intrinsic protein, GLIP, based on its glycerol-transporting function) was first cloned by three different groups, respectively [33–35]. AQP3 is a relatively weak transporter of water but functions as an efficient glycerol transporter. Measurements of the 10-min glycerol uptake of *Xenopus* oocytes after microinjection of 5 ng of AQP3 cRNA and a 24 ~ 27 h incubation at 18 °C indicate that glycerol uptake is remarkably increased compared with control. Glycerol uptake in oocytes expressing AQP0, AQP1, AQP2, AQP4 or AQP5 is not increased significantly compared with control [4]. AQP3 is mainly expressed at the basolateral membrane of epithelial cells in kidney collecting duct, airway and intestine, as

well as in epidermis, urinary bladder, conjunctiva and cornea [36]. As an aquaglyceroporin, AQP3 mediates glycerol permeability in certain organs, tissues and cells. In skin, the stratum corneum (SC) is the most superficial layer whose hydration determines skin appearance and physical properties [37]. Phenotype analysis of AQP3-deficient mice indicates that AQP3 expressed in epidermal keratinocytes plays an essential role in hydration process and maintaining biological function of skin [38, 39]. Study on AQP3 null mice showed that deficiency of skin AQP3 impaired glycerol transport through basal keratinocyte layer into the epidermis and SC, resulting in the reduced glycerol content of epidermis and SC and therefore impairing hydration and epidermal biosynthetic functions [39]. These data provide us compelling evidence that glycerol-transporting property of AQP3 is important for the skin function (Fig. 4.2) [37].

AQP7 is abundantly expressed in human adipose tissue and acts as an adipose glycerol channel (Fig. 4.3) [40], it is found to act as a facilitative carrier for water by tenfold (186 ± 15 μm/s), glycerol by fivefold (the calculated $P_{glycerol}$ was 18.9×10^{-6} cm/s) and urea by ninefold (the calculated P_{urea} was 12.0×10^{-6} cm/s), in *Xenopus* oocytes expressing AQP7 [9].

Rodríguez et al. reported that AQP3 and AQP9 are also expressed in omental and subcutaneous fat depots, in addition to the well-known expression of AQP7 in adipose tissue [41]. AQP3 and AQP9 act as glycerol channels in adipocytes and the liver, respectively, representing novel additional pathways for the glycerol transport in human adipocytes [42, 43]. Studies on AQP7 and AQP9 knockout or knockdown mice demonstrate the pathophysiological relevance of glycerol channels through effects on glycerol metabolism. Impairment or lack of AQP7 function might have a causal role in obesity and diabetes mellitus [43].

AQP9 is mainly expressed in liver, testis, brain, leukocytes, epididymis and spleen [44–46]. By injecting rat AQP9-cRNA into oocytes and determining the permeability profile of AQP9, it is concluded that AQP9 confers high permeability for water as well as other solutes including carbamides, polyols, purines, pyrimidines and monocarboxylates [46]. A subsequent research shows AQP9 mainly facilitates glycerol and urea transport [47]. Further study also shows that AQP9 facilitates glycerol influx and urea efflux in hepatocytes, providing evidence that AQP9 acts as an important solute channel associated with energy metabolism [11].

In human adipocytes, AQP3, AQP7 and AQP9 represent the glycerol channels involved in the regulation of lipid and glucose metabolism [40, 42]. AQP3 is present in the plasma membrane and cytoplasm. AQP7 is expressed predominantly in the cytoplasm upon the lipid droplets. AQP9 is constitutively expressed in the plasma membranes [41]. The role of aquaglyceroporins expressed in adipocytes is to control the transport of glycerol into and out of adipocytes, which are critical steps for lipogenesis and lipolysis [43]. In the lipogenic process, AQP9-mediated glycerol uptake provides glycerol for the glycerol-3-phosphate proceeds, and further involves in the triacylglycerols synthesis of adipocytes (Fig. 4.4) [42]. In the lipolysis, stimulation of adrenergic receptors by catecholamines leads to a translocation of AQP3 and AQP7 to the plasma membrane to facilitate the glycerol release, which parallels with the translocation of HSL to the lipid droplets and its activation, and leptin (via the PI3K/Akt/mTOR signalling cascade) and catecholamines downregulate AQP7 expression, which restrict glycerol release from adipocytes (Fig. 4.3) [42].

AQP10 is abundantly expressed in the duodenum and the jejunum [48], which is subsequently identified as aquaglyceroporin on account of its functional and structural similarity with other aquaglyceroporins AQP3, AQP7 and AQP9 [12]. Using oocytes, isotopic solute uptakes mediated by AQP10 were detected, resulting that the glycerol permeability was significantly increased threefold with AQP10, which was inhibited by $HgCl_2$ [12].

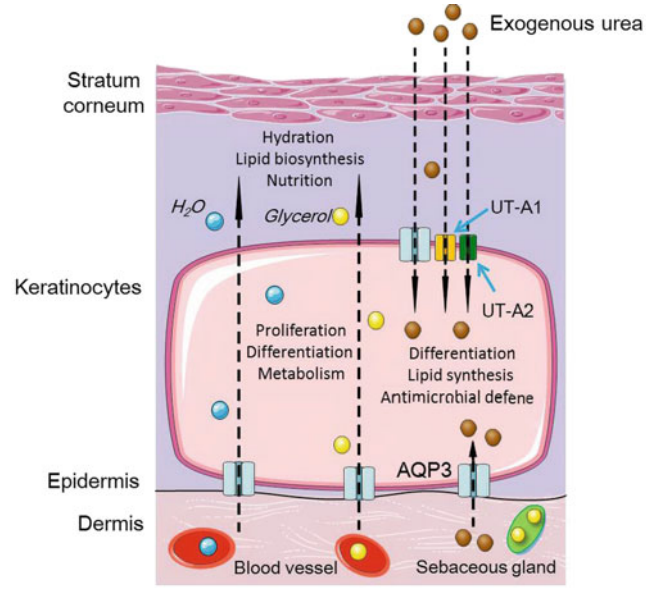

Fig. 4.2 AQP3 mediates transmembrane glycerol permeability in epidermis. Urea transporters UT-A1, UT-A2 and AQP3 facilitate urea uptake in skin, which may induce keratinocyte differentiation and improve barrier and antimicrobial defense function of skin. Moreover, AQP3 facilitates water and glycerol transport from blood and sebaceous glands to keratinocytes

4.3 Urea Transport Mediated by AQPs

Urea is mainly generated from ammonia in liver as a key role in protein catabolism in mammals. As a terminal product, approximately 90% of urea is eliminated in urine by the kidney [49]. In the kidney, urea transport and cycle are vital in urinary concentrating mechanism [50]. Some of human AQPs are permeable to urea including AQP3 [34], AQP7 [51], AQP9 [44], AQP10 [12] and possibly AQP6 [52], but the physiological significance of these aquaporins in urea transport is not fully revealed.

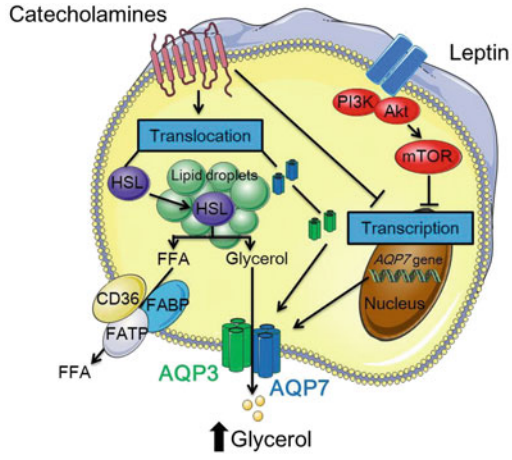

Fig. 4.3 Proposed role of aquaglyceroporins in lipolysis. Translocation of AQP3 and AQP7 to the plasma membrane is led by the stimulation of adrenergic receptors by catecholamines to facilitate the glycerol release. AQP7 expression is downregulated by leptin and catecholamines, which represents a negative feedback regulation in lipolytic states to restrict glycerol release from adipocytes

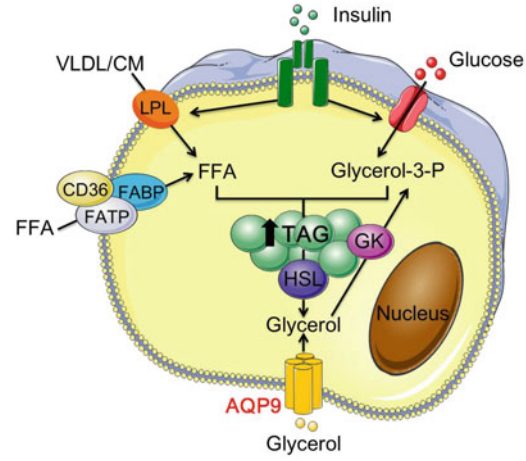

Fig. 4.4 Proposed role of aquaglyceroporins in lipogenesis. Triacylglycerols (TAG) is synthesized from FFA and glycerol-3-phosphate in adipocytes. Glycerol-3-phosphate proceeds from: (1) glucose, (2) glycerol from HSL-dependent lipolysis that is phosphorylated by GK or (3) AQP9-mediated glycerol uptake

Whether AQP3 is one of urea channels remains conflicting. With AQP3 expressing in *Xenopus* oocytes and measurement of the urea permeability, early work suggests that urea uptake is increased to twofold after 30 min incubation with radio-labelled urea, which can be completely blocked by phloretin, the inhibitor of urea transporters [34]. Controversially, subsequent study did not find urea-permeating property of rat AQP3 [53]. The difference may be resulted from the use of diverse concentrations of urea, and the AQP3-mediated urea transport is so low that it does not induce significant change in volume under lower urea concentration (20 mM) [54].

AQP6 possesses water permeability under the activation of Hg^{2+} as described by Yasui et al. [6]. AQP6 was determined to be permeable to urea using AQP6-expressing oocytes, and the uptake of [^{14}C]urea stimulated by $HgCl_2$ was initially large ($P_{urea} = 21.3 \times 10^{-7}$ cm/s) but decreased with time. However, the uptake of [^{14}C]urea into AQP6-expressing oocytes without stimulation of $HgCl_2$ was about three times less [52].

Ishibashi et al. found that urea uptake was increased up to ninefold in 5 min and 16-fold in 10 min with AQP7 expression in the oocytes, whose stimulation effect of urea uptake was much higher than that of AQP3 [9].

AQP9 expressed in cRNA-injected oocytes showed the increase of urea permeability coefficient (P_{urea}) from 1.5×10^{-6} cm/s (water-injected as control) to 23.5×10^{-6} cm/s [45].

Urea permeability of AQP10 was measured in *Xenopus* oocyte expression system, and the result showed urea uptake was significantly increased twofold, which was inhibited by phloretin [12].

AQP3, AQP7 and AQP9 appear to play roles in urea transport in skin. AQP3 and AQP9 are expressed in the differentiating layers of human epidermal skin equivalents [55]. AQP7 localizes to superficial epithelial cells of the gastrointestinal tract [56]. Expression of AQP3, AQP7 and AQP9 could be upregulated by urea [57]. Studies revealed that urea transporters and AQPs transport exogenous urea into keratinocyte, playing a critical role in keratinocyte differentiation, lipid synthesis and maintaining epidermal homeostasis. Moreover, AQP3 is proposed as the important channel of epidermis in which AQP3 facilitates water and glycerol transport from blood and sebaceous glands to keratinocytes involved in proliferation and differentiation of keratinocytes (Fig. 4.2) [16].

AQP3 null mice have nephrogenic diabetes insipidus under normal conditions. When given a urea load, the concentration of urine reaches high level; however, the excretion of other solutes reduces significantly [58]. The capacity of urea to increase the concentration of non-urea solutes relies on AQP3 and its function in transporting both urea and water [59]. AQP10 is only found in duodenum and jejunum [48], and it transports water, urea and glycerol when expressed in *Xenopus* oocytes [12]. Further study of AQP10 in urea transport is less carried out. AQP9 is a urea-permeable protein localized at the basolateral membrane of hepatocytes, since the liver is a major site of urea production [44]. AQP9 is also abundantly expressed in the peripheral leukocytes permeable to water and urea [45].

4.4 Gas Transport Mediated by AQPs

AQPs including AQP1, AQP3, AQP4, AQP5, AQP8 and AQP9 could potentially transport gases such as CO_2, NO, NH_3 and O_2.

4.4.1 Carbon Dioxide

Carbon dioxide (CO_2) is a neutral linear molecule with a diameter similar to water. Early study showed that permeability of CO_2 was significantly increased in *Xenopus* oocytes injected with AQP1 cRNA and proteoliposomes containing purified AQP1 [60, 61], supporting the hypothesis that AQP1 is a pathway for CO_2 transport across the membrane. Also, one study adopting ^{18}O-labelled HCO_3^- to examine the CO_2 permeability of AQP1-null human erythrocytes compared with normal ones verified

that AQP1 is responsible for 60% of the high P_{CO2} of erythrocytes [62], directly suggesting that AQP1 plays the critical role in mediating CO_2 transport. It is suggested that it goes across plasma membrane through central space of tetramer of AQP1 and does not go through water pore (Fig. 4.5).

Further study on cholesterol-containing membranes reconstituted with human AQP1-mediating CO_2 permeability showed significant increase in membrane CO_2 permeability, suggesting that both cholesterol and AQP1 are necessary in CO_2 permeability across biological membranes [63].

When expressed in oocytes, bovine AQP0, human AQP1, rat AQP4-M23, rat AQP5, rat AQP6, rat $AQP6_{N60G}$, or rat AQP9 exhibited significantly increased permeability to CO_2 measured by microelectrode positioned at the surface of the oocytes to detect pH change [8].

However, some studies suggest that AQP1-dependent CO_2 transport has no physiological relevance [64, 65]. Verkman group reported experiments in which physiological consequences of CO_2 transport by AQP1 were studied by comparing CO_2 permeability in erythrocytes and intact lung of wild-type and AQP1 null mice. Results show no difference in CO_2 permeability between AQP1 null mice and wild-type mice, providing direct evidence against physiological significance of CO_2 permeability mediated by AQP1 [64, 65].

4.4.2 Nitric Oxide

As another physiologically important gas, nitric oxide (NO) plays a critical role in cardiovascular, renal, and central nervous system (CNS). The diameter of NO is similar to that of water, so it may be reasonable for NO to pass through water holes. Early studies show that NO is produced by the endothelial cells of the vasculature in which it can relax adjacent vascular smooth muscle cells to regulate blood flow and blood pressure [66–68]. Previous conception that the process of NO transporting from endothelial cells to the vascular muscle cells occurred by free diffusion through the lipid bilayer of the cell membrane was challenged by the discovery of AQP1 in transporting NO. In the vascular system, AQP1 expressed in endothelial cells [69] involved in vascular function. By transfecting AQP1 into CHO cells and reconstituting purified human AQP1 into the lipid vesicles, transport property of NO by AQP1 was measured. In CHO cells expressing AQP1, NO permeability was identified to be correlated with water permeability. The use of AQP1 inhibitor led to a NO transport reduction by 71%, and the NO transport is saturable. In the reconstituted lipid vesicles expressing AQP1, NO influx was increased by 316% [70]. All the above results support the hypothesis that NO is transported by AQP1.

Using AQP1 null mice, Herrera et al. subsequently identified that transport of NO by AQP1 was required in full expression of endothelium-dependent relaxation, though NO free diffusion still occurred in the absence of AQP1 slowly [71, 72].

In addition to AQP1, AQP4 located in brain is also permeable to NO through its central pore, and it even provides a more favourable permeation pathway for gas molecules than AQP1 [73]. Further investigation is required to clarify the role of AQP4 in the control of NO flow in the central nervous system.

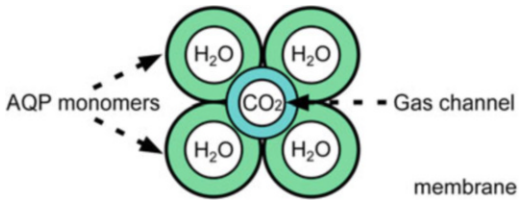

Fig. 4.5 Gas passes through the central space of AQP tetramer in plasma membrane

4.4.3 Ammonia

Ammonia (NH_3) transport by AQPs has not been widely studied. Holm et al. first observed a role of AQPs as NH_3 channels [74]. *Xenopus* oocytes and lipid bilayers expressing AQP1, AQP3, AQP8 and AQP9 have been shown to facilitate

NH_3 transport [74–76]. Another group measured NH_3 permeability of the AQP cRNA-microinjected *Xenopus* oocytes, and their results indicated that human AQP1, rat AQP3, rat AQP6, rat $AQP6_{N60G}$, human AQP7, human AQP8 and rat AQP9 have a significantly increased permeability to NH3 [8]. Nevertheless, the physiological significance of AQPs as NH_3 transporters remains unclear [72].

4.4.4 Oxygen (O_2)

Molecular dynamics (MD) simulations on the AQP1-embedded membranes and on the pure lipid bilayers indicated that the central pore of AQP1 is an ideal channel for the permeation of both CO_2 and oxygen (O_2). The result of MD simulations showed the central pore of APQ1 permeates O_2 with a $-0.4 \sim -1.7$ kcal/M energy well [77].

4.5 Other Molecules Transported by Aquaporins

4.5.1 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) belongs to the group of reactive oxygen species (ROS). ROS are generated in a number of key metabolic processes in cells such as the electron transport chain in the inner mitochondrial membrane [78]. Because of the potential damage of ROS on nucleic acids, proteins and lipids, cells have a number of ROS-scavenging systems to remove these molecules and to maintain a relatively low and constant ROS concentration [79]. Although the formation and scavenging of ROS has been studied thoroughly relatively, little is known about their transport mechanism from the site of origin to the place of action or detoxification [79]. The obvious chemical similarity between water and H_2O_2 suggests that AQPs could likely be candidates for H_2O_2 permeation, and many studies confirmed that certain AQPs could mediate H_2O_2 transmembrane transport [79–83]. H_2O_2 molecules display a dipole moment of 2.26 D, which are slightly greater than that of water molecules (1.85 D). In 2006, human AQP8 was evidenced to facilitate the diffusion of H_2O_2 across membranes adopting fluorescence assay with intact yeast cells and intracellular ROS-sensitive fluorescent dye [79]. And as the H_2O_2 has been revealed to be an important signalling molecule for immune response, growth, differentiation, migration processes, Miller et al. demonstrated that AQP3 and AQP8 promote uptake of H_2O_2 in HEK 293 cells transfected with AQP3 or AQP8 expression vectors, and that intracellular H_2O_2 accumulation can be modulated by endogenous AQP3 expression which influences downstream cell signalling cascades [83]. Another study showed that the AQP3-meidated H_2O_2 uptake is essentially required for the chemokine-dependent T-cell migration during immune response, which revealed a novel physiological role of AQP3-mediated H_2O_2 transport [82].

4.5.2 Some Ions

AQPs were originally regarded as plasma membrane channels that are freely permeable to water or small uncharged solutes but not to ions [84]. But now there is increasing evidence that certain AQPs have ion channel function [84, 85]. In 1996, Yool group reported that AQP1 acts as a cation channel (K^+, Cs^+, and Na^+ and to a lesser degree tetraethylammonium) [86] which was initially controversial. Subsequent researches by Yool group showed that human AQP1 expressed in *Xenopus* oocytes could mediate cationic conductance gated by the activation of cGMP [87] and defined not only the ion channel function but also the detailed molecular mechanisms that govern and mediate the multifunctional capabilities of AQP1 [88]. In AQP1, the central pore at the fourfold axis of symmetry in the tetramer has been proposed as the most likely pathway for cation conduction [85]. AQP1 functions as a non-selective monovalent cation channel when activated by intracellular cGMP, with a large single channel

conductance of approximately 150 pS in standard physiological saline conditions [87].

In other AQPs such as AQP0 and AQP6, the possible role of the intrasubunit pores as ionic conductance pathways is proposed by many research groups [6, 88–90]. As the major protein component of isolated lens junctions, AQP0 shows ion channel activity when reconstituted in bilayers [91]. Bovine AQP0 has a conductance of 200 pS in unilamellar vesicles with 100 mM saline, which supports ion channel activity [88]. The ion channel of AQP is detected to be voltage- and pH-sensitive, open at acidic pH and close permanently at neutral pH [89].

Rat AQP6 was found expressed in intracellular vesicles of renal epithelia. As a gated channel, mammalian AQP6 expressed in oocytes shows intermediate conductance (49 picosiemens in 100 mM NaCl) induced by $HgCl_2$ [90]. At pH lower than 5.5, anion conductance is rapidly and reversibly activated in AQP6 oocytes. The cation/anion selectivity changed with the site-directed mutation of lysine to glutamate at position 72 in the cytoplasmic mouth of the pore leaving low pH activation intact [6]. The studies of Ikeda et al. indicated that AQP6 exhibits a form of anion permeation with significant specificity for nitrate [84]. The functions of AQPs as gated ion channel and as water channel are considered to have physiological and potentially translational relevance [92].

4.5.3 Silicon

Silicon is abundantly and differentially distributed in body. Researchers showed that unlike that silicon transporter exists in plants and algae, human aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) can mediate silicon transport in *Xenopus laevis* oocytes and HEK-293 cells. Further, aquaglyceroporins could act as the relevant silicon permeation pathways in both mice and humans, regulating the Si balance in body. And this study surprisingly found that phloretin stimulated the Si transport of AQP9 [93].

4.5.4 Antimonite and Arsenite

GlpF in the *E. coli* was shown to be responsible for the uptake of the toxic antimonite Sb(III). Deletion of the *fps1* gene in *S. cerevisiae* rendered the yeast cells more resistant to antimonite as well as to arsenite. Trivalent antimonite and arsenite have a pKa of 11.8 and 9.2, respectively. Hence, at physiological pH, these metalloids may pass aquaglyceroporins as neutral hydroxides $Sb(OH)_3$ or $As(OH)_3$, which are structurally similar to glycerol. The mammalian aquaglyceroporins AQP7 and AQP9 may also pass Sb(III) and As(III) [94].

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Non-Transport Functions of Aquaporins

5

Xiaowei Li and Baoxue Yang

Abstract

Although it has been more than 20 years since the first aquaporin was discovered, the specific functions of many aquaporins are still under investigation, because various mice lacking aquaporins have no significant phenotypes. And in many studies, the function of aquaporin is not directly related to its transport function. Therefore, this chapter will focus on some unexpected functions of aquaporins, such the decreased tumor angiogenesis in AQP1 knockout mice, and AQP1 promotes cell migration, possibly by accelerating the water transport in lamellipodia of migrating cells. AQP transports glycerol, and water regulates glycerol content in epidermis and fat, thereby regulating skin hydration/biosynthesis and fat metabolism. AQPs may also be involved in neural signal transduction, cell volume regulation, and organelle physiology. AQP1, AQP3, and AQP5 are also involved in cell proliferation. In addition, AQPs have also been reported to play roles in inflammation in various tissues and organs. The functions of these AQPs may not depend on the permeability of small molecules such as water and glycerol, suggesting AQPs may play more roles in different biological processes in the body.

Keywords

Non-transport function · Aquaporin

5.1 AQPs in Cell Migration

Many studies have found that AQPs are involved in cell migration in different cell types and in AQP-null mice. AQP1 was the first aquaporin found to be involved in cell migration and angiogenesis. Knockout of AQP1 in mice not only slows tumor development and mortality but also slows wound healing [1]. This migration effect extends to chick neural crest cells because they play a key function in development through the extracellular matrix. The migration speed of aortic endothelial cells in Aqp1-deficient mice in response to chemotactic stimuli was lower than that of wild-type cells, and after AQP transfection, cell migration in various cells increased. However, alternative mechanisms are currently being investigated, such as AQP-dependent changes in cell volume during the migration and interaction of AQP with other proteins. The promotion of cell migration by AQP appears to be a general mechanism applicable to tumor metastasis, wound healing, and immune cell chemotaxis.

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Loss of AQP4 slows the migration of reactive astrocytes to chemotactic stimuli and increases glial scar formation [2, 3].

Tumor cells expressing AQP1 have enhanced transvascular exudation and local invasion capabilities [4]. In addition, high levels of AQP expression have been detected in many tumor types (such as glioblastoma) and are related to tumor classification [5].

It has been reported in AQP1-null mice, the volume, blood vessel density, and lung metastasis of polyomas formed by T oncogene (MMTV-PyVT) in the mouse breast tumor virus are all reduced. These results indicate that AQP1 is related to tumor development and lead to the hypothesis of AQP1 as a potential target for adjuvant therapy of solid tumors [6]. In peritoneal macrophages extracted from AQP1-deficient mice, AQP1 ablation affects the morphology, cytoskeletal organization, membrane polarization, and migration of macrophages. It was found that the ablation of AQP1 caused macrophages to extend, axially polarize, and direct membrane lipids transfer to the cell front edge. The number of peritoneal infiltrating macrophages in AQP1-deficient mice was reduced two times [7]. Taken together, these results indicate that the proper expression of AQP1 is necessary for macrophages and possibly for tissue remodeling and wound healing. In further human studies, the pharmacological inhibition of AQP1 may be useful in cancer treatment, and the induction of AQP1 may accelerate wound healing and promote organ regeneration.

Subsequent evidences have shown that AQPs promote cell migration, regardless of AQP and cell type. AQP4 promotes the migration of astrocytes [2, 3], AQP3 promotes the migration of corneal epithelial cells [8] and epidermal cells [9], and AQP1 promotes migration of proximal renal tubule cells [10], melanoma and breast cancer cells [4]. In addition to the angiogenesis defect in AQP1 deficiency, other consequences of AQP promoting cell migration include tumor migration, glial scar formation, and wound healing. AQP1 expression in tumor cells increases their migration across the endothelial barrier, local invasiveness, and metastatic potential [4]. Loss of AQP4 slows down the migration of reactive astrocytes to chemotactic stimuli and increases glial scar formation [2, 3]. AQP3 deficiency causes damage in skin wound healing [11] and corneal wound closure [8]. AQP-dependent cell migration may be involved in other processes, such as organ regeneration and leukocyte chemotaxis, which remains to be studied. In addition, AQPs may also be involved in intracellular vesicle transport, such as secretory granule exocytosis and astrocyte cytokine vesicle secretion in pancreatic acinar cells [12, 13].

Several hypotheses have been proposed to explain the mechanism by which AQPs enhance cell migration. The cell migration of multiple AQPs with different structures was enhanced, indicating that the transmembrane water transport promoted by AQP is a responsible mechanism. Biophysical studies propose that aquaporin polarization to the leading edge of migrating cells facilitates water influx during lamellipodial extension [14]. Actin depolymerization and ion influx increase the osmotic pressure of the cytoplasm at the front end of the migrating cell [15, 16]. These local changes in cytoplasmic osmotic pressure drive the influx of water through the plasma membrane. The water influx facilitated by AQPs causes the adjacent plasma membrane to expand by increasing the local hydrostatic pressure, followed by rapid repolymerization of actin to stabilize the cell membrane protrusion (Fig. 5.1) [1]. AQPs promote the flow of osmotic water through the plasma membrane in the cell protrusions formed during the migration process, thereby promoting the cell migration process.

5.2 AQPs in Central Neural System (CNS)

The role of AQPs in the nervous system has also been revealed in recent years of research. AQPs are widely expressed in the central nervous system, of which AQP4 and AQP1 are the most expressed, and a small amount of AQP9 is expressed in some neurons [17]. In brain tissue, AQP1 is most prominently located in the luminal membrane of the choroid plexus epithelium

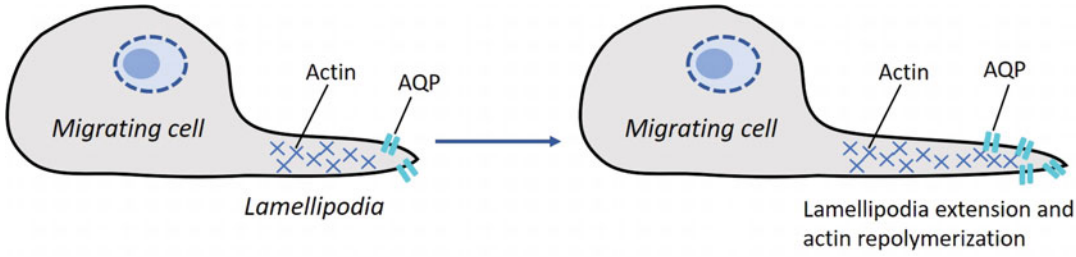

Fig. 5.1 The role of aquaporin in cell migration. Through an osmotic gradient created by actin depolymerization and reactive solute influx (left), water is driven into the cytoplasm primarily through AQPs in the lamellipodia, promoting lamellipodia extension in the direction of cell migration (right)

[18]. Reports have indicated that AQP1 is mostly absent in the brain parenchyma of rodents [19]. In contrast, non-human primates have AQP1 expression mainly in white matter and glial-restricted astrocytes [20]. Although the abundance of AQP1 and AQP9 in human and mouse brain cells is extremely low under physiological conditions [21, 22], AQP1 is upregulated under some pathological conditions [23–25]. AQP4 is expressed in the foot processes of astrocytes in the central nervous system, whose tail feet are in contact with blood vessels related to the blood–brain and brain–fluid interfaces [26]. AQP4 has been proposed to be involved in many aspects of brain physiology and pathophysiology, including brain edema formation, K^+ clearance and related glial cell swelling, glymphatic, ISF (interstitial fluid) flow, volume of interstitial fluid in the brain, formation of astrocytes, optic neuromyelitis, brain tumor growth, and memory [27].

5.2.1 AQP4 in Brain Edema

In the cellular cytotoxic brain edema model (permanent cerebral ischemia and acute water intoxication model), brain swelling was reduced in AQP4-null mice, and neurological outcome was improved, which include water intoxication, focal cerebral ischemia, and bacterial meningitis [28, 29]. The upregulation of AQP4 expression is mainly found in glial cells, choroid plexus, ependymal cells, and hippocampus in the edema area, suggesting that AQP4 expression is related to brain edema after cerebral ischemia, especially cytotoxic edema. MCAO model experiments show that compared with wild-type mice, AQP4 knockout mice have significantly less brain edema [30, 31]. AQP4 deficiency in the cerebral infarction reduced cytotoxic edema after cerebral ischemia. It is confirmed that AQP4 is an important factor in the formation of cytotoxic edema after ischemia [32]. In vitro studies have shown that the inhibition of AQP4 expression in astrocytes by RNA interference technology delayed the edema occurrence.

However, in the vasogenic edema model induced by freezing injury, AQP4-null mice showed more severe brain swelling, including cortical freeze injury, brain tumors, brain abscesses, higher intracranial pressure, and hydrocephalus, which may be due to impaired AQP4-dependent hydrocephalus clearance [33, 34]. Upregulation of AQP4 reduced traumatic brain tissue edema, suggesting that AQP4 plays an important role in the clearance of vasogenic edema [35]. These studies suggest that AQP4 has dual roles in development and resolution of CNS edema, with water flow through AQP4 driving cytotoxic edema development in the early post-injury stage but later clearing vasogenic edema.

5.2.2 AQP4 in Neuroexcitation

In addition to the role of AQP4 in water balance in the brain and spinal cord and astrocyte

migration, evidence indicates that AQP4 is involved in neurostimulation [36–38]. Mice lacking AQP4 have prolonged seizure activity after electrical stimulation [39] and long-term cortical spreading inhibition after mechanical stimulation [40]. Previous studies have found that the decreased expression of AQP4 in the brain of patients with epilepsy suggests that AQP4 is involved in the process of neural excitation [41]. Just like in the brain and spinal cord, AQP4 is expressed in astrocytes adjacent to excitable cells (neurons), and AQP4 is also expressed in supporting cells adjacent to excitable cells in the optic nerve sensory tissue, including Müller cells, Sertoli cells in the inner ear, and Sertoli cells in the olfactory epithelium [42, 43]. AQP4 knockout mice showed impaired evoked potential responses to light [44] sound [45], and olfactory stimuli [46].

Results from AQP4-deficient mouse brain showed that the accumulation of potassium ions (K^+) in the extracellular space (ECS) was reduced during neural excitation [39, 47], and the rate of K^+ clearance in ECS was slowed down after neural excitation [40, 47]. Altered K^+ dynamics in the ECS is believed to be responsible for the neuroexcitation phenotype, and slower K^+ clearance will prolong the duration of seizures [39]. The ECS is a closed water chamber between brain cells and accounts for 20% of the total brain volume [48, 49]. K^+ is released into ECS by neurons in response to membrane depolarization during neural excitation and is mainly cleared by uptake by astrocytes to re-establish the pre-excitation state. K^+ reuptake is mediated by inwardly rectifying K^+ channels, Kir4.1, and other astrocyte K^+ transporters [50, 51] and accompanied by a shrinkage of ECS [52]. The main determinants of K^+ uptake after nerve excitation include electrochemical driving force, astrocyte K^+ permeability, ECS volume, and AQP4 water permeability of unknown mechanism. The mechanism link between the uptake of K^+ by astrocytes in the absence of AQP4 and the water permeability of AQP4 is unclear. A widely speculated possibility indicates the functional interaction between AQP4 and Kir4.1 [53]. However, AQP4 expression did not affect Kir4.1 K^+ channel function in freshly isolated astrocytes [54] and Müller cells [55], or brain [47].

Quantitative ECS volume measurement results showed that the brain ECS volume of AQP4 knockout mice is about 20% higher than that of wild-type mice [56]. Therefore, it is suggested that AQP4 may change the K^+ dynamics by affecting the volume of ECS. One explanation is that the reabsorption of K^+ by astrocytes after nerve excitation drives the inflow of osmotic water and the subsequent contraction of ECS, which will maintain the electrochemical drive force of K^+ re-uptake (Fig. 5.2). Therefore, when AQP4 is deficient, water cannot enter astrocytes through AQP4, resulting in the expansion of the ECS volume, which further slows down the reuptake of K^+ by astrocytes. This hypothesis was confirmed by a mathematical model [57]. Therefore, it is suggested that AQP4-mediated water permeability may directly regulate the process of nerve excitation [58].

Collectively, substantial evidence has indicated that aquaporins play vital roles in cell proliferation through various mechanisms. However, whether the effects of aquaporins on cell proliferation are related to their own water permeability characteristics has not been demonstrated. Aquaporins are responsible for maintaining the balance between cell proliferation and apoptosis by affecting the progression of the cell cycle, as well as through crosstalk with other cellular cascade signals or transcription factors, thereby regulating cell cycle progression or by regulating the biosynthetic pathways of cellular structural components.

5.3 AQPs in Fat Metabolism

In the past few years, the key role of AQP7 in obesity has been proposed. AQP7 is highly expressed in white adipose tissue (WAT), brown adipose tissue (BAT), and testis [59]. AQP7 expressed in the plasma membrane of adipocytes promotes the transport of glycerol across the cell membrane [60]. Studies have found that AQP7 mRNA levels decrease after eating and increase

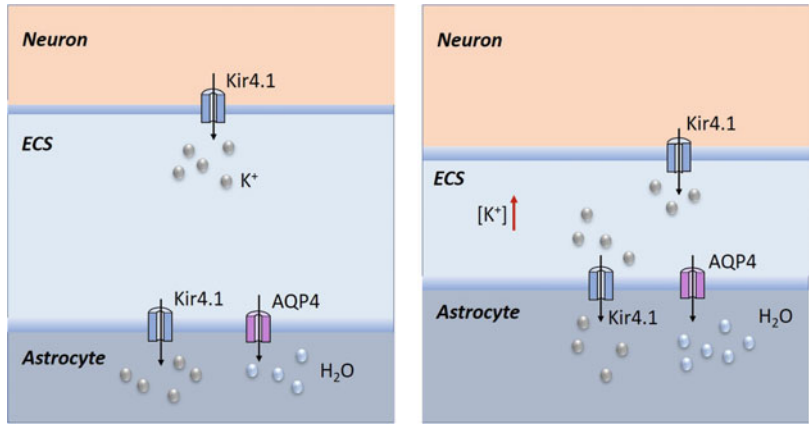

Fig. 5.2 The role of AQP4 in neural excitation. AQP4 promotes entry of water into astrocytes, resulting in extracellular space (ECS) volume contraction and increased ECS K^+ concentration (right panel), which further drives astrocyte K^+ uptake

after fasting [61]. AQP7 expression levels correlate with these nutrient changes and have an opposite trend to plasma insulin levels. In 3T3-L1 adipocytes, insulin dose-dependently suppressed AQP7 mRNA levels. An insulin-negative response element (IRE) was identified in the promoter region of the AQP7 gene [59]. This result suggests that AQP7 transcription and expression levels are tightly regulated by insulin. The storage of glycerol and triglycerides in the adipocytes of AQP7-deficient mice is significantly increased, causing the adipocytes to become hypertrophy, which in turn leads to the development of adult obesity [62]. Overall, these results provide evidence that the increase in fat cell volume may be due to the decreased glycerol permeability of adipocytes and the subsequent accumulation of glycerol and triglycerides. AQP9 is considered to be an important way for the liver to take up glycerol [63]. However, further studies on AQP9 knockout mice have shown that plasma glycerol and triglyceride levels are significantly increased, and glycerol metabolism is insufficient. These results indicate that AQP9 may be a metabolic regulator for hepatic glycerol internalization [64]. Therefore, AQP7 and AQP9 are important promoters of glycerol transmembrane transport and regulators for controlling glycerol metabolism, contributing to investigation on obesity and diabetes [63, 65].

Intra-abdominal visceral fat mainly accumulates in the mesentery. The anatomical distribution of abdominal visceral fat suggests that substances released from visceral fat flow directly into the liver through the portal vein. AQP9 is highly expressed in liver, leukocytes, lung, and spleen [66]. Glycerol, another product of fatty triglycerides produced during lipolysis, flows directly into the liver through the portal vein and becomes a substrate for gluconeogenesis. AQP9 is considered to be the only glycerol channel in the liver and is located in the plasma membrane of the sinusoid facing the portal vein [67]. In conclusion, AQP9 functions as a channel for glycerol uptake in the liver. AQP9 mRNA levels were increased by fasting and decreased by feeding [68]. Likewise, insulin suppressed AQP9 mRNA levels in H4IIE hepatocytes in a time- and dose-dependent manner. Promoter analysis indicated that insulin decreased AQP9 transcript levels through IREs located in the 496/502 promoter region. Fasting induces lipolysis in adipocytes and gluconeogenesis in the liver. AQP7 mRNA levels are elevated, AQP7 translocates to the plasma membrane, and AQP7 acts as an efficient release of glycerol from adipocytes under fasting conditions. Fasting also increases AQP9 mRNA levels in the liver, the increased portal glycerol flows directly into the liver, and AQP9 may contribute to the entry of glycerol into hepatocytes. In the liver, glycerol is one of the substrates for gluconeogenesis. The glycerol cascade from adipose tissue to the liver is maintained by the coordinated regulation of AQP7 and AQP9 in the fasting state. In the fed state, plasma insulin increases, shifting

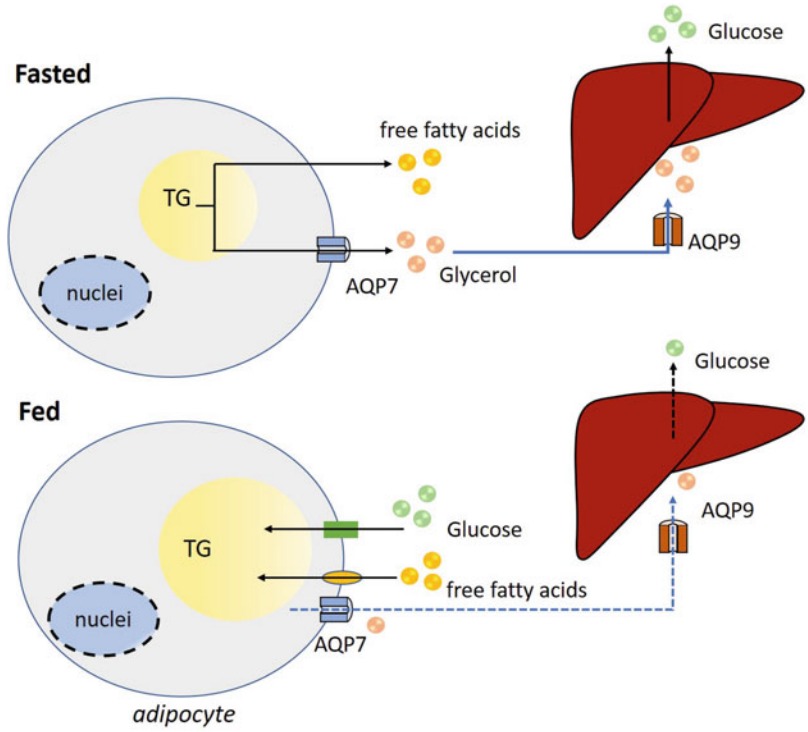

Fig. 5.3 Coordinated regulation of adipocyte AQP7 and hepatic AQP9 in fasted and fed states. In the fasted state (above), AQP7 mRNA levels are elevated, AQP7 translocates to the plasma membrane, and AQP7 acts as an efficient release of glycerol from adipocytes under fasting conditions. Fasting also increases AQP9 mRNA levels in the liver, the increased portal glycerol flows directly into the liver, and AQP9 may contribute to the entry of glycerol into hepatocytes. In the liver, as one of the substrates for gluconeogenesis, glycerol is converted to glucose. In the fed state (bottom), adipose AQP7 mRNA levels are reduced, while glucose transporter 4 (GLUT4) is transported to the plasma membrane and brings glucose into adipocytes. Increased plasma insulin shifts metabolism from lipolysis to lipogenesis in adipocytes and inhibits the production of glucose by the liver. Adipocytes store triglycerides (TG) by esterifying glucose and fatty acids. Portal glycerol levels decreased with a decrease in adipose AQP7 and hepatic AQP9 mRNA

metabolism from lipolysis to lipogenesis in fat cells, and inhibits the production of glucose in the liver. Adipose AQP7 mRNA levels are reduced in the fed state, while glucose transporter 4 transports to the plasma membrane and brings glucose into adipocytes (Fig. 5.3). Adipocytes store triglycerides by esterifying glucose and fatty acids. Portal glycerol levels decreased with a decrease in adipose AQP7 and hepatic AQP9 mRNA. However, despite the presence of hyperinsulinemia, high-fat AQP7 and hepatic AQP9 mRNA levels were observed in obese and insulin-resistant animals. These animals showed an increase in glycerol release from adipose tissue in parallel with an increase in AQP7 mRNA, and also increased glycerol levels in the portal vein. Finally, high glycerol levels in the portal vein will cause gluconeogenesis, leading to hyperglycemia through pathological induction of hepatic AQP9 [68]. Taken together, the coordinated physiological and pathological regulation of organ-specific glycerol channels, adipose AQP7, and hepatic AQP9 may contribute to glycerol and glucose metabolism in vivo.

5.4 AQP and Cell Proliferation

Studies have shown that cells overexpressing AQP1 and AQP3 have larger cell volume, and

proliferation and cell growth are closely related to changes in cell volume [69, 70], thus suggesting that AQP1 and AQP3 promote cell proliferation. Studies have shown that AQP1 expression changes as cells enter different stages of the cell cycle, with higher levels of AQP1 mRNA and protein when cells are in the G0/G1 phase. In contrast, when cells entered S phase and G2/M phase, AQP1 mRNA and protein levels are lower [71]. Besides, it was found that AQP2 accelerates the proliferation and cell cycle progression of renal collecting duct cells by reducing the cells in S phase and G2/M phase and facilitates the increase of cell volume [72]. The direct link between the expression levels of AQPs and the cell cycle may explain the higher proliferation rates in cells overexpressing AQP1 or AQP3, and a series of studies have suggested an important role for AQPs in proliferation. Asynchronous cell cycle analysis in cells overexpressing AQP1 and AQP3 showed that cells overexpressing AQP1 and AQP3 had a higher percentage of cells in S and G2/M phases compared to the distribution of control cells not expressing AQP, and thus the percentage of cells in G0/G1 phase was decreased. Further studies used bioinformatics methods to analyze the whole genome of AQP1-overexpressed cells to reveal the mechanism by which AQP1 promotes cell proliferation. Microarray analysis showed that in cells overexpressing AQP1, more than half of the genes whose expression was altered had cell proliferation-related functions. Numerous transcription factors that promote cell proliferation, such as ZEB2, JUN, JUNB, and NF-kβ2, are upregulated in AQP1-overexpressing cells. Furthermore, it confirmed high expression of the chemokines TNFSF18 and TNF receptors capable of activating the stabilization and translocation of NF-kβ from the cytoplasm to the nucleus. Studies have indicated that the expression levels of proliferative NF-kβ target genes such as ZEB2, cyclin D, and cytokines CXCL9 and CXCL10 are upregulated in cells overexpressing AQP1 [69, 73].

The important role of AQP3 in cell proliferation has been observed in various tissues, including skin, colon, and cornea. AQP3 is essential for the phosphorylation of p38, ERK, and JNK in keratinocytes of skin, and less phosphorylation of p38 and JNK was observed in the epidermis of AQP3-deficient mice, thereby compared to wild-type mice, the wound healing ability in the skin and cornea of AQP3-deficient mice is significantly impaired [8, 74, 75], and colonic epithelial cells are regenerated [76]. Studies have shown that the expression of AQP3 is increased in colorectal cancer [77], human lung cancer [78], gastric adenocarcinoma [79], and human skin squamous cell carcinoma [80]. In addition, glycerol metabolism and biosynthesis are altered in cells derived from AQP3-deficient mice, indicating that the glycerol transport promoted by AQP3 is involved in epidermal cell proliferation and carcinogenesis, and involved with the mechanism of cellular glycerol production of ATP energy. Therefore, AQP3-deficient mouse epithelial cells may reduce the level of cell proliferation through reduced "energy." Decreased cell glycerol concentration and ATP levels were observed in these mice, and lipid biosynthesis and MAPK signal transduction were impaired, which ultimately led to decreased cell proliferation [75]. In addition, a study has confirmed that stable or transient AQP3-expressing cells treated with auphen, a potent inhibitor of glycerol permeability of AQP3, arrest the S-G2/M phase of cell cycle, suggesting that inhibition of AQP3 permeability may arrest cell cycle, thereby slowing cell proliferation [81]. The same mechanism has been observed in breast cancer, where the increased expression of AQP3 promotes the transport of glycerol to cancer cells, thereby facilitating the production of ATP and providing cancer cells with sufficient energy for cancer cell proliferation [82]. Therefore, targeting AQP3 to suppress tumor cell migration and proliferation may become a new strategy for tumor treatment. Currently, studies have revealed that AQP1, AQP3, and AQP5 among all of the AQPs are most frequently associated with human cancers [83–86]. Several studies have suggested a link between AQP and intracellular pathways, but the complete phosphorylation and activation cascade leading to transcription factors and/or cytokines that promote cell proliferation have

not been elucidated [87–89]. The expression of AQP is preferentially associated with the activation of the MAPK cascade signaling pathway, which in turn will lead to the transcription of genes associated with cell proliferation, leading to various human cancers [80]. For example, AQP5 expression was positively correlated with drug resistance factors, and silencing of AQP5 inhibited cell proliferation while reducing MAPK p38 phosphorylation. Furthermore, activation of GSK-3β, ERK, JNK, and p38 MAPK pathways was associated with AQP2 expression levels in lithium-treated renal collecting duct cells [90]. The antiproliferative and antimetastatic activities of the anti-prostate cancer compound Rg3 were associated with p38 MAPK-mediated downregulation of AQP1 [91].

On the other hand, NF-κβ appears to be a key transcription factor regulating the proliferative effect of AQP, on which the effects of AQP converge to promote cell proliferation. In addition, NF-κβ appears to regulate the expression of some AQPs, which in turn controls cell proliferation [92, 93]. Furthermore, hypoxia-inducible transcription factors HIF-1α or 2α may be involved in AQP-mediated proliferation. Overexpression of AQP1, AQP3, and AQP5 has been shown to increase the stability of HIF-2α upon chronic exposure to hypoxia [94, 95], thereby increasing the expression of many genes involved in processes related to tumor growth, such as glucose uptake, cell metabolism, angiogenesis, proliferation, and apoptosis.

5.5 AQPs in Inflammation

Recent findings have revealed that AQPs play roles in the inflammatory, indicating that they can be used as new therapeutic targets for anti-inflammatory therapy in the clinical field. The cells involved in the inflammatory process lose the changes according to the osmotic microenvironment, which include the significant increase in cell water permeability and cell volume and cytoskeletal structure changes [96]. Active resolution of the inflammatory process is essential because it is beneficial to tissue repair after injury, and accumulation of inflammation can lead to continuous tissue damage and progressive fibrosis [97]. Many studies have emphasized that AQPs may be involved in the development of these inflammatory processes, providing new evidence for the importance of AQPs regulating water homeostasis during infection and inflammation. Many data showed that AQPs play roles in maintaining the homeostasis of many physiological processes in tissues related to the secretion and absorption activities of various (such as kidneys, salivary glands, lungs, skin, sweat glands, and intestines).

5.5.1 AQPs and Lung Inflammation

Several AQPs are expressed in lung and bronchopulmonary, where they act as regulators of intercellular water transport [98, 99]. Their dysregulated expression will alter lung physiology and trigger obvious airways inflammation [100, 101]. AQP5 is located in the apical membrane of alveolar epithelial cells and acinar gland cells and provides the main pathway for osmotic-driven water flow in the entire airway system, thereby regulating the hydration of the airway surface [102–104]. AQP5 expression is significantly downregulated during lung inflammation and edema. A recent work has shown that AQP5 function is essential in regulating the bronchoconstriction response. AQP5-deficient mice overreact to cholinergic bronchoconstriction, which is a clinical feature of asthma and a common genetic disease caused by a combination of genetic and environmental factors [105]. In addition, AQP5 expression is reduced in chronic obstructive pulmonary disease and other inflammatory airway diseases [106]. In fact, preclinical data shows that AQP3 and AQP5 are associated with allergic airway inflammation that induces asthma [107]. AQP1 expression is reduced in a model of mechanical ventilation-induced lung injury and edema [108]. LPS, HCl, and ventilation induce lung inflammation in a mouse model resulting in increased pulmonary vessel permeability and inflammatory cell infiltration in bronchoalveolar lavage fluid, ventilation also causes changes in

lung mechanics. These data indicate that the involvement of AQPs in the acute inflammation process depends on the location and type of lung injury. Among the AQP subtypes evaluated (AQP1, AQP4, AQP5, and AQP9), the expression of AQP4 in lung is reduced in HCl and ventilation induction models that primarily target alveolar epithelium, while the expression of AQP5 targets capillary endothelium, and alveolar epithelium is impaired in the LPS induction model [109]. Also research indicated the expression of AQP3 changes in patients with lung cancer and suggested that AQP3 may be involved in tumor differentiation and processes related to the clinical staging of lung adenocarcinoma [110–112].

5.5.2 AQPs and Digestive System Inflammation

The digestive system is the main place for body fluid movement. The widely distributed AQPs in the gastrointestinal tract indicate that they may play an important role in channel-mediated water transport, intestinal permeability, and body fluid secretion/absorption [113]. Interestingly, this is consistent with the potential role of AQP in the pathophysiology of gastrointestinal diseases associated with intense inflammation in organ networks [114–116]. AQP plays an important role in the movement of water across cells and is essential for water absorption in the colon. Inflammatory bowel disease (IBD) is a chronic disease of the gastrointestinal system, characterized by the continuous activation of the immune system leading to chronic and significant inflammation of the intestine [117, 118]. The expression of AQP1, 3, 7, and 8 is significantly reduced in IBD patients, Crohn's disease patients, and ulcerative colitis (UC) patients [119–122], which led to speculation. This decrease is related to the development of these diseases. 5-Fluorouracil (5-FU)-induced mouse diarrhea model showed increased pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-17A, and IL-22), which is related to the decreased transcription levels of AQP4 and AQP8 in the colon [123].

5.5.3 AQPs and Neuroinflammation

AQP4 is the most representative AQP isoform in the brain. Because it is involved in the pathophysiology of a variety of brain diseases, it has been extensively studied [124]. In a model of endotoxemia induced by intraperitoneal injection of LPS in C57Bl/6 mice, resulting in severe central nervous system damage, the expression of AQP4 was increased with cytokine release. Interestingly, dexamethasone attenuates AQP4 expression and IL-6 release, restoring the inflammatory effect of LPS [125]. In addition, microRNA-130a is suggested as a therapeutic target because it inhibits the transcription of AQP4 in the terminal foot of astrocytes, thereby reduced astroedema and neuroinflammation [126]. AQP4 is also an astrocyte proteomic marker, because it was found to be upregulated in delirium caused by sepsis and Parkinson's disease [127, 128]. In addition, studies on rat models of cerebral edema have shown that during transient hypoxia, activation of TLR4 and corticotropin-releasing hormone (CRH)/CRH receptor 1 (CRHR1) signals can upregulate AQP4 and water permeability in the brain. In the same model, LPS treatment itself increases AQP4 and pro-inflammatory cytokines, but cerebral edema can only be achieved by combining LPS treatment with hypoxia. Humans receiving hypobaric hypoxia also showed elevated plasma levels of TNF-α, IL-1β, IL-6, and CRH. These data indicate that systemic inflammation promotes the occurrence of hypoxic cerebral edema, in which AQP4 plays an important role [129].

5.5.4 AQPs and Arthritis

Osteoarthritis is a degenerative disease with irreversible course. It is mainly caused by chondrocyte apoptosis and cartilage matrix degradation.

They are the key factors that regulate the function of articular cartilage by synthesizing the structural components of extracellular matrix and matrix degrading proteases. AQPs have been described in chondrocytes involved in fluid transport and physiological regulation of cartilage [130]. In a rat osteoarthritis model with severe cartilage damage, AQP1 expression is positively correlated with caspase-3 expression and activity, indicating that AQP1 triggers caspase-3 activation, promotes chondrocyte apoptosis, which eventually lead to osteoarthritis [131, 132]. In synovial tissue from patients affected by osteoarthritis and *rheumatoid arthritis*, TNF-α regulates Aqp9 gene and protein expression, indicating that cytokines are modulators of AQPs function [133–135]. Studies have revealed the expression of AQP4 in articular cartilage in adjuvant-induced arthritis(AIA) rat models, proving that AQP4 activation may be involved in the development of AIA in rats [136].

5.5.5 AQPs and Liver Inflammation

Cirrhosis of the liver is another type of severe chronic inflammation associated with pathological water retention. It is the ultimate co-endpoint of various toxic, metabolic, infectious, and autoimmune chronic liver diseases [137]. Several reports have demonstrated that AQP1 is significantly over-expressed in chronic liver disease in humans and rodents [138, 139]. Therefore, the increased expression of AQP1 during cirrhosis promotes angiogenesis and enhances endothelial invasion through the dense extracellular microenvironment associated with the disease [140]. In fact, current studies have shown that AQP1 knockout in vivo has a significant effect on angiogenesis, fibrosis, and portal hypertension after bile duct ligation in mice, further confirming that AQP1 is a convincing treatment target for chronic liver disease treatment [114, 141]. In addition, AQP3 promotes tissue inflammation by increasing the reactive oxygen species through H_2O_2 penetration and stimulating damage processes, such as fibrosis [17]. For many years, researchers have been working to find specific aquaporin inhibitors that are therapeutically effective. According to reports, a new monoclonal antibody reduced CCl_4-induced liver damage through blocking the penetration of H_2O_2 by AQP3 in macrophages [142]. It is hoped that blocking the penetration of H_2O_2 by AQP3 may be more generally applicable to other inflammatory processes involving macrophages that express AQP3.

5.6 Conclusion

In many cases, the elusive roles of many aquaporins in the physiology of normal tissues and organs has not been specifically determined, even with AQP knockout mice, which usually (but not always) show no or mild phenotype. However, more and more in vivo and in vitro studies have shown that the function of aquaporins in normal physiological and disease states are usually not experimentally related to their transporting activity. For example, aquaporins are involved in processes such as cell migration, obesity, inflammation, cancer progression, neurodegenerative diseases, and various inflammatory diseases. Facilitating water and solute movement across membranes might be involved in these processes, but other functions of the aquaporins also play important roles directly, such as their participation in protein–protein interactions with components of the cytoskeleton, as well as with various signal transduction mechanisms and other intracellular pathways. Therefore, investigating the physiological importance of aquaporin function is an ongoing exploration.

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Evolutionary Overview of Aquaporin Superfamily

6

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Abstract

Aquaporins (AQPs) are present not only in three domains of life, bacteria, eukaryotes, and archaea, but also in viruses. With the accumulating arrays of AQP superfamily, the evolutional relationship has attracted much attention with multiple publications on "the genome-wide identification and phylogenetic analysis" of AQP superfamily. A pair of NPA boxes forming a pore is highly conserved throughout the evolution and renders key residues for the classification of AQP superfamily into four groups: AQP1-like, AQP3-like, AQP8-like, and AQP11-like. The complexity of AQP family has mostly been achieved in nematodes and subsequent evolution has been directed toward increasing the number of AQPs through whole-genome duplications (WGDs) to extend the tissue specific expression and regulation. The discovery of the intracellular AQP (iAQP: AQP8-like and AQP11-like) and substrate transports by the plasma membrane AQP (pAQP: AQP1-like and AQP3-like) have accelerated the AQP research much more toward the transport of substrates with complex profiles. This evolutionary overview based on a simple classification of AQPs into four subfamilies will provide putative structural, functional, and localization information and insights into the role of AQP as well as clues to understand the complex diversity of AQP superfamily.

Keywords

NPA box · Domain-based classification · Whole-genome duplication (WGD) · Horizontal gene transfer (HGT)

6.1 Introduction

Aquaporins (AQPs) are present in three domains of life: bacteria, eukaryotes, and archaea with an extension to viruses [1]. In structure, AQP was most likely constructed by a tandem duplication or a fusion of three membrane-spanning helices with an NPA (Asn-Pro-Ala) box (Fig. 6.1) [2]. As this pair of NPA boxes forms the pore, it should be critical for AQP function that has been conserved through evolution. Thus, a pair of NPA boxes is a signature domain for AQP family and domain-based analysis and classification of AQP family will be instrumental to speculate the

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mechanics and selection forces that govern the evolution of AQP family.

In the past, homologous proteins based on a pair of NPA boxes were identified by a PCR cloning using degenerative PCR primers from each NPA box [3]. With increasing availability of public databases from large-scale genome sequencings, many AQP-like sequences have been identified, which sometimes contain highly deviated NPA boxes with overall homology of less than 25% at the level of supergene family [4]. Accordingly, AQP family is more properly called AQP superfamily.

With accumulating arrays of AQP superfamily, evolutional relationships are becoming more and more intriguing with multiple publications on phylogenetic analysis of AQP superfamily. Since the sequences around the two NPA boxes are the important functional domains, they may contain clues to classify AQP superfamily. Indeed, we have identified such key residues for the classification of AQP superfamily into three groups in previous reviews [5, 6]. Such a simplified domain-based classification of AQP superfamily will be useful to obtain an overview of widely distributed AQP family in every kingdom of lives and may provide new insights into the function and subcellular localization.

In addition to general protein and genome databases, a couple of specialized AQP databases are available online such as MIPDB and MIPModDB [7, 8]. Based on a variety of databases, several phylogenetic analyses have been conducted to speculate the evolutional pathways of AQP family [9–14]. However, without new insights into the evolution of AQP family [15] or the combination of other functionally related gene families [16], publishing an original paper on "the genome-wide identification and phylogenetic analysis of AQP family" by a computational analysis of genomic sequence database in certain species will be an easy science. In such studies, the genes analyzed within the phylogenetic tree should be the orthologous genes. However, it is difficult to identify orthologous genes among similar genes (homologous and paralogous genes) and sometimes complicated by polyphyletic lineages. For example, genes with high amino acid identity more than 50% can be non-orthologous genes by convergent evolution, while genes with low amino acid identity less than 30% can be orthologous genes by functional divergence. Moreover, stronger functional effects will be expected by amino acid replacement in the pore-forming region inside the molecule than on the surface of the molecule, although 3D structures are only available in limited numbers of AQPs.

Another way to identify orthologous genes will be the identification of syntenic region where orthologous genes are usually localized. However, the presence of pleural homologous/paralogous genes, genome rearrangement, or duplicated genome with subsequent deletion will make it difficult to identify syntenic region [17]. Therefore, the evolutionary reconstruction deduced from this kind of analysis may not reflect the divergence of assigned orthologous genes, which is expected to coincide with the divergence of species. Furthermore, the presence of horizontal gene transfer (HGT) will produce a phylogenetic tree mismatch between AQP family and species. Similar to prokaryotes with the majority of their genomes composed of genes derived from HGT [18] including possibly an archaeal AQP, AqpM [19], eukaryotic genes have also been acquired by HGT associated with symbiotic or parasitic relationships with bacteria [20, 21] including possibly plant AQPs, NIP, and GIP [22, 23]. Moreover, the results may be complicated by the presence of paralogues and the selection bias under ever-changing environmental conditions where AQP has been expected to play vital roles with possible faster molecular evolutionary rates. On the contrary, some AQP genes may have been lost [15], which may obscure the interpretation of the origin and modes of diversification through the evolution of AQP superfamily.

As many AQPs transport small substrate, the field of AQP research seems to be moving toward complex functions and profiles of substrates transported by AQP rather than a water transport alone, which will uncover and expand more dynamic roles of AQP especially in environmental adaptation. This shift has been accelerated by

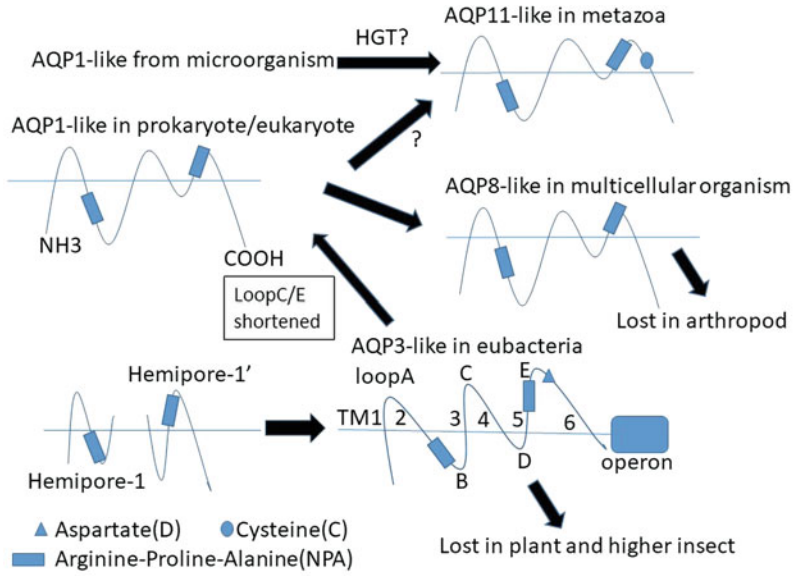

Fig. 6.1 A putative evolutionary pathway of AQP subfamilies. AQP may have been formed by a tandem-repeat or fusion of two genes encoding a hemipore channel with an NPA box (square box). AQP3-like is often located in an operon related to a solute metabolism complex with a conserved Asp (D) residue (triangle) at two residues downstream of the second NPA box. AQP3-like might have been converted to AQP1-like by truncating longer Loop C and Loop E. AQP1-like may have been transformed into AQP8-like in multicellular plants and animals excluding arthropods by widening the ar/R motif selectivity filter (SF). AQP11-like with a conserved Cys (C) residue (circle) at nine residues downstream of the second NPA box may also have been derived from AQP1-like in metazoa either by conversion with low similarities or even by a horizontal gene transfer (HGT) of AQP with deviated NPA boxes from bacteria or protozoa. AQP3-like has been lost in plants and higher insects

the following observations. Firstly, AQP is absent in many microorganisms as revealed by extensive genome projects [24]. Moreover, AQP is also absent in some fungi and a parasitic protozoan, cyst-forming apicomplexan *Cryptosporidium.* Similarly, only a single AQP seems to be sufficient for malaria parasites, another apicomplexan [25]. As non-essential AQP may be antigenic at the cell surface in these parasites as a target for host immunity, only essential AQP may have been preserved in the genome. Therefore, AQP may be dispensable in some microorganisms or harmful as a target from host defense. Secondly, the phenotypes of AQP knockout (KO) mice and humans are generally mild [26, 27] or even normal [28], suggesting AQP may not play essential roles in advanced vertebrates either. Thirdly, AQP has also been identified at the membrane of intracellular organelles including tonoplasts, mitochondria, and the endoplasmic reticulum (ER), where osmotic gradient is relatively small and the intracellular membrane itself probably has a sufficient water permeability without water channel due to a high surface-to-volume ratio except in a low temperature. Within the cell, the production and regulatory transport of osmotic substances will be more important than water transport to control organellar volume as water movement should follow the osmotic gradient produced by osmotically active substances. In other words, water transport by the intracellular AQP may be a safe guard to prevent the development of osmotic gradient inside the cell.

Fourthly, another channel family adopting a similar protein folding in 3D structure of AQP has been identified in bacteria as a formate/nitrite channel (FNT). FNT facilitates anion/H^+ co-transport but not water [29]. However, a

widening mutation of the pore has transformed FNT into a water-selective channel without permeating other substances [30]. Therefore, it is possible that AQP used to be a solute transporter as FNT and later have acquired water permeability. In other words, solute transport may be a more fundamental function of AQP family. More intriguing possibility would be that there could be another water channel other than AQP family with a similar 3D structure but without any homology in the primary sequence with AQP. In particular, this shift of the research interest on AQP will be more relevant to AQP of intracellular organelles and small-sized organisms including bacteria and possibly parasitic protozoa, which may have developed specialized AQP permeating profiles to manage the intracellular milieu [31–33].

This review is an update for our recent reviews on the same topic and detailed sequences around the NPA box of each subfamily are available in these reviews [5, 6].

6.2 Classification of AQP Superfamily

6.2.1 Dichotomy Between Plasma Membrane AQP and Intracellular AQP

AQP superfamily can be divided into two subfamilies based on its subcellular localization: plasma membrane AQP (pAQP) and intracellular AQP (iAQP). This dichotomy will be helpful to speculate its physiological significance because water transport is more important in pAQP while solute transport will be more important in iAQP. As the water permeability induced by AQP is huge, the research on AQP has been centered around this function. Moreover, since the water transport at the plasma membrane is important for cell volume regulation as well as transcellular water transport, pAQP as a water channel was thought to be indispensable for the viability of the organism. However, this preconception is now changing as many pAQPs permeate solutes as well as water and solute transport seems to be more important in several tissues including the adipose tissue and liver.

To be away from the water oriented idea on AQP, it would be appropriate to consider that water transport may be acquired to mitigate quickly the built-up osmotic gradient produced by the solute transport by pAQP to keep cytosolic osmolarity constant. For example, even though a malaria parasite has a single AQP, *P. falciparum* aquaporin (PfAQP) similar to bacterial glycerol channel, GlpF to transport mainly nutrients from housed eukaryotic erythrocytes, it may also be important as pAQP in blood for the survival against osmotic stresses during passing through hypertonic kidney medulla and hypotonic kidney cortex with an additional permeability to water [25]. On the other hand, an enhanced water permeability by pAQP may be harmful to some bacteria living in hypo- or hyper-osmotic environment even protected by the cell wall. Indeed, some bacteria do not have any AQP and their small size may render sufficient water permeability with a higher surface-to-volume ratio. Similarly, the membrane of intracellular organelles may also have sufficient water permeability even in the absence of iAQP, which may have led to the development of different function other than water transport for iAQP.

To provide the basis for the dichotomy between pAQP and iAQP, the mechanisms for AQP trafficking are needed be clarified. However, the mechanism and retention signals for subcellular localization of iAQP have not been clearly identified. On the other hand, the modification of pAQP by intracellular signaling has been shown to regulate its trafficking to the plasma membrane [34]. For example, the trafficking of pAQP is stimulated by cyclic AMP in AQP2, AQP4 or AQP8 [35–37]. The translocation to the plasma membrane has also been reported in AQP7/ AQP10 by isoproterenol and in AQP1 by hypotonia [38, 39]. In fact, targeting such trafficking signals will be promising therapeutic interventions to modulate the expression levels of pAQP at the plasma membrane as shown with AQP4 in brain edema and hypothermia [36, 40].

It should be cautioned, however, that the dichotomy between pAQP and iAQP may not be strictly applicable. As a reservoir, iAQP can be recruited to the plasma membrane on the demand of detrimental environments. Conversely, to meet the demand of intracellular metabolism, pAQP may be endocytosed to be targeted to intracellular organelles as shown with plant pAQP, PIP that is sequestrated intracellularly without further degradation triggered by H_2O_2 [41]. Moreover, the same AQP can be localized both at the plasma membrane and the membrane of intracellular organelle as shown by AQP8 [35]. Obviously, these observations will obscure the dichotomy and indeed the limitation of this classification. Hopefully, the identification of trafficking or retention key signature sequences in AQP will overcome this limitation. Similar limitation, however, has been raised with the functional dichotomy of AQP family depending on the permeating substances: classical aquaporin (water selective) and aquaglyceroporin (glycerol-permeable), due to overlapping permeating substances.

6.2.2 Classification Based on the NPA Box Domain

To evade such limitations, AQP superfamily has been classified based on the primary sequence. We previously classified AQP family into two groups based on the length of residues between the pair of NPA boxes because AQP3-like has longer residues due to longer Loop C and Loop D [42]. Although this classification is still valid for the identification of AQP3-like, further classification will be needed for non-AQP3-like AQPs. Here, AQP superfamily is classified based on the primary sequence around highly conserved a pair of NPA boxes, which turned out to be critical for the function of AQP. Four AQP subfamilies are identified and named after the nomenclature of mammalian AQP family: AQP1-like, AQP3-like, AQP8-like, and AQP11-like (Table 6.1). The separation of AQP8-like from AQP1-like is new in this review as compare with the previous ones [5, 6]. The classification keys for each subfamily are illustrated in Fig. 6.2 and a tentative classification diagram is depicted in Fig. 6.3. As the first NPA box of AQP11-like is not well conserved and sometimes difficult to identify as exemplified in three AQP11-likes in *C. elegans* (Table 6.1), the second NPA box is employed to classify AQP.

As shown in Fig. 6.3, after identifying a pair of NPA boxes, the presence of Asp (D) in the second NPA box following a common R should be screened for AQP3-like as NPAR(D) (Table 6.1 in the bold type). This D is the signature key residue for AQP3-like without exception which is expected to enlarge the pore constriction and makes more hydrophobic by canceling the positive charge of R enabling the permeation of non-ionic larger substances such as glycerol. It should be stressed that this classification is not based on the function although most AQP3-likes are glycerol channels. Thus, naming of non-AQP3-like as glyceroporin or aquaglyceroporin based on the function is confusing because it may have no homology with AQP3-like in the primary structure. For example, a mosquito AQP1-like has been named aquaglyceroporin AgAQP3 from Anopheles based on the function as a member of entomoglyceroporin (Eglyp), AQP1-like [43, 44].

Next, Cys (C) at nine residues downstream of the second NPA box should be sought to identify AQP11-like (Table 6.1 in the bold type). This C is the signature key residue for AQP11-like without exception because it is critical for the function of AQP11 as documented by the result of the point mutation of this residue which produced similar phenotypes of AQP11 knockout mice [45, 46]. It should be cautioned that there are rare exceptional AQPs with exactly located C but should not be included in AQP11-like. For example, although *Chlorobaculum parvum* AQP (WP_012502795) has the second NPA box as NPVRSLAPALV(C), the residue next to the second NPA box is Arg (R), which should be classified as AQP1-like because AQP11-like never has R here (Fig. 6.3). Another way to avoid this misclassification is to focus on the upstream sequences of the first NPA box, which is highly homologous with AQP1-like

Table 6.1 The sequence of NPA boxes in AQP from *C. elegans* and humans

	First NPA box	Second NPA box
AQP1-like		
CeAQP4	-FGHISGGHFNPAVSWAIAGA-	-TGSITGASMNPARSLGPSIIGS-
CeAQP5	–FGKISGGHFNPVVSWAMVLC-	-TASITGTAMNPVRALSPNIVGE-
CeAQP6	-FGGVSGAHINPAVTFGIALV-	-AGAISGASMNPARSFGPNIMGQ-
AQP0	-VGHISGAHVNPAVTFAFLVG-	-GMYYTGAGMNPARSFAPAILTR-
AQP1	-VGHISGAHLNPAVTLGLLLS-	-AIDYTGCGINPARSFGSAVITH-
AQP2	-LGHISGAHINPAVTVACLVG-	-GIHYTGCSMNPARSLAPAVVTG-
AQP4	-FGHISGGHINPAVTVAMVCT-	-AINYTGASMNPARSFGPAVIMG-
AQP5	-LGPVSGGHINPAITLALLVG-	-GIYFTGCSMNPARSFGPAVVMN-
AQP6	-TWKASGAHANPAVTLAFLVG-	-GIHFTGCSMNPARSFGPAIIIG-
AQP3-like		
CeAQP1	-TARMSGGHLNPAVSLLLWSL-	-FGMNIGYPINPAR**D**LGPRLFSY-
CeAQP2	-SAKLSGGHINPAVSFAFLSV-	-FGYNCGYPVNPAR**D**FAPRLFTS-
CeAQP3	-GSKISGAHLNPAVSFFQLTQ-	-LALNAGYAINPAR**D**FAPRLFNL-
CeAQP7	-CSKTSGGHFNPAVSIAFLTL-	-YGMNLGYPINPAR**D**LGPRLFSF-
CeAQP8	-AASVSGGHLNPAISVAQSIL-	-FGANGGFAINPAR**D**FGPRVF**C**L-
AQP3	-AGQVSGAHLNPAVTFAMCFL-	-MGFNSGYAVNPAR**D**FGPRLFTA-
AQP7	-AGRISGAHMNAAVTFANCAL-	-LGMNTGYAINPSR**D**LPPRIFTF-
AQP9	-AGGVSGGHINPAVSLAMCLF-	-LGLNSGCAMNPAR**D**LSPRLFTA-
AQP10	-GGNVSGAHLNPAFSLAMCIV-	-MGANCGIPLNPAR**D**LGPRLFTY-
AQP8-like		
CeAQP12	–VSHLTPAHLNPAISLLQWLR-	-SYPLYGFTSNISLLLVTSTVSY-
AQP8	-LGNISGGHFNPAVSLAAMLI-	-GGPVSGGCMNPARAFGPAVVAN-
AQP11-like		
CeAQP9	-IEFQRDAVAHPCPLVTNCYR-	-GINYTGMYANPIVAWACTFN**C**L-
CeAQP10	-NIFNRGAMTNCAPIFEQFVF-	-LYVVGVPGLNPIVATARLYG**C**R-
CeAQP11	-ALCNRTAFCSPLAPIEQYLF-	-VTFVGDQALDPLVASTLFFG**C**R-
AQP11	-GLTLVGTSSNPCGVMMQMML-	-GGSLTGAVFNPALALSLHFM**C**F-
AQP12	-GVTLDGASANPTVSLQEFLM-	-AGPFTSAFFNPALAASVTFA**C**S-
AQP12L	-GVTLDGASANPTVSLQEFLM-	-AGPFTSAFFNPALAASVTFA**C**S-

NPA box is underlined. Each signature residue for AQP3-like and AQP11-like is in the bold type. CeAQP1~12 for *Caenorhabditis elegans:* 4NP_505512.3, 5NP_505691.2, 6NP_505727.1, 1NP_495510.1, 2NP_495973.1, 3NP_502044.1, 7NP_508515.2, 8NP_001024758.1. 12NP_001022480.1 (updated in 09-Aug-2021). 9NP_001021552.2, 10NP_496105.1, 11NP_499821.2
AQP1~11 from humans

as VSGAHLNPAVT. Thus, even if the signature Cys is identified, the upstream sequences of the first NPA box need to be deviated from AQP1-like to classify as AQP11-like. As AQP3-like is already identified based on D residue, AQP3-like with a coincidentally localized Cys as CeAQP8 in *C. elegans* should not be a problem (Table 6.1).

After both AQP11-like and AQP3-like are identified, the remaining AQP will be AQP1-like or AQP8-like. Further classification of AQP8-like from AQP1-like, however, is not easy because both share highly similar NPA box domains. Based on the previous report [47], His (H) in the transmembrane 2 (TM2) residue in ar/R motif for a selectivity filter (SF) *(*vide infra*)* is tentatively assigned as a key residue for AQP8-like although it is far upstream of the first NPA box (21 residues upstream of the first NPA box)

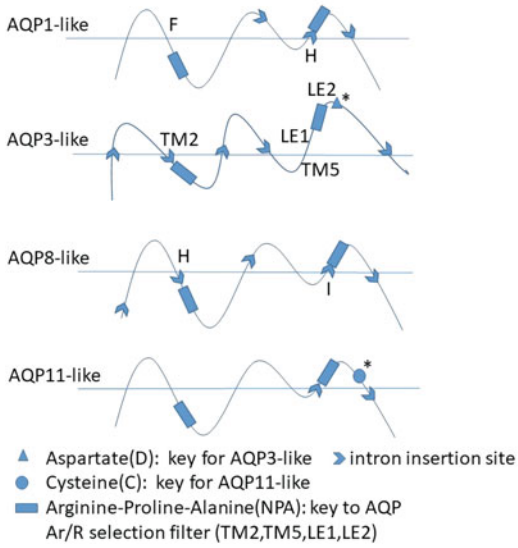

Fig. 6.2 Transmembrane structures of AQP subfamilies. The intron insertion sites for mammalian AQPs are shown by the arrow. The sites of four amino acid residues for the ar/R motif SF are shown in AQP3-like as a representative with the second transmembrane domain (TM2), TM5, Loop E1 (LE1), and LE2. Typical AQP1-like has ar/R motif of "FHXR." AQP3-like always has D in the second NPA box following LE2 as NPAXD. Typical AQP8-like has ar/R motif of "HIXX." AQP11-like always has C at nine residues downstream of the second NPA box as NPAXXXXXXXXC

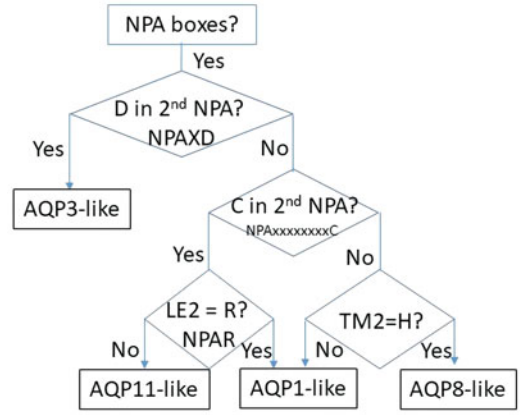

Fig. 6.3 A simplified diagram for the classification of AQPs into four subfamilies. It is based on the presence of a single amino acid residue (D, C, R, and H) around NPA boxes. The characteristics for each subfamily are shown in Fig. 6.2. TM2 (20 residues upstream of the first NPA box) and LE2 (two residues downstream of the second NPA box) are the component of the first and forth residues of ar/R motif SF. See text for details

(Fig. 6.2) and not completely conserved. Similar to the results from whole protein comparison, the diagram identified most plant tonoplast intrinsic proteins (TIPs) as AQP8-like, although His residue is not always identifiable. For example, three AQP8-likes in *Ciona intestinalis* do not have His at TM2 although they are more similar to AQP8 than AQP1 by whole protein comparison (Table 6.2). Conversely, AQP1-like named AX4 or D (GenBank: XM_639170) from a protozoan, amoeba *Dictyostelium discoideum* has His at TM2 [10, 48] simulating AQP8-like although whole protein sequence comparison has revealed that AX4 is more similar to AQP1 than AQP8 (Table 6.2). Moreover, all other AQP1-like from protists [13], fungi [49], and arthropods [43] do not have such His. These observations suggest that AQP8-like may have first appeared in multicellular organisms although arthropods are devoid of AQP8-like (Table 6.2). Obviously, this classification has the limitation to identify AQP8-like, which sometimes requires whole protein sequence comparison to distinguish AQP8-like from AQP1-like definitively.

Indeed, this classification corresponds to previously reported phylogenetically distinct AQP subfamilies by whole protein comparison except for AQP8-like that lacks a reliable key sequence in NPA box domain to be separated from AQP1-like. Roughly speaking, this classification also corresponds to the subcellular localization of AQP: AQP1-like and AQP3-like for pAQP while AQP8-like and AQP11-like for iAQP, although the key sequence for the dichotomy between pAQP and iAQP remains to be identified. In spite of these limitations, above classification is simple and will be helpful to speculate the function and subcellular localizations of AQP based on its subfamily in the face of many uncharacterized AQPs in database.

6.2.3 Correlation to the Selective Filter (SF) of AQP Channel

As the size and the electro-chemical properties of solutes are the main factors determining the

Table 6.2 The distribution of aquaporins in four subfamilies

Organisms	Total	AQP1-like	AQP3-like	AQP8-like	AQP11-like
Bacteria					
E.coli	2	1	1	0	0
S. oligofermentans	3	1	2	0	0
L .plantarum	5	0	5	0	0
M. tuberculosis	4	0	4	0	0
H. pylori	0	0	0	0	0
M. marburgensis	1	1	0	0	0
Protozoa					
T. gondii	1	1	0	0	0
P. falciparum	1	0	0	0	0
D. discoideum	5	5	0	0	0
L. major	5	4	1	0	0
T. cruzi	4	4	0	0	0
T. brucei	3	0	3	0	0
Fungi					
C. neoformans	1	1	0	0	0
S. cerevisiae	4	2	2	0	0
S. pombe	1	0	1	0	0
A. nidulans	5	1	4	0	0
T. harzianum	8	3	5	0	0
Plants					
Moss	23	18	1	4	0
Rice	33	23	0	10	0
Arabidopsis	35	25	0	10	0
Cotton	71	48	0	23	0
Soybean	72	49	0	23	0
Invertebrates					
C. elegans (nematode)	12	3	5	1	3
S. purpuratus (sea urchin)	12	3	5	2	2
C. intestinalis (sea squirt)	7	2	1	3	1
Arthropods					
D. melanogaster (fruit fly)	8	7	0	0	1
B. tabaci (silverleaf whitefly)	7	8	0	0	1
L. salmonis (louse)	9	2	5	0	2
Vertebrates					
Zebra fish	19	7	7	3	2
Mouse	12	6	3	1	2
Pig	13	6	4	1	2
Human	14	6	4	1	3

AQP1-like from the amoeba *Dictyostelium discoideum* AX4 or D (GenBank:XM_639170) has His at TM2 simulating AQP8-like but it should be AQP1-like with whole protein comparison. Plant TIP is included in AQP8-like subfamily as most of them has His at TM2

Strongylocentrotus purpuratus, Ciona intestinalis, Drosophila melanogaster, Bemisia tabaci, Lepeophtheirus salmonis

diffusion through AQP, permeating substances will be predicted from the primary sequence. Indeed, the 3D structure analysis of AQP family has identified the functionally relevant residues for the permeation of AQP, which form two pore constrictions as selectivity filters (SFs): NPA region and ar/R (aromatic residue (ar) and arginine (R)) region [50]. NPA region located at the middle of the channel also serves as a larger electrostatic barrier. On the other hand, ar/R

region located in the outer channel entrance forms a narrower SF inside the channel, which determines the size of permeating solutes by four channel lining residues: two residues each from TM2 (20 residues upstream of the first NPA box) and TM5 (19 residues upstream of the second NPA box), and two residues each from loopE1 (LE1) (three residues upstream of the second NPA box) and LE2 (two residues downstream of the second NPA box), comprising "TM2, TM5, LE1, LE2"(Fig. 6.2). The position of the residue may vary depending on the 3D structure especially TM2 and TM5. In fact, the whole transmembrane sequences of TM2 and TM5 are relatively less conserved and may provide the room for functional fluctuations of permeating substrates as both are closely located to the pore.

As the permeating molecule may be suspected based on a combination of aperture and hydrophobicity of ar/R SF, the validity of the above classification will be examined based on the sequences of ar/R SF for each subfamily. Water selective AQP1-like usually have "FHAR" or "FHCR" with large side-chains [benzene ring in Phe (F) and imidazole in His (H)], which extend to the inner surface of AQP channel restricting the diameter of the pore to ~2.8 Å fitting just to the diameter of a water molecule and the hydrophilic properties of His favors the permeation of water molecules. On the other hand, the absence of the hydrophilic amino acid (His) restricts water movement and the replacement with smaller Gly expands the ar/R SF wider to ~3.4 Å or more permeating small, uncharged solutes such as glycerol, urea, ammonia, or even metalloids including arsenite in AQP3-like as a glycerin channel with "FGYR," "FGCR," or "GGYR." In contrast, AQP8-like has the first two amino acids of ar/R SF with His and Ile as "HIXX," suggesting a wider pore and a higher water permeability with a hydrophilic His. As diagramed in Fig. 6.3, this His is assigned as a signature residue for AQP8-like although it is not highly conserved. Since the 3D structure of AQP11-like is not yet available, the a/R SF sequence is difficult to identify [51]. A putative ar/R SF sequence appears to be highly deviated from other AQP subfamilies with the absence of Arg (R) at LE2 such as "GLGL" or "LAAL" forming a completely hydrophobic filter suggesting a larger pore with poor water permeability in the absence of hydrophilic His. Obviously, as AQP function may not follow the structure of ar/R SF alone, functional studies are needed to define structure and functional relationships.

6.3 Putative Evolution of AQP Superfamily

6.3.1 Overview of Gene Evolution

Multiple lineage-specific expansions including single-gene duplications, splice variations and their losses have been observed through the evolution. Moreover, multiple whole-genome duplications (WGDs) have occurred in selected species associated with obtaining new functions and transformation into pseudogenes leading to gene loss [52]. As AQP family in particular has been regarded as vital for the survival especially in significant life events, it is not surprising that the number of AQP paralogues in different species is highly variable and seems to be correlated with environmental complexities [53].

WGD has been known to play an important role in vertebrate evolution. For example, common ancestors of early vertebrates underwent two rounds of WGD more than 500 million years ago [54, 55]. In addition, teleost, bony fish further experienced another round of WGD about 300 million years ago [54] resulting in 10–19 AQP genes, highest in zebrafish [56, 57]. Salmon and carp even had additional WGD in total of four rounds of WGD [58] about 80 and ten million years ago, respectively [15, 59]. Allotetraploid frogs such as *Xenopus laevis* also had another WGD with three rounds in total [58], while diploid frogs such as *Xenopus tropicalis* had the same number of WGD as mammals with two rounds [54, 55]. Such WGDs have produced multiple homologues (ohnologues) from the orthologue to expand the gene complexity. Recently, a detailed analysis of frog AQP family

has been reported in the context of the vertebrate evolution with careful identification of syntenic regions for the orthologue of each AQP to trace the polyploidization, subsequent pseudogenization and deletion [17].

Another way of gene expansion is tandem duplications or transposon activations to produce paralogues. The newly formed paralogues and homologues initially preserved nearly identical sequences to the original gene (orthologue). Over time, however, paralogues and homologues would have accumulated non-functionalizing mutations to be pseudogenes [60] and eventually lost, or more importantly developed new functions which may have played crucial roles in the survival in harsh environments leading to the evolution of species as well as AQP family [60].

6.3.2 The Relationship Between AQP1-Like and AQP3-Like

From the evolutional point of view, ancestors of AQP might have appeared in bacteria as AQP1-like (similar to AQPZ) or AQP3-like (similar to GlpF) but not AQP11-like nor AQP8-like. Whether they initially had water permeability is a matter of debate. In fact, the function of AQP3-like as a water channel in bacteria is controversial as GlpF has no water permeability with dominant glycerol permeability [61] although mammalian AQP3-like permeates both glycerol and water efficiently [62, 63]. On the other hand, AQPZ has a selective water permeability similar to mammalian AQP1-like [64]. If the water transport by ancient AQPs was a fundamental function, AQP1-like most likely first appeared. Subsequent residue alternations may have transformed the ancient AQP1-like to be permeable to solutes to become AQP3-like as suggested by molecular dynamics simulation studies based on the difference between GlpF and AQPZ [65, 66], in which a double mutant of AQP1 at H180A/R195V of ar/R SF has rendered urea permeability [67]. On the other hand, if the solute transport by ancient AQPs was required for the survival, AQP3-like will be the original AQP. Similarly, a double mutant of GlpF at W24F/F200T of ar/R SF has allowed water to pass with reduced glycerol permeability [68, 69] resulting in the conversion of AQP3-like to AQP1-like.

An archaeal AQP1-like, AqpM has been reported to permeate weakly water and glycerol without urea permeability suggesting that the role of water transport in AQP1-like has been diminished in anaerobic environments at 65 °C with sufficient water permeability without AQP1-like [19]. The crystallography of AqpM has also indicated a narrow pore similar to AQPZ but His in ar/R SF is replaced by isoleucine to be hydrophobic, explaining poor water and glycerol permeabilities [70]. Since archaea have evolved from eubacteria, the results suggest that archaea have developed the intermediate function of AQP1-like and AQP3-like and possibly the transformation from AQP1-like into AQP3-like in structure will have been difficult, although HGT from eubacteria to archaea could explain the acquisition of AqpM [19]. Another observation in favor of AQP3-like as the first ancient AQP will be the truncation of AQP to render water permeability. Wild-type AqpB, AQP1-like in structure, from a protozoan, *Dictyostelium discoideum,* is impermeable to water and glycerol. Interestingly, a mutational truncation of 12 amino acids in Loop D has induced the permeability for water but not for glycerol [71]. As AQP3-like has longer Loop C and Loop E but not Loop D than AQP1-like as indicated by our previous review [42], they need to be truncated to become AQP1-like structure (Fig. 6.2). Although the effect of truncating Loop C or Loop E on the function is unknown, such might have transformed AQP3-like to be water permeable but to be restrictive to glycerol similar to the function of AQP1-like. Moreover, deletion events will be more likely to occur than insertion events. This conversion might be facilitated by the built up of osmotic gradients with the solute transport through AQP3-like, which needs to be balanced rapidly by water transport. Further searches will be required to find the intermediated form of AQP1-like and AQP3-like in bacteria to determine which was the initial ancient AQP.

From the sequenced genomes of 5294 bacteria and 299 archaea, orthologs of AQP1-like and AQP3-like were found in 3315 (60%) prokaryotic species [24]. Among these AQPs, 977 bacteria encode both AQP1-like and AQP3-like, whereas 698 bacteria encode only AQP1-like and 1552 bacteria encode only AQP3-like, indicating AQP3-like is a predominant subfamily in bacteria. Notably, 2067 (40%) bacteria do not have any AQP homologues in their genomes especially in animal pathogenic microorganisms such as *H. pylori* (Table 6.2) and extreme-environment inhabitants suggesting that they do not need any AQP by employing other apparatus for the solute transport. Alternatively, the presence of AQP may be harmful for the survival in the environment with huge osmotic gradients. Similarly, more evolved archaeal phyla living in harsh environments rarely have AQP as indicated by MIPModDB [7] containing only 157 AQPs from Euryarchaeota mostly from methanobacteria possibly for the need of metabolite transports.

In bacteria, AQP3-like but not AQP1-like is often located in operons for glycerol metabolic pathways including glpK encoding glycerol kinase. Accordingly, AQP3-like will be transmitted as a functional complex to the descendants with a conserved function, while AQP1-like is present in the genome in isolation will be more easily mis-localized and transformed or even deleted, which may explain less AQP1-like in bacteria. For example, the *S. oligofermentans* genome carries three AQP homologous genes: a single AQP1-like, *So-aqpA*, and two AQP3-likes, *So-aqpB* and *So-aqpC* (Table 6.2). Only *So-aqpC* is localized in a three-gene operon for glycerol metabolism while *So-aqpA* has been converted to peroxiporin to transport H_2O_2 probably due to a less stringent transmission [72]. Not surprisingly, AQP3-like is more prevalent in some bacteria like *Lactobacillus plantarum* with six glpFs (glpF1 ~ glpF6) without AQP1-like (Table 6.2), which export the metabolic end products such as lactic acid especially by GlpF1 and GlpF4 [73]. Thus, AQP in bacteria may well be for solute transports and not much for water transport.

Interestingly, 27 viral genomes contain AQP genes as shown in MIPModDB [7] including 25 AQP3-like genes. As virus may have taken up partial bacterial genomes, the result may indicate AQP-3 like in bacteria used to be more widespread and possibly have a longer history than AQP1-like, suggesting AQP1-like may have been produced after the emergence of AQP3-like or derived from AQP3-like. Conversely, it could have been the origin of bacterial AQP functioning at the viral envelop or the membrane of the infected bacteria, which may explain the dominant AQP3-like in bacteria. In fact, the AQP3-like (Aqpv1) from dsDNA Chlorovirus has been shown to be functional and expressed in infected tobacco localized to the plastid and plasma membranes with a role of mitigating drought stress responses [1]. Interestingly, a functional potassium channel (Kcv) has also been identified in large dsDNA chlorella viruses which is functional and essential for the virus life cycle [74].

In bacteria, a channel protein, FocA, has been identified with a similar 3D structure to AQP family. Both FocA and AQP have two structural repeats in six transmembrane segments: TM1–TM3 and TM4–TM6. Similar to the NPA box of AQP, the second transmembrane segment of FocA in each repeat (TM2 or TM5) is disrupted by a highly conserved loop named L2 and L5, respectively, although with different sequences from AQP. In spite of the similarity in 3D structure, the tertiary structure and function of FocA are different from AQP. FocA is organized in a homopentamer in contrast to a homotetramer of AQP [75]. FocA is a member of the formate/nitrite transport (FNT) family transporting various anions during anaerobic bacterial growth and localized at the functional operon exporting major end products of anerobic mixed-acid fermentation such as formate, lactate, acetate to prevent their intracellular accumulation [76]. Among anions, HS^- binds to FNT with a higher affinity than formate, Cl^-, or NO_2^- [77]. While AqpZ permeates formate albeit less efficiently, FocA-loaded proteoliposomes have displayed no water channel activity [29]. Surprisingly, a widening mutation of the pore has transformed FocA into a selective water channel without permeating other substances [30]. Speculatively, ancestor

AQPs might have the function as a solute transporter similar to FNT. Unlike FNT, AQP may have acquired a water channel function before evolving to eukaryotes. As FNT family has been lost from the eukaryotic lineage, the function of FNT may be replaced by other channels in eukaryotes and possibly not by AQP. If FNT family or proteins with a similar 3D structure have been transmitted to eukaryotes, they might have acquired the ability to transport water. So far, no such structural homologues of AQP as FNT have been identified yet.

6.3.3 Evolution of AQP3-Like

AQP3-like is widespread in both prokaryotic and eukaryotic organisms [15] playing important roles as shown in trypanosomes where the disruption of all AQP3-likes has made them sensitive to respiratory-inhibitor [78]. However, AQP3-like is not ubiquitous and lost in some protozoa or all plants and insects. Even in vertebrates, a selected AQP3-like has been turned to a pseudogene as AQP7 in primates [15], and AQP10 in cattle [79] and mice [80] or even lost as AQP10 in tortoise and echidna (spiny anteater) [15]. Moreover, KO mice of three AQP3-like each have produced relatively mild phenotypes [27]. As the function of AQP1-like has expanded to transport substrates beyond water and intracellular AQP8-like/AQP11-like may distribute to the plasma membrane, a battery of plasma-membrane-restricted AQP3-like with limited water transport may not be necessary for most of multicellular organisms excluding fungi and small invertebrates where AQP3-like seems to play more important roles as revealed by a gene disruption study in *C. elegans* [81].

In the absence of AQP3-like, land plants have acquired AQP1-like, nodulin 26-like integral protein (NIP) via HGT from bacterial AQP1-like especially from root nodule bacteria [23]. Moreover, bacterial AQP1-like with arsenic detoxification function prevalently located in arsenic resistance operons has been converted in plants to facilitate nutrient transport including glycerol and ammonia through NIP [82]. On the other hand, hemipteran and holometabolous insects have developed a more efficient glycerol channel, Eglp from endogenous AQP1-like [43]. For example, silverleaf whitefly, *B. tabaci* has eight AQPs (BtAqps): seven AQP1-like including Bib, Drip, Prip, and Eglps and a single AQP11-like (Table 6.2). To compensate for the loss of AQP3-like, at least three separate AQP1-likes as Eglp have been obtained by mutating His in ar/R SF of AQP1-like to transport larger substrates such as glycerol and limit water transport by losing hydrophilic His, which is a typical example of an adaptive evolution at the molecular level [83].

6.3.4 The Origin of AQP8-Like and AQP11-Like

AQP11-like and AQP8-like seemed to appear relatively late in evolution, i.e. after the emergence of multicellular organisms (Table 6.2). AQP8-like may have evolved from AQP1-like by converting ar/R SF to become wider with conserved water permeability, which is often localized inside the cell such as plant TIP. As AQP11-like is also present intracellularly, water transport may not be the main function of these subfamilies. Multicellular organisms may have acquired AQP8-like and AQP11-like as iAQP with new functions or regulations to adapt to a new frame of multicellular life style. For example, as the tonoplast is so huge that it requires a water channel for efficient water transport through cytoplasm as well as for the accumulation of substrates inside the tonoplast, which may have facilitated for plants to obtain multiple and huge amounts of TIPs (Table 6.2) [84]. In fact, TIP appeared relatively late in plant evolution following algae [85]. On the other hand, metazoa seem to have a few AQP8-like in the genome and arthropods even have lost it. For example, a nematode, *C. elegans* has a single AQP8-like (Tables 6.1 and 6.2), and more evolved *Ciona intestinalis* (sea squirt) has three AQP8-likes (Table 6.2). The increase of AQP8-like may not be related to larger sizes of organisms as human has only one AQP8-like. Interestingly, a diploid frog, *Xenopus tropicalis* has two AQP8-likes, and

one of them has been lost through evolution leading to a single AQP8-like of tortoise and chicken in the genome [17]. As coelacanth (lobe-finned fish) also has a single AQP8-like, the reason for the expansion of AQP8-like in amphibians compared with other vertebrates is intriguing, which may be related to the landing of tetrapods.

Unlike AQP8-like sharing high similarities with AQP1-like, AQP11-like has been highly mutated from AQP1-like possibly due to a loose restriction in less harsh intracellular milieu than extracellular environments. Alternatively, such a wide deviation is unusual among other AQP subfamilies as exemplified by three AQP11-likes in *C. elegans* (Table 6.1) showing almost no homology even with each other suggesting the individual evolution from separate ancestors. Such an outstanding diversity of NPA box domains of AQP11-like may suggest that AQP11-like has been obtained by HGT from AQP1-like of intracellular microorganisms or parasites as they sometimes have highly deviated NPA boxes as well as the replacement of R at the LoopE2 of ar/R SF. Moreover, only some bacterial AQPs share a similar hydrophobic ar/R SF as "IVLL" or "IAAV" with AQP11-like as "LVAL." Interestingly, intracellularly localized protozoal AQP1-like [13, 31] and the ER-localized plant short intrinsic basic protein (SIP) [86] also have the replacement of R in LoopE2 although they belong to AQP1-like but not to AQP8-like based on the absence of His at TM2 and on the whole protein comparison, even not to AQP11-like in the absence of the key Cys. Plant AQP8-like, TIP but not mammalian AQP8 has the replacement of R in LoopE2 as well. Accordingly, the absence of R next to the second NPA box as NPAX instead of NPAR may be a key sequence for iAQP. In the absence of AQP3-like and AQP11-like, plants may have developed complex arrays of AQP1-like including plasma membrane intrinsic protein (PIP), NIP, SIP, uncategorized X intrinsic protein (XIP), large intrinsic protein (LIP), and hybrid intrinsic protein (HIP). For example, XIP also present in protozoa and fungi has been shown to be located at the plasma membrane and impermeable to water but permeable to boric acid, glycerol, hydrogen peroxide, and urea [87].

Although homologues of AQP1-like and AQP3-like in lower animals are surprisingly well conserved, AQP11-like is highly deviated among them. In particular, first half of AQP11-like in lower animals is more deviated from that of AQP1-like or AQP3-like, while the second half is relatively conserved. This may be due to the fact that the second half mainly defines the entrance selective filter forming ar/R SF. Accordingly, the second half may be constrained through evolution resulting in higher conservation while the first half may be more freely changeable possibly enabling to obtain new functions. For example, salmon lice have unusual members of AQP11-like with very low homology (11%–18%) which are localized intracellularly and difficult to be expressed in *Xenopus oocytes* [12]. More deviated three AQP11-likes are also found in the *C. elegans* genome, which have not yet been tested in *Xenopus* oocyte expression system (Table 6.1). Interestingly, AQP11-like in mosquito is very highly expressed intracellularly in a pupae stage and functions as a mercury-sensitive water channel when expressed in cultured cells although the physiological role is unknown [88]. The comprehensive analysis of phenotypes in genetically modified animals with disrupted AQP11-like will be a clue to identify new functions as well as novel permeating molecules. So far, only a limited number of AQP11-like knockout studies have been reported including zebrafish and mammals. AQP11 disruption in zebrafish has produced a phenotype of body axis curvature [89] while AQP12 knockout mice have appeared normal although they have developed severe acute pancreatitis when stimulated by secretagogues [90]. On the other hand, AQP11 knockout mice have suffered from polycystic kidney disease (PKD) with intracellular vacuoles in the kidney and liver [45, 91]. The mechanism for the development of PKD in AQP11 knockout mice seems to be related to the failure of PKD1 trafficking to the plasma membrane from the ER [92] which may be caused by ER disfunction due to the absent of AQP11 at

the ER membrane possibly regulating H_2O_2 transport [93]. Curiously, conditional AQP11 knockout mice from postnatal 12 days onward have not produced the defect in renal development even without PKD [94]. The role of AQP11 in the development of the kidney remains to be clarified.

6.4 Perspectives

When viewed from the evolutionary perspective, AQP family is highly diversified already in small animals like a nematode, *C. elegans* which has 12 AQPs: three AQP1-likes, one AQP8-like (NP_001022480.1 updated in 2021: the second NPA box is not clear probably due to a wrong exon connection, but N-terminal half is similar to AQP8 without His in TM2), five AQP3-likes and three AQP11-likes (Table 6.1). Not only the number of AQPs is similar to that of human, but its complexity is also comparable to human who has 14 AQPs: six AQP1-likes, four AQP3-likes, one AQP8-like, and three AQP11-likes (primate-specific AQP12L is included). Therefore, the evolution of AQP complexity has already been achieved in nematodes and subsequent evolution seems to be shifted toward increasing the number rather than the complexity by single-gene duplications and WGDs or even HGT to enable the tissue specific distribution and regulation [53]. Accordingly, the research on these small organisms may provide the answer for the fundamental question of the purpose of AQP in life. Their strategies for adapting to ever-changing environments will also be useful for us and may open a new avenue for AQP research with novel therapeutic applications.

The view that the major role of AQP family is water transport may not hold in bacteria. Moreover, AQP may be important in protozoa and fungi as osmolyte channels such as a glycerol channel, where osmoregulation is critical and AQP will be required for cell survival under harsh conditions [95]. On the other hand, as AQP is highly expressed in almost all fluid-related structures in metazoa, the evolution of AQP seems to be tightly linked to fluid homeostasis as well as osmoregulation. In addition to the regulation of transcellular water transport by AQP, the possibility of AQP as an osmosensor by facilitating cell volume change has been proposed and there is accumulating information to support this hypothesis [96]. For example, the association between transient receptor potential vanilloid 4 (TRPV4) and AQP5 has been shown to control the regulatory volume decrease (RVD) in salivary gland cells exposed to hypotonicity [97].

The dichotomy between pAQP and iAQP will shift the research more toward the role of AQP in the regulation of intracellular milieu. As prokaryotes have few intracellular organelles if any, the role of iAQP will be negligible. On the other hand, free-living protozoa may need iAQP for the adaptation to hypotonic environments to expel the water accumulated in the cytosol from outside. For example, in a ciliated Paramecium, the presence of water channel on the membrane of contractile vacuole has been documented as AQP1-like, AQP1. The AQP1-GFP fusion protein has clearly demonstrated the subcellular localization of AQP1 on the contractile vacuole complex [98]. The regulation of water accumulation together with energy-consuming osmolyte transporters seems to be facilitated by an efficient water transport by AQP initiated by intracellular signaling. In fact, cells in multicellular organisms may also face similar osmotic stresses and need a similar mechanism to survive. Such mechanisms may also be involved in exocytosis facilitated by AQP [90, 99]. Even a cancer cell as a single cell organism may take advantage of this role of AQP to move around in our body [100, 101].

Obviously, hormonal controls of AQP function and expression will be most relevant to human physiology and pathophysiology. To elucidate the mechanisms orchestrating AQPs in concert to overcome environmental challenges will be one of the ultimate goals of AQP research, which requires specific inhibitors [102] and conditional knockout [94] in healthy and diseased conditions. These will be detailed in other chapters. Moreover, cooperative evolutions of

AQPs and regulating hormones as well as associated proteins including channels, transporters, and pumps will also be intriguing.

Finally, this evolutionary overview based on a simple classification of AQP into four subfamilies will provide putative structural, functional, and localization information, and hopefully insights into the role of AQP as well as clues to understand the complex diversity of AQP superfamily.

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Aquaporins in Nervous System

7

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Abstract

Aquaporins (AQPs) mediate water flux between the four distinct water compartments in the central nervous system (CNS). In the present chapter, we mainly focus on the expression and function of the nine AQPs expressed in the CNS, which include five members of aquaporin subfamily: AQP1, AQP4, AQP5, AQP6, and AQP8; three members of aquaglyceroporin subfamily: AQP3, AQP7, and AQP9; and one member of superaquaporin subfamily: AQP11. In addition, AQP1, AQP2, and AQP4 expressed in the peripheral nervous system are also reviewed. AQP4, the predominant water channel in the CNS, is involved both in the astrocyte swelling of cytotoxic edema and the resolution of vasogenic edema and is of pivotal importance in the pathology of brain disorders such as neuromyelitis optica, brain tumors, and neurodegenerative disorders. Moreover, AQP4 has been demonstrated as a functional regulator of recently discovered glymphatic system that is a main contributor to clearance of toxic macromolecule from the brain. Other AQPs are also involved in a variety of important physiological and pathological process in the brain. It has been suggested that AQPs could represent an important target in treatment of brain disorders like cerebral edema. Future investigations are necessary to elucidate the pathological significance of AQPs in the CNS.

Keywords

Aquaporins · Nervous system · Brain disorders

7.1 Introduction

Water homeostasis in the central nervous system (CNS) is of pivotal physiological and clinical importance, since about 80% weight of brain is water [1]. Water transport is linked to a number of brain functions such as production and drainage of cerebrospinal fluid, cell volume regulation, and controlling of the dimensions of the extracellular space [2–4]. In a pathophysiological context,

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water transport plays important role in cerebral edema, which may lead to ultimately cerebral herniation and death due to progressive increase in brain water content [5–7].

In the brain and other organs, water passes through plasma membranes by three distinct mechanisms: mere diffusion through the lipid bilayer, cotransport with organic or inorganic ions, and by way of specialized water channels (aquaporins, AQPs) [2, 8, 9]. It is recognized that AQPs are seen to mediate water flux between the four distinct water compartments existing in the brain: intracellular fluid (ICF), interstitial fluid (ISF), cerebrospinal fluid (CSF), and blood [10, 11], which are driven by osmotic and hydrostatic pressure gradient [12].

At present, nine AQPs have been identified at distinct brain sites, including AQP1 [13, 14], AQP3 [15, 16], AQP4 [17, 18], AQP5 [15, 19], AQP6 [20, 21], AQP7 [22–24], AQP8 [15, 25], AQP9 [26, 27], and AQP11 (Fig. 7.1) [28, 29]. A number of studies have reported the unexpected roles for the three members of this family (AQP1, AQP4, and AQP9) in physiology and pathology of CNS such as cerebral edema [30, 31], tumor angiogenesis [14, 32], autoimmune disease [33], neurodegenerative disorders [34], glial scar formation [35], and neuro-excitation [36]. To date, little is known about the function and regulation of AQP3, AQP5, AQP6, AQP7, AQP8, and AQP11 in the CNS [10]. This chapter will provide an update of recent findings in these rarely reviewed AQPs, and further the field of AQPs in the nervous system, and in particular the potential pathophysiological role of AQP4 in the CNS.

7.2 Expression of AQPs in Nervous System

7.2.1 AQPs in the Central Nervous System

7.2.1.1 AQP1

AQP1 is primarily distributed at the apical membrane in epithelial cells of the choroid plexus where the transcellular water movement via AQP1 contributes 25% of CSF production as shown by study on AQP1 null mice [37]. AQP1 has also been found in small diameter sensory neurons in dorsal horn of the spinal cord and trigeminal and nodose ganglia, with a strong

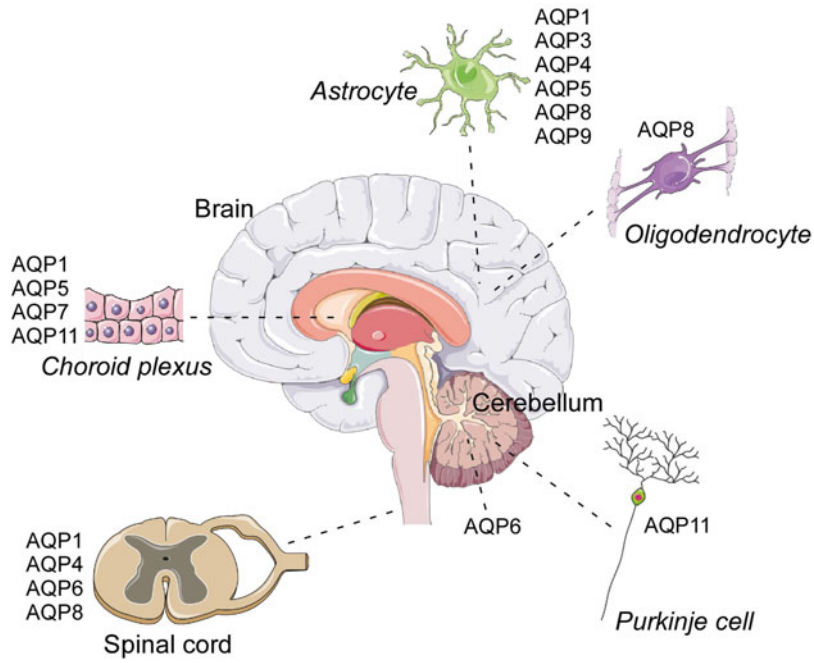

Fig. 7.1 Distribution of AQPs in the central nervous system. Astrocytes express AQP1, AQP3, AQP4, AQP5, AQP8 and AQP9. AQP1, AQP5, AQP7 and AQP11 are expressed in the choroid plexus. AQP1, AQP4, AQP6 and AQP8 are found in the spinal cord. AQP6 is expressed in the cerebellum. AQP11 is found in the cerebellar Purkinje cell. Oligodendrocytes express AQP8

implication that AQP1 may be involved in pain signaling [38, 39]. Moreover, intensive AQP1 expression is detected in neuronal filaments in the septum after juvenile traumatic brain injury [40]. In addition to these locations, AQP1 is expressed in astrocytes in the white matter and the glia limitans, and neurons innervating the pial blood vessels in the non-human primate and human brains [41, 42].

Besides these hereinbefore expression, AQP1 also abnormally distributes at specific sites in some brain disorders. For instance, AQP1 is localized in vascular structures of glioblastomas, microvascular endothelia and astrocytoma, and metastatic carcinomas [14, 43]. Moreover, in combination with NKCC1 (the Na-K-2Cl cotransporter 1), AQP1 is identified in meningioma cells and capillaries invading the dura [44]. These findings suggest that AQP1 may be involved in the tumor spread [45]. More recently, the alteration of AQP1 expression has been detected in the temporal neocortex of patients with Parkinson's disease (PD) and Alzheimer's disease (AD), indicating that astrocytes-involving water homeostasis is disturbed along with the neurodegenerative progression [46, 47].

7.2.1.2 AQP3

AQP3, permeable to glycerol and urea, was first found in brain meningeal cells in the CNS [48]. Studies show that similar to AQP5 and AQP8, AQP3 is expressed in astrocytes and neurons of piriform cortex, hippocampus, and dorsal thalamus [18, 49]. However, no expression of AQP3 is found in pig brain [23]. It seems that the distribution of AQP3 in the CNS shows a species-specific model. The role of AQP3 in the CNS remains scarcely investigated, earlier research has demonstrated that AQP3 expression is upregulated within the first 6 h after ischemia, suggesting a role of AQP3 in the early formation of the cerebral edema and the neuronal swelling [16]. Consistently, mRNA expression of AQP3 increases in human cerebral cortex neurons during edema, as assessment by a single cell digital PCR [50]. A recent study showed rare duplications of AQP3 in SCZ patients, suggesting a correlation between abnormal AQP3 expression and SCZ. Conditional knockout of neruofascin155 (NF155), a key protein of paranodal junction in oligodendrocytes, significantly decreases AQP3 expression in mouse brain. By contrast, overexpression of AQP3 in motor cortex neurons of in NF155 conditional knockout mice results in a significant increase in caspase 3-dependent neuronal apoptosis in AQP3-transduced cells [51]. These data indicate that targeting AQP3 expression may provide new therapeutic approaches for SCZ.

7.2.1.3 AQP4

AQP4 is the principal water channel in the CNS, primarily expressed in perivascular astrocyte foot processes throughout the brain structures [52, 53], the spinal cord [54], retina and optic nerve [55], periventricular organs [56], ependymal cells that line the lateral ventricles and cerebellum [17], hypothalamic magnocellular nuclei [57], dentate gyrus [58], and temporal neocortex [46]. The extensive distribution of AQP4 between the brain and various fluid compartments suggest its role in the brain water homeostasis [59]. Interestingly, the expression of AQP4 coincides the location of the potassium channel 4.1 (Kir4.1) [60].

AQP4 expression shows heterogeneous region-specific expression pattern with highest in the cerebellum [53]. AQP4 is also abundant in osmosensory areas, including the supraoptic nucleus and subfornical organ [18]. In the hippocampus, AQP4 expression exhibits laminar-specific pattern, with highest expression in the CA1 stratum lacunosum moleculare and the molecular layer of the dentate gyrus [58]. Activated astrocytes increase AQP4 expression in the whole astrocyte elements, causing AQP4 depolarized from the vascular end feet to parenchymal process, which occurs in the aging brain and a variety of neurological pathological conditions [61–65]. Notably, the polarity of AQP4 in brain is under circadian control. The perivascular polarization of AQP4 is highest during the rest phase, facilitating movement of CSF. By contrast, the awake brain has less perivascular AQP4 with low interstitial space volume. Deletion of AQP4 in mice eliminates circadian CSF distribution [66]. The molecular clock in

astrocytes may regulate the AQP4 gene expression, but the exact mechanism warrants further investigation [67]. In addition to circadian rhythm, an interesting finding reported that brain interleukin33 (IL33) is required for regulation of AQP4 expression in astrocytes, especially those at neuron-facing membrane domain. IL33 deletion in mice causes a loss of AQP4 in astrocyte process facing neurons after middle age, which coincides with exacerbated tauopathy and neurodegeneration [68].

Moreover, AQP4 can form both homo and hetero tetramers, with the hetero tetramers formed by a longer AQP4-M1 isoform and a shorter AQP4-M23 isoform. The M23-containing tetramers could assemble into orthogonal arrays of particles (OAPs), acting as a critical component of blood–brain barrier (BBB) [69–71]. Functionally, OAPs might be involved in the development and maturation of the BBB and serve to increase water permeability, enable the modulation of AQP4 membrane distribution [72–74]. Consistent with this concept, the ratio of M1 to M23 tends to decrease during the day that a time for rodents to rest. These data indicate that the expression of AQP4 in brain not only exhibits regional heterogeneity, but also undergoes dynamic changes on membrane domains in astrocytes; and various factors participate regulation of AQP4 expression and polarity, suggesting that AQP4 has complex biological functions.

7.2.1.4 AQP5

AQP5 expression in the CNS is similar to that described for AQP3, AQP4, and AQP8, mainly expressed in the astrocytes and neurons of choroid plexus, piriform cortex, hippocampus, and dorsal thalamus [75], and could expand to the nucleus caudatus putamen and globus pallidus in rat ischemic hemisphere [16, 76]. Whether AQP5 facilitates the highest water transport in the body remains uncertain [77, 78].

AQP5 might be an important water channel in astrocytes that is differentially expressed during various brain injuries [76]. AQP5 expression in brain is upregulated both after permanent focal cerebral ischemia [16] and following preterm intraventricular hemorrhage [79]. Upregulation of AQP5 after scratch injury is polarized to the astrocyte processes and cytoplasmic membrane in the leading edge of the scratch-wound, and facilitated astrocyte process elongation [76]. AQP5 expression is also detected near the ischemia-induced infarct border in the rat brain, and AQP5 level could be regulated by hypoxia [18] and protein kinase A (PKA) [19]. Recent research has also demonstrated that AQP5 expression is associated with the development and intensity of peritumoral edema in meningioma patients [80].

7.2.1.5 AQP6

AQP6 mRNA has been observed in neonatal and adult mouse cerebellum by using reverse transcription PCR [20]. AQP6 gene was found in mouse hind brain (involving cerebellum) and spinal cord [21], while AQP6 protein was detected at synaptic vesicles in rat brains [81]. The role of AQP6 in the CNS remains unknown. Since AQP6 mRNA expression is regulated in a tissue-specific and age-related way, it is likely that AQP6 plays a role in mouse development [20]. In addition, the location of AQP6 in synaptic vesicles might participate in their swelling and secretion [82]. Further investigation is needed to understand the function of AQP6 in the CNS.

7.2.1.6 AQP7

By using northern blot analysis, a weak band of AQP7 was first detected in rat brain. [83] Function as a glycerol channel mainly in fat metabolism, AQP7 is largely localized in the choroid plexus in brain of mice [22, 24]. Similarly, signals of AQP7 mRNA are also detected in pig brain [23]. Recent study shows that AQP7 expression is found to be restricted to the apical membrane of choroid plexus epithelial cells (CPECs) and endothelial progenitor cells (EPCs), in parallel with previous study [22], suggesting that AQP7 could be involved in CSF secretion [24].

7.2.1.7 AQP8

AQP8 was early detected in astrocytes, neurons, and oligodendrocytes [18], and in ependymal cells lining the central canal in spinal cords [55]. AQP8 is expressed in the cytoplasm of

astrocytoma cells in piriform cortex, hippocampus, and dorsal thalamus; weakly in ependyma and choroid plexus [25]. AQP8 is also present in the Bergmann glial cells of cerebellum, cochlear nucleus, and trapezoid nuclei [76]. AQP8 may play an important role in the development of brain disorders (edema and tumor), and can be used as a potential therapeutic drug for astrocytoma and glioma. For instance, AQP8 expression level is upregulated both along with the severity grade of astrocytoma and gliomas [25, 84]. In addition, AQP8 expression is upregulated after brain ischemia, suggesting that AQP8 contributes to the early formation of edema [16]. Even though AQP8 null mice show surprisingly mild phenotype [83], its role in the CNS seems to be pivotal.

7.2.1.8 AQP9

AQP9, a channel permeable to water, glycerol, urea, and monocarboxylates, has been evidenced in rodent and primate brains [41]. AQP9 is present in the ependymal cells lining the ventricles and the tanycytes of hypothalamus [26], astrocytes, endothelial cells of pial vessels, catecholaminergic neurons [27, 85], and the intracellular distribution of AQP9 is in mitochondrial inner membranes of brain cells [86]. It has also been reported that AQP9 is expressed in malignant astrocytic cells and leukocytes, which infiltrate the tumor in glioblastoma [87]. AQP9 knockout mice do not show severe abnormalities [88]. However, silencing of AQP9 in astrocyte cultures contributed to decreased glycerol uptake and increased glucose uptake and oxidative metabolism [88]. In addition, AQP9 expression was decreased under hypoxia and recovered with reoxygenation [18]. It has been suggested that signal transduction via PKA pathway may regulate the expression of AQP9 by some factors induced by dibutyryl-cAMP [19].

7.2.1.9 AQP11

AQP11 is found to be expressed in the CNS in rats and mice [89, 90], appearing in hippocampal and cerebral cortical neurons, purkinje cell dendrites in rat brains [28], and epithelium of the choroid plexus and endothelium of the brain capillary in mouse brains [29, 91]. The brain of AQP11 null mice appears normal, without any morphological and functional abnormalities [29]. However, AQP4 expression at the BBB is reduced by half in AQP11 null mice, suggesting AQP11 may functionally interact with AQP4 [29]. In agreement with this, acute hypernatremia increases AQP4 but decreases AQP1 expression in heterogeneous AQP11 null mice, whereas decreases AQP1 and AQP11 by half without changing AQP4 expression in WT mice [92]. It has been proposed that, when osmotically challenged, AQP11 may reduce its expression to protect the brain [91]. This view is further supported by a recent study that AQP11 may serve as a neuroprotective target of miR-27a-3p in the CNS, because miR-27a-3p mimic can suppress the upregulation of AQP11 in the perihematomal area and in brain microvascular endothelial cells, and protect against the BBB disrupt and brain injury intracerebral hemorrhage [93].

Since AQP11 has a unique high affinity mercury ion binding site (tri-cysteine motif site), AQP11 distributed in Purkinje cells may interact with the cations like mercury in autism, and be the therapeutic target for this cognition-related disorders [94]. Future investigations are necessary to elucidate the physiological role of AQP11 in the CNS.

7.2.2 AQPs in the Peripheral Nervous System

In the peripheral nervous system (PNS), three AQPs (AQP1, AQP2, and AQP4) are localized to neurons or glial cells in the ganglia and visceral plexuses.

7.2.2.1 AQP1

AQP1 is mainly localized to the cytoplasm and cell membrane of some medium and small-sized trigeminal or dorsal root ganglion (DRG) neurons [38, 94]. The expression pattern of AQP1 in the primary afferent sensory neurons suggests involvement in the specific somatosensory transduction including pain signal transduction [39, 95–97]. Furthermore, a recent study suggests

that AQP1 is mediated in DRG axonal growth and regeneration [98]. Additionally, AQP1 has been found in peripheral trigeminal axons and spinal nerve axons of humans and mice [99]. Interestingly, differences in the cellular localization of AQP1 in the central trigeminal root between humans and mice were detected. AQP1 is specifically expressed in astrocytes of humans, but is restricted to nerve fibers within the central trigeminal root and spinal trigeminal tract and nucleus in mice [99]. In the visceral plexuses, strong AQP1 expression is localized to satellite cells rather than neurons of humans [100]. In contrast, the localization of AQP1 protein in a particular neuronal subtype has been observed in the enteric nervous system of rats [101, 102]. Together, these morphological evidences have revealed a species difference of AQP1 expression in the PNS, but the underlying mechanisms remain to be determined. Apart from pain signal transduction, AQP1 may regulate DRG axonal growth and regeneration, thus can serve as a therapeutic target of peripheral nerve injuries [98]. A recent study demonstrated that AQP1 is expressed by Schwann cells in human and mouse skin, which might be involved in neuropathy and pain hyperalgesia [103]. The exact pathophysiological roles of AQP1 in the PNS disorders need to be further studied.

7.2.2.2 AQP2

An early study reported AQP2 expression in rat trigeminal ganglion neurons, with strong labeling in the medium- and large-sized types and weak labeling in the small-size type. After formalin treatment, there was a marked increase of AQP2 expression in small-sized neurons and a decrease in medium- and large-sized neurons [104]. Another study shows that AQP2 expression is not detectable in the DRG of normal rats, but remarkable increase in small-sized DRG neurons in response to chronic constriction injury treatment. These data suggest that AQP2 is involved in pain transmission in the PNS [105]. The cellular localization of AQP2 in the human PNS has not been studied yet.

7.2.2.3 AQP4

Compared to extensive studies of AQP4 in the normal CNS and neuropsychological diseases, little is known about its expression and function in the PNS. A study by Thi et al. (2008) identified AQP4 protein expression in the myenteric and submucosal nerve plexuses of mice and rats [106]. There are about 12% myenteric neurons positive for AQP4 in the myenteric plexus, while nearly 80% neurons are positive for AQP4 in the submucosal plexus of colon. Glial cells in the rat and mouse enteric plexuses are immunonegative to AQP4. Kato and colleagues reported that AQP4 is exclusively localized to satellite glial cells surrounding the cell bodies of the primary afferent sensory neurons in the trigeminal ganglia and DRG of mice [107]. Jiang and colleagues reported that there are different patterns of AQP4 expression in the enteric nervous system of human, guinea pig, rat, and mouse colon mucosa. In rat and mouse, AQP4 is expressed at a small subpopulation of neurons, while in the guinea pig and human AQP4 is localized to enteric glial cells [108]. Increasing evidence suggests that apart from NMOSD, anti-AQP4 antibodies are also involved in peripheral demyelination [109–112]. The cellular localization and function of AQP4 in the PNS including in the trigeminal and dorsal root ganglia need further study.

7.3 Functions of AQPs in Nervous System

7.3.1 CNS Water Balance

Phenotypic analysis of AQP4-knockout mouse model [31, 113–116] has shown that AQP4 facilitates a detrimental cellular water uptake as well as a protective clearance of extracellular fluid in cerebral edema following stroke [30], traumatic brain injury [117, 118], transient focal cerebral ischemia [119], spinal cord injury [120, 121], brain tumors [122], bacterial meningitis [123], and brain metabolic disturbances such as hyponatremia and water intoxication

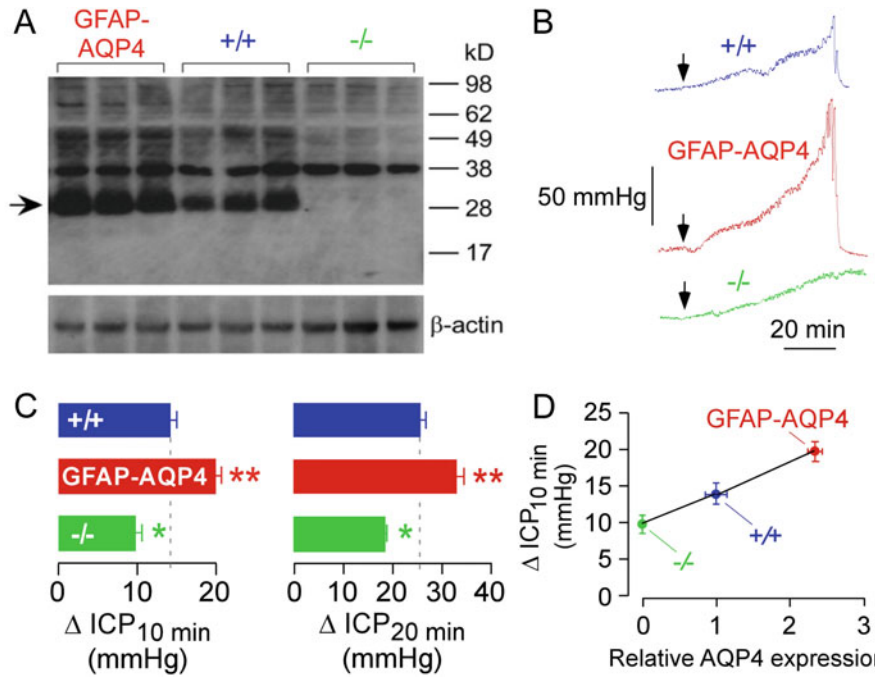

Fig. 7.2 Role of AQP4 in cytotoxic brain swelling. (**a**) Brain AQP4 protein expression detected by immunoblot analysis of whole brain homogenates from AQP4-overexpressing mice (GFAP-AQP4), wild-type mice (+/+) or AQP4 knockout mice (–/–). (**b**) Representative intracranial pressure (ICP) curves for mice with indicated genotype in a water intoxication model of cytotoxic brain edema. (**c**) Summary of ICP curve analysis: ΔICP at 10 and 20 min, (*$p < 0.05$, **$p < 0.01$ *vs.* +/+ mice). (**d**) ΔICP at 10 min determined from ICP curve analysis plotted against AQP4 protein expression determined by immunoblot analysis (S.E.). Adapted from [125]

[30, 124]. In accordance with this dual role of AQP4, its overexpression in glial cell accelerates cytotoxic brain swelling in transgenic mice (Fig. 7.2) [125].

The high AQP4 polarization at blood-brain and blood-CSF interfaces is crucial for rapid transport of water into and out of the brain parenchyma [59, 126]. The AQP4 deletion causes a slightly increase in the baseline water content in the brain and spinal cord of adult mice [120, 121, 127–129], which further supports that AQP4 may facilitate water efflux from the brain parenchyma into the brain vessels, ventricles and subarachnoid space. AQP4 also facilitates the elimination of excess brain water following vasogenic edema [31, 130–132]. However, there is also evidence indicating that AQP4 is responsible for rapid water movement into the brain [133]. AQP4 null mice have reduced brain swelling and improved survival when compared with wild-type littermates following water intoxication, focal cerebral ischemia or controlled cortical impact brain injury [30, 118]. These studies together suggest that AQP4 is a bidirectional water channel that facilitates water transport into and out of the brain.

Apart from maintaining brain water balance under physiological and pathophysiological conditions, AQP4 is also involved in the establishment of brain water homeostasis during the development. Early studies reported that AQP4 expression coincides with the BBB differentiation in the cerebellum of postnatal rat and the optic tectum of embryonic chicken [134–136]. Subsequent studies revealed that increased AQP4 expression levels partially relate to decreased brain water content in postnatal mice [137]. Systemic or conditional AQP4 knockout mice show a significant delayed decrease in brain water content during the postnatal development,

providing the direct evidence for a role of AQP4 in postnatal brain water uptake [127, 138].

7.3.2 Clearance of ISF Substances

An imbalance between the production and clearance of beta-amyloid (Aβ) and Tau has been regarded as the central event in AD pathogenesis [139]. Data have accumulated to support that AQP4 is necessary for clearance of interstitial solutes, including Aβ, Tau and α- synuclein through the glymphatic system [64, 65, 140, 141]. The lymphatic system, also entitled perivascular pathways, is responsible for tissue homeostasis clearance via clearance of excess fluid and interstitial solutes. The lymphatic vessels are present throughout all parts of the peripheral tissues. The CNS has long been regarded as lack of lymphatic network because no conventional lymphatic vessels are found within brain parenchyma. However, this view has been challenged by recent studies that reveal the clearance of ISF with its constituent proteins and other solutes along the perivascular space [142–145]. On the basis of in vivo two-photon imaging of small fluorescent tracers, Iliff et al. reported that CSF tracers rapidly enter brain parenchyma along the cortical pial arteries, and then influx into the Virchow-Robin spaces along penetrating arterioles [140]. The tracers rapidly distribute into brain parenchyma and subsequently exit the CNS primarily along the central deep veins and lateral ventral caudal rhinal veins [140]. The ISF within the perivenous space flows into dural lymphatic vessels, and eventually drains toward the deep cervical lymph nodes [145]. The perivascular pathways within brain parenchyma mainly include periarterial space, pericapillary space, and perivenous space, all of which are surrounded by astrocyte vascular end feet [146]. These astrocyte end feet have 50 nm gaps each other, creating the outer wall of the perivascular space and forming a donut-shaped tunnel surrounding the vasculature. These unique perivascular pathways not only provide efficient routes for rapid interchange of CSF and ISF, but also for clearance of soluble proteins and metabolites from the brain [147].

Particularly, Iliff et al. found that intrastriatal injected fluorescent or radiolabeled $A\beta_{1–40}$ is rapidly cleared from the mouse brain along the glymphatic perivenous efflux pathway [140]. Moreover, AQP4 null mice exhibit slowed CSF influx through this system and a ~65% reduction in ISF clearance and a ~45% reduction in clearance of intrastriatal injected radio labeled $A\beta_{1–40}$ [140]. These data highly suggest that AQP4-dependent astroglial water fluxes couple the clearance of interstitial solutes, including soluble Aβ from the brain. Further studies have revealed that the perivascular clearance pathways are impaired in the aging brain [64]. Compared to young controls, old mice show dramatic decreases in the efficiency of exchange between subarachnoid CSF and brain parenchyma and clearance of intraparenchymally injected Aβ. Apart from aging brain, impairment of glymphatic pathway function has been observed in mouse models of traumatic brain injury, ischemic stroke, AD and PD [65, 141, 148]. Impairments of glymphatic transport in the above pathological conditions are mainly due to mis-localization of AQP4 from the perivascular feet to the soma and non-perivascular processes of reactive astrocytes. These results imply that specific expression of AQP4 on the perivascular end feet of astrocytes is necessary for glymphatic system-mediated ISF bulk flow. Indeed, recent studies have shown that voluntary exercise improves astrocytic AQP4 polarization in aged mice and APP/PS1 mice, which facilitates glymphatic clearance of Aβ [149]. AQP4 deletion in APP/PS1 mice eliminates the alleviating effect of voluntary exercise in AD-like pathology [150]. All of these evidences suggest that targeting AQP4 polarization may be an effective strategy to prevent brain homeostasis disorders.

7.3.3 Spatial Buffering of Extracellular Potassium

Astrocytes mediated potassium (K^+) homeostasis is of critical importance for the regulation of

neuronal excitability. Synaptic activity causes release of K^+ into the extracellular space (ECS). The ECS K^+ is efficiently taken up by astrocytes through the inward rectifier potassium channel Kir4.1, then redistributed through the astroglial syncytium via gap junctions, thereby stabilizing neuronal activity [151]. The early study reported that AQP4 is co-localized with Kir4.1 in the end feet of retinal Müller cells, indicating their functional interaction [60, 152]. By contrast, the subsequent studies on AQP4 null mice provide evidence against functional interaction between AQP4 and Kir4.1 in retinal Müller cells [153]. However, the deletion of AQP4 in mice does impair extracellular K^+ clearance, which subsequently affects neuro-excitation with reduced seizure threshold and increased seizure duration [154–156]. These results support that AQP4 contributes to K^+ clearance, although the underling mechanism remains unclear.

Neuronal activity is associated with a shrinkage of the ECS around the active synapses [157, 158], which may be dependent on AQP4-mediated rapid water movement. AQP4 facilitates water entry into astrocyte processes surrounding the synapse, transports water through the astroglial network, and releases distantly into the ECS surrounding micro-vessels, thus subsequently produces a local shrinkage of ECS during the synaptic activity. Certainly, the AQP4-mediated rapid transport of intercellular water would drive reuptake of the ECS solutes including K^+ by astrocytes, because water serves as a transport medium for these substances.

7.3.4 Calcium Signal Transduction

Calcium (Ca^{2+}) signaling serves as a mediator of bidirectional interactions between neurons and astrocytes. Impaired Ca^{2+} signaling plays a critical role in the progression of brain edema [159]. Recent evidence suggests an involvement of AQP4 in astrocyte Ca^{2+} signaling. Deletion of AQP4 reduces hypo-osmotic stress-evoked Ca^{2+} signaling in astrocytes [160]. Subsequent functional studies revealed that AQP4 and TRPV4, a polymodal nonselective cation channel, synergistically regulate cell volume, and Ca^{2+} homeostasis [161]. Coimmunoprecipitation and immunohistochemistry further demonstrated that AQP4 and TRPV4 co-localize within astrocytes and retinal Müller glia [161, 162]. Functional analysis of an astrocyte-derived cell expressing TRPV4 but not AQP4 shows that cell volume control, and intracellular Ca^{2+} response can be reconstituted by transfection with AQP4 but not with AQP1 [162]. These data indicate that a TRPV4/AQP4 complex that constitutes a molecular system that finely regulates astroglial volume via integrating Ca^{2+} signaling and water transport, and might exacerbate the pathological outcome when an edema develops.

7.3.5 Regulation of Neurotransmission

Glutamate is the most prominent excitatory neurotransmitter in the CNS. Astrocytes absorb extracellular glutamate via excitatory amino acid receptors [163]. Glutamate uptake is also accompanied by water transport, which causes astrocyte processes to swell around the synapses, subsequently reducing the extracellular synaptic space during synaptic transmission and processing [164]. To restore ECS volume, astrocytes rapidly transport water into the surrounding capillary via AQP4 located in the perivascular end feet. Previous studies demonstrate that the AQP4 deletion downregulates glutamate transporter 1 expression in astrocytes and impairs their ability of glutamate uptake [165–168]. Previous studies also suggested that AQP4 is involved in the metabolism of dopamine, serotonin, and other neurotransmitters [169, 170].

7.3.6 Synaptic Plasticity

There is growing evidence that astrocytes play a role in long-term potentiation (LTP) and long-term depression (LTD) [171–173], which could be regulated by AQP4 [174, 175]. Experiments using mice with a deletion of the astrocyte-specific channel AQP4 on hippocampal synaptic

plasticity and spatial memory function has been investigated by Skucas et al. [176]. The mechanism appears to be related to neurotrophins, and especially brain-derived neurotrophic factor (BDNF) because pharmacological blockade of neurotrophin Trk receptors or scavenging BDNF restores synaptic plasticity [176]. However, the underlying mechanism for AQP4 modulating synaptic plasticity still needs more research.

7.3.7 Adult Neurogenesis

A previous study demonstrated that in corticosterone-treated model, AQP4 deficiency aggravates decreased proliferation and survival of new-born cells in the dentate gyrus [177]. Recent studies suggest that the development of depression-like behavior in corticosterone-treated models is paralleled by hippocampal neurogenesis, and adult hippocampal neurogenesis buffers stress responses and depressive behaviors [178, 179]. Thus, the aggravated neurogenesis inhibition in the hippocampus could also contribute to the exacerbated depressive behaviors in AQP4 null mice. This is consistent with the previous in vitro studies demonstrating that deletion of AQP4 impairs proliferation, migration and neuronal differentiation of adult neural stem cells (ANSCs) [180].

The lack of AQP4 could change the intrinsic property of ANSCs and enhance the injurious effects of corticosterone to ANSCs [181]. It has been revealed that AQP4 is essential for the initiation of intracellular Ca^{2+} event, including Ca^{2+} spikes and Ca^{2+}oscillation [160], AQP4 deficiency results in abnormal expressions of Ca^{2+} handling proteins in skeletal muscle cells and cardiac muscle cells [182, 183], and it has been suggested that AQP4 modulates the effects of corticosterone on ANSCs by regulating Ca^{2+} signaling [181]. However, the exact mechanisms still are not fully explored yet.

7.3.8 Energy Metabolism

AQP9 permeability to various molecules suggests a role in energy metabolism in addition to water homeostasis [74]. AQP9 plays a role in normal cell metabolism, under physiological conditions, and also increase cell stress tolerance, under pathological conditions [184]. For instance, AQP9 expression is upregulated by decreased insulin concentration in diabetic rats [185], after transient focal cerebral edema [27], in astrocytic tumors [186], after permanent middle cerebral artery occlusion [187], and with hirudin treatment after intracerebral hemorrhage [188]. All these findings would suggest that AQP9 is involved in astrocyte energy metabolism and the malignant progression of astrocytic tumors. Changes in AQP9 expression may be the consequence of glial cell attempt to response to hypoxic and ischemic conditions via facilitating clearance of glycerol and lactate [10]. In addition, a recent study reports that an upregulation of AQP9 expression in the brain plays a compensatory role in response to intracranial hemorrhage, via promoting brain angiogenesis, and preventing subsequent neuronal death and deterioration of neurological outcome [189].

7.3.9 Other Functions

AQP4 is known to be associated with astrocyte migration in glial scar formation [35, 190], and involved in facilitating gas diffusion [191] and cell adhesion between astrocytes [192, 193]. In terms of metal intoxication, AQP4 may act as either a neuro-protector or a mediator during the development of oxidative stress in the brain [194]. Furthermore, interactions of AQP4 and TRPV4 (transient receptor potential isoform 4) could function as an osmoregulatory complex in astrocytes [195].

7.4 AQPs in Diseases in Nervous System

It has been observed that AQP4 is clearly upregulated in several pathological conditions including brain tumors [196, 197], cerebral ischemia [198, 199], traumatic brain injury [40, 200, 201], and neuroinflammation [202]. In general terms, the upregulation of AQP4 in astrocytes is associated with edema resolution [1, 40, 202,

203]. Most cases, the increase and redistribution of the AQP4 is detected near the lesion site because of reactive astrogliosis [40, 196, 197]. Indeed, decreased AQP4 expression also occurs in some pathological conditions like severe traumatic brain injury [204, 205], epilepsy [206], and depression [207], which may be related to atrophy of glial cells or loss of processes. Moreover, in the same disease, such as AD, changes in the expression levels of AQP4 are variable among different brain areas and different stages [208]. The above heterogeneity of AQP4 expression is related to the different states of astrocytes.

7.4.1 Cerebral Edema

The role of AQP4 in cerebral edema has been extensively established by using AQP4-knockout models [52, 59, 143]. Considering the timeline of the newly observed AQP4 changes in ischemia, some researchers propose that cerebral edema should be divided into three major types: anoxic, ionic, and vasogenic edema, to replace two traditionally categories: cytotoxic and vasogenic edema [1, 209]. The initial anoxic edema, currently used, is characterized as the induces of ions into cells, accompanied by water entry and astrocyte swelling, while ionic edema occurs due to further alternations of the endothelial cell's transcapillary flux of sodium ion [210]. The development of ionic brain edema is associated with upregulation of AQP4 [1, 198, 199]. The final step termed as vasogenic edema, occurs with the disruption of the tight junction between the cerebrovascular endothelial cells, which comprise the BBB. At this time, a second increase of AQP4 expression is observed [198, 209], the presence of AQP4 is to facilitate clearance of excess fluid in vasogenic brain edema (Fig. 7.3) [31].

The dynamic spatial distribution of AQP4 at the astrocyte membrane is one of the two major modulation following injuries. AQP4 becomes more uniformly distributed on the astrocyte plasmalemma, termed as "dysregulation," which seems occur in parallel with cytotoxic edema to counteract early edema formation [40, 211, 212]. Interestingly, the ratio of AQP4-M1 and AQP4-M23 is increased in the ischemic hemisphere [199], the physiological role of this change remains unclear. Dysregulation of AQP4 may be produced via the reduction of the perivascular laminin, agrin, and ß-dystroglycan, which facilitate AQP4 to diffuse freely throughout the astrocyte membrane [212, 213]. The true function of AQP4 dysregulation remains largely unknown [213].

The activity of ion transporters or channels that induce AQP4-mediated cytotoxic and ionic

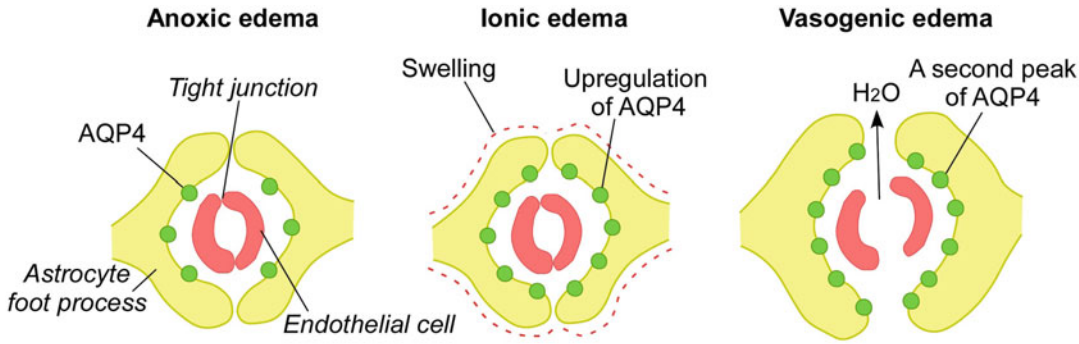

Fig. 7.3 Schematic drawing of AQP4 in 3 different edema phases: anoxic, ionic, and vasogenic edema. Anoxic edema is characterized as a swelling of the astrocytes caused by a disruption of the cellular ionic gradients and the entry of ions followed by water entry and leading to cellular swelling. During the ionic edema, astrocytes become swollen, AQP4 is upregulated. Vasogenic edema is a result of disruption of the tight junctions between the endothelial cells, leading to increased expression of AQP4 and permeability of the cerebral blood vessels, further contributing to swelling of astrocytes

edema is the other major modulation following injury [213]. Besides, AQP4 probably integrates with other astrocyte proteins like connexin-43 (Cx43) and the potassium channel Kir4.1 to eliminate the excess fluid [1]. SiRNA to silence the AQP4 expression, used as a potential drug to block AQP4, contributes to reduction of the edema formation after post-traumatic brain injury [214, 215]. An early study suggests that there is no convective solute flow in the pathology of acute brain edema, as proposed in "glymphatic" system [216]. However, this view is corrected by a recent study reporting that CSF entry into the brain along the glymphatic pathway is the principal mechanism for edema formation and ion perturbation during acute ischemic stroke. Spreading edema depends on AQP4 expression because deletion of AQP4 significantly suppresses ischemia-induced CSF tracer influx into the cortical cortex [217].

7.4.2 Neuromyelitis Optica

AQP4 specific antibodies have been identified as the therapeutic target for neuromyelitis optica (NMO), an autoimmune inflammatory disease of CNS that develops to paralysis and loss of vision [33, 218–220]. The binding of AQP4-IgG to AQP4 on astrocyte end feet is involved in activation of the complement cascade, a classical inflammatory response that occurs with pronounced granulocyte and macrophage infiltration, followed by oligodendrocyte damage, demyelination, and even neuron death (Fig. 7.4) [220]. To date, this complement-dependent cytotoxicity may be the most accepted hypothesis for NMO pathogenesis [221]. AQP4 IgG generally has greater binding affinity to OAPs than individual AQP tetramers [222–224], the structural changes in the AQP4 epitope upon array assembly greatly increases complement activation [224]. However,

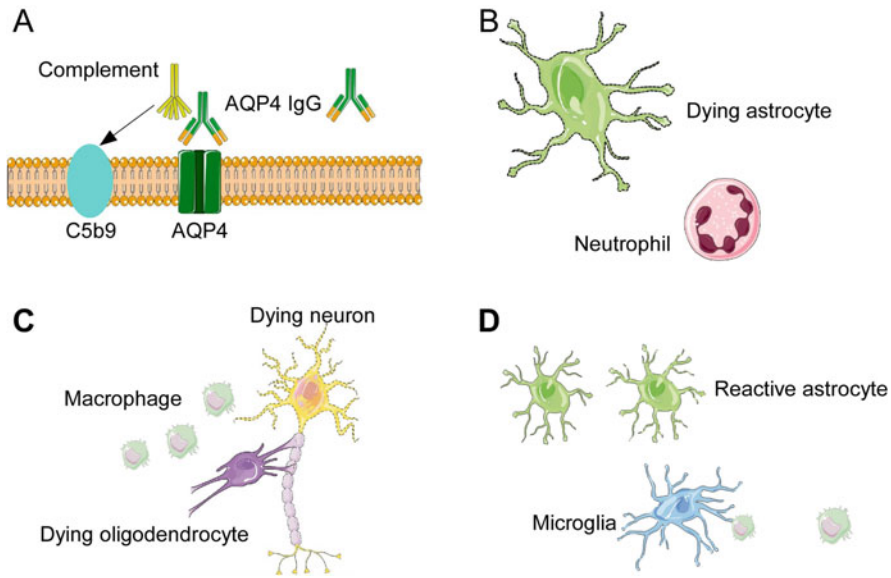

Fig. 7.4 AQP4 and the pathogenesis of neuromyelitis optica. (**a**) AQP4-IgG binds to AQP4 on astrocyte foot processes. Complement is activated via the classical pathway with deposition of C5bC9 complexes in astrocyte cell plasma membranes. (**b**) Activated complement components attract peripheral neutrophils into the lesion, which causing astrocyte death. (**c**) Dying astrocytes attract macrophages, causing death of oligodendrocytes and neurons. (**d**) Microglia enter the lesion as well as reactive astrocytes. The lesion core is necrotic with a macrophage infiltrate

AQP4 water permeability and the size of OAPs are not altered by binding to NMO-IgG [225].

A novel NMO therapeutics that target AQP4, involves using aquaporumab, a monoclonal antibody that blocks the binding of AQP4-IgG to AQP4 without cytotoxic side-effects [226]. Another approach to block the binding of AQP4-IgG and AQP4 is via a small-molecule blocker strategy [227]. AQP-IgG-targeted enzymatic therapeutics involves bacteria-derived endoglycosidase S (EndoS) and the enzyme IdeS, which neutralizes NMO-IgG pathogenicity [228, 229]. Other potential therapeutic strategies for NMO include reducing the entry of AQP4-IgG into the CNS or the expression of AQP4 on astrocytes, as well as preventing the formation of OAPs, or upregulating complement inhibitor proteins such as CD59 [6].

Recently, AQP4 specific antibody was applied for the diagnosis of NMO by using AQP4 extracellular loop-based carbon nanotube biosensor [230]. Since AQP4-targeted therapies are quite selective, new drugs (like aquaporumab, sivelestat, and eculizumab) entered into clinical trials need to be proved effective for NMO. [203] Moreover, there exist many important unsolved questions about the relationship of AQP4-lgG and NMO. For instance, the role of AQP4-lgG in the classification of NMO remains uncertain [231]. Furthermore, it is largely unknown about the reason why peripheral AQP4-expressing organs cannot be damaged by AQP4-IgG. Further studies in patients worldwide could help to identify more genetic susceptibility factors for NMO [232].

7.4.3 Brain Tumor

It has been clearly established that AQP1 expression is upregulated in brain astrocytomas [14, 45, 233] and positively correlated with the grade of malignancy, which is associated with angiogenesis and tumor invasion [7, 32, 234]. In this case, the AQP1 polymorphisms could be used as a survival prognosticator in patients suffering from glioblastoma multiforme [235]. One possible mechanism could be the induction of cell migration mediated by the water permeation of AQPs in "Osmotic Engine Model" [236], or the water influx into the cells leading to an expansion of their lamellipodia [237]. AQP1 expression is also upregulated in other brain disorders including choroid plexus tumors [238], subependymomas [239], and neoplastic invasiveness [240, 241].

AQP4 is expressed in astrocytoma cells and around the tumor [197]. And its expression is upregulated in astrocytoma and glioblastoma [12, 197]. A role for AQP4 in cell migration and cell–cell adhesion suggest its involvement in promoting glioblastoma cell migration, glioma invasion, and glioblastoma cell apoptosis [35, 190, 192, 242–244].

The possible mechanism is that AQP4 induces cell morphological changes via polarizing to cell lamellipodia and inducing an increased number or size of lamellipodia in migrating cells [242–244]. Structure of AQP4 (including OAPs) suggest its role in channel-mediated cell adhesion [192]. However, absence of such abnormalities in AQP4 knockout mice raises the argument about whether AQP4 plays a role in cell–cell adhesion [113]. Once, data against involvement of AQP4 in cell adhesion were demonstrated [245]. However, recent experiments display that the larger AQP4-M23 rich OAPs could bind with adhesion complexes [83]. So it could be speculated that whether AQP4 plays a role in cell adhesion is determined by the involvement of OAPs.

7.4.4 Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative disease among the elderly and characterized by Aβ plaque deposition, neurofibrillary tangles, and neuronal and synapse loss in learning and memory related regions [246]. It has been adequately reported that AQP1 plays important role in neurodegenerative disease like AD [47, 247–249] and Parkinson's disease (PD) [46]. AQP1-expressing fibrillary astrocytes are closely around Aβ plaques in patients with AD [47] or prion plaques in GSS disease [250],

suggesting an involvement in plaque formation. AQP1 is abnormally expressed by neurons in the brain of AD patients [247] and transgenic AD mice [251–253]. Upregulated expression of AQP1 in neurons may inhibit Aβ production by reducing the interaction between β-amyloid precursor protein and β-secretase [248, 254].

As mentioned earlier, activated astrocytes accompanied with altered polarization of AQP4 occur in the brain tissues of patients with AD and several AD models [61, 62, 247], indicating an involvement in AD pathology. A recent study reported that the AQP4 gene deletion in APP/PS1 transgenic AD model mice impairs Aβ clearance from brain parenchyma and exacerbates spatial learning and memory defects associated with more severe Aβ plaque deposits and synaptic protein loss [255]. This finding provides the direct evidence for a key role of AQP4 in the pathogenesis of AD. Actually, accumulatively direct and indirect evidences have indicated that AQP4 affects the onset and progress of AD via various mechanisms, such as Aβ clearance, glutamate transduction, synapse plasticity, Ca^{2+} signal transduction, neuroinflammation, and neurotrophic factor secretion [64, 65, 140, 148, 162, 255–258]. For example, reactive gliosis with loss of perivascular AQP4 polarization impairs the glymphatic pathway function, causing reduction in CSF-ISF exchange and Aβ deposition in cortical and leptomeningeal vessels [139].

In agreement with animal experiment results, glymphatic transport ability of AD patients has been found to be lower than that of healthy controls of the same age, via assessment of perivascular fluid movement with diffusion tensor magnetic resonance imaging [259]. Loss of perivascular AQP4 localization is associated with increased Aβ deposition [260]. Perivascular AQP4 localization is preserved among individuals older than 85 years who remains cognitively intact, highlighting a critical role of AQP4 polarity in maintaining cognitive function during the age process [260]. This review is further supported by the fact that alterations in AQP4 expression and localization in the fronto-temporal lobe are associated with AD status and pathology. A noncoding *Aqp4* SNPs may contribute to these changes [261]. In addition, a recent study reported that functional variants in AQP4 modulate deep non-rapid eye movement sleep and cognitive consequences of prolonged wakefulness [262]. These data suggest that AQP4 SNPs may affect sleep quality that is one of key factors in the occurrence and progression of patients with AD. More evidence is necessary in order to confirm this presumption. Together, these results from both animal and human studies indicate that AQP4 is vital for glymphatic clearance, thus serving as a hopeful target for prevention and treatment against AD.

7.4.5 Other Diseases in Nervous System

AQP1 is also upregulated expression in other brain disorders including spinal cord injury [263], cerebral edema [264, 265], neoplastic invasiveness [232, 233], NMO-spectrum disorders [266], Gerstmann-Sträussler-Scheinker (GSS) disease [259], and Creutzfeldt-Jakob disease [248]. Moreover, it seems that there is a possible link between AQP1 and neuropathic pain sensation, since pain responses are decreased in consistence with reduced AQP1 expression [39, 263]. Based on these findings, AQP1 inhibitors could be used as potential drugs in treatment of these brain diseases [7, 37, 258]. Interestingly, AQP1 could be inhibited by melatonin in rodents with spinal cord injury and agmatine in cerebral edema [263, 264], suggesting that melatonin and agmatine agonists could be used as such potent agents.

AQP4 null mice demonstrate that AQP4 deficiency reduces neuroinflammation, in support of a deleterious role of AQP4 in the pathophysiology of multiple sclerosis [267]. Similarly, AQP4 deletion is neuroprotective after severe global cerebral ischemia [268] and micro-traumatic brain injury in mice [269]. However, the absence of AQP4 shows more hyperactive microglial inflammatory responses, potentially increasing the severity of PD [46, 270]. Moreover, AQP4 knockout in mice produces several impairments in neuro-excitation phenomena including

hearing, vision, olfaction, epilepsy, and cortical spreading depression [271]. Besides, AQP4 deficiency impairs synaptic plasticity and associative fear memory in the lateral amygdala [167], causes an impairment of blood–retinal barrier [272], and increases capillary density in the brain [273].

7.5 Modulators of AQPs in the CNS

It has been proposed that AQP4 modulators have potential utility in the treatment of AQP4 related brain diseases [274, 275]. AQP4 inhibitors such as vasopressin, melatonin, PKC, mercury (Hg^{+}), trombin, dopamine, hypoxia, tetraethylammonium (TEA), bumetanide, acetazolamide (AZA), siAQP4, curcumin, and H_2S may be regarded as potential therapeutic drugs for cytotoxic brain swelling, seizure, glial scar [215, 275–279]; while the AQP4 enhancers including glutamate, syntrophin, dystrophin, connexin 43 (Cx43), K^{+} (Na^{+}, K^{+}-ATPase; NKCC1), Kir4.1, lead (Pb^{2+}), cyclic AMP, and lactic acid have therapeutic potentials in reducing vasogenic brain swelling [154, 275, 280, 281]. AQP4 modulators could offer new therapeutic options for many brain disorders like preventing tumor malignancy in glioblastoma [10]. Notably, many of the AQP4 modulators have been experimentally examined in isolation. However, these factors are likely to interact after injury [213]. A recent study confirmed that TGN-020, the novel AQP4 inhibitor, inhibits glymphatic CSF lymphatic-ISF exchange and tau protein clearance from the brain [282]. These studies further support AQP4 as a promising druggable target for regulation of the glymphatic system function and related neurological diseases.

7.6 Future Directions

AQPs are involved in a variety of important physiological process in the CNS, by coordinating water and solutes trafficking among the different fluid compartments [10]. Specific upregulation of some AQPs expression along with their involvement in brain edema formation has been consistently investigated by many scientists. It has been strongly suggested that AQPs could represent important targets in treatment of cerebral edema, brain tumor [14, 32], CNS autoimmune disease [33], neurodegenerative disorders [34], and psychiatric disorders [283, 284]. However, we are still far from having a full comprehension of the physiological and pathological significance of all AQPs in the CNS [285]. Although AQP inhibitors or agonists have been used to treat brain edema in laboratory animals, their clinical application is still a long way off. To date, the major challenge is still to facilitate drug delivery across the BBB [286]. Considering the importance of AQPs in brain disorders, it will be of great achievement to find out novel drugs capable to cross the BBB and to selectively suppress AQP upregulation [10].

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Aquaporins in Cardiovascular System

8

Lu Fan, Pin Wu, Xuejun Li, and Lu Tie

Abstract

Recent studies have shown that aquaporins (AQPs) are involved in the regulation of cardiovascular function and the development of related diseases, especially in cerebral ischemia, congestive heart failure, hypertension, and angiogenesis. Therefore, further studies are needed to elucidate the mechanism accounting for the association between AQPs and vascular function-related diseases, which may lead to novel approaches to the prevention and treatment of those diseases. Here we will discuss the expression and physiological roles of AQPs in vascular tissues and summarize recent progress in the research on AQPs related cardiovascular diseases.

Keywords

Aquaporin · Cerebral ischemia · Congestive heart failure · Hypertension · Angiogenesis

8.1 Introduction

The cardiovascular system (CVS) comprises the heart and blood vessels, including arteries, capillaries, and veins [1, 2]. In the embryo, the CVS is the first functional organ system to develop. The main functions of CVS are transporting oxygen, active compounds (nutrients, metabolic intermediates, hormones, etc.), and waste products throughout the body. Once damaged, CVS has a very limited capacity for regeneration [3]. Therefore, exploring CVS diseases and regulatory mechanisms has always attracted great attention. Identification of novel therapeutic targets would provide opportunities for developing more effective strategies.

Aquaporins (AQPs), a family of transmembrane proteins present in almost all species including viruses [4], mediate the permeability of water and some small molecules across cell membranes driven by osmotic or concentration gradient. Since the first AQP was identified in red blood cells in 1991, 13 AQPs (AQP0–12) have been identified in mammals [4, 5], which regulate various important biological functions in the kidney, brain, lung, digestive system, eye, skin, etc. Consequently, dysfunction of AQPs leads to a diverse range of diseases. Studies have shown that AQPs are also involved in the regulation of cardiovascular function and the development of related diseases, especially cerebral ischemia, congestive heart failure, hypertension, and angiogenesis. Meanwhile, because of the intrinsic undruggable aquaporin pore compounded by issues with the reproducibility of current assays, the development of AQP drugs made little progress [6]. Therefore, further

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studies are needed to elucidate the mechanism accounting for the association between AQPs and cardiovascular diseases, which may lead to novel approaches to the prevention and treatment of those diseases.

8.2 Expression and Physiological Function of AQPs in the Cardiovascular System

AQP1, AQP4, AQP7, and AQP9 have been found in the cardiovascular system. They distribute in the heart, endothelial cells, and vascular smooth muscle cells [7, 8], and participate in the transportation of water, glycerol, and lactic acid, which play an important role in vascular physiological function. AQP function may be related to the pathological process of CVS diseases. AQPs have a specific distribution pattern in cardiomyocytes, but whether their presence is essential for proper (electro)physiological cardiac function has not intensively been studied [9].

8.2.1 AQP1

AQP1 is widely distributed in the body where it plays a role in the regulation of water transport. AQP1 is also essential in angiogenesis, cell migration, and cell growth of microvessels in the kidney, lung and airways, secretory glands, skeletal muscle, pleura, and peritoneum. AQP1 was also expressed in endothelial cells in the cornea and lacteals of the small intestine [10, 11].

The cardiac AQP presence and expression depend on various species, sex, development, and aging, with data sometimes being contradictory between species, such as the expression of AQP1 is high in the rat embryonic heart, but its expression level was substantially reduced after birth, whereas mice showed an even further decrease postnatally. Aging increased AQP1 and AQP4 expression levels in the myocardium of *db*/*db* mice [9, 12, 13]. AQP1 facilitates transendothelial water movement in osmotically driven membrane processes. More and more data show that AQP1 can mediate the transport of small molecules such as urea, CO_2, NH_3, H_2O_2, nitric oxide (NO), $Sb(OH)_3$, $As(OH)_3$ [5, 14–16]. As the main isoform in cardiac myocytes, AQP1 expression level correlates with the severity of hypertrophic remodeling. A recent study by Montiel et al. showed that cardiac hypertrophy is caused by AQP1-mediated transport of H_2O_2 [12]. Extracellular H_2O_2 was a major involvement in the hypertrophic response. NADPH oxidase-2 (NOX2) can produce superoxide anions in the extracellular space and then rapidly dismutated by superoxide dismutase 3 to H_2O_2. AQP1 has been found to be colocalized with the $p47^{phox}$ subunit of NOX2 at the peripheral plasma membrane, which facilitated H_2O_2 transport and activated the oxidant-sensitive kinases (ERK/MAPK) signaling pathway in myocardial hypertrophy (Fig. 8.1). Therefore, inhibition of AQP1 may provide a new direction toward the effective treatment of hypertrophic cardiomyopathies. In CVS diseases, AQP1 regulates the entry of NO into endothelial cells and affects vascular tension and blood pressure by controlling NO levels, bioavailability, and diffusion distance [9, 17]. In liver cirrhosis, AQP1 is upregulated in the fibrotic diaphragm of the liver and promotes angiogenesis by enhancing endothelial invasion/proliferation [18].

AQP1 knockout attenuated the angiogenesis, fibrosis, and portal hypertension that follows bile duct ligation in mice [19, 20]. In retinal vascular endothelial cells, AQP1 is involved in hypoxia-inducible angiogenesis through a VEGF signaling pathway independent manner [21, 22]. However, in oxygen-induced retinopathy microvessel proliferation was not affected in AQP1 knockout neonatal mice [23, 24].

Moreover, AQP1 is highly expressed in microvascular endothelial cells in malignant tumors. Inhibited tumor growth and reduced vascularity with extensive necrosis were found in AQP1 knockout mice after subcutaneous or intracranial tumor implantation [25, 26]. Our previous study demonstrated that a carbonic anhydrase inhibitor acetazolamide could inhibit AQP1 protein expression and angiogenesis in tumor tissues (Fig. 8.2) [27]. AQP1 DNA immunity based on the ubiquitin-proteasome system can directly

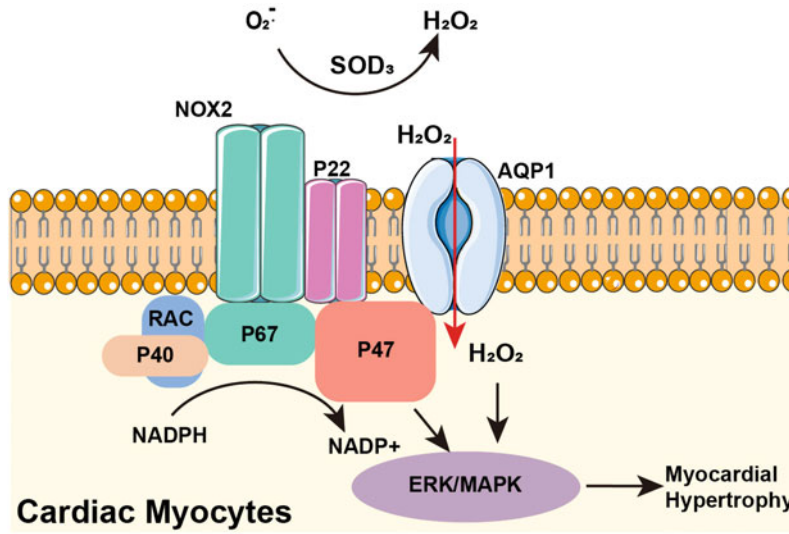

Fig. 8.1 AQP1 and myocardial hypertrophy. AQP1 has been found to be colocalized with NOX2 at the peripheral plasma membrane, facilitated H_2O_2 transport, and activated the oxidant-sensitive kinases (ERK/MAPK) signaling pathway in myocardial hypertrophy [12]

damage the vascular system of melanoma tumors, thereby inhibiting tumor growth in mice [28, 29]. Therefore, targeting to regulate AQP1 expression in vascular endothelial cells may play a positive role in tumor angiogenesis and treatment.

8.2.2 AQP4

AQP4 is mainly expressed in the central nervous system and is highly expressed in the brain, spinal cord, and optic nerve [30–34]. AQP4 mainly exists in astrocytes surrounding cerebral capillaries and is distributed in the astrocytic foot processes, external glial limiting membrane, ependyma, and subependymal internal glial. Most scientists do not think that AQP4 is expressed in cerebrovascular endothelial cells. However, Amiry-Moghaddam and colleagues demonstrated that AQP4 is expressed in brain endothelial cells by using immunogold electron microscopy, at lower levels than in astrocytes [35, 36]. A selective knockout of the AQP4 in the astrocytic foot

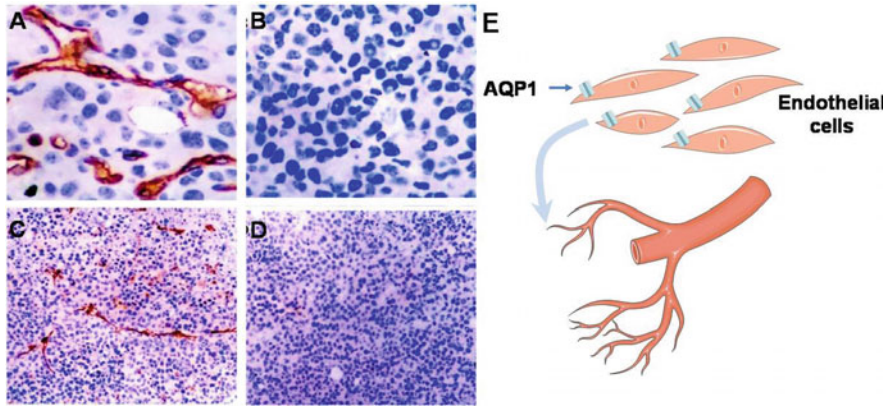

Fig. 8.2 AQP1 and angiogenesis. Expression of AQP1 in capillaries (**a**, **b**) and postcapillary venules endothelial cell (**c**, **d**) of the primary tumor; (**a**) and (**c**) untreated group, (**b**) and (**d**) treated with acetazolamide. (**e**) AQP1 in endothelial cells could assist cell migration and promote angiogenesis

processes delayed cerebral edema, despite the presence of a normal complement of endothelial AQP4. But whether the endothelial AQP4 is involved in maintaining water balance in the brain is still elusive.

AQP4 plays an important role in the blood–brain barrier and blood–cerebrospinal fluid barrier by maintaining the balance of brain water in the central nervous system [37]. The highly polarized AQP4 expression (in glial membranes that are in direct contact with capillaries and pia) indicates that AQP4 mediates the flow of water between glial cells and the cavities filled with CSF and the intravascular space. AQP4 deficiency impairs the migration of mouse astrocytes. In addition, AQP4 deficiency impairs the migration of astrocytes cultured in vitro, which shows that another role of AQP4 is to promote astrocyte migration and neural signal transduction. [38, 39]. In the heart, the content of AQP4 in mouse cardiomyocytes is higher than that in rat cardiomyocytes [10].

8.2.3 AQP7

AQP7, a water/glycerol transporting protein, regulates adipocyte glycerol efflux and influences lipid and glucose homeostasis, mainly distributed in adipose tissue, testis, cardiac and striated muscle, and renal proximal tubules. A microarray study showed that the heart was the second biggest expression tissue of AQP7 mRNA after adipose tissue [40], but studies on the cardiac role of AQP7 are limited. In 2009, Hibuse and colleagues demonstrated that AQP7 knockout mice have lower cardiac glycerol and ATP content than those of wild-type mice [41]. Thomas Hospital's solution 2 (STH2) is usually used to protect the myocardium during surgery, but AQP7 deficiency does not affect the protective efficacy of STH2 [42]. Under basal conditions, AQP7 knockout mice had normal cardiac histology and morphology; when injections of isoproterenol or subjected to transverse aortic constriction (TAC), AQP7 knockout mice developed advanced hypertrophy and lower survival than wild-type mice, indicating the importance of glycerol as a cardiac energy substrate [41]. In addition, AQP7 was expressed in capillary endothelial cells of adipose tissue, but its functions remain to be fully elucidated [43, 44]. Therefore, it is necessary to clarify the physiological and pathological significance of cardiac and endothelial AQP7 in the future.

8.2.4 AQP9

AQP9 is also an aquaglyceroporin and has permeability to water, monocarboxylate, glycerol, urea, and other small neutral solutes. AQP9 has two isoforms: a short isoform located on the inner membrane of mitochondria, and a long isoform located within the cell membrane [45–47]. AQP9 is distributed in the brain, liver, spleen, testis, and other tissues. The expression of AQP9 is also detected in the endothelial cells of pial blood vessels [48, 49]. AQP9 is believed to contribute to extracellular water homeostasis, and its functional effects are similar to AQP4 [50]. AQP9 might participate in brain energy metabolism. It is also expressed in neuronal mitochondria and glucose-sensitive neurons, and its expression could be negatively regulated by insulin [49]. AQP9 is involved in the transport of lactate and ketone bodies across the blood–brain barrier. It has been suggested that AQP9 may participate in the clearance of excess lactate and other metabolites during cerebral ischemia [45]. Moreover, AQP9, as an aquaglyceroporin, is expressed in many types of immune cells and plays important role in tumor initiation and progression. However, the relationship between AQP9 and tumor-infiltrating cells, and its prognostic value in cancers still require comprehensive understanding [51].

8.3 AQPs and Cardiovascular Disease

8.3.1 Cerebral Ischemia

Stroke is a complex and destructive neurological disease with limited treatment options. In

ischemic stroke, edema is the main factor leading to the aggravation of stroke, and brain edema is a serious complication of stroke. Therefore, edema is an important target for the treatment of stroke. Early edema formation can significantly contribute to infarct formation and thus represents a promising target. Seven AQP subtypes, including AQP1, AQP3, AQP4, AQP5, AQP8, AQP9, and AQP12, have currently been identified in the brain. Among them, AQP1, AQP4, and AQP9 are the most abundant AQPs in the brain. The expressions of AQP4 and AQP9 were changed during cerebral edema after ischemic stroke, but AQP1 expression was unchanged [52, 53]. AQP4 expression was found to be increased on astrocyte endfeet in the core and the border of the lesion 1 h after cerebral ischemia, and increased in astrocytes in the border of the lesion over the whole cell for 48 h after ischemia; both were coinciding with the peak of cerebral edema [53]. AQP4 was more abundant in the early stage of cerebral ischemia [54, 55]. A novel Ser111Thr variant exists in AQP4 in cerebral ischemia patients [56]. Various studies have shown that AQP4 deficiency mice exhibited a significant reduction in infarct volume and improvement in neurological prognosis after cerebral ischemia. AQP4 deficiency can partially prevent the destruction of the blood–brain barrier and reduce neuroinflammation caused by cerebral ischemia [57–59]. Hastings and colleagues demonstrated that cerebral hemispheric edema was reduced in AQP4 null mice 1 hour after ischemia [57]. In addition, Hirt and colleagues reported that AQP4 absence on behavioral outcomes and lesion volume was not associated with the reduction of edema formation on days 3 and 7 after ischemia [60].

The expression of AQP9 was significantly induced 24 h after ischemia and increased gradually with time, which had nothing to do with cell swelling [53]. Its functional roles remain to be fully elucidated. A few studies have examined the associations between AQP3, AQP5, or AQP8 and cerebral ischemia. Yang and colleagues demonstrated that the expression of AQP3, AQP5, and AQP8 enhanced until 24 h after cerebral ischemia in the border region but decreased 6 h after ischemia in the ischemic core, suggesting their involvement in edema formation after cerebral ischemia [61, 62]. Therefore, the selective regulation of aquaporin may provide a new and effective strategy for the treatment of cerebral ischemia.

8.3.2 Congestive Heart Failure (CHF)

The end-stage manifestation of most heart diseases is heart failure, of which the most typical end-stage manifestation is congestive heart failure (CHF), accompanied by disturbance of water excretion. Acute CHF aggravation stimulates the pituitary gland, resulting in activation of the renin-angiotensin-aldosterone system (RAAS) and increased release of adrenocorticotropin (ACTH) and arginine vasopressin (AVP). Subsequently, sodium and water retention are induced. The kidney is essential for the reabsorption of water and the retention of water and sodium. AVP increases the water permeability of the kidney collecting tube cells, allowing more water to be absorbed back into the blood from the urine of the collecting tube. In addition, AVP acts on the V2 receptor in the renal collecting duct, thus regulating the expression and transport of AQP2 [63–65]. AQP2 is a promising marker of the concentrating and diluting ability of the kidney. AVP triggers a reversible translocation of AQP2 from intracellular storage vesicles into the apical plasma membranes (APM) over several minutes, and AQP2 protein levels could be elevated by AVP over a period of hours to days [63, 66, 67]. Renal AQP2 expression is significantly increased in CHF rats, whereas other subtypes of AQP expressions (such as AQP1 and AQP3) were unaltered. Besides its expression, urinary excretion of AQP2 was also markedly increased in CHF patients [68, 69]. There is a close correlation between plasma AVP levels, renal AQP2 expression, and the severity of CHF. Administration of V2 receptor antagonist tolvaptan could downregulate renal AQP2 protein levels in CHF rats [70]. Tolvaptan was approved in 2009 by

FDA to treat hyponatremia associated with CHF, however, the ideal responders to tolvaptan have not yet been identified. AQP2 may be served as an ideal predictor of response to tolvaptan and guide its treatment in the future [71].

8.3.3 Hypertension

Hypertension is a common cardiovascular disease, that can lead to heart disease, stroke, as well as hypertensive retinopathy, and chronic kidney disease. Blood pressure (BP) is affected by various factors, including peripheral resistance, vessel elasticity, blood volume, and cardiac output. Therefore, the mechanism of hypertension is too complex. Recently, much interest focused on the role of AQPs in the pathophysiology of hypertension. In spontaneously hypertensive rats (SHR), AQP2 expression in renal tubule epithelial cells is upregulated, along with activation of the cAMP pathway induced by AVP [72, 73]. Same results have been found in DOCA-salt hypertensive rat model [74]. In addition, treatment with AVP V2 receptor antagonist would lower BP and urinary osmolarity, and alleviate urinary AQP2 levels both in control and SHR, indicating AQP2 and AVP are involved in the pathogenesis of hypertension in spontaneously hypertensive [75]. In addition to AQP2, the expression of AQP1 and AQP3 in the medulla of SHR also increased significantly compared with the corresponding control group (WKY rats), while the expression change of AQP4 was not significant [73].

Klein Fukuoka and colleagues demonstrated that medullary AQP2 expression was decreased in response to angiotensin II or norepinephrine-induced acute hypertension [76, 77]. Alterations in the expressions of AQPs in the brain were also found during hypertension. AQP1 expression was increased in the choroid plexus epithelium of SHR, and elevated AQP4 expression was found in the frontal cortex, striatum, and hippocampus of SHR compared to control WKY rats [78]. The increased AQPs expression may modulate the fluid exchange between the blood–brain barrier and blood–CSF barrier, and evoked an acute increase in blood pressure and impairment of the blood–brain barrier.

In 2007, Herrera and colleagues reported that AQP1 mediated transfer of NO at a $K_{1/2}$ (the concentration of NO that produces half of the maximum transport rate) of 0.54 μmol/L, and knockdown of AQP1 by siRNA could prevent NO release by 44% in endothelial cells [11]. They further (2007) demonstrated that AQP1 facilitated transport of NO out of endothelial cells and influx into vascular smooth muscle cells, and got involved in endothelium-dependent vascular relaxation [9]. However, humans with AQP1 deletion and AQP1 knockout mice do not suffer from hypertension, so the role of AQP1 in hypertension requires further investigation [79, 80].

8.3.4 Pulmonary Hypertension

Hypoxic pulmonary hypertension (HPH) is characterized by excessive proliferation and migration of endothelial and smooth muscle cells. AQP1 has been revealed to play a prominent role in the proliferation and migration of pulmonary artery smooth muscle cells. However, its potential role in the pathogenesis of pulmonary hypertension (PH) has not been addressed so far. Studies have found that AQP1 directly drives hypoxia-induced vascular remodeling, increased pulmonary arterial pressure and right ventricle hypertrophy. AQP1 deficiency could directly reverse dysregulation of pulmonary artery smooth muscle cells (PASMCs) and primary mouse lung endothelial cells induced by hypoxia [81]. AQP1 promotes the proliferation and migration of PASMCs via upregulation of β-catenin protein levels [82]. Higher endothelial AQP1 expression leads to an increase in the critical pressure of subendothelial intimal compression and a decrease in arterial wall hydraulic conductivity [83]. These findings thus offer novel insights into the pathogenetic understanding of HPH and propose AQP1 as a potential therapeutic target.

8.3.5 Angiogenesis

Angiogenesis plays a critical role in both physiological functions and disease pathogenesis. Excessive angiogenesis can promote neoplastic diseases and retinopathies, while inadequate angiogenesis can lead to aberrant perfusion and impaired wound healing. Angiogenesis is regulated by many factors, such as VEGF, PDGF, TGF-β, FGF, ANG, NOTCH, WNT, etc. Recently, AQPs have been shown to be involved in angiogenesis, especially in tumor angiogenesis. Tumor angiogenesis includes three procedures: (1) matrix breakdown (2) proliferation, migration, and differentiation of endothelial cells, (3) supplement of periendothelial cells [84].

In brain cancer and glioblastoma, AQPs expression is positively correlated with tumor histological differentiation [85–87]. In other cancers, such as breast cancer, brain cancer, and multiple myeloma, high expression of AQPs results in localized edema that aggravated matrix breakdown [26, 88, 89].

AQP-dependent cell migration has been found in a variety of cell types both in vitro and in vivo (Fig. 8.2). Saadoun et al. found that upregulated expression of AQP1 in tumor microvascular endothelial cells could assist cell migration and its expression was positively correlated with tumor microvascular density. AQP1 deletion reduces endothelial cell migration and inhibits tumor angiogenesis and growth [25, 90]. Tumor cells with high expression of AQP1 have stronger metastatic potential and increased local invasion. When AQP1 was deleted, the migration ability of proximal tubular epithelial cells was impaired. In addition, impaired cell migration was also observed in corneal epithelial cells, intestinal cells, and skin keratinocytes with AQP3 deletion [90], while AQP4 deletion mainly slowed down the migration of astrocytes, thus impaired glial scar formation after brain injury [87]. The mechanism of AQPs and cell migration is not clear, and further research is needed.

The study of aquaporin and tumor angiogenesis provides a theoretical basis for tumor treatment. AQPs-mediated water influx may affect the proliferation, migration, metastasis, and angiogenesis potential of cancer cells. Provides a new direction for tumor treatment and helps to improve the treatment of various tumors and their poor prognosis [91, 92].

8.4 Summary

Due to the diversity and complexity of the AQP family, the cardiovascular system may require a variety of AQP subtypes to collaborate to finish their normal physiological function. It is necessary to study the expression and function of AQPs in the cardiovascular system further from the level of integration. AQPs are involved in many related disease occurrences, development, and cardiovascular function regulation. It has important clinical significance to understand the accurate correlation between AQPs expression variation and cardiovascular diseases, which can provide new ideas and methods for cardiovascular disease treatment.

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Aquaporins in Respiratory System

9

Linlin Wang, Jian Wang, Xiaodan Zhu, Chunxue Bai, and Yuanlin Song

Abstract

Aquaporins (AQPs) are water channel proteins facilitating fluid transport in alveolar space, airway humidification, pleural fluid absorption, and submucosal gland secretion. In this chapter, we mainly focus on the expression of four AQPs in the lungs, which include AQP1, AQP2, AQP4, and AQP5 in normal and disease status, and the experience of AQPs function from various model and transgenic mice were summarized in detail to improve our understanding of the role of AQPs in fluid balance of respiratory system. It has been suggested that AQPs play important roles in various physiology and pathophysiology conditions of different lung diseases. There still remains unclear the exact role of AQPs in lung diseases, and thus continuous efforts on elucidating the roles of AQPs in lung physiological and pathophysiological processes are warranted.

Keywords

Aquaporins · Lung disorders · Fluid transport

9.1 Introduction

Respiratory system by definition includes respiratory center located in brain stem; respiratory muscle including external and internal intercostal muscle, sternocleidomastoid muscle, and diaphragm; airways including upper airway and lower airway; alveolus and surrounding pulmonary and systemic circulation. Each part has specific function and mainly carries the function of ventilation and oxygenation with coordination of ventilation and pulmonary circulation that provides adequate oxygen delivery to distal organs. However, the lungs also have metabolism, defending, immune, and fluid transport function. The fetus lung is filled with fluid before the fetus is delivered, and the fluid inside of the lungs is absorbed immediately to keep the lungs relatively dry to maintain adequate ventilation and oxygenation after delivery. When the lungs or airways were insulted, it may bring fluid transport disorders, such as airway and lung edema,

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pleural effusion, etc. However, if there is extra fluid absorption, the airway may become relatively dry and induce thick sputum and subsequent airway inflammation. Thus, it is critical to keep fluid balance in alveolus, interstitial space, airway and pleural space to maintain normal respiratory function.

The fluid transport follows few rules: the osmotic fluid transport due to osmotic gradient; the Starling mechanism due to hydrostatic pressure; and the fluid pinocytosis. It has been a long history for the researchers to discover that the cell membrane expresses a water channel aquaporin (AQP) to control fluid transport [1]. Since the first report of AQP1 in red blood cells, there were numerous publications addressing expression and function of AQPs in various organs including respiratory system. So far, there are four AQPs expressed in the lungs, including AQP1 in the vascular endothelium and pleural membrane, AQP3 in epithelium of large airway, AQP4 in epithelium of small airways, and AQP5 in alveolar type I cells and submucosal glands. In this chapter, the expression of above mentioned AQPs in normal and disease status, and the experience of AQPs function from various model and transgenic mice were summarized in detail to improve our understanding of the role of AQPs in fluid balance of respiratory system.

9.2 Expression of AQPs in Lungs and Airways

There are four AQPs expressed in the lungs including AQP1, AQP3, AQP4, and AQP5. AQP1 is expressed in the endothelium of pulmonary capillary, vein, and artery [2, 3], the apical and basolateral membrane of the microvascular endothelium within pleural membrane, including inner and outer membrane [4]. AQP3 is located in the basolateral membrane of basal cells of the tracheal epithelium and in submucosal gland cell membranes in rodents and in apical membrane of bronchioles and type II alveolar epithelial cells (ACEs) of adult humans, while AQP4 is expressed in the basolateral membrane of columnar cells in the bronchi and trachea of rats and in type I AECs in humans [5–8]. AQP5 is expressed in apical membrane of type I ACEs, as well as apical membrane of serous cells of upper airway submucosal glands, it has also been detected in type II AECs in mice [8, 9]. Some studies show AQP5 is also expressed at apical membrane of ACEs [10].

Levels of AQPs expression depend on timing of lung development and pathological conditions. There is a dramatic difference of AQPs expression in airway and alveolar epithelium before and after birth delivery. The underlying mechanism might be the accommodation of fluid transport because airway epithelium and alveolar epithelium play an important role in fetal lung fluid secretion before delivery and turn to absorption function after delivery to clear lung fluid for oxygenation. Most of the studies about AQPs in fetal lungs are derived from animal experiments. Fetal sheep have been used as an important animal model for lung developmental studies, particularly of factors regulating the physiological development of the fetal lung [11, 12]. Sheep fetal lungs express AQP1, AQP3, AQP4, or AQP5 in mRNA and protein levels during midterm gestation [13]. Rat fetal lungs express very little AQPs before birth, and only AQP1 and AQP4 in rats has been detected at present before birth [14–16]. Although AQP1 expression in mRNA and protein levels in the lungs of fetal and neonatal rats is increased when treated with syn- thetic glucocorticoids [7, 15], little is known about the physiological factors to control its expression before birth. Besides, Ya sui et al. [15] found that AQP4 could be induced to increase by corticosteroids and β-adrenergic agents. However, AQP5 mRNA expression in very low level was detected before birth in mice [13].

The deletion of one or more AQP genes in the studies of mice suggested that AQPs are not essential for neonatal survival [17]. However, what is true in mice may not be true for all species, including humans [18]. Because the expression and distributions of different AQPs in the lungs vary from the different species, it is difficult to make a consistent conclusion about the physiological role of AQPs in fetal lung

development and the transition to extra-uterine life at birth, especially in the species with long-gestation such as humans.

9.3 Functions of AQPs in Lung Fluid Transport

Besides ventilation and oxygenation, the lungs exert other biological functions such as lung fluid transport, metabolism, cell migration, immune defense, etc. Herein, lung fluid transport refers to the alveolar fluid balance, airway hydration, pleural fluid transport, and submucosal glands secretion.

9.3.1 Alveolar Fluid Balance

Fluid transport between alveolar and capillary endothelium presents with several forms including the osmotic fluid transport, blood–gas barrier disruption induced fluid leakage and hydrostatic fluid transport. AQP1 and AQP5 are mainly expressed at apical membrane of capillary endothelial cells and type I AECs [8, 9, 19] (Fig. 9.1). The location of these two AQPs suggests possible roles in facilitating water transport. As stated before, AQP expression varies during gestation time, 45 min immediately after delivery do not shown difference of lung wet/dry weight ratio between wild-type and AQP1, 4, 5 knockout mice [21], suggesting slow fluid absorption does not require AQP facilitation, plus these AQPs do not have full expression at the time point of experiment. Several studies have showed that knockout AQP1 and AQP5 could significantly reduce osmotic fluid transport [17, 22]. However, deletion of AQP1 or AQP5 did not alter lung edema formation and resolution difference in acute lung injury model [23, 24], in which increased capillary permeability leads to the fluid accumulation in interstitial and alveolar tissue. This might be explained that AQP-mediated fluid transport is slower than fluid transport through enlarged capillary leakage, and fluid transport through cell membrane is little [23, 24]. Similarly, to study the effects of AQP5 on hydrostatic pressure induced lung edema, high pressure infusion plus blockage of outflow from left atrium are designed to mimic left heart failure induced lung edema. Deletion of AQP5 did not affect lung edema induced by high pulmonary pressure infusion [22]. These studies further indicate that AQP1 and AQP5 mainly facilitate osmotic fluid transport through the apical membrane of capillary endothelial cells and AECs, but they may not participate in fluid transport driven by capillary permeability and hydrostatic pressure changes.

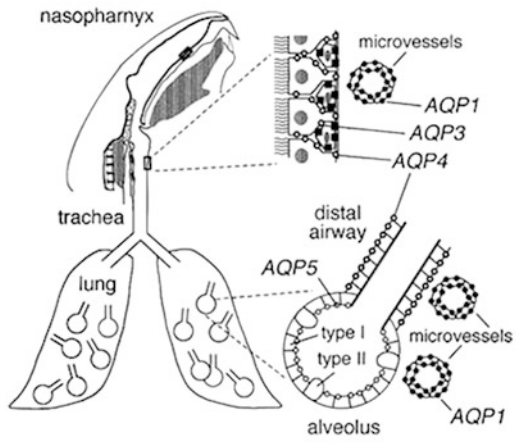

Fig. 9.1 AQP1, AQP3, AQP4, and AQP5 expression in capillary, airway, and alveolar space [20]

Peri-bronchial edema formation was found to decrease in AQP1 mutation patients after bonus saline infusion, for capillary network formation defects after AQP1 mutation, and thus it is unlikely that AQP1 could contribute to hydrostatic pressure induced fluid accumulation [25]. Besides, deletion of AQP4, which is expressed on the epithelium of small airways close to alveolar spaces, does not significantly affect fluid transport compared to wild-type mice. However, AQP4 deletion displays a more decrease in osmotic fluid transport compared with AQP1 knockout in mice, suggesting AQP4 acts as the main role in facilitating fluid transport through small airway epithelium [25]. The potential effect of AQP4 is under covered by AQP1, because function of AQP4 appeared more significant when AQP1 is deleted.

9.3.2 Airway Fluid Balance

Airway must keep high humidity to protect airway epithelial cells that work together with submucosal glands to secret fluid to facilitate cilliary movement to expel inhaled exopathogens. Although AQP3 and AQP4 has been found to be expressed on apical membrane of ciliated epithelial cells [20] (Fig. 9.1) and studies showed that AQPs play minor role in airway humidification, ASL hydration, and isosmolar fluid absorption in AQP3 and AQP4 knockout mice [26]. By calculating fluid transport rate, the fluid movement across airway epithelium challenged by dry air is relatively slower compared to salivary gland secretion where AQP5 facilitates fluid transport. Furthermore, the minor effect of AQP3 and AQP4 in airway physiology suggests slow fluid movement does not rely on water channel necessarily unless it is challenged by osmotic fluid movement [26].

A recent study showed AQP3 deletion reduce airway re-epithelialization [27], the possible role is reduced epithelial cell migration due to water and glycerol transport reduction [28]. The role of AQP3 in airway epithelial growth provide potential role of AQP in tissue repair.

9.3.3 Pleural Fluid Balance

The pleural space plays an important role in pleural fluid secretion and absorption and lubricating visceral and parietals membrane of pleural space to facilitate lung extension. The fluid is filtered through capillary within visceral membrane and reabsorbed by parietal lymphatic duct located on parietal membrane. In some malignancy, these lymphatic ducts can be blocked to result in fluid accumulation within pleural space. AQP1 is expressed at apical membrane of visceral and parietal pleura, and apical membrane of endothelial cell within visceral membrane [4]. Our group found that AQP1 could facilitate the osmotic fluid transport within pleural space, and deletion of AQP1 could significantly reduce osmotic fluid transport. However, AQP1 did not take part in pleural isosmolar fluid clearance [29, 30]. Similarly, there is no relationship of AQP1 with clinically relevant mechanisms of pleural fluid accumulation or clearance [4].

9.3.4 Submucosal Gland Secretion

Submucosal glands are located at upper and lower airway submucosal area, where capillary and nerves are surrounded to keep normal function for gland secretion. In general, when glands are stimulated with nerve or chemical through muscarinic receptors, increased cytosolic cAMP level will activate CFTR function, to induce chloride secretion, and sodium will increase in cell to follow the electronic neutralization through intracellular and paracellular pathway, and then water will come out of the cells following the ionic osmotic gradient mainly through AQP5 water channel. This phenomenon was evidenced in airway submucosal glands and salivary glands [31] (Fig. 9.2). Deletion of AQP5 significantly reduced gland fluid secretion and thus made the secreted fluid more viscous [32]. There are few studies showing that dry mouth due to salivary glands radiation or Sojoren syndrome are associated with abnormal distribution of AQP5 [33, 34], suggesting AQP5 modulation may potentially improve dry moth syndrome through correction of saliva secretion. It is therefore

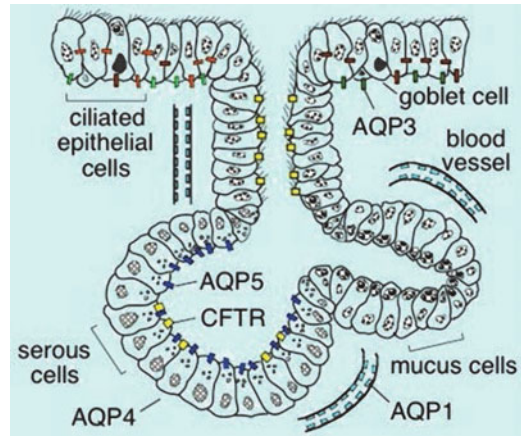

Fig. 9.2 AQP1, AQP3, AQP4, and AQP5 expression in airway submucosal glands [32]

interesting to test whether AQP5 modulation could be useful to promote airway mucus clearance in COPD or bronchiectasis patients.

9.4 AQPs in Respiratory Diseases

9.4.1 Lung Cancer Development

Several studied found that AQP1, AQP3, AQP4, and AQP5 are over-expressed in lung cancer [35–38]. The expression of AQP1 is higher in lung adenocarcinoma (ADCs) and bronchoalveolar carcinoma than that in lung squamous cell carcinoma and normal lung tissue [36]. AQP1 is located in the endothelial cells of capillaries within lung cancer tissue and responsible for tumor angiogenesis [39, 40]. AQP1 is also involved in invasion of lung cancer cells, and reducing AQP1 expression by AQP1-shRNA could inhibit lung cancer cell invasion and migration [40]. Moreover, AQP1 expression is correlated with high postoperative metastasis ratios and low disease-free survival rates in ADCs, especially with micropapillary ADC components [35]. These studies suggest that AQP1 could be a significant prognostic index for stage and histologic differentiation of lung cancer.

AQP3 is over-expressed in non-small cell carcinoma (NSCLC), especially ADCs, well differentiated bronchioloalveolar carcinomas and papillary subtypes. Some studies found that AQP3 might regulate biological functions of lung cancer cells, in the early stage of lung ADC [35], and even involve in angiogenesis of lung cancer through HIF-2α-VEGF pathway and lung cancer cell invasion partly by the AKT-MMPs pathway, mitochondrial ATP formation and cellular glycerol uptake [41]. The anticancer effect of shRNA- targeting AQP3 is confirmed in experimental NSCLC models, and further is confirmed in preclinical studies [41]. Besides, AQP4 wis involved in the invasion of lung cancer cells [40]. Higher transcript and protein levels of AQP4 in well differentiated lung ADCs suggest an association with a better prognosis [37].

The expression of AQP5 was also detected to dramatically increase in lung ADCs and correlated with poor prognosis of patients with NSCLC [42]. AQP5 promoted cell migration and angiogenesis in NSCLC as demonstrated in H1299 cell line [43], and silencing of AQP5 mRNA inhibited the growth in vitro and in vivo for A549 lung cancer cells [44]. AQP5-expressed cells exhibited a loss of epithelial cell markers and activation of c-Src through SH3 binding domain to promote epithelial to mesenchymal transition (EMT) which might be responsible for the promote metastasis of lung cancer [42]. Over-expressed AQP5 could facilitate lung cancer cell growth and invasion through the activation of the EGFR/ ERK/p38 MAPK pathway [42, 45]. The cAMP- protein kinase (PKA) consensus site in AQP5 is also preferentially phosphorylated and promoted cell proliferation ability in tumor. The phosphorylation S156 in PKA consensus site is demonstrated to play an important role in tumor proliferation and invasion [46]. Therefore, S156 in AQP5 may provide a potential therapeutic target by developing small molecules as an inhibitor. Moreover, developing specific monoclonal antibody targeting AQP5 will also be another approach.

9.4.2 Lung Inflammation/Lung Injury

Several studies have shown that both AQP1 and AQP5 are down-regulated after lung injury [23, 24, 47]. Deletion of AQP1 does not show significant phenotype changes while AQP5 deletion shows worsened lung injury after *P. aeruginosa* challenge [21, 24]. The mechanism may be that AQP1 was expressed in pulmonary capillary endothelium cells, and deletion of AQP1 impairs osmotic fluid transport but not near isosmolar fluid transport during capillary leaking due to increased permeability changes. AQP1 mutation in human does not cause morphology changes, but results in retarding fluid accumulation around airways [23]. The underlying mechanism could be a change in capillary networks. It is believed that hydrostatic force could affect

isosmolar fluid transport through water channels. Besides, the worsened lung injury in AQP5 null mice after *P. aeruginosa* challenge could be due to airway surface liquid property changes [24], in which AQP5 deficiency leads to reduced mucin production in lung. Moreover, and declined activation of mitogen-activated protein kinase and nuclear factor-kappa B before and after PA infection.

Considering that AQP 1 and AQP5 are expressed at blood–gas barrier, and both of them facilitate osmotic fluid transport, it has been though that AQP1 and AQP5 may play an important role in acute lung injury, especially in the pulmonary edema. Several studies showed that AQP1 and AQP5 are significantly downregulated after lung injury [23, 24], and deletion of AQP1 does not show any difference of lung edema formation or resolution in LPS induced acute lung injury, suggesting slow fluid transport or fluid leakage from paracellular pathway may not require AQPs for intracellular fluid transport in acute lung injury. Meanwhile, AQPs may facilitate osmotic fluid transport but not near isosmolar fluid movement. Studies also found prolonged mechanical ventilation deregulated AQP1 and AQP5 mRNA expression, and increased lung water. The effect of pulmonary protective ventilation strategy on expression of AQP1 and AQP5 was relatively small [48]. Moreover, studies found that lipoxin A4 and Fasudil, a selective rho kinase (ROCK) inhibitor, could restore the expression of AQP5 to eliminate LPS induced lung edema, suggesting AQP 5 may play protective role in LPS induced ALI [49, 50].

Besides AQP knockout mice, AQPs inhibitor has been implicated in research. TGN-020, a specific AQP4 inhibitor, has been used in an acute lung injury model induced by LPS. Results showed protective effect of AQP4 inhibition and this effect is associated with inhibition of IL-17A [51]. The exact mechanism of AQP 4 and lung inflammation need to be further investigated although AQP4 is associated with neuroimmunological abnormalities on astrocyte in brain [52].

9.4.3 Asthma

Asthma is featured by increased airway constriction, eosinophilic infiltration, hypersecretion of airway mucus, and small airway epithelium edema formation. Immunostaining study shows AQP1 expressed not only in alveolar type I and type II cells, as well as in airway epithelium. AQP1 and AQP5 levels in the sputum of asthma patients were proposed as a diagnostic marker in mild to moderate adult-onset asthma [53]. OVA-induced Asthma animal model shows an increase in expression of AQP1 and AQP5 compared to control group, suggesting AQP1 and AQP5 may participate in airway epithelium edema formation [54]. Bronchial provoke test usually shows hyperactivity and hyperresponsiveness to methacholine [55]. AQP5 knockout mice study shows deletion of AQP5 increased airway reactivity challenged by inhalation of methacholine accompanying with increased airway resistance [10]. It is not clear why deletion of AQP5 decreases airway challenge threshold. Besides, same loci of AQP5 and other asthma gene located at chromosome 12q and mouse chromosome 15 further indicated potential role of AQP5 in asthma development [10].

9.5 Summary

AQPs are water channel proteins supposed to facilitating fluid transport in alveolar space, airway humidification, pleural fluid absorption, submucosal gland secretion, and cell migration. Previous studies suggested the roles of AQPs in various physiology and pathophysiology condition of different lung disease in vivo or vitro. It still remains unclear the exact role of AQPs in lung diseases, and thus continuous efforts on elucidating the roles of AQPs in lung physiological and pathophysiological processes are warranted.

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Aquaporins in Digestive System

10

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Abstract

In this chapter, we mainly discuss the expression and function of aquaporins (AQPs) expressed in digestive system. AQPs are highly conserved transmembrane protein responsible for water transport across cell membranes. AQPs in gastrointestinal tract include four members of aquaporin subfamily: AQP1, AQP4, AQP5, and AQP8, and three members of aquaglyceroporin subfamily: AQP3, AQP7, and AQP10. In the digestive glands, especially the liver, we discuss four members of aquaporin subfamily: AQP1, AQP4, AQP5, and AQP8, three members of aquaglyceroporin subfamily: AQP7, AQP9, and AQP12. In digestive system, the abnormal expression of AQPs is closely related to the occurrence and development of a variety of diseases. AQP1 is involved in saliva secretion and fat digestion and is closely related to gastric cancer and chronic liver disease; AQP3 is involved in the diarrhea and inflammatory bowel disease; AQP4 regulates gastric acid secretion and is associated with the development of gastric cancer; AQP5 is relevant to gastric carcinoma cell proliferation and migration; AQP7 is the major aquaglyceroporin in pancreatic β cells; AQP8 plays a role in pancreatic juice secretion and may be a potential target for the treatment of diarrhea; AQP9 plays considerable role in glycerol metabolism and hepatocellular carcinoma; Studies on the function of AQP10 and AQP12 are still limited. Further studies are necessary for specific locations and functions of AQPs in digestive system.

Keywords

Aquaporins · Digestive system · Gastrointestinal tract · Water electrolyte balance · Glycerol metabolism · Diabetes

10.1 Introduction

Digestive system includes the digestive tract and digestive gland. The digestive tract is composed of oral cavity, pharynx, esophagus, stomach, small intestine, colon, and anus. The digestive glands include large digestive glands and plenty of small digestive glands spreading over the wall

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of the digestive tract. The large digestive glands, such as three-pair salivary glands, pancreas and liver, have secretary portion and ducts formed by gland cells to drain the excreta into the digestive tract. Moreover, pancreas can also perform as an endocrine gland, as A-cells excrete glucagon, B-cells excrete insulin, D-cells excrete somatostatin, and PP-cells excrete pancreatic polypeptide. These endocrine hormones regulate blood glucose and the movement of gastrointestinal tract. The total amount of digestive juice secreted by various digestive glands can reach 6–8 L/day. During a meal, after the primary digestion of saliva, the osmolarities of the food we eat can change rapidly from zero (water) to several hundred million moles (solid meal). In response to the rapid change of the osmolarity in gastrointestinal tract, gastric juice or other kind of digestive fluids will be secreted to balance the osmolarity of gastric content [1]. In addition, the digestive juices secreted by digestive glands provide a suitable pH environment to maintain digestive enzyme activity. Apart from secretion, absorption is an important function for digestive systems, especially for gastrointestinal tract. When the content comes to small intestine, most water will be absorbed with solutes and nutrition. When it comes to colon, the content is further dehydrated and forms feces. Totally, about 7.5 L of fluid is secreted into the tract, which includes saliva, gastric secretions, bile, pancreatic juice, and intestinal secretion, and about 9 L fluid is absorbed each day [1, 2]. Moreover, the liver is related to substance metabolism.

Aquaporins (AQPs) are expressed and play physiological roles in the digestive system [1]. The distributions of AQPs are relevant to their functions. Basolateral water channels AQP3 and AQP4 are more expressed in secretive epithelia (e.g., stomach), whereas apical water channels are more localized in absorbing epithelia (e.g., small intestine). In the colon, which can both absorb and secrete water, both apical and basolateral AQPs are expressed [3]. AQP9, which is an aquaglyceroporin, correlates with the absorption of glycerol and are expressed in the liver, involved in fat metabolism. Here we describe some important isoforms of AQPs in digestive system, and mention others that are not very clearly studied. The general distributions of AQPs in digestive system are summarized in Fig. 10.1 [4–6]. Figure 10.2 presents the possible pathways for transepithelial water transport in digestive system. It mainly consists of paracellular pathway, transcellular pathway, diffusion, and osmolality-dependent AQP pathway. Figure 10.3 presents the biological functions of AQPs in the digestive system. Considering the constant phenotype of specific AQP knockout mice regarding the fluid secretion [7, 8], the function of AQPs in the digestive system might be limited.

10.2 Expression of AQPs in the Gastrointestinal Tract and Digestive Glands

10.2.1 Aquaporin Subfamily

10.2.1.1 AQP1

In gastrointestinal tract, AQP1 is diversely expressed on the endothelial barriers, while there is no expression in the epithelia and mucosa. It is more expressed in the body of the stomach, duodenum, and ascending colon than the pyloric antrum [9]. A moderate amount of AQP1 was also observed in the stromal tissue of the anus, but it is difficult to identify the specific location [10].

In digestive glands, AQP1 is localized to the basolateral membrane of the gallbladder, intrahepatic cholangiocytes, hepatic ducts, labial glands, endothelial barriers in the liver, and pancreatic ducts and centroacinar cells in the pancreas [10, 11]. Moreover, AQP1 is specifically expressed in the intralobular and interlobular ducts, modulating the water transport through the cells [1].

In human tissues, AQP1 was demonstrated on the endothelial cells of the lymphatic vessels in the submucosa and lamina and capillary endothelial cells in the smooth muscle layer throughout the gastrointestinal tract. For other species,

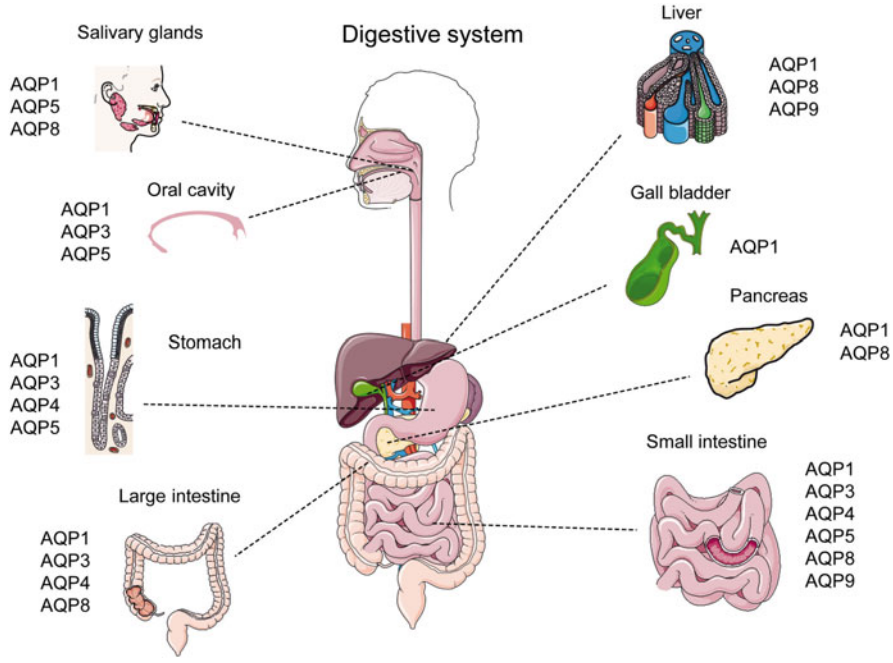

Fig. 10.1 Distribution of aquaporins in the digestive system. AQP1, AQP5, and AQP8 are expressed in salivary glands. AQP1, AQP3, and AQP5 are present in oral cavity. In the stomach, AQP1 is expressed in the endothelial cells of capillaries and small vessels; AQP3 is expressed in the basolateral membrane of surface mucous cells; AQP4 is expressed in the basolateral membrane of parietal cells, and AQP5 is present at the apical membrane of parietal cells. Small intestine expresses AQP1, AQP3, AQP4, AQP5, AQP8, and AQP9. AQP1, AQP3, AQP4, and AQP8 are expressed in large intestine. AQP1, AQP8, and AQP9 are expressed in the liver. AQP1 is diversely expressed in gallbladder, bile duct, and pancreas, while AQP8 is present in the pancreas as well

abundant expression of AQP1 was detected in endothelium of capillaries and small vessels in digestive system [12–16].

10.2.1.2 AQP4

AQP4 is selectively expressed in the basolateral membrane of parietal cells of the stomach, especially at the base of gastric pits, which is also expressed in the basolateral membrane of the crypt cells located at the bottom of the crypt in small intestine and the basolateral membrane of surface epithelial cells in the colon.

The salivary glands are involved in the secretion of saliva. For a long time, the presence of AQP4 in the glands remained controversial. Sabrina Lisi's group confirmed the presence of AQP4 in human salivary glands through immunohistochemistry, high-resolution confocal microscopy, quantitative image analysis, Western blot, and real-time RT-PCR [17].

10.2.1.3 AQP5

AQP5 is typically expressed in glandular tissues, which include salivary glands, lacrimal glands, and pancreas. In addition, the expression of AQP5 was shown by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis in the liver [2]. In salivary gland, AQP5 is present at the apical membrane, including the intercellular secretory canaliculi of acinar cells [1]. In pancreas, AQP5 is located at the apical membrane of centroacinar and intercalated ductal cells [18].

In digestive tract, it is present in the stomach and duodenum in rat. For stomach, it is expressed in apical membrane of secretory cells of the pyloric gland, and there is almost no expression in the

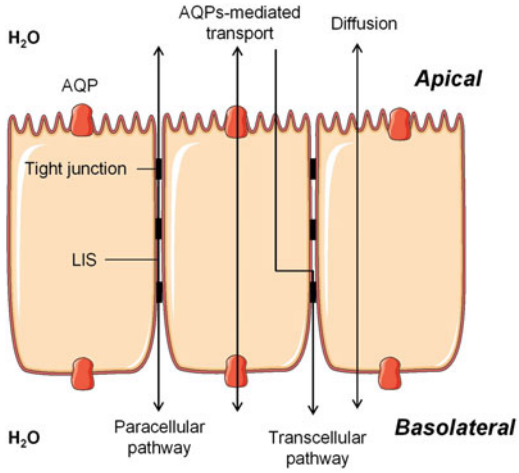

Fig. 10.2 Possible pathways for transepithelial water transport in the digestive system. There are four pathways for transepithelial water transport. Water can traverse through the cell by paracellular pathway via tight junctions; it can also diffuse through the apical/basolateral membranes. These two pathways are bidirectional. AQP-mediated pathway is also available. In absorptive epithelia, the osmolality is higher in lateral intercellular space (LIS) than in the cell. Therefore, water first transports into the cell, then to blood via LIS, which is also a possible method for water transport

fundic gland. In the duodenum, AQP5 is present along the apical membrane of secretory cells in duodenal gland [19]. AQP5 is not detected in other tissues of digestive system by immunohistochemistry.

10.2.1.4 AQP8

AQP8 transcript is widely expressed in the digestive system, including the salivary glands, small intestine, colon, pancreas, and liver. In digestive tract, it is mainly present at the subapical intracellular sites of epithelial cells in the duodenum, jejunum, and colon [1, 20, 21]. Studies have found that AQP8 is also expressed on the human gallbladder mucosa [22]. For digestive glands, AQP8 is mainly expressed in parotid, salivary glands, liver, and pancreas. AQP8 is present in myoepithelial cells around the acini and the intercalated duct rather than the acinar or ductal cells of rat parotid, submandibular, and sublingual cells [23].

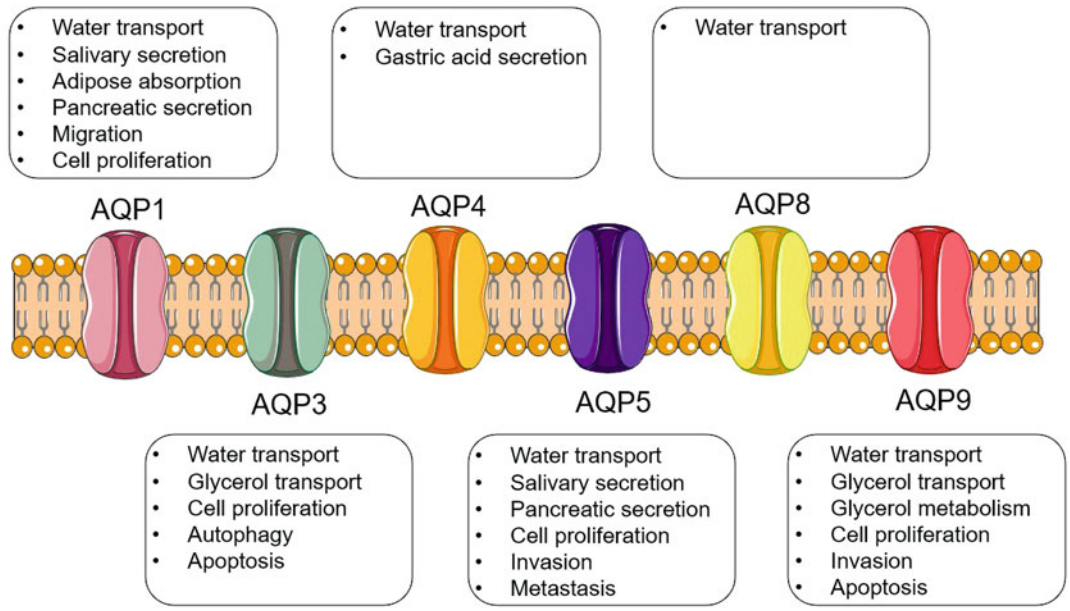

Fig. 10.3 Biological functions of AQPs in the digestive system

10.2.2 Aquaglyceroporin Subfamily

10.2.2.1 AQP3

In digestive system, AQP3 is highly expressed in the esophagus, proximal, and distal colon in particular [21]. According to immunohistochemical results from rat digestive tract, AQP3 is also present in the oral cavity, forestomach, and anus, where AQP3 situates at the basolateral membrane.

10.2.2.2 AQP7

In human gastrointestinal tract, AQP7 is detected on the superficial epithelial cells throughout the small intestine and colon [24]. For rats, it is present on the apical region of the enterocytes in the villi; epithelial cells of the colon and caecum suggests its involvement in rapid fluid movement through the villus epithelium.

In addition, AQP7 is the major aquaglyceroporin in pancreatic β cells, which is a regulator of glycerol kinase activity, β-cell mass, and insulin production and secretion [25].

10.2.2.3 AQP9

AQP9 is expressed on the basolateral membrane of mouse ileal goblet cells. In the human digestive system, AQP9 is distributed on the cytoplasmic surface of small intestine and liver [26].

10.2.2.4 AQP10

It has been reported that AQP10 mRNA has been detected in the pyloric antrum of the human stomach, but the protein expression and specific location has not yet been determined [1]. In human intestine, AQP10 was found to be expressed on the apical membrane of epithelial cells, which was expressed higher in duodenum and jejunum than in ileum [26–29]. Moreover, AQP10 performs as a pseudogene in some kinds of species [1].

10.2.3 Superaquaporin Subfamily

10.2.3.1 AQP12

AQP12 is selectively expressed in the pancreas, especially in acinar cells, and it is proved to be expressed inside the cell. Previous research with double or even triple knockout mice for AQP1, AQP8, or AQP12 were not observed evident abnormalities [30].

10.3 Functions of AQPs in the Digestive System

10.3.1 Fat Digestion

AQP1 is present in endothelial cells of central lacteals in the villi of small intestine, which produces chylomicrons when digesting food. Therefore, AQP1 might be involved in the fat digestion process. AQP1 null mice showed a defect of dietary fat [13], which indicates that the invalidation or downregulation of AQP1 is responsible for malabsorption [1].

10.3.2 Digestive Fluid Secretion

AQP4 is acknowledged to play the main role in modulating the secretion of the acid. AQP4 null mice were applied to find the role of AQP4 in gastric acid secretion by Verkman's group [7]. There is no apparent difference in morphology in the parietal cells within the gastric pits for AQP4 null mice. And the deficiency of AQP4 shows no difference to the rates of basal or stimulated acid or gastric fluid secretion. Nor did it affect the pH level and fasting serum gastrin concentration in the stomach. These data suggest that AQP4 has little influence on gastric acid production [8].

In the AQP5 knockout mice, when compared to wild-type mice, the saliva production was reduced and was hypertonic, which shows that AQP5 plays a main role in saliva secretion [1]. In Sjögren's syndrome models, AQP1 expression was increased and AQP5 expression was decreased, suggesting new pathways to explain the disease [31].

In salivary glands, strong transcripts were detected while AQP8 could not be found at protein level by immunofluorescence or immunoblot analysis. Furthermore, salivary secretion was not affected by AQP8 deficiency, according to the comparison about the phenotypes between

AQP8 knockout mice and wild-type mice, nor was it affected in the comparison of AQP8/AQP5 double knockout mice and AQP5 knockout mice.

10.3.3 Water Absorption

It is suggested that AQP4 is involved in colonic fluid transport. However, in AQP4 null mice, the water permeability was decreased in the proximal colon but not the distal colon, while the water content of the feces has no difference compared to wild-type mice. All in all, AQP4 in surface epithelial cells has no influence on feces dehydration and colonic fluid secretion [1].

In small intestine, AQP8 knockout model made no difference in cholera toxin- or agonist-stimulated maximal fluid secretion. In colon, AQP8 knockout model had little effect on the colonic fluid absorption or fecal dehydration. And water content in stool changed little in AQP8 knockout mice. Only mild phenotype differences between the wild-type and AQP8 knockout mice were found. And the function of AQP8 in the water absorption and secretion of small intestine and colon is limited [32].

Studies have shown that AQP3 plays an important role in colonic water absorption. In human colon, AQP3 is predominantly expressed in the mucosal epithelial cells [33, 34], which indicates its important role in water transport. It is reported that the inhibition of AQP3 in the colon leads to diarrhea. AQP3 inhibitor ($HgCl_2$ and $CuSO_4$) applied for more than 1 h, the fecal water content increased to approximately four times that in the control group. And severe diarrhea was observed [35, 36]. The stimulant laxative, such as bisacodyl, works by promoting the peristaltic movements of the bowel. When it was applied to rats, AQP3 expression was found downregulated, and severe diarrhea was observed without osmotic pressure changes. However, several laxatives present a laxative effect by the upregulation of AQP3 expression. For osmotic laxatives such as magnesium sulfate, previously thought to work by increasing the osmotic pressure in the intestinal tract, AQP3 expression was found upregulated, suggesting that osmotic laxative might play its role in response to the increased AQP3 expression. Contrary to diarrhea, AQP3 expression is also involved in the constipation. Morphine is clinically used as a narcotic analgesic with usual adverse effect of constipation, which is caused by the decrease of peristaltic movements of the bowel. In this model, AQP3 expression is upregulated, which might take part in the constipation. Generally speaking, deeper investigation for the mechanism of AQP3-involved water transport may provide candidates for new laxatives and antidiarrheal drugs in the future [33].

10.3.4 Intestinal Barrier

AQP3 is involved in regulating the integrity of the intestinal barrier [34]. Looking into the AQP3 null mice, the intestinal barrier integrity was impaired based on previous work [37]. The results show that the AQP3 deletion induces a dramatic increase in *E. coli* C25 translocation, which exists in the colon and its translocations is relevant to the impairment of intestinal barrier, and the reduction of claudin-1 and occludin expression, which are compositions of the tight junction, indicating that it might open the tight junction complex of paracellular pathway and enhance paracellular permeability in the process.

10.3.5 Insulin Secretion

AQP7 is the major aquaglyceroporin in pancreatic β cells, which is a regulator of glycerol kinase activity, β-cell mass, and insulin production and secretion [25]. Studies have found that AQP7 knockout mice exhibit adult-onset obesity, impaired insulin secretion, and insulin resistance compared with control mice. However, loss of AQP7 function in humans has not been associated with obesity or type 2 diabetes [38]. A recent study found that metformin can promote the influx of glycerol into pancreatic β cells by

inhibiting the MAPK signaling and upregulating the expression of AQP7 in pancreatic β cells, ultimately promoting the secretion of insulin in type 2 diabetes [39].

10.3.6 Glycerol Metabolism

AQP9 is also associated with glycerol metabolism in liver. Glycerol, as a product from adipose triglycerides during lipolysis, flows into the liver through the portal vein. And it takes part in gluconeogenesis later. AQP9 is verified as the only glycerol channel in the liver, which selectively localizes at the sinusoidal plasma membrane facing the portal vein. Thus, AQP9 is considered to be the channel for glycerol uptake in the liver [40–44].

AQP9 knockout model was constructed to study its role in glycerol metabolism. The results revealed that AQP9 null mice had evident hyperglycerolemia and hypertriglyceridemia compared to AQP9 heterozygous mice. When AQP9 null mice crossed with $Lepr^{db}$/$Lepr^{db}$ mice, a model of obese and type 2 diabetes, it showed that $Lepr^{db}$/$Lepr^{db}$ AQP9 null mice had lower blood glucose levels than $Lepr^{db}$/$Lepr^{db}$ AQP9 heterozygous mice. AQP9 null mice had lower plasma glycerol levels than AQP9 heterozygous mice. These results suggest the possible role of AQP9 in the hepatic glycerol absorption as well as glucose metabolism [40].

10.4 AQPs and Digestive Diseases

10.4.1 Cancer

Studies have shown that biological behavior of cancer cells depends on the transport of water molecules across the membrane. AQP1, which is widely distributed in the gastrointestinal tract, may play an important role in the development of gastric cancer [45]. Previous studies have found that the mRNA and protein levels of AQP1 in the tumor tissues of patients with epithelial gastric neoplasms are upregulated and are associated with high recurrence rates, suggesting that AQP1 may be a potential prognostic biomarker for gastric cancer [46].

AQP5 could promote the rapid transmembrane water transport and the progression and invasion of several cancers [47]. It is upregulated in a variety of cancers and associated with the clinicopathological characteristics of patients, which include colon cancer, lung cancer, chronic myelogenous leukemia, breast cancer, and biliary tract carcinoma. In gastric carcinoma, AQP5 is relevant to the tumorigenesis and progression, such as differentiation, lymph node metastasis, and lymphovascular invasion [3, 47], which shows that AQP5 may be a potential therapeutic target for cancer.

Studies have found that AQP5 is highly expressed in hepatocellular carcinoma cell lines. Downregulation of AQP5 could suppress tumor metastasis and epithelial-mesenchymal transition (EMT) process by inhibiting NF-κB signaling pathway. This suggests that AQP5 may serve as a potential therapeutic target for hepatocellular carcinoma.

AQP3 is also involved in the development of gastrointestinal tumors. Studies have found that when AQP3 is knocked down, the proliferation reduces and proliferation elevates in gastric cancer cells [48].

AQP9 has been proved to be the major routes of arsenite uptake into the mammalian cells, whose accumulation might result in hepatocellular damage and hepatocellular carcinoma. A recent study found that AQP9 mRNA level was significantly reduced in hepatocellular carcinoma tissues, which was positively correlated with the survival rate of HCC patients. Overexpression of AQP9 can inhibit the proliferation, invasion, and migration of HCC cells through the Wnt/β-catenin pathway and inhibit tumor growth in vivo. Another study showed that AQP9 inhibits the expression of hypoxia-inducible factor 1α in a hypoxic tumor environment, which suppresses the invasion of hepatocellular carcinoma [49].

10.4.2 Sjogren's Syndrome

Studies demonstrated that the abnormal expression or lack of AQP1 can affect the transportation of water, leading to the occurrence of digestive system diseases. The AQP1 expression in the labial glands of patients with Sjogren's syndrome was downregulated and the expression level of AQP1 in epithelial cells increased after rituximab treatment, suggesting that AQP1 may be involved in the secretion of saliva [11].

Studies found that the expression level of AQP4 was downregulated in myoepithelial cells in the salivary glands of patients with primary Sjögren's syndrome, and the water permeability in myoepithelial cells was changed, suggesting that AQP4 may be used as a new target for the treatment of xerostomia [17].

10.4.3 Pancreatitis

A previous study showed that AQP1 expression is upregulated in the apical and lateral pancreatic duct membranes of patients with autoimmune pancreatitis. This phenomenon may be due to the compensatory upregulation of AQP1 stimulated by reduced pancreatic secretion.

In pancreas, AQP5 is located at the apical membrane of centroacinar and intercalated ductal cells [18]. AQP5 might be involved in the diabetes and pancreatitis.

In the model of acute pancreatitis induced by the caerulein, the pathological damage of pancreas in AQP12 knockout mice was more severe than that in WT mice [50]. Further research is needed in its function studies.

10.4.4 Liver Disease

When AQP1 knockout mice undergo bile duct ligation, it leads to a decrease in angiogenesis and fibrosis and less portal hypertension, indicating that AQP1 may play an important role in the development of chronic liver disease [51].

10.4.5 Inflammatory Bowel Disease (IBD)

Studies have shown that the lack of AQP4, which is expressed on the basolateral membrane of colonic epithelial cells, can alleviate experimental colitis in mice induced by dextran sodium sulfate. This result suggests that blocking AQP4 may be a novel therapeutic approach for ulcerative colitis [52].

In 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis model, which mimics human Crohn's disease, AQP8 expression is downregulated with the increase of inflammation and injury [53], indicating that AQP8 is possibly involved in inflammatory bowel disease. A recent study found low AQP8 expression in intestinal epithelial cells of patients with collagenous colitis, which is strongly associated with higher stool frequency in patients with collagenous colitis. It is suggested that AQP8 may be a potential target for the treatment of diarrhea [54].

Previous studies have shown that the expression of AQP3 is also closely related to the development of intestinal diseases. In TNBS-induced colitis, AQP3 expression is downregulated in accordance with AQP8, accompanied with intestinal inflammation and injury. After small bowel resection and improvement of intestinal functions in IBD rats, AQP3 is upregulated during the adaptation [33]. The evidence indicates that AQP3 might involve in the pathogenesis of inflammatory bowel disease [21, 53].

10.5 Conclusion

AQPs is widely expressed in the digestive tracts and glands. AQP1 is involved in saliva secretion and fat digestion and is closely related to gastric cancer and chronic liver disease. AQP3 is involved in diarrhea, constipation, and inflammatory bowel disease. AQP4 regulates gastric acid secretion and is associated with the development of gastric cancer, while its deletion can alleviate experimental colitis in mice induced by dextran sodium sulfate. AQP5 can promote the secretion

of saliva and the progression and invasion of several cancers. AQP7 is the major aquaglyceroporin in pancreatic β cells, which is a regulator of glycerol kinase activity, β-cell mass, and insulin production and secretion. AQP8 plays a role in pancreatic juice secretion and may be a potential target for the treatment of diarrhea. AQP9 plays considerable role in glycerol metabolism, urea transport, and hepatocellular carcinoma. Studies on the function of AQP10 and AQP12 are still limited. Further investigation is necessary for specific locations and functions of AQPs in digestive system.

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Aquaporins in Urinary System

11

Zhiwei Qiu, Tao Jiang, Yingjie Li, Weiling Wang, and Baoxue Yang

Abstract

There are at least eight aquaporins (AQPs) expressed in the kidney. Including AQP1 expressed in proximal tubules, thin descending limb of Henle and vasa recta; AQP2, AQP3, AQP4, AQP5, and AQP6 expressed in collecting ducts; AQP7 expressed in proximal tubules; AQP8 expressed in proximal tubules and collecting ducts; and AQP11 expressed in the endoplasmic reticulum of proximal tubular epithelial cells. Over years, researchers have constructed different AQP knockout mice and explored the effect of AQP knockout on kidney function. Thus, the roles of AQPs in renal physiology are revealed, providing very useful information for addressing fundamental questions about transepithelial water transport and the mechanism of near isoosmolar fluid reabsorption. This chapter introduces the localization and function of AQPs in the kidney and their roles in different kidney diseases to reveal the prospects of AQPs in further basic and clinical studies.

Keywords

Water · Urine concentrating mechanism · Polyuria · Knockout mouse · NDI

11.1 Introduction

The urinary system includes the kidneys, ureters, bladder, and urethra. The upper urinary tract is composed of kidneys, while the lower urinary tract is composed of other structures [1]. Urine formed in the kidneys flows into the renal pelvis, ureters, and bladder. Finally, the urine stored in the bladder is expelled through the urethra. Throughout the urinary system, the kidney is the central organ that reabsorbs water and concentrates urine.

The kidneys concentrate and dilute urine by regulating water excretion and reabsorption. The water permeability of the proximal tubules, descending limbs of Henle, late distal tubules, and collecting ducts are important during water excretion and reabsorption. Conversely, those persistently impermeable segments such as ascending limbs of Henle are also required to establish osmotic gradients from the renal cortex to the inner medulla. Antidiuretic hormone

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(ADH) alters the water permeability of late distal tubules and collecting ducts, which modulates urine concentration.

During urine concentration and dilution, the water permeability of certain segments of renal tubules, collecting ducts, and vasa recta are mediated by aquaporins (AQPs), the water channels in the plasma membrane of epithelial and endothelial cells.

11.2 Expression and Localization of AQPs in Urinary System

Kidney expresses at least nine AQPs, including AQP1~8 and AQP 11. AQP1 is expressed in the proximal tubule, thin descending limb of Henle, and vasa recta, AQP2~6 are expressed in the collecting duct, AQP7 is expressed in the proximal tubule, AQP8 is expressed in the proximal tubule and collecting duct, and AQP11 is expressed in the endoplasmic reticulum of proximal tubule cells (Fig. 11.1) [2–6].

Interestingly, studies reported that AQP2 and AQP3 could transport water and solutes in urothelium, although mammalian urothelium is generally regarded as a urine component-impermeable barrier [7, 8].

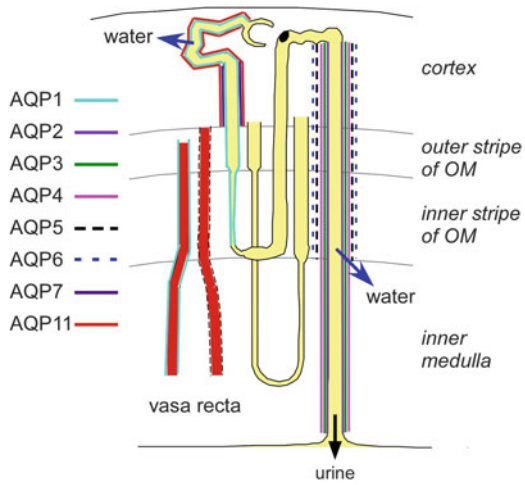

Fig. 11.1 Expression of AQPs in kidney. AQP1 in the proximal tubule, thin descending limb of Henle, and vasa recta; AQP2-6 in the collecting duct; AQP7 in the proximal tubule; AQP8 in the proximal tubule and collecting duct and AQP11 in the endoplasmic reticulum of proximal tubule

11.2.1 AQP1

AQP1 is expressed at the apical and basolateral plasma membranes of the proximal tubule and the thin descending limb of Henle (TDLH), and the microvascular endothelium of the descending vasa recta (DVR) of medulla [9, 10]. In details, AQP1 is localized in the apical and basolateral membranes of epithelial cells from S1 (except for the earliest part of S1) to S3 [11, 12]. In ureter and bladder, AQP1 is also localized in capillary endothelial cells and arteriole endothelial cells [7, 13].

11.2.2 AQP2

AQP2 is expressed at the principal cells of collecting duct, and localized from the connecting tubule through the papillary duct. In detail, AQP2 traffics between the intracellular vesicular compartment and the apical plasma membrane of the cell, which is regulated by vasopressin [14–16]. In ureter and bladder, AQP2 is circumferentially localized in the epithelial cell membranes (except for the apical membrane of the epithelial cells adjacent to the lumens) [17].

11.2.3 AQP3

AQP3 is expressed at the basolateral membrane of collecting duct epithelium in cortex and outer medulla [18]. In the basal and intermediate layers of urothelium, AQP3 is also expressed intensely at cell borders [17, 19].

11.2.4 AQP4

AQP4 is expressed at the basolateral membrane of epithelial cell and is localized in the epithelium of inner medullary collecting duct and the S3 region of proximal tubule [20–23]. The rat AQP4 expressed by Chinese hamster ovary

(CHO) cells will form orthogonal arrays of particles (OAPs) in basolateral membrane, which could be found by freeze-fracture electron microscopy (Fig. 11.2) [23, 24].

11.2.5 AQP5

AQP5 is expressed at the apical membrane of type B intercalated cells in collecting duct [25].

11.2.6 AQP6

AQP6 is expressed primarily at the membrane of the intracellular vesicles of type A intercalated cells and is localized in the collecting duct [26]. And some AQP6 is colocalized with H^+-ATPase [27].

11.2.7 AQP7

AQP7 is expressed at the apical membrane of epithelial cell in the S3 segment of proximal straight tubules [28, 29].

11.2.8 AQP8

AQP8 is expressed in the apical, central, and basal cytoplasmic domains of epithelial cell in proximal tubule and collecting ducts [30].

11.2.9 AQP9

AQP9 is only found in the urinary concentration system of birds [31].

11.2.10 AQP11

As an unorthodox AQP, AQP11 is expressed at the endoplasmic reticulum (ER) [32, 33] of epithelial cells in the proximal tubules [34].

11.3 Functions of AQPs in Urinary System

11.3.1 AQP1

As early as 1998, Verkman's group constructed AQP1 knockout mice. Except for mild growth retardation, there was no significant difference between AQP1 knockout mice and wild-type mice in survival, gross physical appearance, and organ morphology [35–39].

Further, Verkman's group investigated the effect of AQP1 knockout on water permeability of proximal tubule. They used a raffinose gradient to drive water out of the tubule lumen and measured transepithelial osmotic water permeability (P_f) in isolated microperfused S2 segments of proximal tubule. Results showed that the P_f value of AQP1 knockout mice was nearly five-fold lower than that of wild-type mice. It suggested that in the perfused S2 segment of the proximal tubule, water transport was primarily transcellular, and this process was mediated by AQP1.

Stop-flow measurements also showed that the purified apical plasma membrane vesicles isolated from proximal tubules of AQP1 knockout mice had nine-fold lower P_f values at 10 °C than that of wild-type mice. Moreover, this low water permeability in vesicles of AQP1 mice was not affected by mercurial agents. AQP1 knockout mice had a P_f value of 0.033 cm/s, and if assuming a folding factor of about 10 to account for membrane redundancy in the proximal tubule, and equal apical and basolateral membrane water permeability, then at 37 °C, the intrinsic membrane P_f was about 0.006 cm/s. Such low water permeability was almost identical to that of the lipid portion of the membrane. Thus, these findings suggested that the water permeability of the proximal tubule was mainly mediated by AQP1 and that other aquaporins or non-aquaporin transporters represented little influence on the water permeability of this tubular segment. In addition, it also indicated that osmotically driven paracellular transepithelial water transport in proximal tubule was less than 20%.

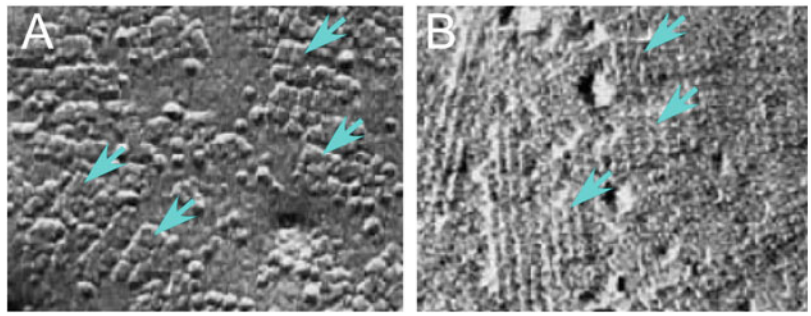

Fig. 11.2 Freeze-fracture electron micrographs of collecting duct principal cell basolateral plasma membranes. (**a**) OAPs in the basolateral membrane P-face of a collecting duct principal cell. (**b**) E-face of the basolateral plasma membrane of a collecting duct principal cell showing the appearance of imprints left in this membrane leaflet by the P-face OAP arrays (Data cited from [24])

The urinary flow rate was significantly increased in AQP1 knockout mice despite normal distal delivery, suggesting that AQP1 knockout induced diuresis was primarily due to decreased fluid absorption in the collecting ducts. Since AQP1 is highly expressed in TDLH and DVR under normal physiological condition, AQP1 knockout may result in a defective countercurrent mechanism that prevented the formation of a hyperosmolar medullary interstitium. Consistent with this speculation, it is found that in water-deprived AQP1 knockout mice, dDAVP stimulation made urinary and medullary interstitial osmolality nearly equal, but not increased urine osmolality [40]. However, unlike nephrogenic diabetes insipidus (NDI), AQP1 knockout mice did not experience sharp drop in urine osmolarity that NDI mice did, although it had significantly increased urine output. AQP1 knockout mice had slightly concentrated urine due to functional salt transporters and water-permeable collecting ducts.

To directly explore the effect of AQP1 on water transepithelial transport in TDLH, Chou et al. compared the P_f values of isolated perfused segments of TDLH from wild-type and AQP1 knockout mice. Results showed that P_f values were significantly reduced in AQP1 knockout mice (wild-type mice, 0.26 cm/s; AQP1 knockout mice, 0.031 cm/s) [41]. Indicating that AQP1 is the principal water channel in the TDLH, and the osmotic equilibration along the TDLH maintained by AQP1-mediated water transport plays a key role in the renal countercurrent concentration mechanism.

How much water reabsorption and solute entry contribute to the osmotic equilibrium along the TDLH has been debated [42]. Loss of AQP1 resulted in a severe defect in urine concentration capacity and reduced TDLH water permeability, suggesting that high water permeability in TDLH is required for urine concentration. Although it has not been demonstrated whether TDLH are permeable to NaCl and urea, they are unlikely to be affected by AQP1 expression levels, as AQP1 has been shown to be impermeable to NaCl or urea. Thus, these findings suggested that osmotic water transported out of the TDLH lumen was important for the countercurrent proliferation mechanism and that solute entry is not sufficient to form the maximum concentration of urine.

To further determine whether AQP1 is necessary for urine concentration, Verkman's group conducted a 36-h water deprivation experiment. The results showed that wild-type mice lost 20–22% of body weight with mean serum osmolality of 311–325 mosm/kg H_2O after water deprivation. The average urine osmolality in wild-type mice increased from 1400 mosm/kg H_2O to 3000 mosm/kg H_2O after water deprivation. But it remained active, which was no different from that before water deprivation. In contrast, AQP1 knockout mice lost 35% of their body weight with mean serum osmolality of

517 mosm/kg H_2O after water deprivation. The osmolality gradient cannot be established without AQP1 (Fig. 11.3); therefore, the urine osmolarity of AQP1 knockout mice was very low (only 580–610 mosm/kg H_2O) and did not increase after water deprivation. Besides, four out of five AQP1 knockout mice had low concentrations of sodium (less than 10 mM) in their urine. AQP1 knockout mice were unresponsive and became lethargic after water deprivation. Intraperitoneal injection of V2 receptor agonist dDAVP did not restore the urine osmolality of AQP1 knockout mice, indicating that the decrease in urine osmolality induced by AQP1 dysfunction was related to kidney but not central osmoreceptor sensing.

In summary, the inability of AQP1 knockout mice to form concentrated urine may be caused by defects in synergism. After AQP1 knockout, the permeability of TDLH and DVR is reduced and the proximal tubules are unable to reabsorb water, leading to fluid overload in the distal nephron and damage to the medullary countercurrent multiplication mechanism [43, 44].

11.3.2 AQP2

AQP2 is localized mainly in intracellular vesicles of collecting duct epithelial cells in the basal state. Upon stimulation with ADH, AQP2 is translocated from the intracellular compartment to the apical plasma membrane by exocytic fusion of AQP2-bearing vesicles [45, 46]. The transport of AQP2 to the apical membrane can regulate the water permeability of apical membrane [47–50]. AQP3 and AQP4 are expressed in the basolateral membrane of principal cells. Therefore, once AQP2 is transported to and appears on the apical membrane, water will be reabsorbed easily. Since mutations in AQP2 lead to NDI, AQP2 may be important in urine concentration [51, 52].

Rojek et al. generated a gene-edited mouse with collecting duct-specific knockout of AQP2 while junctional tubules expressing AQP2. These mice were found to have a severe defect in urine concentration ability. In contrast, globally knockout of AQP2 caused severe dehydration, and these mice will die within 2 weeks [53]. These results suggest that AQP2 plays important roles in the kidney.

In normal conditions, AQP2 is stored in intracellular vesicles under water-saturated conditions. However, when dehydration or hypernatremia occur, the secretion of the pituitary antidiuretic hormone arginine vasopressin (AVP) by the pituitary increases. AVP binds to vasopressin V2 receptor in the basolateral membrane, thereby activating the cAMP-PKA signaling pathway [16, 54–56]. This process triggers phosphorylation at serine 256 of the C-terminal AQP2 protein, which promotes AQP2 trafficking to the apical membrane [57–59].

Phosphorylation at serine 256 of AQP2 promotes its transport to the apical membrane upon AVP stimulation [60, 61]. But the localization of AQP2 is also affected by phosphorylation at serine 261, 264, and 269 [62–65].

Phosphoproteomics analysis also showed that AQP2 was phosphorylated at serine 261 in rat inner medullary collecting duct epithelial cells [66, 67]. In addition to inducing monophosphorylation at serine 256, ADH also induced double diphosphorylation at serine 256 and serine 261. These findings indicated that phosphorylation of both sites was involved in AQP2 trafficking [68]. Interestingly, AQP2 phosphorylated at serine 261 was mainly localized intracellularly and distinct from the endoplasmic reticulum, Golgi apparatus, and lysosomes [69]. Phosphorylation at serine 261 does not affect AQP2 regulation and constitutive trafficking [70].

AQP2 also undergoes constitutive recycling, which is independent of vasopressin. Li et al. reported that activation of bile acid receptor Takda G protein-coupled receptor 5 (TGR5) promoted the expression of AQP2 by upregulating PKA signaling pathway, which reversed the defection of urinary concentration in NDI mouse model [71]. Luo et al. reported that hydrogen sulfide (H_2S) activated the cAMP-PKA signaling pathways, which inhibited the downregulation of AQP2 and polyuria in lithium-induced NDI mouse model [72]. Lei et al. identified $MnCl_2$ as a potent AQP2 trafficking regulator. In LLC-PK1

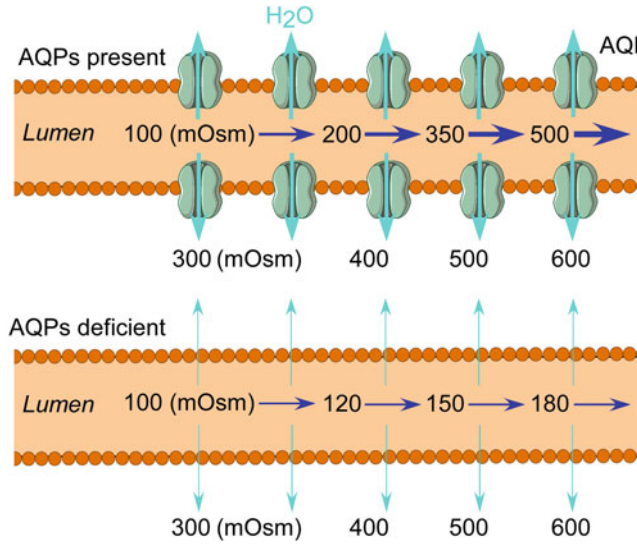

Fig. 11.3 Osmolality gradient inside or outside the lumen with or without AQP. Top, the osmolality gradient in the lumen was established by water reabsorbing via AQP. Bottom, the osmolality gradient could not be established without AQP

cells, $MnCl_2$ promoted the internalization and intracellular accumulation of AQP2 without affecting its phosphorylation. But $MnCl_2$ inhibited vasopressin-induced inhibition of AQP2 phosphorylation at serine-256, -264, and -269 residues and dephosphorylation at serine-261. $MnCl_2$ promoted the polymerization of F-actin and downregulation of RhoA, which inhibited membrane accumulation of AQP2. In C57BL/6 mice, $MnCl_2$ treatment caused polyuria and urinary concentration reduction, which could not be corrected by vasopressin [73].

In addition to AVP/cAMP/PKA signaling pathway, expression, phosphorylation, and trafficking of AQP2 are also regulated by other signal pathways. Ando et al. reported that Wnt5a increased the apical membrane localization of AQP2 by activating calcium/calmodulin/calcineurin signaling pathways, which reversed the decrease of urine osmolality in NDI mouse model [74]. Cheung et al. reported that erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, promoted the phosphorylation of AQP2 at Ser-256 and Ser-269 and reduced the endocytosis of AQP2, which enhanced the plasma membrane accumulation and water reabsorption function of AQP2 [62]. Jung et al. found that C/EBPβ was involved in the transcriptional regulation of CREB on *Aqp2*, which illustrated that the effect of CREB on *Aqp2* was indirect [75]. Besides, other transcription factors, such as AP-1, NF-κB and NFAT, also regulate the expression of AQP2 [18].

The redistribution of AQP2 results in increased transcellular water permeability and urine concentration. Once the correct water balance is restored, AQP2 is internalized and redirected to storage vesicles or targeted for degradation through ubiquitin-mediated endocytosis.

11.3.3 AQP3

AQP3 knockout did not affect perinatal survival and postnatal growth of mice. But AQP3 knockdown resulted in a significant increase in fluid consumption and urine production in mice. It is reported that AQP3 knockout mice consumed and excreted ten-fold of fluid than wild-type mice [76]. The mean urine osmolality of AQP3 knockout mice was about 262 mosm/kg H_2O, which was much lower than that of wild-type mice (1270 mosm/kg H_2O). Researchers performed 36-hour water deprivation experiment or dDAVP administration to investigate the urine concentration ability of AQP3 knockout mice. Results showed that urine osmolality in AQP3 knockout mice was significantly increased after water deprivation and dDAVP administration, although the increase was still much smaller

than that in wild-type mice. These results indicate that the countercurrent exchange in the kidney of AQP3 knockout mouse remains basically intact, but the osmolality of the renal medulla is lower than that of wild-type mice due to the diuresis washout. These findings suggest that AQP3 deficiency causes NDI by a different mechanism than AQP1 and that dysfunction in both countercurrent exchange and collecting duct can cause NDI [77].

When the osmotic water permeability (P_f) of the basolateral membrane of the cortical collecting duct was measured by spatial filtration microscopy. Results showed that the volume of the cortical collecting duct changed rapidly with a half-time ($t_{1/2}$) for osmotic equilibration of 1.1 s in wild-type mice. However, osmotic equilibration in the collecting ducts of AQP3 knockout mice was markedly slow with a $t_{1/2}$ of 2.7 s. The rate of solution exchange in the tubules of wild-type mice may be underestimated because the time of solution exchange in the system is finite. Therefore, AQP3 knockout resulted in at least a three-fold decrease in water permeability in the basolateral membrane of the cortical collecting duct.

The impaired urine concentration ability of AQP3 knockout mice confirmed that the water permeability of the basolateral membrane of the collecting duct may act as a rate-limiting barrier when AQP3 is absent, suggesting that AQP3-mediated water transport across the basolateral membrane of collecting duct epithelium is important for concentrated urine formation.

11.3.4 AQP4

In the basal state, there was no significant difference in urine osmolality, serum sodium concentration, and serum osmolality between AQP4 knockout mice and wild-type mice [78, 79]. However, the results of 36-hour water deprivation experiment showed that the maximum urine osmolality of AQP4 knockout mice decreased significantly after water deprivation, and desmopressin could not effectively restore the osmolality of QP4 knockout mice [80, 81]. These findings suggest that AQP4 knockout mice have a mild urine concentrating defect.

The researchers also compared P_f values of perfused IMCD segments isolated from wild-type mice and AQP4 knockout mice. The results showed that in the condition of 18–48 h water deprivation and presence of vasopressin, the transepithelial P_f values of microdissected IMCD in wild-type mice and AQP4 knockout mice were 0.056 cm/s and 0.013 cm/s, respectively. These results suggested that AQP4 was a major player in water movement in the basolateral membrane of IMCD.

Although the water permeability of IMCD in AQP4 knockout mice was greatly reduced, their urine concentration ability was only slightly impaired. This may be related to the normal distribution of water transport along the collecting duct. Because the amount of water reabsorbed by cortical collecting duct is far more than that of medullary collecting duct [82], and AQP4 is mainly expressed in medullary collecting duct. Therefore, the reduction in water absorption caused by AQP4 knockdown is limited.

11.3.5 AQP5

There was no difference in renal function between AQP5 knockout mice and wild-type mice [83]. Therefore, the role of AQP5 in the kidney remains to be explored.

11.3.6 AQP6

Although AQP6 is a classical aquaporin, it is quite different from other aquaporins. Hg^{2+}, a well-known AQP inhibitor, causes aquaporin dysfunction, but increases the permeability of AQP6 to water and anions. In addition, acidic conditions also induce enhanced AQP6 function [84–87]. These phenomena suggest that AQP6 might be involved in the acid secretion process of collecting duct.

However, the role of AQP6-mediated permeability of water and anions in renal tubules remains unclear. Intercalated cells are the main acid-secreting cells in distal tubules and collecting ducts. Intercalated cells contain a large number of mitochondria, which provide energy for cellular activities [88]. Intercalated cells rely on intracellular vesicles containing the H^+-ATPase to transport H^+ [87]. And AQP6 localizes to these vesicles. It has been found that H^+-ATPase could be transported from intracellular vesicles to apical membrane in response to acid–base changes. However, AQP6 was not found in the plasma membrane of intercalated cells [89, 90]. These findings suggested that AQP6 functioned only at the intracellular sites. Current opinions suggest that AQP6 may play a role in maintaining acid–base balance in cellular regulation [91, 92], without effects on direct fluid transport in renal tubules. But these views need further confirmation.

11.3.7 AQP7

AQP7 contributed little to the permeability of proximal straight tubes, as the water permeability of the outer medullary vesicles of AQP7 knockout mice was 18×10^{-3} cm/s, it is only slightly lower than that of wild-type mice, which is 20×10^{-3} cm/s [93]. And it is reported that the water permeability of AQP7 in proximal straight tubules is only 1/8 of that of AQP1 protein [35].

It is reported that AQP7 knockout mice did not show defect in urine concentration, but AQP7 knockout significantly increased the urine output of AQP1 knockout mice. The 24-hour urine output of AQP1 knockout mice was about 5.7 mL and the 24-hour urine output of AQP1 and AQP7 double knockout mice was about 7.3 mL. These results suggest that the water permeability of AQP7 in proximal straight tubules contributes to reabsorption.

The serum glycerol concentration of AQP7 knockout mice was about 0.036 mg/ml, which was slightly lower than that of wild-type mice of 0.042 mg/ml. However, the urine glycerol concentration of AQP7 knockout mice was 1.7 mg/ml, much higher than the 0.005 mg/ml of wild-type mice. These results suggested that the primary role of AQP7 in the proximal straight tubules is to reabsorb glycerol. Moreover, there might be no other glycerol reabsorption system in the kidney to complement the impaired glycerol reabsorption caused by AQP7 knockout [94–96]. It also suggested that AQP7 played a secondary role in renal water reabsorption.

Studies also found that AQP7 can penetrate ammonia [97, 98]. However, the physiological role of AQP7-mediated ammonia permeability has not been elucidated. As we know, glutamine metabolism occurs in the proximal tubules, which produces HCO_3^- and NH_4^+. The HCO_3^- and NH_4^+ will be excreted into the renal tubules, and some NH_4^+ may exit from the proximal tubule cells to the renal tubule as NH_3, where it is protonated [99]. The researchers speculated that AQP7 may be involved in the secretion of NH_3 and NH_4^+, thereby mediating tubular regulation of ammonia concentrations on both sides of the membrane over a shorter period of time.

11.3.8 AQP8

AQP8 has no essential role in renal urine concentration function. Studies found that AQP8 knockout did not affect the urine osmolality both in basal and 36-hour water deprivation condition in mice [100]. Researchers generated AQP1 and AQP8 double-knockout mice, and the results showed that there was no significant difference in urine osmolality between AQP1 knockout mice and AQP1 and AQP8 double-knockout mice [98, 101–103]. These findings suggested that AQP8 had no effect on urine concentration.

Besides, Molinas et al. found that AQP8 was expressed in the inner mitochondrial membrane of HK-2 cells. After knockout of AQP8, the rate of ammonia release from HK-2 cells decreased, and the expression of AQP8 in HK-2 cells was significantly upregulated when exposed to acidic medium [104]. These studies suggest that AQP8 may be involved in the transport of ammonia in the kidney.

11.3.9 AQP11

AQP11 is involved in renal oxygen homeostasis. Previous study reported that AQP11 knockout mice showed upregulation of NOX2 and enhancement of oxidative stress in kidney, which accompanied by macrophage infiltration [105]. Moreover, due to severe renal failure, AQP11 knockout mice started dying within 2 weeks, which suggested that this isoform was of fundamental importance [34].

11.4 AQPs in Renal Diseases

11.4.1 Nephrogenic Diabetes Insipidus

AQP2 plays a critical role in the progress of NDI [106]. Acquired NDI is mainly caused by abnormalities of chemical substances and electrolyte, or obstructive uropathy [107–109]. Lithium-induced NDI is a disorder characterized by the inability of the renal collecting duct to concentrate urine in respond to ADH. Studies found that the expression level of AQP2 was significantly decreased after lithium induction [110–112]. Whereas, the mutation of AQP2 is also an important cause of congenital NDI [113–116]. Two modes of inheritance of AQP2 mutations are known, autosomal recessive and autosomal dominant. In autosomal recessive AQP2 mutation, the AQP2 protein loses its function as a water channel and is mislocalized in the endoplasmic reticulum. In autosomal dominant AQP2 mutation, AQP2 is abnormally localized in intracellular compartments such as the Golgi apparatus, late endosomes, and lysosome, or in the basolateral membrane [76].

Elucidating the regulatory mechanism of AQP2 is of great significance for the treatment of NDI and the development of potential drugs [117]. Therefore, researchers generated an inducible mouse model of recessive NDI by AQP2-T126M gene mutation [118–120]. Western blot results showed that the molecular size of AQP2 bands was about 34~40 kDa (Fig. 11.4a), indicating complex glycosylation of fully processed AQP2 [121]. In addition, there were also bands of unglycosylated AQP2 (about 29 kDa). However, the AQP2 bands were about 31 kDa in AQP2-T126M mutation mice, which were endoplasmic reticulum-retained, core-glycosylated form of AQP2-T126M. If treated with endoglycosidase H, the 31 kDa bands will largely disappear.

The urine output of AQP2-T126M mutant mice was seven times higher than wild-type mice. After 18 h of water deprivation, the urine osmolality of wild-type mice increased from 1840 mosm/kg H_2O to 2872 mosm/kg H_2O, while that of AQP2 knockout mice did not increase, but that of AQP2-T126M mutant mice increased to 1027 mosm/kg H_2O. These results indicated that AQP2 in AQP2-T126M mutant mice still retained a certain water permeation function. In addition, Hsp90 is a molecular chaperone of AQP2, which can regulate the activity of AQP2. Studies have shown that the HSP90 inhibitor 17-AAG can partially reverse the increased urine output induced by AQP2-T126M mutation. These results suggested that Hsp90 inhibitors were potential therapeutics for NDI (Fig. 11.4b, c) [121].

PKA signaling pathway was found to play an important role in regulating AQP2 in NDI. Genetic or pharmacological inhibition on adenylate cyclase 6 or glycogen synthase kinase 3β (GSK3β) significantly decreased the level of cAMP, which downregulated AQP2 and caused NDI [122, 123]. Ando et al. reported that 3,3′--diamino-4,4′-dihydroxydiphenylmethane (FMP-API-1) and its derivatives showed inhibitory effect on NDI through upregulating AQP2 by activating PKA [124]. Gao et al. reported that prostaglandin E2 receptor EP4 decreased AQP2 expression by activating cAMP-PKA pathways in renal collecting ducts, which impaired urinary concentration and caused NDI [125]. These findings implied that PKA signaling pathway was potential target for NDI treatment. However, inhibition of cAMP-PKA pathways not only exhibited limited efficacy but also accompanied with a large number of side effects, denying its potential in NDI treatment [18].

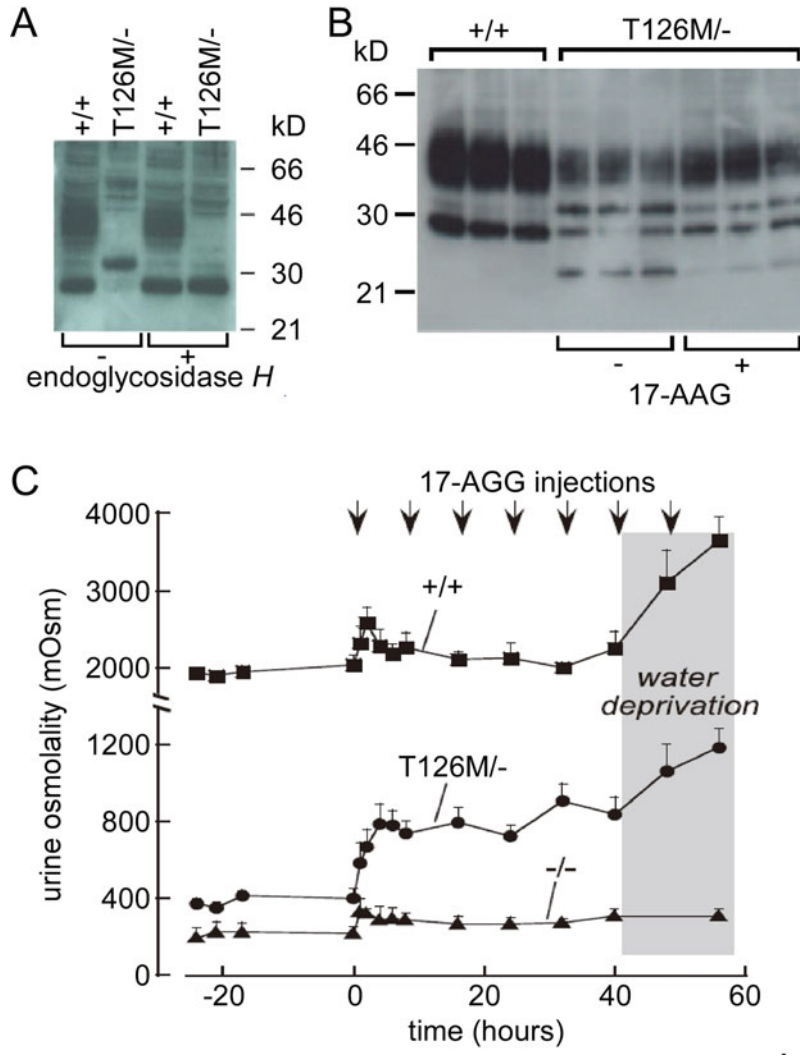

Fig. 11.4 17-AAG partially corrects defective urinary concentrating function in AQP2-T126M mutant mice. (**a**) Representative Western blots of AQP2 protein from wild-type (+/+) and AQP2-T126M mutant (T126/–) mice. (**b**) Representative Western blots of AQP2 protein from the kidney homogenates of wild-type and AQP2-T126M mutant mice treated without or with 17-AAG. (**c**) Urine osmolality in wild-type, AQP2-T126M mutant, and AQP2 knockout (–/–) mice before and after 17-AAG treatment. (Data cited from [121])

AQP2 is also regulated by many other factors. Suzuki et al. reported that in the *Keap1*$^{-/-}$; *Nrf2*$^{flox/flox}$; *K5*-Cre mice, dysfunction of KEAP1 caused the abnormal activation of NRF2 in the kidney, which downregulated AQP2 and led to defects in water reabsorption. These data suggested that abnormal activation of NRF2 in the kidney caused NDI by reducing AQP2 expression [126]. Hatem-Vaquero et al. reported that integrin-linked kinase (ILK), as a scaffold protein that linking ECM to intracellular signaling pathway, upregulated AQP2 by activating ILK/GSK3β/NFAT signaling pathways. Dysfunction of ILK caused NDI. These findings suggested that regulating ECM may be an effective therapy for NDI [127]. Bonfrate et al. found that statins could upregulate the expression of AQP2 in the kidney, which increased water reabsorption and remedied NDI [128].

In addition, deficiency of AQP1, AQP3, or AQP4 causes NDI in mouse models. AQP1 knockout mice showed increased urinary flow rates with low urinary osmolality [35]. The diuresis of AQP1 knockout mice came mainly from the reduced fluid absorption in the proximal tubule [35]. Therefore, there was no abnormality found in AQP1-deficient human patients not subjected to water-deprivation stress [129]. AQP3 null mice

showed polyuric and collecting duct function defect. But the countercurrent exchange in the kidney of AQP3 knockout mouse is basically intact, although diuresis washout made medullary interstitial osmolalities lower than that in wild-type mouse [76, 77]. AQP4 deficiency caused very mild defect in urine-concentrating ability, although the water permeability of IMCD was greatly reduced [78–81].

11.4.2 Polycystic Kidney Disease

Autosomal dominant polycystic kidney disease (ADPKD) is a human inherited disease with an estimated prevalence of between one in 2500 to one in 1000 individuals. It is characterized by progressive enlargement of fluid-filled cysts derived from renal tubular epithelial cells. Massive cysts gradually compress renal parenchyma, destroy the normal renal structures, and eventually cause the loss of kidney function. The formation and growth of renal cysts are mainly caused by the abnormal proliferation of cyst epithelial cells and the secretion of fluid, but the underlying mechanism still need to be clarified.

In human ADPKD patients, AQP1 was found to be expressed in 71% of renal cyst epithelial cells, and 44% of them were derived from proximal tubules [130]. Wang et al. found that AQP1 was important in retarding renal cyst expansion [131]. AQP1 knockout significantly promoted renal cyst formation and growth in kidney-specific *Pkd1* knockout mouse (Fig. 11.5a). And the excess cysts were concentrated in proximal tubule. The MDCK cyst model is an in vitro model that simulates the formation and growth of renal cysts. It is reported that overexpression of AQP1 in MDCK cells significantly inhibited MDCK cyst formation in MDCK cyst model (Fig. 11.5b).

The inhibitory effect of AQP1 on cyst formation and growth may be derived from its inhibitory effect on β-catenin and cyclin D1, which leads to the downregulation of Wnt signaling pathway. The results of co-immunoprecipitation suggested that AQP1 could interact with β-catenin, GSK3β, LRP6, and Axin1. And subcellular fractionation experiments also showed that β-catenin, GSK3β, and Axin1 co-existed in the cytosolic and membrane fractions, whereas LRP6 and AQP1 were detected only in the membrane fractions (Fig. 11.5c).

It is hypothesized that the interactions between AQP1 and GSK3β, βLRP6, Axin1, serine/threonine kinases (CK1), APC could stabilize "destruction signaling complex" on the plasma membrane (Fig. 11.5d). These processes promoted β-catenin phosphorylation. β-TrCP recognizes and ubiquitinates phosphorylated β-catenin, causing β-catenin degradation. AQP1 knockout decreased the stability of "destruction signaling complex," which blocked the ubiquitination and degradation of β-catenin. Excessive β-catenin will be translocated into the nucleus and found a β-catenin/TCF complex by binding to TCF, which promoted the transcription of Wnt target genes. These findings suggested that AQP1 may be a therapeutic target for ADPKD.

It is found that AQP2 was expressed in two-thirds of the cyst epithelial cells [132, 133], and AQP2 dysregulation occurs in ADPKD [16]. Aboudehen et al. reported that in the HIF-1α mutated mice, AQP2 was overexpressed and mislocalized in the collecting duct cell cytoplasm, which promoted renal cyst formation and urinary concentration defect [134]. Noitem et al. reported that steviol facilitated the cyst growth by downregulating AQP2 [135]. These findings implied the potential role of AQP2 in ADPKD treatment.

AQP3 plays an important role in promoting the progress of ADPKD. It was reported that in the MDCK cyst model, overexpression of AQP3 significantly upregulated the expression of HIF1-α and glucose transporter 1 (GLUT1) (Fig. 11.6a), which promoted glucose uptake and accelerated cyst expansion (Fig. 11.6b). Consistent with these, in kidney-specific *Pkd1* knockout mouse model and inducible *Pkd1* knockout mouse model, AQP3 knockout significantly reduced the kidney volumes and renal cyst indices (Fig. 11.6c). Moreover, AQP3

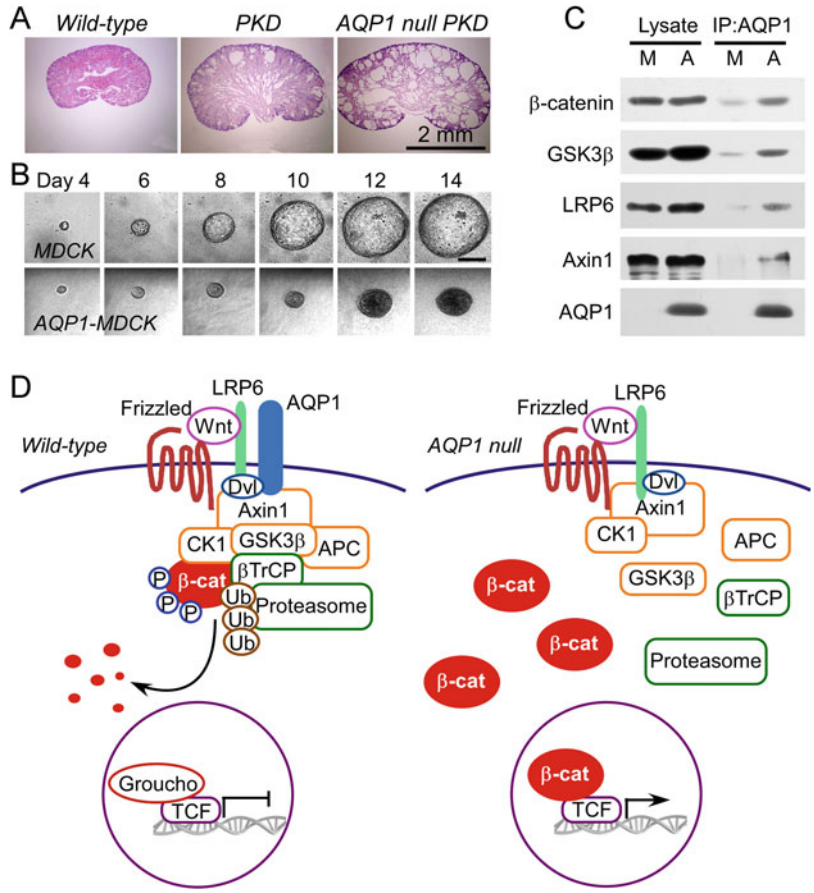

Fig. 11.5 AQP1 inhibits renal cyst development in polycystic kidney disease. (**a**) Representative images of wild-type, *Pkd1*$^{-/-}$ and *Aqp1*$^{-/-}$*; Pkd1*$^{-/-}$ kidneys. (**b**) Representative images of MDCK cysts formed by non-transfected MDCK cells and AQP1-MDCK cells. (**c**) Representative coimmunoprecipitation images of AQP1, β-catenin, GSK3b, LRP6, and Axin1 in AQP1-MDCK cells. (**d**) Schematic of proposed β-catenin regulation by AQP1. (Data cited from [131])

dysfunction significantly reduced ATP content in the kidney of *Pkd1* knockout mouse model (Fig. 11.6c), which promoted the phosphorylation of AMPK and dephosphorylation of ERK and mTOR. These processes finally retarded renal cyst development (Fig. 11.6e–g) [136].

Studies have shown that AQP11 knockout mice exhibited the same phenotype of enlarged kidneys, polycystic, and anemia as human PKD patients. However, renal cysts of AQP11 knockout mice mainly generated form cortex but no medulla (AQP11 is highly expressed in cortex) [137].

It is reported that AQP11 knockout mice showed vacuolization and ER lumen enlargement in proximal tubule cells [34]. Among these abnormal cells, there were a large number of TUNEL-positive cells and cleaved caspase-3-positive cells, suggesting an enhancement of apoptosis [138]. In the abnormal ER, the expression of ER stress-related genes such as Hspa5 and Hsp90b1 were significantly increased, suggesting ER stress. In addition, the expression of Ki-67 and epidermal growth factor receptor were also significantly increased, suggesting an abnormality in cell proliferation. Atochina et al. also reported that in the kidneys of *AQP11* knockout mice, the expression of ER stress marker Bip and apoptosis marker cleaved caspase-3 were significantly increased, accompanied by mitochondrial damage [139]. These results suggest that AQP11 knockout causes ER stress which induces renal injury.

Besides, Inoue et al. found significant upregulation of PC1 and significant downregulation of PC2 in the kidneys of AQP11 knockout mice, which were accompanied by a significant increase in primary cilia of

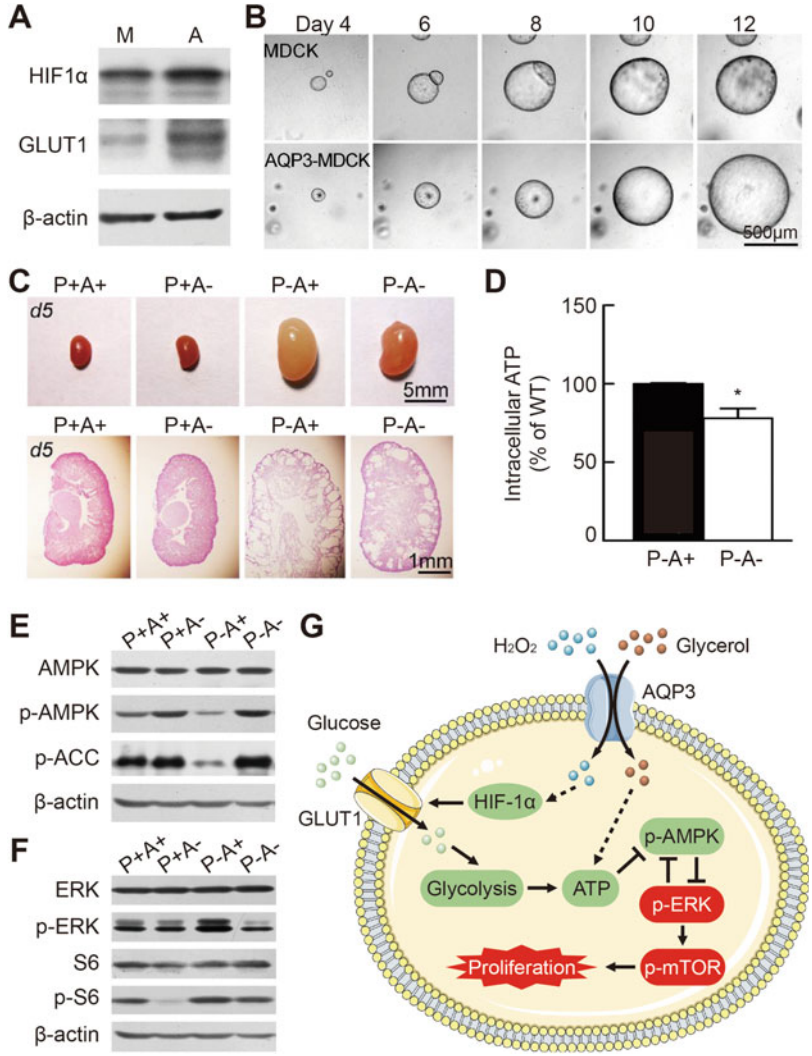

Fig. 11.6 AQP3 promotes renal cyst development in polycystic kidney disease. (**a**) Representative Western blots of HIF1α and GLUT1 protein from *Pkd1*$^{-/-}$; *Aqp3*$^{+/+}$ and *Pkd1*$^{-/-}$; *Aqp3*$^{-/-}$ mice. (**b**) MDCK cysts formed by normal MDCK cells and AQP3-transfected MDCK cells. (**c**) Representative images of wild-type, *Aqp3*$^{-/-}$, *Pkd1*$^{-/-}$ and *Pkd1*$^{-/-}$; *Aqp3*$^{-/-}$ mouse kidneys. (**d**) ATP content in the kidney of *Pkd1*$^{-/-}$; *Aqp3*$^{+/+}$ and *Pkd1*$^{-/-}$; *Aqp3*$^{-/-}$ mice. (**e**) Representative western blots of AMPK and p-AMPK in the kidney of wild-type, *Aqp3*$^{-/-}$, *Pkd1*$^{-/-}$ and *Pkd1*$^{-/-}$; *Aqp3*$^{-/-}$ mice. (**f**) Immunoblots of ERK, p-ERK, S6 and p-S6 in the kidney of wild-type, *Aqp3*$^{-/-}$, *Pkd1*$^{-/-}$ and *Pkd1*$^{-/-}$; *Aqp3*$^{-/-}$ mice. (**g**) The suggested mechanism of AQP3 in promoting the progress of ADPKD. (Data cited from [136])

proximal tubule epithelial cells, similar to the phenotype observed in polycystic kidney [33]. These findings suggest that ER dysfunction may induce abnormal N-glycosylation of PC1, leading to impaired transport of PC1 to the cilia. And AQP11 knockout may lead to polycystic kidney by affecting the function of PC1 [140]. However, how AQP11 affects the progression of ADPKD is still unclear. Further elucidation of the function of AQP11 is expected to provide a new idea for the study of ADPKD.

11.4.3 Acute Kidney Injury

AQP1 plays a functional role in acute kidney injury (AKI). Wang et al. reported that loss of AQP1 promoted the endotoxin-induced acute kidney injury in AQP1 knockout mice [141]. Liu et al. reported that macrophage M2 polarization was likely the cellular mechanism for the anti-AKI property of AQP1, and that PI3K activation was involved in AQP1-induced M2 phenotype switch [142].

AQP2 plays a functional role in renal ischemia/reperfusion (I/R) injury-induced AKI. Hussein et al. reported that the expression of

AQP2 was decreased in I/R mice, which caused urinary concentration defect [143]. Asvapromtada et al. reported that in the bilateral and unilateral renal I/R rats, release of urinary exosomal AQP2 was decreased [144]. Fan et al. reported that AQP2 was decreased as the occurrence of pyroptosis in renal I/R mouse model and overexpressed AQP2 in the HK-2 cells partially reversed hypoxia-reoxygenation-induced pyroptosis [145]. Besides, the urinary AQP2 level is a potential biomarker of AKI in coronary care unit patients with acute decompensated heart failure [146].

AQP3 was involved in I/R-induced kidney injury. Lei et al. reported that 25 min of I/R did not result in abnormal changes in the kidneys of wild-type mice. But the levels of serum creatinine, urea, and LDH in AQP3 knockout mice were significantly increased after I/R, and the contents of MPO and MDA in the kidney were also increased, while SOD activity was decreased. HE staining showed no obvious change in the kidneys of wild-type mice after I/R. But dilated lumens of collecting ducts, incomplete tube walls, and swollen epithelial cells were found in AQP3 knockout mice after I/R (Fig. 11.7a). F4/80 staining showed that the infiltration of macrophages in collecting ducts was significantly increased in AQP3 knockout mice after I/R. TUNEL staining showed that AQP3 knockout significantly enhanced I/R-induced apoptosis. Consistent with this, the ratio of Bax to Bcl-2, cleaved-caspase-3 to caspase-3, and p-p53 to p53 heightened by I/R were also enhanced in AQP3 knockout mice (Fig. 11.7b). AQP3 knockout downregulated the MAPK signaling pathway in physiological conditions, but it enhanced the I/R-induced abnormal activation of MAPK signaling pathway (Fig. 11.7c). Similarly, AQP3 promoted MDCK cell proliferation and migration in the basic condition. Overexpression of AQP3 significantly alleviated the hypoxia/reoxygenation-induced cell death by reducing the ratios of Bax to Bcl-2, cleaved-Caspase-3 to Caspase-3, and p-p53 to p53 and suppressing apoptosis. At the same time, AQP3 enhanced the activity of MAPK signaling pathway in MDCK cells and inhibited the changes of ERK, P38 and JNK caused by hypoxia/reoxygenation or cobalt chloride stimulation (Fig. 11.7d) [147].

Besides, a report showed that AQP3 dysfunction occurred in cisplatin-induced kidney injury animal model [148].

11.4.4 Epithelial–Mesenchymal Transition and Fibrosis

AQP1 is involved in the process of epithelial–mesenchymal transition (EMT). Li et al. reported that, in HK-2 cells, aristolochic acid I (AA-I) induced EMT by activating TGF-β/Smad-independent signaling pathways, including β-catenin, Ras-Raf-ERK1/2 signaling pathways. These processes upregulated the expression of AQP1, which could be reversed by ERK1/2 inhibitor PD98059 [149]. Lovisa et al. reported that AQP1 was down-regulated in the kidneys of unilateral ureteral obstruction (UUO) mice, which could be reversed by suppressing EMT. These findings supported the important role of AQP1 in EMT [150].

AQP2 has been proven to function in UUO-induced renal fibrosis. Ampawong et al. reported that AQP2 was upregulated in hydronephrosis mice [151]. Wang et al. reported that the downregulation of AQP2 in UUO mouse contributed to the urinary concentrating defect [152]. Activating AQP2 and AQP4 by cholecalciferol cholesterol emulsion also significantly inhibited UUO-induced renal fibrosis [153]. Therefore, activators that target to AQP2 have enormous potential in renal fibrosis treatment.

AQP4 plays a functional role in promoting renal fibrosis. Liu et al. reported that AQP4 was upregulated in UUO animal models. Cholecalciferol cholesterol emulsion significantly suppressed the expression of AQP4, which retarded UUO-induced renal fibrosis. These results implied a functional role of AQP4 in promoting renal fibrosis [153]. MacManes et al. reported that AQP4 was downregulated in a desert-adapted animal model with acute dehydration, suggesting that AQP4 might prevent kidney injury by handling water [154].

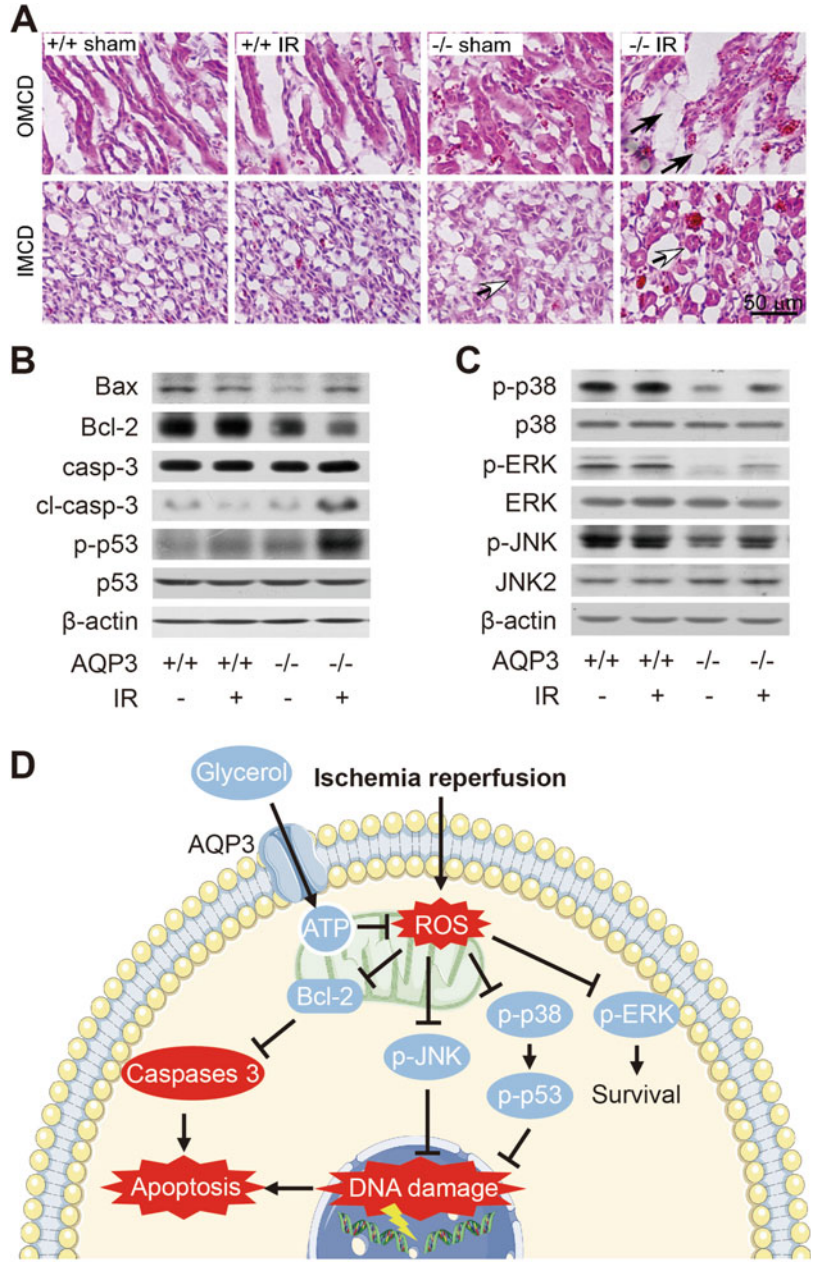

Fig. 11.7 AQP3 promotes ischemia/reperfusion-induced kidney injury. (**a**) Hematoxylin- and eosin-stained pictures of kidneys from wild-type and AQP3 knockout mouse treated without or with I/R. (**b**) Representative western blots of Bax, Bcl-2, Caspase-3, cleaved-Caspase-3, p-p53, and p53 proteins in the kidneys of wild-type and AQP3 knockout mice treated without or with I/R. (**c**) Representative western blots of p-p38, p38, p-ERK, ERK, p-JNK, and JNK2 proteins in the kidneys of wild-type and AQP3 knockout mice treated without or with I/R. (**d**) The suggested mechanism of AQP3 in inhibiting I/R-induced kidney injury. (Data cited from [147])

In addition, studies have confirmed that the expression level of AQP4 is negatively correlated with the degree of renal injury [155]. AQP4 was upregulated and AQP1 and AQP2 were downregulated in the salt-sensitive hypertension patients [156].

11.4.5 Tumors

AQP1 has been shown to be associated with a varity of tumors, especially those originating in water-permeable organs, such as kidney and bladder [157–159]. It has been confirmed as an enhancer of cell growth and migration, which promotes tumor angiogenesis [160, 161]. Because

of the heterogeneity of its distribution and expression, AQP1 could be used as a biomarker of tumors [162, 163].

AQP3 plays an important role in tumor. Yu et al. reported that AQP3 was upregulated in arsenical cancers and arsenic-treated keratinocytes, accompanied by an increase of autophagy flux. Inhibiting AQP3 by aquaporin inhibitors $AgNO_3$ or RNA interference significantly suppressed the arsenic-induced autophagy, implying the functional role of AQP3 in promoting autophagy [164].

Studies have shown that the expression of AQP6 changes with the development of renal cell carcinoma (RCC) and oncocytoma, suggesting that AQP6 could be used as a diagnostic marker for renal cancer [165, 166].

11.4.6 Pyelonephritis and IgA Nephropathy

Increase of urinary AQP2 excretion was found in pyelonephritis and IgA nephropathy patients. The reduction of AQP2 was regarded as the main cause of polyuria [167, 168]. Landegren et al. reported that targeting AQP2 or its upstream molecules by autoantibodies resulted in tubulointerstitial nephritis in patients with autoimmune diseases [169], suggesting AQP2 as the therapeutic targets for these diseases.

11.4.7 Chronic Kidney Diseases

AQP5 may play a functional role in diabetic nephropathy (DN). AQP5 was found in kidney biopsies from ND patients, while was not found in normal controls [170]. Mechanistic studies suggest that upregulated AQP5 may lead to polyuria by affecting AQP2 membrane localization. These results suggest that AQP5 in urine may serve as a biomarker of ND [171].

DN is also affected by AQP11. The results of a prospective cohort study on patients with DN suggest that AQP11 rs2276415 variant affects the prognosis of DN patients. The integrity of AQP11 function dose-dependently affects cumulative events-free survival, in which the A allele of AQP11 gene (GA + AA) increased the risk of CKD progression [172].

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Aquaporins in Reproductive System

12

Hang Zhang and Baoxue Yang

Abstract

AQP0-12, a total of 13 aquaporins are expressed in the mammalian reproductive system. These aquaporins mediate the transport of water and small solutes across biofilms for maintaining reproductive tract water balance and germ cell water homeostasis. These aquaporins play important roles in the regulation of sperm and egg cell production, maturation, and fertilization processes. Impaired AQP function may lead to diminished male and female fertility. This review focuses on the distribution, function, and regulation of AQPs throughout the male and female reproductive organs and tracts. Their correlation with reproductive success, revealing recent advances in the physiological and pathophysiological roles of aquaporins in the reproductive system.

Keywords

Reproductive system · Spermatogenesis · Oogenesis · Fertility

12.1 Aquaporins in the Male Reproductive System

12.1.1 Water Transport in the Male Reproductive System

The male reproductive system of mammals consists of paired testes, efferent ducts, epididymides, vas deferens, urethra, penis, and accessory gonad (seminal vesicle, prostate, bulbourethral gland, ampullary gland, preputial gland, etc.), of which ampullary gland and preputial gland are unique to rodents such as mice and rats.

The transport and metabolism of water in the male reproductive system are more active and have obvious particularities. Germ cells require the transport, secretion, and reabsorption of large amounts of water during development. Spermatogonia develop into spermatids after multiple divisions in the seminiferous tubules of the testis, and then spermatids will flow through the seminiferous tubules, testicular rete, efferent tubules with fluid in the testis, and finally store in the epididymis and further mature. In the above processes, especially the stage when round spermatids are transformed into elongated spermatids, the significant reduction in cell volume due to osmotically driven fluid outflow is one of the most significant morphological changes. The distal parts of the outflow duct system, especially the epididymis, undergo

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massive fluid resorption resulting in a significant increase in their internal sperm concentration.

Aquaporins (AQP) expressed in spermatogenic cells and epithelial cells of the reproductive tract regulate the movement of water and are responsible for balancing the intraluminal environment of spermatogenesis. In addition, AQPs play an important role in the vas deferens, seminal vesicles, and prostate, which have secretory and resorptive functions and produce nutrient-rich fluids to maintain sperm maturation.

12.1.2 Aquaporins in Testis

Testis is one of the important male internal reproductive organs, which can produce sperm and androgen and promote the emergence of secondary sexual signs and the development of other sexual organs. There are AQP0, AQP1, AQP3, AQP4, AQP7, AQP8, AQP9, and AQP11. These eight AQPs are expressed in different cells in testicular tissue, including stromal cells, Sertoli cells (SCs), and spermatogenic cells (Fig. 12.1).

AQP0 has been found in SCs and Leydig cells, but not in the efferent duct or epididymis [1]. AQP1 is mainly localized in the plasma membrane of epithelial cells and microvascular endothelial cells of the testis [2]. AQP3 is expressed in mouse and rat SCs [1, 3]. AQP4 is present in the tunica media of human seminiferous tubules [4] and rat SCs [5]. AQP5 is localized in the Leydig cells [6]. Double-labeling, confocal microscopy showed co-expression of AQP5 with capillary AQP1 in the testis [6]. AQP7 is expressed in elongated spermatids and sperm tails in the testis [6]. A concurrent study also described AQP7 expression on rodent spermatocytes [7]. Immunolocalization showed that AQP8 was located in the plasma membrane of SCs [8, 9]. AQP9 expression has been detected in human SCs, primary spermatocytes, and haploid germ cells within the enucleation [10, 11]. Evaluation of AQP11 expression in rat testes by immunohistochemistry showed signals in germ cells and only in the distal tail of elongated spermatids and residual bodies within SCs, possibly caused by inactive elongated spermatid phagocytosis and intracellular organelles [12].

Histological sections of rat seminiferous tubules showed that AQP0 expression levels in SCs correlated at different stages of the seminiferous epithelial cycle. Higher AQP0 expression was observed at intermediate stages (V-VIII) [1]. In fact, AQP0 expression in SC is associated with the process of detachment of elongated spermatids from these somatic cells into seminiferous tubule. AQP1 is directly involved in regulating the transport of fluid in the testicular microvascular endothelial cell membrane [13]. During the development of the rat testicular outflow duct, the structural and functional expression of AQP1 is vulnerable to neonatal estrogen exposure and may be a direct effect [14]. AQP3 is thought to mediate the transport of glycerol, which is an essential substrate for germ cell development and spermatogenesis [3]. In rodent SCs, AQP4 and cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel [5], interacts, which indicates that AQP4 and CFTR can regulate the steady state of water fine tube.

The transcripts of AQP7 are detectable at 23–25 days postpartum, when round spermatids begin to appear [15]. However, studies showed no alterations in male fertility in AQP7 knockout mice compared to wild-type mice, especially no abnormalities in daily spermatogenesis, motility, or even offspring number [16]. AQP9 functional studies on mouse SCs have shown that estrogen treatment results in downregulation of the AQP9 gene with a concomitant reduction in glycerol transport [3]. In rats, AQP9 is thought to mediate water and uncharged solutes between cells and blood vessels and/or interstitial spaces, which helps to maintain the homeostatic dynamics of cells [17, 18]. AQP11 plays a role in recovering the remaining cytoplasmic fraction of elongated spermatids and maintaining SC capacity [12]

12.1.3 Aquaporins in Sperm

In the male reproductive tract, spermatogenesis is associated with massive fluid secretion and absorption [19–21]. The role AQPs play in

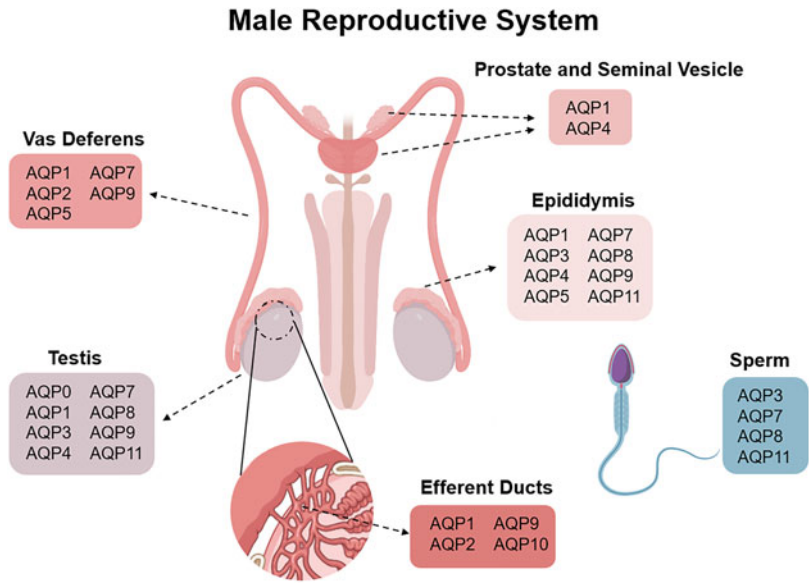

Fig. 12.1 Expression of AQPs in male reproductive system

sperm is mainly associated with changes in cell volume during spermatogenesis and changes in the osmotic pressure of the surrounding environment when sperms are motile in the male and female reproductive tracts.

Human spermatozoa have been shown to express four aquaporins, AQP3, AQP7, AQP8 and AQP11, which localize specifically to the plasma membrane and intracellular organelles. In human spermatozoa, AQP3 expression was localized in the tail of spermatids by immunofluorescence staining [22], localization of which is consistent with that found in other mammals [22–24]. The first aquaporin found in human sperm cells is AQP7, which is localized in the midpiece and front part of the sperm [25]. AQP8 protein is localized in the middle piece of sperm, where it is more pronounced in the mitochondria [10, 26]. The results of immunohistochemical staining showed intense labeling of AQP11 in the distal quarter of mouse and rat sperm tails [12, 26]. The presence of AQP11 protein in humans was confirmed by Western blot and immunostaining [27, 28], which was localized in vesicular structures in the sperm tail and cytoplasm.

Due to the hypertonic environment of the cauda epididymis (~415 mOsm/kg in mouse) and an isotonic environment of the uterine body (~310 mOsm/kg in mouse), osmotic adaptation is essential during the transition of sperm from the male to the female reproductive tract [22]. Studies have shown that AQP3-null mice show normal spermatogenesis and normal motor activation caused by hypotonic stimuli, but have increased vulnerability to cell swelling and tail bending caused by hypotonicity, and specifically, deletion of AQP3 was found to result in tail deformation, formation of hairpin-like structures due to mechanical membrane stretching, resulting in impaired migration of sperm into the fallopian tube, which in turn leads to reduced fertilization ability [22, 29, 30]. AQP3 may form a molecular complex with other ion channels (such as volume-sensitive chloride channel CLC-3), which can detect the osmotic change and trigger the subsequent volume regulation process [22, 29, 31]. Recently, it has been found that AQP3 plays an important role in human sperm motility and mitochondrial membrane potential integrity [32].

AQP7 has been studied extensively in mouse and human sperm. About 20% of infertile patients have low sperm motility and low AQP7 expression [25]. Studies on AQP7 knockout mice have shown no difference in testicular and epididymal

morphology, sperm quality, fertilization capacity, and offspring compared with wild-type mice [16]. Data suggest that AQP7 knockout mice neither have abnormality in sperm function and morphology nor develop infertility. The experiments with another AQP7-null mouse model showed that compensatory upregulation of AQP8 may guarantee normal water perfusion and eversion capacity of mouse spermatozoa [21]. While emphasizing the importance of AQP8 for sperm water transport in mice during osmoregulation, this precludes a critical role for AQP7 in regulating sperm volume change necessary for fertilization in vivo [21]. AQP7 functions primarily as a solute channel rather than a water channel in spermatozoa. Immunofluorescence showed that AQP7 was expressed in the pericentriolar area, midpiece, equatorial segment, and weakly in the tail of normal sperm, while abnormal sperm had a diffuse low intensity of AQP7 expression in coiled tail and head [33]. In addition, a correlation between AQP7 localization and percentage of normal morphology and forward motion was demonstrated [33]. In another study, Yang and colleagues compared fertile controls and infertile patients and also found that the relative content of AQP7 in sperm detected by flow cytometry correlated with sperm motility. The relative content of AQP7 was higher in fertile donors than in infertile patients [10]. It has also been shown that AQP7 acts as an aquaglyceroporin to promote glycerol transport in spermatozoa for use as an energy substrate [26]. Taken together, the current evidence supports the notion that AQP7 plays a crucial role during spermiogenesis and epididymal maturation, glycerol metabolism in sperm, and changes in sperm cell volume [10, 34].

In humans, AQP8 levels were found to be inversely correlated with the degree of sperm tail curling [10]. When researchers used two inhibitors ($HgCl_2$ and phloretin) to determine the role of AQP8 in sperm volume regulation, they found that $HgCl_2$ but not phloretin was effective in blocking quinine-induced swelling [10]. Considering these evidences, it can be assumed that AQP8 is the main permeation pathway for water in the sperm midstream [21]. However, sperm from AQP8-null mice did not show significant difference in number or morphology compared with wild-type mice, and fertility remained normal [35]. In a recent study, the expression of AQP8 in the mitochondrial membrane of human spermatozoa was confirmed [27], which highlights the role of AQP8 involved in ROS processing in spermatozoa [36].

AQP11 is present in the terminal stages of elongated spermatids and localizes to the distal sperm tail, suggesting that AQP11 plays a role in the terminal stages of spermatogenesis and in promoting the elimination of residual caudal cytoplasm during spermiogenesis [12]. Studies on changes in the testicular transcriptome in Syrian hamsters during photoperiodic stimulation to regulate fertility indicated that AQP11 was significantly associated with testicular weight and testicular fertility markers, supporting the critical role of AQP in fertility control [37]. The possible role of AQP11 in the elimination of H_2O_2 and other metabolic waste products in mitochondria and other organelles makes AQP11 a possible modulator of redox homeostasis and signaling [38]. Currently, a study using porcine sperm cells, which have expression patterns similar to those observed in human spermatozoa, noted that AQP11 expression is associated with higher sperm quality [27]. Spermatozoa with higher AQP11 expression also had higher membrane integrity and motility, further demonstrating the relevance of AQP11 for sperm function.

After spermiogenesis, in the process from seminiferous tubules to oviduct, it is necessary to regulate its own volume to adapt to the reproductive tract microenvironment of males and females with different osmotic pressures. This osmotic adaptation can prevent sperm swelling and avoid affecting the bending movement of its tail, so as to ensure sperm motility and maintain the maximum fertilization ability [30]. This adaptation depends not only on ion channels but also on water channels [10]. Therefore, various AQPs are expressed in spermatozoa and play the role and function related to water and solute transport and energy metabolism, ultimately enabling successful fertilization of spermatozoa.

12.1.4 Aquaporins in Efferent Ducts

The efferent ductules of mammals are a group of fine ducts that connect the testis to the epididymis and are entrapped by adipose tissue. The efferent tubule epithelium consists of nonciliated principal cells and ciliated cells containing kinocilia as well as basal cells. Its main function is to transport sperm and reabsorb the water, ions, and proteins of the testis. The plasma membrane on its surface expresses AQPs and water-ion transporters, which also contributes to the reabsorption of testicular fluid by the efferent tubules and maintain the homeostasis of testicular fluid.

AQP1 expression in the efferent tubules was confirmed in mice, rats, marmosets, and adult dogs [39–42]. In adult dogs, AQP2 is expressed in the efferent duct [42]. AQP9 has been found to be expressed in the microvilli of principal cells without cilia in rat efferent ducts [9] and in the apical membrane of principal cells in humans [43]. It has been reported that AQP10 is also present in ciliated and non-ciliated cells of the rodent efferent duct [1].

AQP9 may contribute to the entry of glycerol and other neutral solutes into the lumen [44]. It was found that in the efferent duct of the epididymis, the expression of AQP9 protein was increased by 300% in the animals treated with diethylstilbestrol, while there was no difference in AQP9 mRNA expression [45]. Moreover, the expression of AQP1 and AQP9 in the efferent tubules may be regulated by estrogen, as AQP expression of these two isoforms is significantly reduced in the efferent tubules of estrogen receptor-deficient mice [46].

12.1.5 Aquaporins in Epididymis

The epididymis can be divided into three distinct parts: head, body, and tail, which, as part of the vas deferens, not only functions to deliver sperm but is also associated with sperm maturation. Immature spermatids undergo a series of developmental processes in the microenvironment of the epididymis, acquiring motility and the ability to fertilize with oocytes [47].

AQP1 is expressed on endothelial cells of epididymal vascular [9, 48]. AQP3 expression was demonstrated in basal cells in rodent epididymis [1]. AQP4 is expressed in columnar epithelial cells of the rat epididymal duct. AQP5 expression has not been described in human tissues but is present in principal cells of rat epididymal body and tail sections [49]. AQP7 is expressed in the basolateral membrane of adjacent principal cells in the head of the rat epididymis and also in the plasma membrane of principal cells in the cauda epididymis [50]. AQP8 expression has also been demonstrated in rat epididymal basal cells [8]. AQP9 appears to be one of the most abundant AQP in the epididymis, which is expressed in all sections of the epididymal epithelium, especially in the apical microvilli of principal cells [49], and its expression has been described in humans with a similar expression pattern as reported in rodents [43]. Similarly, AQP11 expression has been demonstrated in microvilli of principal cells in the caudal as well as more distal regions of the rat epididymis [51].

AQP1 can assist the final reabsorption of water into the circulation in epididymal vascular [1]. The expression of AQP4 in epididymal parietal membrane is significantly stronger than that in basal parietal membrane, which is the most important part of epididymal canal fluid secretion. The high expression of AQP4 in lumen surface suggests that AQP4 is related to epididymal canal fluid secretion and plays a role in sperm maturation. AQP9 expression during epididymal development in neonatal rats is downregulated by diethylhexylestradiol, GnRHa, ethinyl estradiol, and utamide, these effects are mediated by estrogen and prevented by testosterone [52]. CFTR, acting as a cAMP-activated chloride channel [53], and AQP9 [43] co-expressed in the membrane of rat and human epididymal principal cells, which play an important role in the formation and storage of the mature luminal fluid of sperm, especially in the cauda epididymis [46, 54].

12.1.6 Aquaporins in Vas Deferens

The vas deferens is a direct continuation of the epididymal duct, which begins at the caudal end of the epididymis and terminates in the ejaculatory duct and is divided into three different parts: the proximal, the middle and the distal, which are about 50 cm long in adult males. After maturation in the epididymis, spermatozoa are guided to the vas deferens. AQP-mediated transepithelial water reabsorption in the vas deferens plays an important role in maintaining the environment during sperm maturation [55].

It has been found that AQP1 is expressed in the membrane of the distal principal cells and the basolateral membrane of the rat vas deferens [40]. In adult rats, AQP2 is expressed throughout the vas deferens, where it is more strongly expressed in principal cells in the middle and distal parts compared to those in the principal cells of the bullae part [56]. The variable expression of AQP1 and AQP2 in different regions of the vas deferens may result from differences in principal cell architecture along the vas deferens [57]. AQP5 localizes to the basal cells of the vas deferens [6]. AQP7 was also expressed in the vas deferens of adult dogs [42]. However, AQP9 is expressed in the principal cells of the entire segment of the rodent vas deferens [43, 47], which may be to ensure rapid glycerol uptake by sperm cells and thus energy metabolism [44].

AQP1 is responsible for the movement of large amounts of water and may be used to concentrate sperm cells in the vas deferens, keeping with the physiological function of the efferent duct itself [47]. Interestingly, AQP2 expression levels change during postnatal development in rats, showing the expression pattern detected in adult animals at 4 weeks of age. The studies suggest that AQP2 expression is coordinated through translational or posttranscriptional mechanisms [49]. A study using AQP9 knockout mouse model showed elevated glycerol concentrations in serum despite the fertility of these animals, highlighting the ability of AQP9 to transport this substrate. Furthermore, the role of AQP9 in the efflux products associated with its peroxidase activity is not excluded.

12.1.7 Aquaporins in Male Accessory Glands

Expression of AQP1 and AQP4 was found in both the prostate and seminal vesicle, and AQP1 was also expressed in the plasma membrane of ventral prostate and seminal vesicle epithelial cells [2]. These AQPs play important functions during fluid secretion and reabsorption [4, 40, 43].

12.2 Aquaporins in the Female Reproductive System

In the female reproductive system, AQPs are widely distributed in various reproductive organ tissues, including uterus, ovary, fallopian tube, and vagina (Fig. 12.2). AQPs play important roles in the physiological function of female reproductive organs and the pathological process of gynecological diseases. In addition, changes in vaginal secretions, follicle ovulation, menstruation formation, amniotic fluid during pregnancy, malignant tumor development, or benign gynecological diseases are related to the fluid flow, uterine cavity or follicular cavity volume, in which AQPs play important roles.

12.2.1 Aquaporins in Vagina

Vagina is the organ of sexual intercourse in women, the conduit for expulsion of menstrual blood, and delivery of the fetus and is an organ with fluid secretion ability that maintains the vagina moist and has a self-cleaning effect. Vaginal lubrication increases during sexual activity and can also play a role in carrying sperm after ejaculation in men. So, vaginal epithelial cells express a variety of AQPs that assist in the production of vaginal fluid.

The available data for AQP0 only concerns its presence in the rodent vagina, where it is abundantly expressed in the rat vaginal epithelium [58]. AQP1, AQP2, AQP3, AQP5, and AQP6 were expressed in rat and human vaginal mares [59–61]. In addition, rat vaginal epithelium also

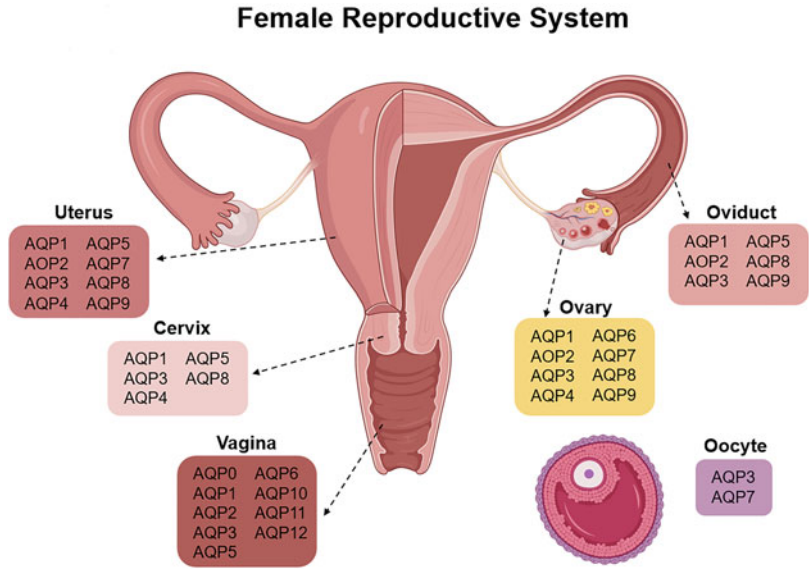

Fig. 12.2 Expression of AQPs in female reproductive system

expresses AQP10, AQP11, and AQP12, which have not yet been demonstrated in human vaginal tissues [58].

It was shown that AQP1 and AQP2 expressed in rat vaginal endothelial cells were transferred from the cytoplasm to the membrane compartment immediately after nerve stimulation and decreased 5 min after nerve stimulation [60]. The protein expression levels of AQP2, eNOS, and nNOS were significantly reduced after ovariectomy, and returned to pretreatment levels with 17β-estradiol administration [62]. Expression of AQP10, AQP11, and AQP12 is decreased after ovariectomy in rat vaginal epithelium, possibly due to a significant reduction in estrogen (E2) secretion, but the specific function of these AQPs is still not clear [58].

12.2.2 Aquaporins in Cervix

The uterine cervix is a complex heterogeneous organ that undergoes extensive changes throughout pregnancy and delivery [63]. These changes include the character and amount of cervical mucus secreted by endocervical glands, which are associated with AQPs [64].

It is reported that AQP3, AQP5, and AQP8 are expressed in the mouse cervix [65]. The localization patterns of these AQPs depend on the cell type and gene expression and change during pregnancy in mice. AQP1 is expressed in cervical vascular endothelial cells. AQP3 and AQP8 were found in the cervix of women with mild cervicitis and cervical cancer [66].

In mouse, AQP3 is expressed in basal cell layer of mouse cervical epithelium, whose expression is low in the non-pregnant and mid-pregnant cervix, with peak expression at gestational day 19 and postnatal day 1 [65]. In addition, AQP3 plays an important role in the increased water content of cervical tissue in pregnant mice, as well as in the process of promoting cervical ripening [67]. AQP4, 5, and 8 were mainly expressed in apical cell layers, whose content also changed in varying degrees during pregnancy [65]. AQP3, 4, 5, and 8 regulate distinct aspects of cervical water balance during pregnancy and parturition [65].

The expression of AQP3 and AQP8 in human cervical cancer and HPV-transformed cells,

respectively, suggests that AQPs may play a role in human cervical hyperplasia and carcinogenesis [68]. The expression of AQP8 was positively correlated with Bcl-2 in human cervical cancer. The AQP3 expression in hTERT positive cervical cancer was confirmed by real-time RT-PCR and immunohistochemistry, indicating that AQP3 may become a molecular biomarker for the diagnosis of cervical cancer [68].

12.2.3 Aquaporins in Uterus

The uterus is an organ that is located in the pelvic cavity and has thick muscular cavity wall. It breeds embryos and fetuses during pregnancy. Its endometrium thickens and falls off periodically with the change of hormone level in the body to form menstruation. AQPs have been detected in the uterus. AQP1 is the first water channel found in the reproductive system [69]. It is expressed in mouse, rat, dog, pig, and human uteri [70–75]. AQP2 is present in mouse, dog, and human uteri [70, 74, 76]. AQP3 and AQP4 were expressed only in the mouse uterus [70, 71, 77]. AQP5 is present in mouse, rat, pig and human uteri [71, 74, 75, 78, 79]. AQP7 and AQP8 are expressed in mouse and human uteri [70, 77, 79], whereas AQP9 is expressed in rat and pig uteri [75, 78]. Accumulating evidence suggests that ovarian steroids hormones may influence the expression of several AQPs in the female genital tract [70, 74, 75, 80].

In humans, AQP1 and AQP2 are expressed in the endometrium [76, 81, 82]. AQP1 mRNA expression can be detected in the endometrium during the proliferative and secretory phases of the normal menstrual cycle. AQP1 protein is expressed in capillaries of endometrial stroma and endothelial cells of small vessels [82]. AQP2 is localized in endometrial and glandular epithelial cells [76, 82]. AQP2 expression in the endometrial glandular epithelium was higher in the secretory phase than in the proliferative phase, with the highest in the mid-secretory phase, which is consistent with the production of endometrial edema. AQP2 may be involved in human endometrial water transport and plays an important role in cyclic changes in the endometrium. The expression of AQP2 was related to the concentrations of estradiol and progesterone and regulated by estrogen and progesterone [76]. Recently, the estrogen response element in the promoter region of AQP2 gene has been successfully identified [80]. Studies have shown that complexes of estrogen and its receptor can bind to estrogen response elements located in the promoter region of the AQP2 gene, thereby activating AQP2 expression.

AQP3 is expressed in the smooth muscle of the uterus, endometrial glands, and uterine tissue [83]. In mice, AQP3 is involved in the efflux and influx of water from the uterine cavity of the neutron during the estrous cycle. It is worth mentioning that the mouse uterus has an AQP3 expression pattern similar to that described in humans [83, 84], demonstrating that the effects described in mouse models may be homologous to humans, although humans present menstrual cycles rather than estrous cycles.

Several studies have also provided evidence confirming the role of AQPs in embryo implantation and pregnancy. In mice, greater intensity of AQP4 on the epithelium was found [71]. Interestingly, uterine compartmentalization is normal during development in AQP4 knockout mice. However, these knockout mice have reduced uterine hypertrophy and endometrial thickness compared to wild-type mice, which could explain the subfertility exhibited by these animals [85]. One of the important effects of estrogen on the endometrium is to promote the absorption of water by the endometrium. Estrogen mainly acts through estrogen receptor type α (ERα). Mice with knockout of ERα are not fertile. Further studies showed that estrogen acts by directly regulating the AQP5 gene through ERα [86]. The estrogen response element in the AQP5 promoter region provides a direct regulation of the AQP5 gene by estrogen. High expression of AQP5 occurred at the time of embryo implantation in the rat uterus [78].

In mouse uterine tissue, AQP8 was found to be present in stromal cells of the endometrium and uterus, which is thought to modulate water distribution in these tissues and to be involved in

uterine edema formation in the stromal layer [70]. AQP9 is expressed in endometrial epithelial cells of pigs and pregnant rats [6, 78]. In the latter, AQP9 expression is enhanced at implantation and may contribute to uterine volume reduction and uterine cavity closure, which are specific features of this gestational stage [78].

12.2.4 Aquaporins in Oviduct

The passage from the ovary to the uterus, known as the fallopian tube, is the conduit that transports eggs into the uterus. The organ can be divided into three different sections: the canyon (section close to the uterus), the ampulla (middle section), and the infundibulum (section close to the ovary). The fluid produced and secreted by the oviduct enables gamete transport, fertilization, and early embryonic development. Oviductal fluid volume fluctuates during the estrous cycle, indicating that water supply is hormonally controlled [87].

AQP1 was detected in female rat and pig oviducts [6, 59, 88]. AQP2 in the human fallopian tube is located at the apical or intracellular side of the ovarian cortical cell [83, 89]. AQP3 is expressed within the human fallopian tube, on both non-ciliated and ciliated cells, but is more pronounced in the latter [83]. AQP5, AQP8, and AQP9 are expressed in rat and pig oviducts [6, 90].

In rats, the expression of AQP1 was also confirmed in the mesothelial cells of the outer surface of the tube and in the membrane of the smooth muscle cells of the muscular myosalpinx [59]. It has been reported that AQP1 assists in regulating the volume of smooth muscle cells and thus assists in regulating the diameter. The process is thought to be important for controlling transport of the fertilized egg through the fallopian tube into the uterus [59]. Ciliated cells are responsible for secreting nutrients into the oviduct and reaching the uterine cavity [83]. AQP3 expressed in ciliated cells assists in the transport of glycerol and other neutral nutrients, which are necessary for the insemination process as an energy source for sperm and fertilized eggs.

AQP5 is expressed in the cytoplasm, parietal membrane, and basolateral membrane of mouse secretory non-ciliated cells and is more abundant in the infundibulum and ampulla of oviduct [91]. AQP5 is responsible for assisting secretion and resorption of oviductal fluid before and after ovulation, respectively, given the expression pattern of the AQP5 gene throughout ovulation [91]. In the rat oviduct, AQP8 expression is present in epithelial cells throughout the ampulla and the canyon [90]. In humans and rats, the location of AQP9 is mainly restricted to epithelial cells [92]. In order to achieve fertilization, sperm require a lot of energy to promote activity so that they can push themselves through the zona pellucida. Glycerol release mediated by AQP9 may be important for the end of this process and help sperm reach the oocyte and achieve fertilization [93].

12.2.5 Aquaporins in Ovary

The main functions of the ovary include the production and expulsion of eggs and the secretion of sex hormones to promote the development and maintenance of female sexual characteristics. AQPs expressed in human ovarian tissues include: AQP1, AQP2, AQP3, AQP4, AQP6, AQP7, AQP8, AQP9 [94, 95].

In humans, AQP1 is present in vascular cells, theca cells, and granulosa cells that surround the theca layer [96]. In granulosa cells surrounding the oocyte, AQP1 transcript is increased after post-ovulatory follicle rupture, which suggests that AQP1 may play a role in the process of follicular to luteal transformation [96]. Researchers suggest that low AQP1 expression is a manifestation of luteal phase insufficiency [97]. AQP3 mainly distributes in primary oocytes, corpora lutea, and oviducts [83]. Research found that secondary follicles with knockdown of AQP3 gene expression were less prone to follicular lumen than those without knockdown [98]. Another study showed that AQP3 expression continued to increase in developing follicles, but decreased after complete follicular maturation, indicating that AQP3

contributes to follicular development of mature follicles [99]. AQP4 is expressed on theca and granulosa cells, albeit at a lower intensity compared to the previously mentioned AQPs. AQP4 mRNA expression increases during the early stage of ovulation, and AQP4 may be involved in the production of the follicular antrum [96]. In short, AQP1 increased in granulosa cells during and after ovulation, and AQP2 and AQP3 increased significantly during early ovulation, while AQP4 decreased from before ovulation to early ovulation. These changes indicated that AQP1 played a role in the process of luteogenesis, while AQP2 and AQP3 played a role in the process of follicular rupture.

In addition, AQP6 [100] and AQP7 are expressed in the cytoplasm and plasma membrane of human ovarian epithelial tissues and parietal granulosa cells, respectively. Interestingly, AQP7 expression in granulosa cells is positively correlated with female fertility and normal folliculogenesis [95].

AQP8 is expressed in granulosa cells of mouse and rat ovaries, but is found in ovarian tissue of healthy women [101], which has only been reported to be present in epithelial tumor tissues [100]. In AQP8 knockout mice, ovarian granulosa cell apoptosis was reduced and the number of mature follicles was increased, compared with wild-type mice [102]. Subsequent studies found that AQP8 knockout mice had a significantly increased number of multiple oocyte follicles (MOFs), which are MOFs that contain multiple oocytes in one follicle [103]. Further research shows that the number of antral follicles in the ovaries of AQP8 knockout mice was significantly increased, and the formation of follicular cavities in AQP8 knockout follicles cultured in vitro was significantly increased, indicating AQP8 plays an important role in the development and maturation of follicles. The mechanism may be related to the increase of intracellular space caused by the defect of proliferation and migration of granulosa cells [104].

Studies on ovine follicles have shown that AQP3 and AQP9 play an important role in the process of antral follicle formation, while AQP7 has only appeared before antral formation and has little association with antral follicle formation [105]. During the observation of follicular maturation in rats, it is found that AQP9 only appeared in follicles in proestrus, while it disappeared in estrous follicles, indicating that AQP9 expression is not required during follicular maturation [106].

12.2.6 Aquaporins in Oocyte

Oocytes undergo meiosis during oogenesis, including primary oocytes and secondary oocytes. Oocyte maturation is accompanied by expansion and spread of the cumulus cell-oocyte complex (COC). AQP3 and AQP7 are expressed in human and mouse oocytes [107], are permeable not only to water but also to small neutral solutes, such as glycerol and urea [108]. This is important for oocyte cryopreservation. On comparison of the expression of AQP3 between oocytes matured and cultured in vitro and immature oocytes in vivo by superovulation treatment of ICR female mice, the results showed that the mRNA levels of AQP3 significantly increased during oocyte maturation [99].

It was found that AQP8 knockout mice had significantly increased COC, enhanced follicular development, and increased litter size and improved reproductive performance of offspring of knockout mice [102, 109]. Some experts have found that there was a significant difference in the expression of rat *Aqp9* gene between immature and mature follicles, the permeability of immature follicles to water was significantly higher than that of mature follicles. After follicular maturation, AQP9 mRNA expression also disappeared correspondingly [106].

12.3 Aquaporins and Sperm Cryopreservation

It was found that AQP3 and AQP7 increase cryopreservation ability for porcine sperm, as evaluated by recovery of sperm survival and motility after thawing [110]. In cattle, both AQP3 and AQP7 are associated with sperm cold tolerance [111]. Studies found that the relative

content of AQP11 is not only related to the higher resilience of sperm to cryopreservation but also to the fertilization results of frozen-thawed sperm [112]. As far as equine spermatozoa are concerned, AQP3 and AQP11 are related to their cold tolerance. Fresh ejaculates with higher relative levels of AQP3 and AQP11 are more likely to have higher viability after thawing [113].

12.4 Aquaporins and Reproductive Diseases

12.4.1 Varicocele

In Leydig cells, AQP1 acts as a key reabsorption factor in reducing intracellular and extracellular abnormal fluid retention matrix [114]. The findings suggest that AQP1 reduced excessive intratubular and extratubular fluid caused by varicocele. A study on the testis of adolescents with varicocele showed that AQP9 was absent in the testis of these adolescent patients [11]. The authors concluded that AQP9 may play a role in the transport of lactate from SCs to developing germ cells, leading to lactate deprivation in developing germ cells of varicocele patients [11]. Lactate is known to be the preferred substrate for energy production by developing germ cells [115]. Therefore, lactate deficiency due to AQP9 downregulation may explain the slow rate of spermatogenesis reported in patients with varicocele [11].

12.4.2 Endometriosis

Endometriosis (EMS) is defined as the presence of active endometrial cells in sites other than endometrial coverage, with three subtypes: superficial EMS, ovarian EMS, and deep infiltrating EMS. The recognized mechanism of EMS is the occurrence of offsite adhesion-invasion-angiogenesis and abnormal immune system function in the eutopic endometrium. It was found that the expression levels of AQP2, AQP5, and AQP8 in eutopic endometrial cells were significantly higher than those in ectopic endometrial cells, and the migration activity of eutopic endometrial cells was higher than that of ectopic endometrial cells, suggesting that AQP promotes the migration of eutopic endometrial cells [79].

During the exploration of the specific mechanism for single AQP, it was found that AQP2 could also regulate the migration and invasion of intimal cells by altering the expression of F-actin and annexin 2 as well as the reorganization of F-actin [80]. In addition, estrogen is key to EMS regulation. AQP2, on the other hand, is regulated by estrogen, so changes in the expression level of estrogen in endometrial cells can affect the migration ability of cells [116]. It has been found that AQP2 can inhibit cell invasion by decreasing the activity of estrogen. AQP2 knockdown can downregulate the expression of estrogen, while estrogen can induce morphological change in endometrial cells [117]. In addition, AQP4 knockdown can also reduce estrogen and progesterone levels, which in turn inhibit cell invasion [118]. These reports suggest that AQP2 and AQP4 may affect cell migration by regulating the expression of estrogen, while estrogen is regulated and secreted by neurotransmission, so there may be a relationship between AQP and neurotransmission.

The generation of new blood vessels is key to the development of EMS, and high AQP1 expression was found in ectopic lesions of EMS patients, preliminarily demonstrating that AQP is closely related to angiogenesis in EMS. In recent years, it has been found that hypoxia-inducible factor family is widely involved in tumor formation. High expression of hypoxia-inducible factor under hypoxic conditions can lead to tumor development. The expression level of AQP is closely related to the stability of hypoxia-inducible factor 1α, while low expression of AQP1 and AQP5 and their mRNA ensures the stability of hypoxia-inducible factor 1α [119]. Therefore, it is speculated that AQP1 and AQP5 may promote the formation of blood vessels in ectopic endometrial tissues by interfering with the stability of the hypoxia-inducible factor family. The vascular endothelial growth factor/nuclear factor κB signaling pathway can promote the development of miR-138-

induced exosome-mediated inflammation and apoptosis in the blood and tissues of EMS patients [120]. It has been found that the secretion of vascular endothelial growth factor is positively correlated with the expression of AQP5, so AQP5 gene silencing may reduce EMS angiogenesis ability and thus inhibit EMS formation [121].

After ectopic implantation of endometrial cells, abnormal expression of excessive proliferation is formed through a series of changes in proliferation and secretion. Proliferative nuclear antigen is an indispensable factor in the DNA synthesis period of cells [122]. Compared with eutopic endometrium, there are a large number of proliferative nuclear antigens in ectopic endometrium. AQP5 gene silencing inhibits vascular cell adhesion molecule-1, thereby reducing the proliferation and migration of ectopic endometrial glandular epithelial cells, and the mechanism may be related to protein kinase B activation. AQP3 is highly expressed in epithelial cells and glandular epithelial cells. Studies have shown that there is a correlation between AQP3 expression and tumor grade. AQP3 inhibits cell differentiation and promotes cell proliferation [108]. AQP5 is able to stimulate estrogen secretion, facilitate ectopic endometrial cell implantation, promote ectopic cell proliferation in EMS, and then promote the formation of ectopic lesions [123].

12.4.3 Cervical Cancer

Cervical cancer, the most common of gynecological malignancies, is one of the leading factors in the cause of cancer death in women worldwide. Human papillomavirus (HPV) infection and other carcinogenic factors play a key role in the development of cervical cancer. The important factor is the downregulation of telomerase catalytic subunit human telomerase reverse transcriptase (hTERT) expression, increasing telomerase activity. AQP1 is expressed in capillaries of human endometrial stroma and endothelial cells of small vessels. The decrease of AQP1 in the endometrium of patients with dysfunctional uterine bleeding may lead to poor angiogenesis. AQP1 may be involved in the development of the disease [124]. A study found that the ratio of AQP1/IMD (intratumoral microvessel density) was high in the endometrial adenocarcinoma group, followed by the endometrial dysplasia group, and low in the normal proliferative endometrium group. In endometrial adenocarcinoma, the ratio of AQP1/IMD was significantly correlated with the grade of adenocarcinoma, surgical stage, invasion, and extrauterine metastasis. The expression of AQP1 was positively correlated with the expression of IMD and vascular endothelial growth factor (VEGF). It is speculated that AQP1 may be involved in the angiogenesis of tumors and thus related to the development of endometrial adenocarcinoma. Additional studies have found that decreased expression of AQP1 can reduce the formation of uterine spiral arteries. AQP1 may be used as a marker of tumors [125]. AQP1 was expressed on capillaries and small vessel endothelial cells in normal ovarian tissue, while AQP5 was expressed in ovarian tumor cells. As ovarian epithelial tumors progress from benign to malignant, the expression of AQP1 and AQP5 gradually increases. The expression of AQP1 in ovarian cancer with clinical stage III and IV was significantly higher than that in stage I and II. The expression of AQP1 and AQP5 was positively correlated with ascites volume. The high expression of AQP1 and AQP5 was related to the occurrence, development, and prognosis of ovarian cancer, which may be the main cause of ascites in ovarian cancer [89].

12.5 Conclusions and Prospects

AQPs are widely distributed in the male and female reproductive tract and germ cells and play key roles in the regulation of water and solute concentrations in the reproductive tract, which affects fertility. Differences in AQP expression patterns between healthy and sick individuals may be applied as potential biomarkers for evaluating male and female reproductive health. The current understanding of the function and regulation of AQPs in the human male and female reproductive tract and gametes is still in its infancy. Further studies on humans and

transgenic animal models will provide new information of some reproduction-related diseases related to water and energy metabolism and novel strategies for clinical treatment of reproductive diseases.

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Aquaporins in Immune System

13

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Abstract

Recent studies have shown that at least six aquaporins (AQPs), including AQP1, AQP3, AQP4, AQP5, AQP7, and AQP9, are expressed in immune system. These AQPs distribute in lymphocytes, macrophages, dendritic cells, and neutrophils, and mediate water and glycerol transportation in these cells, which play important roles in innate and adaptive immune functions. Immune system plays important roles in body physiological functions and health. Therefore, understanding the association between AQPs and immune system may provide approaches to prevent and treat related diseases. Here we will discuss the expression and physiological functions of AQPs in immune system and summarize recent researches on AQPs in immune diseases.

Keywords

Water · Immune cells · Macrophage · Dendritic cells · Neutrophils

13.1 Introduction

The immune system is a network of interactions among lymphoid organs, cells, humoral factors, and cytokines. It provides defense against pathogens and functions to maintain tissue homeostasis for the life of the organism. The immune system can be divided into innate immunity and adaptive immunity, although in practice there is much interaction between them. Innate immunity bases on physical, chemical, and microbiological barriers, and also includes the elements of the immune system (neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins), which provide immediate host defense. Adaptive immunity response consists of antigen-specific reactions through T lymphocytes and B lymphocytes. Innate immune cells respond quickly, while adaptive immune cells have a delayed response and may take days to fully develop but continue to form immunological memory [1, 2].

The ability of immune cells to communicate and to shift shape is critical to their function, such

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as the secretion of chemokines and cytokines, migration, phagocytosis, and antigen uptake [1]. These diverse functions of immunity are mostly dependent on cell membrane for signal transduction and maintaining the homeostasis of the microenvironment. In fact, responding to the external states, the cells regulate their internal microenvironment. Failure on regulating fluid movement across plasma membrane leads to intense alterations of cell physiology. AQPs are a class of protein channels that are expressed on cell membrane and mediate water and small uncharged molecules (such as glycerol or hydrogen peroxide) across the membrane. At present, 13 AQP homologous molecules (AQP0-AQP12) have been identified in mammals, which are widely distributed in various tissues and organs of the body. According to their transportation capacity, they are divided into three subgroups: water selective AQPs (AQP0, 1, 2, 4, 5, 6, 8), aquaglyceroporins (AQP3, 7, 9, 10), and superaquaporins (AQP11, 12) [3]. A number of AQP isoforms are expressed in a variety of immune cells and participate in processes such as phagocytosis and migration.

13.2 Expression of AQPs in Immune System

There are six AQPs expressed in the immune cells including AQP1, AQP3, AQP4, AQP5, AQP7, and AQP9. They distribute in lymphocytes, macrophages, dendritic cells, and neutrophils, participate in water and glycerol transportation (Table 13.1), which play important roles in innate and adaptive immune response.

13.3 Functions of AQPs in Immune System

13.3.1 Immune Cell Priming

AQPs isoforms were shown to be upregulated during immune cell priming and activation. In human, AQP1, AQP3, and AQP5 are expressed in activated B and T lymphocytes. AQP3 and AQP5 are expressed in immature dendritic cells. However, none of these AQPs is expressed in inactivated B or T lymphocytes [4]. In human leukocytes, AQP1 was detected and upregulated after in vitro lipopolysaccharide (LPS) stimulation. AQP1 expression was induced at the onset of sepsis and was further increased in leukocyte during septic shock [18]. In addition, AQP9 expression is enhanced in activated polymorphonuclear leukocytes from patients with systemic inflammatory response syndrome [19]. The gene expression analysis of blood from patients with infective endocarditis also revealed that AQP9 is upregulated and significantly associated with the occurrence of acute heart failure. AQP9 is also expressed on macrophages and plays a role in the process of *Pseudomonas aeruginosa* infection [26]. The *P. aeruginosa* infections increase and relocalize AQP9 expression to the leading and trailing regions in macrophages, which changes cell area and length. These processes are related to water flux across cell membrane through AQP9 [20, 21].

13.3.2 Migration, Phagocytosis, and Antigen Uptake of Immune Cells

Macrophages take up residence in all tissues of the body and are mostly relatively long-lived. They are adept phagocytic cell, capable of migrating to the infection sites and swallowing invading pathogens, foreign substances, and apoptotic cells. Macrophages induce inflammation by producing cytokines and chemokines that both attract and activate other immune cells to the site of infection. Mouse resident peritoneal macrophages (mRPMs) express the AQP3 in plasma membrane. It has been shown that AQP3 is involved in the phagocytosis and migration of macrophages mediated by the water- and glycerol-transporting functions. In the model of bacterial peritonitis, AQP3 null mice showed remarkably reduced survival than wild-type mice. Compared to the wild-type mRPMs, AQP3 null mRPMs reduced migration speed and impaired phagocytic activity and energy

Table 13.1 Expression of AQPs in immune cells of human and mouse

AQPs	Species	Immune cells	References
AQP1	Human	Lymphocytes	[4]
	Mouse	Macrophages	[5, 6]
		RAW264.7 cells	[7]
AQP3	Human	Lymphocytes	[4]
		Dendritic cells	[4, 8]
	Mouse	Macrophages	[9, 10]
		THP-1 monocytic cells	[11]
		Dendritic cells	[12, 13]
		T cells	[14]
AQP4	Mouse	T cells	[15]
AQP5	Human	Lymphocytes	[4]
		Dendritic cells	[4]
	Mouse	Dendritic cells	[16]
AQP7	Human	Dendritic cells	[8]
	Mouse	Dendritic cells	[12, 17]
AQP9	Human	Leucocytes	[18, 19]
		Macrophages	[20, 21]
		Dendritic cells	[8]
		Neutrophils	[22, 23]
		Neutrophil-like HL60 cells	[23]
	Mouse	Neutrophils	[23, 24]
		Mast cells	[24]
		Dendritic cells	[12]
		T cells	[25]

metabolism. In the AQP3 null mRPMs, glycerol, glucose, and ATP contents were lower than those of wild-type mRPMs. Besides, incubation of AQP3 null mRPMs with glycerol significantly increased the cellular glucose, ATP content, phagocytosis, and migration [9].

AQP1 are also associated with macrophage migration. The effect of AQP1 on macrophage migration cannot be simply summarized as promoting or inhibiting, but depends on external stimuli. Macrophage, a heterogeneous cell population, can switch phenotype depending on environmental conditions. There are two main phenotypes: classically activated macrophages (M1) and alternatively activated macrophages (M2). M1 macrophage exhibits pro-inflammatory, anti-tumor, and anti-microbial properties. M2 macrophage shows anti-inflammatory activity and is instead involved in tissue remodeling, healing, and repairing [27]. M1 and M2 macrophages have different features in cell shape, cytoskeletal organization, and migration [28]. For undifferentiated macrophages (M0), ablation of AQP1 spontaneously induces macrophage elongation, axial polarization, and membrane lipid orientation to the leading edge via Src/PI3K/Rac signaling pathway to promote migration and switch M0 to M2 phenotype. However, for M1 macrophage, the ablation of AQP1 has the opposite effect on macrophage migration. In the acute bacterial peritonitis model, AQP1$^{-/-}$ mice has a decreased number of infiltrating macrophages [5].

Neutrophils are regarded as short-lived effector cells of the innate immune system, playing a major role in acute inflammation and fighting extracellular pathogens. These cells are able to act as phagocytic cells, releasing lytic enzymes from their granules and producing reactive oxygen intermediates (ROI) with anti-microbial potential. Neutrophils are usually the first cells to extravasate into tissue in response to noxious stimuli. They are highly reactive to a variety of stimuli, especially pathogen- and

damage-associated molecular patterns (PAMPs and DAMPs, respectively). Once in the tissue, they undergo degranulation responses, oxidative burst and NETosis, promoting recruitment of additional granulocytes and phlogistic monocytes to promote inflammation and the clearance of pathogens. They also engage in complex bidirectional interactions with macrophages, mesenchymal stem cells, dendritic cells, natural killer cells, and B and T cells, and contribute to the activation, orientation, and expression of adaptive immune responses [29, 30].

AQP9 plays a key role in neutrophil motility. Targeting AQP9 with anti-AQP9 antibodies and with low concentrations of $HgCl_2$ or tetraethyl ammonium (TEA) can block chemoattractant-stimulated shape change and subsequent motility. AQP9 channels were preferentially localized at the leading edge of morphologically polarized cells, and co-localized with N-formyl peptide receptor [22]. Further research showed that the phosphorylation of AQP9 on serine 11 is essential for its proper plasma membrane localization, which is accomplished through a Rac1-dependent pathway [23].

The involvement of AQP9 in the migration of neutrophils has also been demonstrated in disease models. Hapten-induced contact hypersensitivity (CHS) can be used as a murine model of allergic contact dermatitis (ACD). Neutrophils are important for both the sensitization and elicitation phase of CHS. In $AQP9^{-/-}$ mice, ear swelling, the hallmark of CHS, was decreased as compared to wild-type mice after the challenge with hapten dinitrofluorobenzene (DNFB). AQP9 deficiency decreased the accumulation of neutrophils in skin draining lymph nodes (dLNs) during the sensitization phase of CHS. Neutrophil recruitment to dLNs after sensitization was modulated by AQP9. The efficiency of sensitized neutrophil migration toward CCR7 ligands was markedly impaired in AQP9 null cells compared with wild-type cells. These results suggest that AQP9 may have a prominent role in neutrophil migration by participating water transport in neutrophils during chemotaxis [24].

Innate immunity presenting information to adaptive immune cells requires antigen-presenting cells (APCs). Dendritic cells (DCs) are APCs that are able to induce primary immune responses, thus permitting establishment of immunological memory. In the bone marrow, DC progenitors promote circulating precursors back to tissues, where they reside as immature DC cells with high phagocytic capacity. Precursor DCs are patrolling through blood, lymphatics, and lymphoid tissues. Upon pathogen recognition, they release large amounts of cytokines, e.g. IFN-α, to limit the spread of infection. Immature DCs possess high endocytic and phagocytic capacity. After antigen capture, immature DCs migrate to lymphoid organs where, after maturation, they express high levels of costimulatory molecules permitting antigen presentation, thus communicate the presence of pathogens to the adaptive immune system thereby initiating long lasting, antigen-specific responses [31, 32].

Four AQPs have been identified in DCs, including AQP3, AOP5, AQP7, and AQP9 [4, 8, 12, 17]. Previous studies suggested the involvement of AQPs in antigen uptake in DCs. Immature monocyte-derived DCs have a high endocytic capacity and capture antigens via distinct mechanisms [33]. The endocytic activity is stronger in the immature stage and is lost as DCs mature and migrate to the secondary lymphoid organs. Among them, soluble antigens are represented by receptors or micropinocytosis. Macropinocytosis requires DCs rapidly exchanging water across plasma membrane. AQP3 and AQP7 are expressed in immature DCs and are downregulated after maturation. Functional inhibition of aquaporins in DCs decreases uptake and concentration of macrosolutes and leads to dramatic cell swelling. It indicates that AQPs play an essential role in the process of antigen uptake and concentration via fluid phase micropinocytosis [8]. AQP3 and AQP7 are also expressed in epidermal Langerhans cells and dermal DCs. Although AQP3 expression had little effect on skin DC function, AQP7 is involved in macropinocytosis and/or phagocytosis, specifically antigen uptake. Compared to wild-type cells, water/glycerol transport was impaired and the uptake of LY (Lucifer yellow CH potassium salt), FITC (fluorescein isothiocyanate),

FITC-dextran, and FITC-ovalbumin was significantly reduced in AQP7 null DCs [17].

AQP5 and AQP9 are also expressed in DCs and participate in antigen presentation. AQP5 null DCs reduce endocytosis of antigen, and if plasmids expressing AQP5 were transfected to the AQP5 null DCs, endocytosis ability is reversed. In addition, AQP5 null DCs have less expression of CD80 and CD86 than wild-type DCs before being stimulated with LPS [16]. Another study showed that AQP9 is the most frequently expressed AQP in murine bone marrow-derived dendritic cells (mBMDCs). There is a significant increase of AQP9 expression at sixth hour after LPS administration. AQP9 inhibition and AQP9 null BMDCs reduce inflammatory cytokines secretion. The anti-inflammatory cytokine IL-10 was increased in the supernatant of AQP9 null BMDCs [12].

While studying the effect of AQPs on DC antigen presentation, researchers also found that LPS administration in the absence of AQP9 results in a decreased release of chemokines [12]. Chemotaxis was significantly impaired in AQP7 null Langerhans cells (LCs) and dermal DCs (dDCs) compared to wild-type cells [16]. These data indicate that AQPs are involved in DC migration.

Regulated T cell migration and trafficking are essential for both steady-state T cell homeostasis and active immune responses. AQPs were found to be expressed in T cells and involved in adaptive immune regulation.

AQP3 is expressed on skin-infiltrating T cells and participates in skin immune regulation by affecting cell migration. In response to chemotactic signals, T cells reorganize their actin cytoskeletons and polarize in the direction of the chemoattractant gradient, which leads to chemotaxis and T cell trafficking. In AQP3 null T lymphocytes, F-actin polymerization and the Cdc42 activation in response to CXCL12 were impaired and chemotaxis efficiency reduced. These processes are related to the decrease of intracellular H_2O_2 content caused by AQP3-mediated H_2O_2 uptake but not the canonical water/glycerol transport. Moreover, AQP3 null mice showed resistance to CHS development, which is based on the trafficking of T cells to regional sites. In conclusion, AQP3 regulates T cell migration by mediating the transport of H_2O_2, and plays an important role in skin immunity [14].

AQP4 is expressed in naive and memory T cells. AQP4 blockade with a small molecule inhibitor prolongs murine heart allograft survival at least partially through inhibiting early infiltration of endogenous memory $CD8^+$ T cells and $CD4^+/CD8^+$ T cell proliferation and effector functions. CTLA4-Ig can block T cell activation and T cell-dependent B cell function. The synergistic effect of AQP4 inhibition and CTLA4-Ig prolongs the survival of heart allografts [34]. Further research investigated that how AQP4 function impacts T cells in the absence of antigen stimulation. AQP4 blockade can down-regulate transcription factor KLF2 and reduce the expression of chemokine receptors S1PR1 and CCR7 involved in T cell circulation, resulting in decreased chemotaxis of their respective ligands S1P and CCL2. Without systemic T cell depletion, AQP4 inhibition transiently reduced the number of circulating $CD4^+/CD8^+$ T cells in naïve non-transplanted mice. The experimental results indicate that AQP4 affects the normal migration and trafficking of T cell [15].

13.3.3 Inflammatory Activation

Inflammation is an important player in the immune response. AQP3 is involved in macrophage inflammation mediated by H_2O_2 transport. In a model of liver injury and fibrosis produced by CCl_4, AQP3 null mice showed a significant reduction in the secretion of chemokines and inflammatory cytokines and the elevations in serum AST and ALT in liver. AQP3 expression also affects cellular ROS levels and oxidative stress during acute liver injury. Extracellular addition of H_2O_2 produced higher intracellular H_2O_2 concentration in wild-type macrophage than AQP3 null macrophage, which acted as a secondary messenger for NF-κB activation [10].

The inflammasome is an important player in the immune response in macrophage. More

recently, reactive oxygen species (ROS), lysosomal damage, intracellular potassium (K^+) efflux, and fast cell reswelling have been considered to be related to NLRP3 activation and consequent IL-1β secretion [35]. Inhibition of AQPs in macrophages specifically during the regulatory volume decreases NLRP3-mediated inflammation [6]. AQP3, which transports glycerol and hydrogen peroxide in THP-1 cells, is involved in cell swelling induced NLRP3 activation. Moreover, AQP3 may affect nigericin induced IL-1β release by facilitating cellular K^+ efflux [11]. In terms of AQP3-mediated H_2O_2 transport, intracellular ROS rising with subsequent inflammasome activation should also be considered in the further researches.

AQP1 is also associated with macrophage inflammation. In a model of acute lung inflammation induced by crystals, AQP1 ablation in macrophages was associated with a marked reduction in NLRP3 inflammasome activation and IL-1β release and neutrophilic inflammation in the lung [6]. In LPS-induced acute kidney injury (AKI) model, macrophage phenotype has changed over time. With the increase of pro-inflammatory cytokines, the expression of AQP1 decreased. In vitro, experiments have found that the silencing of AQP1 in RAW264.7 cell enhanced the activation of p38/MAPK pathway induced by LPS [7]. However, another study showed that the comparable LPS-induced p38/MAPK activation, iNOS expression, and IκBα phosphorylation in wild-type and AQP1 null macrophages [5].

13.3.4 Other Aspects

One study investigated the role of AQP3 in development, subtypes, and activation of DCs. AQP3 depletion did not affect the development of BMDCs by GM-CSF or the Flt3 ligand and the level of expression of CD86 on unstimulated and LPS-stimulated BMDCs. However, the frequency of $CD4^+CD8^-$ cDCs was significantly lowered in the spleen of AQP3 null mice. There was higher CD103 expression in $CD4^-CD8^+$ subpopulation of splenic cDCs obtained from AQP3 null mice than in those from wild-type mice. These results suggest that AQP3 depletion may not affect the maturation of DCs but the composition of DC subtypes [13].

In addition, AQP9 is expressed in memory $CD8^+$ T cells and is essential for the T cell memory. IL-7 plays an important role in lymphopoiesis and peripheral T cell survival. Research shows that IL-7 induces AQP9 expression in virus-specific memory $CD8^+$ T cells, but not naive cells. AQP9 promotes glycerol import into memory $CD8^+$ T cells for fatty acid esterification and triglyceride (TAG) synthesis and storage, which is beneficial to the survival and homeostasis of $CD8^+$ memory T cells [25].

13.4 AQPs and Immune Disease

Allergic contact dermatitis (ACD) is one of the most prevalent skin diseases, which has two phases including sensitization and elicitation. It is classified as a delayed-type hypersensitivity response. Murine contact hypersensitivity (CHS) is one of the most frequently used animal models of ACD. The important role of AQPs in immune cells in ACD have been implicated from studies on CHS. The CHS response to hapten dinitrofluorobenzene (DNFB) was impaired in AQP7 null and AQP9 null mice compared with wild-type mice. AQP7 is expressed in mouse DCs and involved in antigen uptake and chemokine-dependent cell migration. AQP7 deficiency decreases accumulation of antigen-retaining DCs in the LNs after antigen application to the skin [17]. AQP9, which expressed in neutrophils, is important for the sensitization phase of CHS. AQP9 null neutrophils showed a reduced CCR7 ligand-induced migration efficacy and decreased IL-17A production by dLN cells [24]. These findings suggest that blocking AQPs by the use of topical drugs might be a treatment strategy for ACD.

13.5 Conclusion and Prospect

AQPs are involved in a variety of important physiological processes in the immune cells by coordinating water and solutes trafficking. In a variety of immune cells, AQPs are involved in cell migration and immune response. However, we are still far from having a full comprehension of the physiological and pathological significance of all AQPs in the immune system. In view of the important role of immune system in various diseases, we believe that it is necessary to further explore the function of AQPs in immune cells. Targeting AQPs may be one of the feasible strategies for many diseases.

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Aquaporins in Eye

14

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Abstract

The major part of the eye consists of water. Continuous movement of water and ions between the ocular compartments and to the systemic circulation is pivotal for many physiological functions in the eye. The movement of water facilitates removal of the many metabolic products of corneal-, ciliary body-, lens-, and retinal metabolism, while maintaining transparency in the optical compartments. Transport across the corneal epithelium and endothelium maintains the corneal transparency. Also, aqueous humor is continuously secreted by the epithelia of the ciliary body and maintains the intraocular pressure. In the retina, water is transported into the vitreous body and across the retinal pigment epithelium to regulate the extracellular environment and the hydration of the retina. Aquaporins are a major contributor in the water transport throughout the eye.

Keywords

Aquaporins · Eye · Water transport

14.1 Introduction

Precise regulation of ocular fluids is necessary for the optimization of visual function, as the greater part of the eye is comprised of water [1]. Continuous movement of water and ions between the ocular compartments and to the systemic circulation is essential. The movement of water facilitates removal of the many metabolic products of corneal-, ciliary body-, lens-, and retinal metabolism, all while maintaining transparency in the optical compartments [1]. The transport of water across the corneal epithelium and endothelium maintains corneal transparency. Rapid changes in the water content of the iris stroma facilitate changes in shape during pupil constriction and dilatation. In the retina, water is transported transcellularly across the retinal pigment epithelium into the choroid preventing subretinal edema and retinal detachment [1, 2]. The intraocular pressure (IOP) is maintained by the aqueous humor [3]. Aqueous humor is secreted by the pigmented and nonpigmented epithelia of the ciliary body in a concerted action involving active membrane proteins and passive ion and water channels [3]. Aquaporins (AQPs) are expressed in several of these structures and facilitate the transport of water (Fig. 14.1). AQP0 contributes to lens transparency, and AQP1 is involved in the secretion and drainage of aqueous humor. AQP3 and AQP5 have corneal and conjunctival barrier functions, and AQP4 plays an important role in retinal water

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homeostasis [2, 4, 5]. AQP9 has been suggested to provide neurons with lactate and glycerol for energy metabolism [6]. AQP7 and AQP11 have also been localized to various epithelia in the human eye with unclarified function [7].

14.2 Expression of AQPs in Eye

14.2.1 Cornea

The cornea is the first light refractive medium when light hits the eye and the transparency of the cornea relies on the precisely organized stroma consisting of collagen fibrils and glycosaminoglycans. Precise regulation of the water content is required to keep the stroma neatly packed avoiding light scatter. The water content of the cornea is 78% by weight and negatively charged glycosaminoglycans and proportionate cations make the cornea slightly hyperosmolar compared to the aqueous humor [8, 9]. Consequently, the continuous transport of solutes and steady water expulsion away from the cornea is needed. The corneal endothelium expresses AQP1 and is responsible for transport of the major part of water out of the corneal stroma [5, 8, 10]. The corneal endothelium contains active transporters (Na^+/K^+ -ATPase, $Na^+/K^+/2Cl^-$, HCO_3^-/Cl^-) that pump solutes from the stroma into the aqueous humor. Water is transported passively, driven by the osmotic gradient, into the anterior chamber partly through AQP1 but also paracellularly [8, 10]. In addition, AQP1 expressed by the keratocytes has been suggested to facilitate volume changes in the keratocytes in response to changing corneal hydration [5, 11].

The outer stratified epithelium of the anterior corneal epithelium expresses AQP3 and AQP5, and facilitates water transport away from the cornea. Deletion of AQP5 in mice increases the corneal thickness and reduces the osmotic water permeability across the corneal epithelium [5, 8]. However, when exposed to hyperosmolar stress no noticeable changes occur during swelling and recovery. Mainly the corneal endothelium maintains the hydration of the cornea, therefore, transport via AQP5 across the epithelium does not affect the overall transparency of the cornea [8].

Maintenance of the corneal epithelium is crucial in providing a smooth and transparent refractive surface [2, 12]. The stratified corneal epithelium expresses the water- and glycerol transporting AQP3 [5, 12]. During reepithelialization AQP3 facilitates the water and glycerol transport in the corneal epithelial cell migration and proliferation [12]. AQP3-facilitated cell migration has also been demonstrated in wound healing of the skin [13]. In the migration phase of reepithelialization, marginal cells extend lamellipodia and filopodia at the wound's leading edge and AQP3 provides for the local water transport here. Corneal epithelial cell migration also requires mobilization of energy stores, particularly glycogen [12]. Defective glycerol transport in AQP3 deficiency may impair the glycogen synthesis or utilization by direct or indirect effects on glycolysis [13]. AQP3 deletion in mice demonstrated reduced glycerol permeability but the steady state corneal epithelial glycerol content was not significantly affected [12]. However, a significant delay in resurfacing was found and the AQP3-deficient corneas were thinner suggesting impaired proliferation in AQP3 deficiency.

Furthermore, during corneal wound healing AQP1 may facilitate keratocyte migration through AQP-facilitated water influx into lamellipodia at the leading edge of migrating cells [14]. Also, AQP5 may promote corneal wound healing through higher levels of cell migration and proliferation [15]. AQP7 has been localized in the corneal epithelium and endothelium and AQP11 is expressed in the basal cells of the anterior epithelium the corneal-limbal region, however, the function of these aquaporins here is not known [7].

14.2.2 Trabecular Meshwork

AQP1 is expressed by the endothelial cells of the trabecular meshwork (TM) and Schlemm's canal [5, 16, 17]. In the conventional outflow pathway, fluid drainage is predominantly paracellular and

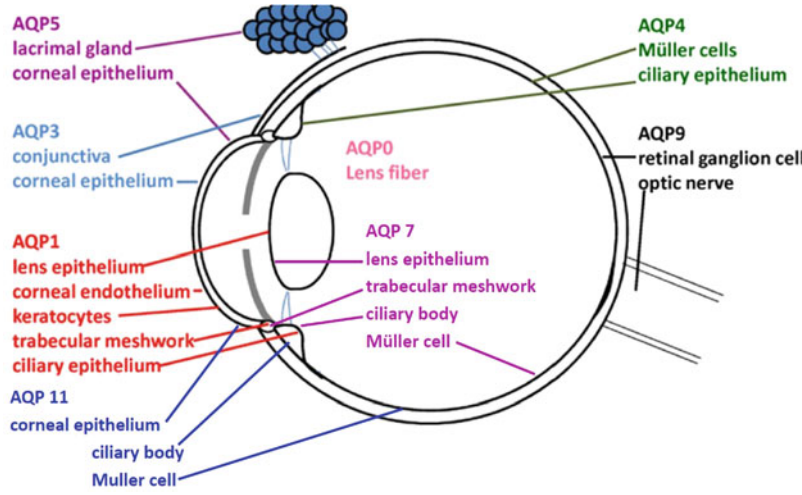

Fig. 14.1 Schematic drawing of the cellular localization of AQPs in eye

therefore AQP1 might not directly regulate the aqueous humor outflow [17]. Rather, AQP1 may regulate the TM endothelial intracellular volume and indirectly modulate the paracellular drainage [16]. Also, AQP1 may contribute to endothelial cell survival to resist the mechanical strain during the passage of aqueous humor [17]. A study showed that downregulation of AQP1 by endothelin-1 resulted in glaucomatous changes such as actin fiber reorganization, collagen production, extracellular matrix deposition, and contractility alteration of TM cells [18]. AQP7 is also expressed in the trabecular meshwork endothelium [7].

14.2.3 Ciliary Body

Aqueous humor provides nutrients and removes metabolic waste product from the avascular structures in the anterior eye, namely the cornea and the lens. Furthermore, aqueous humor maintains the intraocular pressure (IOP) [3]. Aqueous humor is secreted by the ciliary body through combined actions of active pumps and AQPs in the ciliary epithelia [19]. The ATP-consuming pumps and channels transport ions and major solutes from the ciliary capillaries into the posterior chamber. AQP1 and AQP4 facilitate the major part of the passive water transport in the apical and basolateral membrane of ciliary nonpigmented epithelial cell [5, 19]. AQPs are not expressed in the ciliary pigmented epithelium, and water may be actively cotransported into the pigmented epithelial layer together with major solutes [20]. AQP7, AQP9 and AQP11 are all expressed in the ciliary nonpigmented epithelium [7].

14.2.4 Lens

The lens is another important refractive structure and transparency of the lens is equally important. The lens is avascular, and therefore, transport of oxygen, nutrients and ions occur by diffusion and active transport across the epithelial layer from the aqueous humor [21, 22]. The transport of water into the lens is mediated by AQP1 in the epithelial cells and AQP0/MIP (major intrinsic protein) expressed by the lens fibers [21, 22]. AQP0 either functions as a water channel or a structural protein depending on the molecular changes and post-translational modifications occurring during shifts in the spatial location of the lens fibers [22].

In the cortical fibers, AQP0 interacts with other proteins such as connexins, filensin, phakinin, and crystallins and AQP0 mediates passage of water. However, in the nuclear fibers cleaving of AQP0 results in conformational changes and closes the water transporting pores

of AQP0. The AQP0 function switches from water channel to adhesion molecule, since AQP0 no longer interacts with the surrounding proteins [21, 22]. Instead, AQP0 forms tight junctions and assists in maintaining minimal space between the fibers. Thus, AQP0 facilitates microcirculation and also interfiber adhesion within the lens and consequently contributes to maintaining transparency of the lens. AQP7 is also expressed in the lens epithelium [7].

14.2.5 Retina

In the retina, considerable amounts of water are produced during the large metabolic turnover [4, 23]. Cotransport of water during uptake of metabolic glucose and lactate from the blood also contributes to the water content [23, 24]. Furthermore, hydrostatic forces driven by the intraocular pressure also push water to enter the retina from the vitreous body. Therefore, significant amount of water has to be cleared to maintain local balance of ions for effective signal transduction [4, 25].

In the inner retina the major glial support cells, the Müller cells, redistribute water, and ions [4, 23]. Neuronal activity accompanying synaptic transmission results in a transient increase in $[K^+]$ in the plexiform layers of the retina and a decrease in the extracellular $[K^+]$ in the subretinal space. Rapid removal of K^+ is important to maintain neuronal excitability during prolonged light stimulation. The Müller cells regulate the K^+ balance by uptake of K+ through Kir4.1, the inwardly rectifying potassium channel, and siphoning K^+ into the vitreous body or retinal capillaries [4, 23]. AQP4, co-localized with Kir4.1, facilitates the accompanying osmotic water transport in response to the K^+-flux and together they maintain the spatial buffering of $[K^+]$ [4].

The RPE cells are responsible for clearing metabolic waste product, neurotransmitters, excess ions and water from the outer retina, and subretinal space through active transport and solute-linked transport [26, 27]. Cotransport proteins known to facilitate transcellular ion movement elsewhere in the body also function as molecular water pumps in the retina in the absence of an osmotic gradient [20]. AQPs may also contribute to ion- and water elimination in the RPE cells. However, AQP1 has only been identified in the cell membrane of cultured cells [5, 28]. Interestingly, AQP1 expression has been found in RPE cells overlying retinal drusen suggesting that fluid transport is altered across the drusen [29].

AQP9 is expressed by the retinal ganglion cells (RGC) [5, 30, 31], and has formerly been found in the brain, primarily in the astrocytes [6]. AQP9 has been suggested to provide neurons with lactate and glycerol for energy metabolism [6, 23, 32]. The presence of AQP9 in both the brain and retina is not surprising given their close connection. Accordingly, AQP9 may have a similar function in the retina and brain and may facilitate the uptake of lactate or glycerol into the RGCs and photoreceptors [31, 33]. Animal models show that downregulation of AQP9 negatively affects RGC survival [34, 35]. In the retina, the Müller cell endfeet showed AQP7 and AQP11 labeling [7].

AQP6 has been localized to the outer plexiform layer in the rat retina, but not confirmed in human retina [36].

14.2.6 Lacrimal Gland

In the lacrimal gland, AQP5 is expressed in the apical membranes of the acinar and duct cells, AQP4 in the basolateral membranes of the acinar cells, and AQP1 in microvascular endothelia. AQP3 is expressed in the basolateral membrane of the acinar cells [5]. AQPs expression are essential in exocrine glands such as the lacrimal gland, and salivary and sweat glands [2]. However, knock out mice lacking AQP1, AQP4, AQP3 or AQP5 does not show decreased tear production [37]. It is suggested that as the fluid secretion by the lacrimal gland is substantially lower than salivary glands the secretion does not rely on AQP-dependent water transport. AQP5 may therefore regulate the osmolality of tears rather than tear production [37].

14.3 Functional Abnormality of AQPs in Eye and Diseases

AQPs are present in all structures in the eye that have a water regulating function and this poses the question whether dysfunction or inhibition of AQPs lead to pathology, especially in diseases where oedema is a prominent clinical observation. In Fuch's endothelial dystrophy and pseudophakic bullous keratopathy, oedema of the cornea is the central problem. Downregulation of AQP1 in the corneal endothelium has been demonstrated in these keratopathies [38]. Healing of corneal abrasions is linked with AQP3 expression by the corneal epithelium, as deletion of AQP3 delays epithelial cell resurfacing and results in a thinner epithelium [12, 13].

The role of AQP0 in the lens has been clearly demonstrated and several mutations in AQP0 or any of the molecules interacting with AQP0 result in cataract formation [21]. Complex coordination of the lens proteins is required, as each protein contributes to lens transparency, appropriate refractive index, and accommodation. All characterized mutations in AQP0 result in autosomal dominant bilateral cataract [21, 22].

In glaucoma, several structures in the eye are involved. The ciliary body, the trabecular meshwork, the retina, and the optic nerve may be coupled with the development of glaucoma. Deletions in AQP1 and AQP4 result in modest reductions in IOP and aqueous humor production [39]. AQP1 and AQP4 null mice show preserved anterior chamber morphology. Wu et al. (2020) showed that specific AQP1 deletion in the ciliary body through gene therapy resulted in significant IOP reduction in both normal eyes and in a glaucoma model [40].

AQP9 has been shown to be downregulated with increased intraocular pressure [31]. Downregulation of AQP9 expression by RGC has been shown to be coupled with RGC metabolism and apoptosis [31, 35, 41].

Diabetes mellitus and age-related macular degeneration (AMD) are two major reasons for impaired vision. Severe stages of both diseases involve neovascularization resulting in retinal oedema due to leakage from the newly formed capillaries. The excess fluid exceeds the capacity of the glial cells and RPE cells in clearing fluid from the retina. Changes in the expression of ion channels and transporters, and of AQPs may intensify the retinal oedema. Additionally, AQP expression has been demonstrated to be altered in a complex pattern in the diabetic rat retina [42].

Finally, in Sjögrens syndrome a modified AQP5 distribution, and presence of anti-AQP5 autoantibodies has been described [43, 44].

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Aquaporins in Skin

15

Zhuming Yin and Huiwen Ren

Abstract

The skin is the largest organ of our body and plays a protective role against the external environment. The skin functions as a mechanical and water permeability barrier, assisting with thermoregulation and defending our body against a variety of stresses such as ultraviolet radiation, microbial infection, physical injuries, and chemical hazards. The structure of the skin consists of three main layers: the hypodermis, the dermis, and the epidermis. Aquaporins (AQPs) are a family of integral membrane proteins whose function is to regulate intracellular fluid hemostasis by facilitating the transportation of water, and in some cases small molecules, across the cell membranes. Up to six different AQPs (AQP1, 3, 5, 7, 9, and 10) are expressed in a variety of cell types in the skin. The AQP family plays an important role in these various locations, contributing to many key functions of the skin including hydration, wound healing, and immune responses. The involvement of different aquaporin family members in skin is discussed.

Keywords

Aquaporins · Epidermis · Skin · Psoriasis · Skin in cancer

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15.1 Introduction

Skin is the largest organ of our body and plays a protective role against the external environment. The skin functions as a mechanical and water permeability barrier, assisting with thermoregulation and defending our body against a variety of stresses such as ultraviolet radiation, microbial infection, physical injuries, and chemical hazards [1]. Skin is also the first organ seen by others, such that some skin abnormalities or diseases are often immediately apparent and can negatively affect our social interactions.

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Aquaporins (AQPs) are a family of integral membrane proteins whose function is to regulate intracellular fluid hemostasis by facilitating the transportation of water, and in some cases small molecules, across the cell membranes [2]. Up to six different AQPs (AQP1, 3, 5, 7, 9, and 10) are expressed in a variety of cell types in the skin (Fig. 15.1) [3]. AQP1 is mostly localized in the walls of skin blood capillaries, while AQP3 has been identified mostly in the epidermal layer of the skin. AQP5 is mostly observed in the secretory glands of skin and sometimes in the epidermis of the thick skin of the palms [4]. AQP7 is located primarily in fat tissues in the hypodermis and dendritic cells in dermal layer [5]. Both AQP9 and 10 are located in the epidermis. The AQP family plays an important role in these various locations, contributing to many key functions of the skin including hydration, wound healing, and immune responses. Thus it is necessary to clarify how the localization and function of this channel are regulated in order to find out effective treatment strategies for relative skin diseases.

15.2 Expression of AQPs in Different Layers of Skin

The structure of skin consists of three main layers: the hypodermis, the dermis, and the epidermis (Fig. 15.1). The hypodermis is the deepest layer of skin and is mainly comprised of adipose tissue that serves as a calorie reservoir and an insulator of the body [6]. The dermis is located in between the layers of epidermis and hypodermis. Its main role is to provide support and nutrition to the epidermis. It is composed primarily of connective tissue that is rich in extracellular matrix and fibroblasts. The hair follicles, sweat glands, sebaceous glands, capillaries, and nerves are also located in this layer. The epidermis is the outermost layer of the skin, which is comprised of a variety of cell types. The keratinocytes are the main constituents of the skin barrier, accounting for approximately 90% of the epidermal cells. They can be further divided into four sublayers from deep to superficial: the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. An additional layer called the stratum lucidum, located in between the stratum granulosum and stratum corneum, exists in areas of thick skin such as the palms of hands and soles of feet. The epidermal melanocytes are pigment-generating cells that synthesize the melanin to absorb the harmful environmental ultraviolet light energy and protect the skin from oxidative stress and DNA damage [7]. The Langerhans cells in the epidermis are antigen-presenting dendritic cells, while the Merkel cells assist in pressure sensation by the skin.

The epidermal turnover process is extremely important for normal skin function, as well as wound healing and some skin diseases. It takes about 4 weeks for the unmature keratinocytes to keratinize and form an impermeable layer of the stratum corneum. The physiological process is basically the proliferation, differentiation, and maturation of keratinocytes. It is initiated by the basal progenitor cells in the stratum basale adjacent to a collagenous basement membrane. The basal stem cells proliferate and migrate superficially to regenerate other cells in the epidermis [8] Apart from the characteristics of proliferation, the basal layer also expresses the immature keratins keratin 5 and 14, which dimerize and combine as the cytoskeleton to provide mechanical stability to the keratinocytes [9]. After leaving the stratum basale, the keratinocytes migrate upward into the stratum spinosum layer. The spine-like cells stop proliferating and begin to express mature keratins such as keratin 1 and keratin 10, which make up keratin intermediate filaments that provide mechanical stability to the epidermis [8]. In addition, there is an upregulation of desmosomes and involucrin, leading to increased intercellular adhesion in the stratum spinosum.

15.2.1 AQPs in Epidermis

AQP3 is the most abundant aquaporin in the epidermal layer of the skin and has been studied comprehensively and deeply. AQP3 is an aquaglyceroporin, able to transport glycerol and hydrogen peroxide in addition to water [10–12]. AQP3 is mainly expressed on the plasma

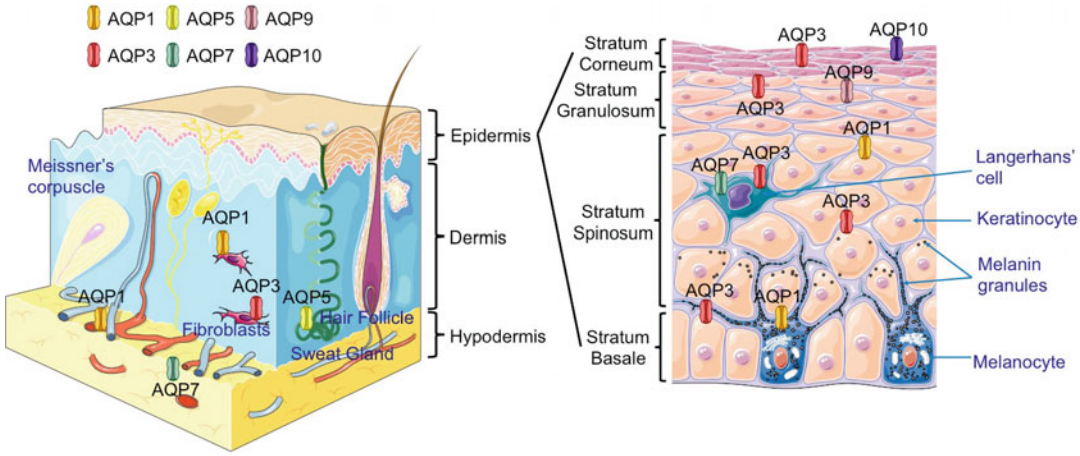

Fig. 15.1 Summary of skin structure and localization of AQPs in the skin. The structure of the skin consists of three main layers: the hypodermis, the dermis, and the epidermis (from deep to superficial). The hypodermis is mainly comprised of adipose tissue. The dermis is composed primarily of connective tissue which is rich in fibroblasts. The keratinocytes are the main constituents of the epidermis. They can be further divided into four sublayers: the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum. There are at least six different AQPs (AQP1, 3, 5, 7, 9, and 10) are expressed in the skin. The localization of each AQP in various cells of the skin is illustrated in this figure

membrane and intracellular compartment of keratinocytes in the basal layer [13]; in fact, however, AQP3 has also been detected to localize on the plasma membrane of keratinocytes in the stratum spinosum [14–17] and even in the stratum corneum [18]. The membranous expression is consistent with the findings in the in vitro differentiation test [19], but its aberrant expression and localization may cause a series of skin disorders [20].

AQP1 is the first-discovered member of AQP family with the well-established role in maintaining tissue water balance and osmotic gradients. In the epidermis, it has been detected in melanocytes and keratinocytes [21]. AQP5 is predominantly expressed in the plasma membrane of keratinocytes in the stratum granulosum of thick skin areas [4]. AQP9 is another type of AQP family that is located in many tissues throughout the human body, including the epidermis. It is specifically located in the layer of stratum granulosum above the level where AQP3 is commonly seen [22]. AQP10 is found in the keratinocytes of the epidermis. It is commonly localized in the outmost layer of the skin, the stratum corneum and may play a role in the water permeability barrier formation [3, 18].

15.2.2 AQPs in Dermis and Hypodermis

To begin with, AQP1 and AQP3 are often found in dermal fibroblasts and vascular endothelial cells [21, 23]. AQP5 is specifically expressed in the sweat glands of the skin, modulating the secretion and absorption of sweat. Moreover, there are a variety of immunocytes in the layer of dermis, in which the AQP family also plays a key role in immune modulation. AQP7 is expressed in Langerhans cells and dermal dendritic cells of the skin and involved in primary cutaneous immune responses.

The hypodermis is mainly comprised of adipose cells, on the membrane of which AQP7 is located. Adipocytes regulate lipogenesis and lipolysis to store and provide energy. When there is an excess of nutrition supply, adipocytes absorb glucose from the blood and metabolize and combine it with fatty acids to synthesize

triglycerides in a process called lipogenesis. In times of starvation or exercise when energy is needed, adipocytes initiate lipolysis, hydrolyzing triglycerides to glycerol and fatty acids so that the body may use them for energy.

15.3 Functions of AQPs in Different Layers of Skin

15.3.1 Functions of AQPs in Epidermis

AQP3 is an aquaglyceroporin, able to transport small solutes such as glycerol. Within the layer of the epidermis, AQP3 firstly plays an essential role in skin hydration by regulating epidermal glycerol percentage [24, 25]. This idea has been proved by the phenotypes of AQP3-knockout mice, which exhibit decreased stratum corneum hydration as measured by changes in skin surface conductance and stratum corneum water content [24, 26]. The hydration difference between AQP3-knockout and wild-type mice is not significant under the condition of low (10%) environmental humidity [26] and is not accompanied by differences in stratum corneum morphology, thickness, lipid content, or levels of metabolites such as amino acids, lactic acid, glucose, or urea [24]. However, the dysfunction can be reversed by pharmacologic administration of glycerol (via a topical, intraperitoneal or oral route), indicating that the epidermal glycerol level is correlated with stratum corneum hydration [24–26].

AQP3 also affects the proliferation and migration of keratinocytes in vitro and epidermal layers in vivo [24, 27–30]. AQP3 overexpression increases the glycerol uptake, keratin 5 and 14 expression, and cell growth in keratinocytes, whereas AQP3 knockdown inhibited cell proliferation stimulated by CCL17 [30]. AQP3 knockdown decreased keratinocyte proliferation and increased the expression of several differentiation markers (keratin 10, involucrin, and filaggrin) [31]. AQP3-knockout mice also show a similar inhibition of epidermis proliferation, even treated with retinoic acid [27]. The mechanisms include regulation of cellular ATP levels and uptake and metabolism of glycerol. Keratinocyte proliferation induced by wounding is also weakened by AQP3 deficiency, and glycerol supplementation can correct the cell cycle arrest [28]. On the other hand, AQP3-knockout mice exhibit reduced tumor formation in a mouse model of carcinogenesis [29, 32]. Besides, the 12-O-tetradecanoyl phorbol 13-acetate (TPA)-induced epidermal hyperplasia and keratinocyte proliferation are mitigated in AQP3-knockout versus wild-type mice [29]. AQP3 also plays a role in keratinocyte migration through regulating the permeability of water [32]. Decreased glycerol uptake, scratch wound healing, and transwell migration are observed in AQP3-knockdown keratinocytes [28]. Keratinocytes isolated from AQP3-knockout mice also show reduced migration compared with wild-type control, while the adenoviral-mediated expression of AQP3 is able to restore normal capability of cell migration.

Thirdly, AQP3 participates in the regulation of keratinocyte differentiation [33, 34]. The keratinocytes in the stratum basale need to undergo growth arrest and maturation before they move to the next layer. Upregulated AQP3 expression is observed in the high-cell-density-induced human keratinocyte differentiation, concomitant with increased keratin 1 levels, indicating a pro-differentiation role of AQP3 [31]. In addition, AQP3 re-expression in AQP3-knockout keratinocytes increased the expression of several representative differentiation markers such as keratin 10, either alone or together with differentiation promotors such as agonists of the nuclear hormone receptor PPARγ [35–37]. AQP3 knockdown results in downregulation of E-cadherin, β- and γ-catenins, which are related to adherens junctions [38, 39], and reduced phosphorylation of phosphoinositide 3-kinase (PI3K), which is critical for the survival of keratinocytes in the stratum spinosum [38, 40]. These results are again consistent with the effect of AQP3 on the early keratinocyte differentiation [41] and provide strong evidence for the important role for AQP3 in maintaining keratinocyte viability during the differentiation process. However, the influence of AQP3 on the keratinocyte maturation process is still controversial. Hara-Chikuma et al. [27] found insignificant effect of AQP3

knockdown on the expression of differentiation markers in both calcium-induced human keratinocyte differentiation and AQP3-knockout mouse epidermis. The controversial roles for AQP3 in keratinocyte differentiation versus proliferation may lie in a lipid-metabolizing enzyme phospholipase D-2 (PLD-2) [19, 42] PLD-2 can produce the phospholipid, phosphatidylglycerol (PG) using glycerol, which is transported by AQP3 [20]. PG is well acknowledged as a second messenger that can induce the growth arrest and differentiation of epidermal keratinocytes [33, 34]. Thus, the activity of PLD-2 and the amount of PG are regulated by AQP3 by orchestrating the intracellular glycerol levels.

An increase in AQP1 expression has been reported in melanocytes under osmotic stress [3]. It has also been shown that AQP1 can regulate the keratinocyte migration by mediating the transmembrane water permeability indicated in the "osmotic engine model" [28, 43]. Influx of water through either AQP1 or AQP3 polarization provides the hydraulic pressure discrepancy which is necessary for cell movement. Apart from the changes in expression level, the mislocation of AQP1 may be another cause of human diseases. AQP1 is dominantly expression on the cellular and intracellular membranes of multiple kinds of human cells. We recently have found that AQP1 is aberrantly co-localization in triple negative breast cancer cells with the receptor-interacting protein kinase 1 (RIPK1), which is exclusively located in the cytoplasm (Fig. 15.2) [44]. Further studies proved that the cytoplasmic AQP1 is one of the main reasons for the aggressive phenotype of triple negative breast cancer and the cytoplasm is the main field where the AQP1-RIPK1 complex assembles and functions.

A recent transcriptome sequencing study demonstrated that AQP5 is able to regulate the balance of proliferation and differentiation of epidermal stem cells in skin aging changes [45]. However, the mechanisms how AQP5 contributes physiologically to the formation of the water permeability barrier in thick skin remains to be further explored.

The retinoic acid downregulates the expression of AQP9 but upregulates AQP3 in keratinocytes [22, 46], indicating the different functions and mechanisms of AQP9 compared to AQP3. Apart from water transport, AQP9 also facilitates the transport of glycerol, urea and hydrogen peroxide [22, 47]. The intracellular urea can regulate the differentiation, lipid synthesis, and antimicrobial reaction of keratinocytes [48].

AQP10 is believed to share similar functions as AQP3 in the epidermis and may also be involved in pompholyx [49]. The specific mechanism by which AQP10 is involved in the regulation of keratinocyte proliferation and differentiation still needs further investigation.

15.3.2 Functions of AQPs in Dermis and Hypodermis

The main function of AQP1 in the fibroblasts and vascular endothelial cells is to exchange water between the blood and dermis to maintain skin hydration during hypertonic stress. Thus, AQP1 may contribute to skin inflammation, vasculopathy, and dermal fibrosis by regulating tissue edema and cell proliferation and migration.

AQP3 enhances the oxidative stress by transporting the hydrogen peroxide into dermal fibroblasts of the bleomycin-treated mice. AQP3 knockout mice showed reduced dermal thickness/fibrosis, hydrogen peroxide levels, TGF-β1 abundance, and collagen expression in bleomycin-induce fibrosis models [50]. In addition, AQP3 mediates epidermal growth factor receptor (EGFR)-induced dermal fibroblast migration, displayed by delayed scratch wound healing in vitro [51]. EGFR activation also increases the expression of AQP3 through mitogen-activated protein kinase (MAPK) signaling pathway and PI3K activity. Finally, AQP3 protects dermal fibroblasts from ultraviolet-induced cell death by upregulating the anti-apoptotic protein BCL-2 [52]. These results suggest a potential involvement of AQP3 in dermal fibroblast proliferation and migration and collagen synthesis, although future studies are still needed to determine the exact function of AQP3 in fibrotic diseases.

AQP5 is necessary for the process of sweat secretion, as absence of AQP5 has been shown

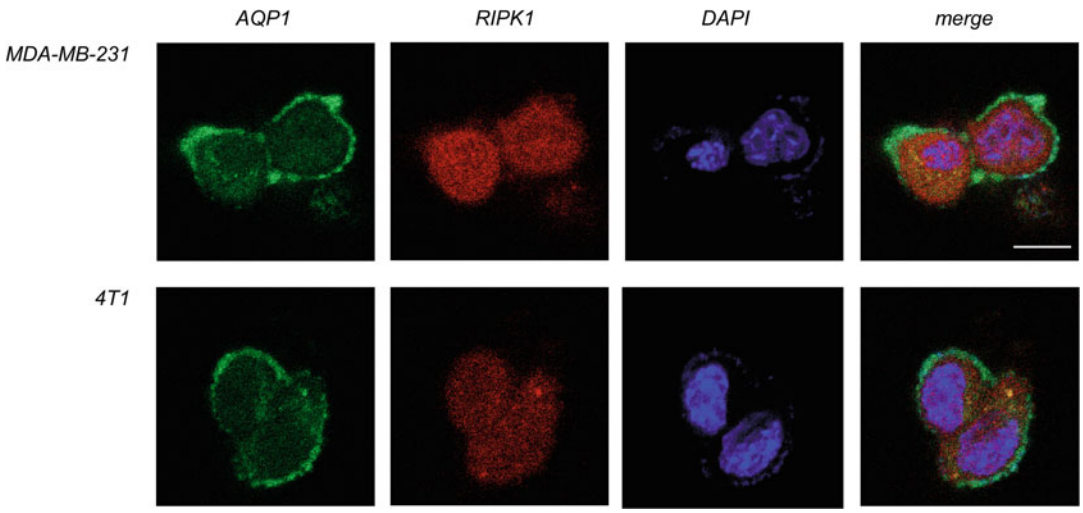

Fig. 15.2 AQP1 mislocation in triple-negative breast cancer cells. AQP1 is commonly seen on the cell membrane of normal breast tissues. But it is aberrantly expressed in the cytoplasm of triple negative breast cancer (TNBC) cells. This figure shows representative images of anti-AQP1 (green) and anti-RIPK1 (red) immunofluorescence staining of MDA-MB-231 (human TNBC cell line) and 4 T1 (mouse TNBC cell line) cells stably overexpressing AQP1and RIPK1. Orange/yellow fluorescence in the merged images represents the co-localization of AQP1 and RIPK1. These results demonstrates that cytoplasmic AQP1 is one of the main reasons for the aggressive phenotype of triple negative breast cancer and the cytoplasm is the main field where the AQP1-RIPK1 complex assembles and functions to promote the progression and metastasis of TNBC. *Scale bar*: 20 μm

to lead to a large decrease in active sweat glands [53, 54]. Humans have an estimated two to four million eccrine sweat glands located throughout the body [6]. They function as a pathway of water and electrolytes out of the body, regulating the internal environment of human body, maintaining body temperature, and protecting the skin from harmful microbe. In the subcellular level, AQP5 is located in both the apical membrane and the basolateral membranes of the secretory coils of the eccrine sweat glands. It moves to the apical membrane during the active process of sweating to increase plasma membrane water permeability [55]. Acetylcholine is the main regulator of sweating and increases intracellular calcium, followed by the apical translocation of AQP5. Therefore, active chemicals modulating AQP5 activity may be a potential and promising therapeutic strategy for patients suffering from sweating disorders.

AQP7-knockout mice show impairment of contact hypersensitivity response and decreased sensitization [5]. Further experiments indicate that AQP7 is required for the ability of dermal dendritic cells to take up antigens for presentation, while AQP7 knockout dendritic cells demonstrate reduced internalization of antigens of various sizes, indicating an impairment of micropinocytosis in these cells. AQP7 is also necessary for dendritic cell chemotaxis, with the AQP7 knockout dendritic cells exhibiting reduced migration in response to C-X-C motif chemokine ligand 12 (CXCL12, also known as stromal-derived factor-1 or SDF-1) or C-C motif chemokine ligand 21 (CCL21) [5]. Together, these results indicate an involvement of AQP7 in cutaneous dendritic cell function, and thus hypersensitivity reactions in the skin.

AQP9 has also been recently shown to play a role in contact hypersensitivity. Despite showing comparable numbers of immune cell types, AQP9 knockout mice exhibited significantly lighter ear edema in contrast to the wild-type animals upon a sensitization model [56]. Further studies found decreased numbers of infiltrating CD4-positive and CD8- positive T cells and neutrophils, but

not mast cells. The ear swelling could be reversed in AQP9 knockout mice by replace the bone marrow with wild-type one, indicating that the relevant cell type in this system seems to be an immune cell rather than keratinocytes. In particular, the response could be restored by reconstituting AQP9 knockout mice with wild-type neutrophils, but not T cells, and was decreased upon antibody-mediated neutrophil depletion (in wild-type mice) [56], suggesting the importance of neutrophils in this contact hypersensitivity response. Skin draining lymph nodes isolated from AQP9 knockout mice models also exhibited reduced secretion of interleukin-17A (IL-17A), with no effect on interferon-gamma (IFN-γ) levels. In addition, AQP9 knockout mouse-derived neutrophils demonstrated reduced chemokine-induced cell migration [56]. Together these results suggest that the AQP9 expressed in neutrophils contributes to the induction of contact hypersensitivity by allowing chemokine-mediated recruitment of IL-17A-producing neutrophils to sites of skin sensitization.

During the process of lipolysis, AQP7 plays a crucial role by transporting glycerol out of the adipocytes to allow maintained triglyceride breakdown [57]. In silent period, AQP7 is found in the cytoplasm near the nucleus, but when lipolysis is triggered, epinephrine acts on adrenergic receptors to increase the levels of cAMP, which subsequently activate protein kinase A to result in the stimulation of hormone-sensitive lipase activity. The lipase hydrolyzes triglycerides to yield glycerol and fatty acids, and AQP7 is then translocated to the cell membrane to allow for the transport of glycerol out of the cell and into the blood for gluconeogenesis. AQP7 expression and localization are found to be regulated by insulin. Thus, AQP7 are upregulated when lipolysis is induced by starvation and declined amount of insulin [57].

AQP7 is also a target of peroxisome proliferator-activated receptor-gamma (PPAR-γ), a regulator of many genes in adipose tissue, and is upregulated when PPAR-γ is activated. PPAR-γ is the main target of the drug class of thiazolidinediones used in diabetes to decrease insulin resistance. These drugs have been shown to sensitize adipocytes to insulin, probably due to the increased expression of AQP7 [57]. On the other hand, absence of AQP7 has been shown to lead to obesity and insulin resistance for the reason of glycerol accumulation and subsequent adipocyte hypertrophy. Adipocytes in AQP7-knockout mice exhibit increased intracellular glycerol, enhanced uptake of fatty acids, and accelerated triglyceride synthesis [58]. Due to these findings, modulation of AQP7 has been suggested as a possible therapy for obesity as well as diabetes [47].

15.4 AQPs in Skin Diseases

15.4.1 Xeroderma and Skin Dryness

The xeroderma is a disease characterized by dryness and roughness and a fine scaly desquamation of the skin, which is largely correlated with the content of glycerol [24]. The glycerol is extremely important for maintaining the skin function, supported by a study using an asebia mouse model. The deficient mice carry a mutation in the gene encoding stearoyl-CoA desaturase-1 and exhibit reduced production of sebum (rich in triacylglycerols and phospholipids) by hypoplastic sebaceous gland [59]. The defect in sebum production subsequently results in decreased epidermal glycerol content and abnormal stratum corneum hydration, which in turn leads to hyperkeratosis (epidermal thickening), epidermal hyperplasia, and mast cell activation. This dysfunction can also be corrected by topical administration of glycerol. The glycerol is also routinely added to skin lotions and wound salves, improving the skin hydration and enhanced wound healing [60]. In addition, AQP3 is regulated in a circadian pattern, and the skin hydration levels changes in accordance with the cyclical expression of AQP3 [61]. The xeroderma is a kind of skin disease with the manifestation of skin dryness and hardness, which is usually observed in diabetes. In a streptozotocin (STZ)-induced diabetes mouse model, downregulation of epidermal AQP3 has been revealed in concomitant with a

reduction in the transepidermal water loss (TEWL) [62]. The decrease in AQP3 expression started to be significant 1 week after the model establishment and seemed not to be the result of elevated serum glucose levels. Meanwhile, the expression of several circadian rhythm regulators (Bmal1, Clock, and Dbp) were reduced at 2 weeks (but not 1 week) after STZ administration, suggesting that the disturbance of the circadian rhythm may be the true reason for AQP3 downregulation in diabetic mice. Consequently, the above results support the important effect of glycerol on skin hydration as well as the critical function of AQP3 in maintaining epidermal glycerol content and stratum corneum water-holding capacity. Since AQP3 improves the efficiency of entry of glycerol into keratinocytes, increased AQP3 levels may enhance the beneficial effect of this agent in the skin. This may lead to further research on novel agents to induce AQP3 expression, aiming to improve epidermal hydration and other aspects of skin function [16, 62–64]. In addition, skin dryness associated with aging has also been linked to AQP3. Thus, decreased AQP3 contributes to both extrinsically (sun-exposed) and intrinsically (age-related) aged human skin [65, 66], leading to epidermis atrophy and poor wound healing. Similar phenotype is observed in mouse skin models and keratinocytes [62, 67], indicating that AQP3 overexpression may be effective for dry skin pathology.

Another disease characterized by skin dryness is the Sjogren's syndrome. Patients with Sjogren's syndrome has their AQP5 mislocated in the cytoplasm of the sweat secreting cells, leading to significantly decreased saliva and tear production [68]. Moreover, the abundance of urea transporter A1 (UT-A1) and UT-B1 in uremic sweat glands was significantly increased in patient skin tissues, while the expression of AQP5 was decreased [69], indicating again the regulatory role of AQP5 in sweat secretion.

15.4.2 Psoriasis

Psoriasis is a commonly seen skin disease that brings patients decreased quality-of-life and a negative impact on social communication [34, 70]. It is characterized by hyperproliferation and abnormal differentiation of keratinocytes [71]. Mechanically, although the immune dysfunction is involved in psoriasis, accumulating evidence has been obtained regarding the effect of AQP3 on the development of psoriasiform skin lesions in vitro and in vivo. Decreased or mislocalized AQP3 has been found in psoriatic lesions [15, 41, 72]. AQP3-knockout mice exhibit reduced psoriasiform lesion development and epidermal hyperplasia compared with wild-type mice in two mouse models of psoriasis [12], while overexpression of AQP3 exacerbates psoriasiform acanthosis [73]. However, some semiquantitive studies have indicated increased expression of AQP3 in psoriatic skin lesions [74, 75]. Intriguingly, unchanged AQP3 level has also been reported in psoriasis [12]. Thus, AQP3 in keratinocytes seems to play an important role in psoriasis; however, whether AQP3 is up- or downregulated in psoriasis remains controversial. Possible explanations include that proliferating keratinocytes with increased AQP3 expression comprise a greater proportion of psoriatic epidermis specimen than of the normal skin tissues, but AQP3 expression is decreased with later differentiation and keratinization [19] and mislocated in a cytoplasm-dominant staining pattern [15]. Nevertheless, the previous data support critical involvement of this channel in the disease. Meanwhile, AQP3 might play a modulating role by regulating the permeability of glycerol in psoriasis because glycerol can ameliorate psoriasis symptoms alone or in conjunction with other treatments [76].

15.4.3 Vitiligo

A reduction in AQP3 levels is also revealed in depigmented vitiligo skin of both patients and AQP3 knockout mice, as AQP3 in epidermal keratinocytes is involved in maintaining the health of the melanocytes by regulating the amount of E-cadherin and the activity of phosphoinositide 3-kinase (PI3K) [24, 36]. Specifically, acral vitiligo skin differs from non-acral

skin in showing gradual fading of AQP3 expression through the epidermis and weaker AQP3 expression in the stratum spinosum in perilesional compared to lesional skin specimens [77].

15.4.4 Dermatitis

In atopic dermatitis, discrepant results suggest either up- or down- regulation of AQP3 [3, 78]. Nonetheless, these results indicate a role for AQP3 in the pathological process of atopic dermatitis. Epidermal AQP3 levels are also elevated in babies with erythema toxicum neonatorum [14]. Besides, its transportation function of water and glycerol is involved in the initiation and development of pompholyx, a type of eczema characterized by vesicles or blisters on the hands and feet [49]. Aberrant high AQP1 level has been found in the pathological epidermis of infants with erythema toxicum neonatorum [14]. However, the causative relationship between the amount of AQP1/3 and the common neonatal disease is largely unknown. AQP7 and AQP9 are involved in the immune cells (neutrophils, T cells, etc.) of the skin tissue, thus also regulating inflammatory and infectious diseases in the skin.

15.4.5 Skin Tumors

In basal cell carcinoma and squamous cell carcinoma, nonmelanoma skin cancers characterized by excessive proliferation and downregulated differentiation, AQP3 abundance reduces in tumor cells compared with the normal epidermis [15]. In addition, an increase in AQP1 expression has been reported in melanocytic skin tumors [79]. However, the mechanisms why the abundance of AQPs change and how they functions in skin tumors remains unclear.

15.4.6 Palmoplantar Keratoderma

Mutations in AQP5 have been identified in patients with a form of diffuse nonepidermolytic palmoplantar keratoderma a disease characterized by thickened skin of the soles and palms and a defective water permeability barrier [4, 80, 81]. The involved areas are negatively affected upon the water exposure, resulting in a white spongy appearance of the skin [4, 80, 82]. However, the mechanism why these mutations can cause the annoying keratoderma remains to be further explored. Moreover, topical urea has been shown to improve skin barrier function and is used for a variety of conditions such as hyperkeratotic or xerotic skin [48, 83]. Thus, by regulating the transport of urea in keratinocytes, AQP9 plays a role in both antimicrobial defense and barrier permeability function of the epidermis.

15.4.7 Systemic Sclerosis

In the dermal fibroblasts and endothelial cells of patients suffering from scleroderma/systemic sclerosis (SSc), a multisystem autoimmune disease characterized by vasculopathy and fibrosis, AQP1 is upregulated partially due to autocrine transforming growth factor-β (TGF-β) stimulation and Fli1 deficiency [84]. AQP3 in dermal fibroblasts may also play a role in SSc. In a bleomycin-induced mouse model of SSc, AQP3 is upregulated in mice injected with bleomycin compared with control, as well as in fibroblasts isolated from the dermis of the same animals [50]. However, another study showed paradoxical results that the expression of AQP3 decreases in dermal fibroblasts isolated from the skin of SSc patients compared with normal dermal fibroblasts [85]. Possible explanations on discrepancies of different studies may include that AQP3 is downregulated in SSc patients as negative feedback in an attempt to reduce a profibrotic effect of this channel. It is also possible that the results of those two studies cannot be directly compared because of the apparent differences on species (mouse vs. human) and model establishment methods (acute vs. chronic SSc) between these studies model differences.

15.4.8 Wound Healing

AQP3 exerts a key role in skin wound healing, especially for the epidermis regeneration. AQP3-knockout mice exhibit delayed wound healing of full-thickness skin wounds. The delay in skin wound healing can also be restored by glycerol supplementation, which is often included in wound ointments [28]. Moreover, AQP3 expression is reduced in the wounds of diabetic rats with impaired wound healing [86], indicating AQP3 is indispensable for normal skin regeneration. However, there is an argument that the conclusion drawn from a rodent model may not be extended to human skin wound healing, because rodent wounds heal predominantly by contraction, whereas human wounds heal primarily by re-epithelialization. Unfortunately, it is still unknown whether AQP3 is also involved in human skin wound healing. Nevertheless, the data showing the ability of AQP3 to promote proliferation and migration of human keratinocytes may in part support the potential of this channel in human skin wound healing as well.

15.5 Summary

The skin is the largest and outmost organ in human body, serving as a mechanical and biological barrier, assisting with thermoregulation, protecting against ultraviolet radiation and chemical hazards, and allowing tactile sensation. Aquaporins, channels that transport water and sometimes other small molecules, are expressed in a variety of cells in different layers of the skin. Accumulating data in the literature indicate a key role for AQPs in skin homeostasis, with its dysregulation, dysfunction, and mislocation contributing to multiple skin disorders. They contribute to many key functions of the skin including hydration, formation of the water permeability barrier, keratinocyte maturation, sweat secretion, wound healing, lipolysis, and immune responses. However, continued studies are still warranted to better understand the role of each aquaporin in both normal skin tissues and abnormal skin disorders.

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Aquaporins in Glandular Secretion

16

Giuseppe Calamita and Christine Delporte

Abstract

Exocrine and endocrine glands deliver their secretory product, respectively, at the surface of the target organs or within the bloodstream. The release of their products has been shown to rely on secretory mechanisms often involving aquaporins (AQPs). This chapter will provide insight into the role of AQPs in secretory glands located within the gastrointestinal tract, including salivary glands, gastric glands, duodenal Brunner's glands, liver, gallbladder, intestinal goblets cells, and pancreas, as well and in other parts of the body, including airway submucosal glands, lacrimal glands, mammary glands, and eccrine sweat glands. The involvement of AQPs in both physiological and pathophysiological conditions will also be highlighted.

Keywords

Aquaporins · Exocrine glands · Endocrine glands · Secretion · Function · Expression

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16.1 Role of AQPs in Secretory Glands Located within the Gastrointestinal Tract

Aquaporins (AQPs) are expressed to several secretory glands located within the entire length of the gastrointestinal tract including salivary glands, gastric glands, duodenal Brunner's glands, liver, gallbladder, intestinal goblets cells, and pancreas. Figure 16.1 summarizes the involvement of AQPs in the secretory gland functions that is detailed in the following sections.

16.1.1 Salivary Glands

Major salivary glands, namely parotid, submandibular, and sublingual glands, and minor salivary glands contribute to whole saliva secretion [1, 2]. The secretory structure of the glands consists into several lobes subdivided into lobules. Lobules are made of secretory units namely acini (consisting into the association of multiple acinar cells) connected through a network of ducts formed of ductal cells. Myoepithelial cells surround the secretory epithelia [3]. The acinar cells are either serous, mucous or seromucous, based on their secretory products and characteristics [3]. The ductal system can be subdivided into intralobular (intercalated and striated), interlobular, interlobar (excretory) ducts. Saliva secretion relies on a two

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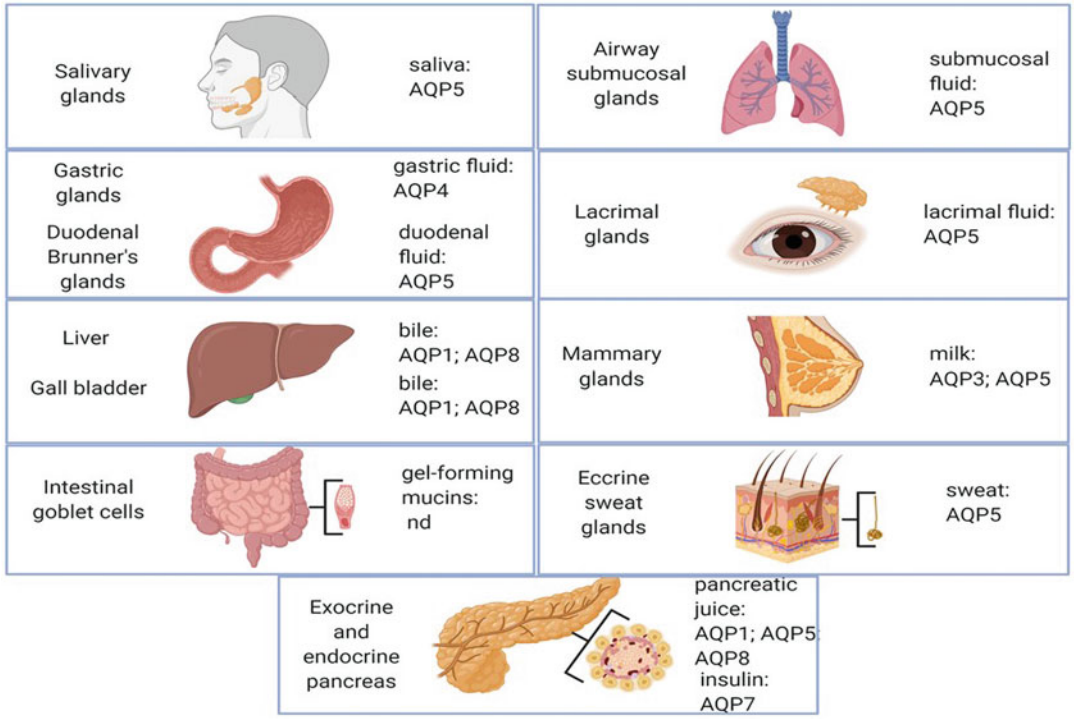

Fig. 16.1 Involvement of AQPs in secretory gland functions

steps mechanism in which acinar cells secrete an isotonic-like fluid rich in NaCl and water and ductal cells reabsorb some NaCl and secrete bicarbonate [4, 5]. These two steps mechanism results into the secretion of a final hypotonic saliva into the oral cavity.

In the first step, water flows to the lumen of the acini through the apically-located AQP5 thereof playing a major role in saliva secretion (Fig. 16.2) [6, 7]. Indeed, a 60% decrease in pilocarpine-stimulated saliva secretion, and a more viscous and hypertonic saliva have been observed in AQP5 knockout mice [6, 7]. Furthermore, substantial decrease in water permeability of parotid (65%) and sublingual (77%) acinar cells has been shown in AQP5 knockout mice [7]. Therefore, studies infer that AQP5 is responsible for acinar water movement [4, 5, 8, 9]. However, it has been suggested that AQP5 could act as an osmosensor controlling the tonicity of the transported fluid by mixing transcellular and paracellular water flows [10]. In response to muscarinic stimulation inducing intracellular calcium increase, AQP5 traffics from intracellular vesicles to plasma membrane [11–13]. Concomitantly to its physiological role, AQP5 expression is mostly confined to the apical membrane of serous acinar cells from all human salivary glands [14, 15] and from submandibular and parotid glands in rats [15–18] and mice [11, 19, 20]. The AQP5 expression reported in rat and mouse ductal cells [11, 18, 21, 22] is difficult to explain on a physiological point of view considering ductal cells are water impermeable [23]. Noteworthy, a naturally occurring point mutation of AQP5 has been identified in rats and associated with decreased AQP5 expression and saliva secretion [24]. Until now to our knowledge, no AQP5 mutation has been associated with saliva flow dysfunction in humans.

The use of knockout mice models has not been able to show the involvement of other AQPs, i.e. AQP1, AQP4, and AQP8, in saliva secretion [6, 25, 26]. Therefore, AQP1 expressed in mouse salivary gland endothelial and myoepithelial cells [27] is not involved in saliva secretion. AQP1 is also expressed in human myoepithelial [28] and

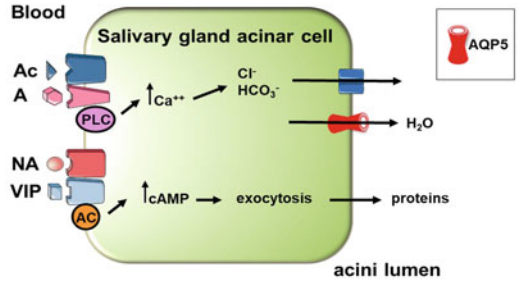

Fig. 16.2 Proposed mechanism of AQP-mediated water transport in saliva formation in salivary gland acinar cells. Upon nerve stimulation, acetylcholine and adrenalin bind to muscarinic receptors M1 and M3 and α1-adrenergic receptors leading to phospholipase C activation and subsequent intracellular calcium increase, while noradrenalin and vasoactive intestinal peptide bind to β1-adrenergic and VIP receptors leading to adenylyl cyclase activation and subsequent intracellular cyclic adenosine monophosphate (cAMP) increase. cAMP leads to protein kinase C activation and exocytosis of proteins, while intracellular calcium increase leads to Cl^- and HCO_3^- secretion driving water transport though AQP5 into the acini lumen. *AC* acetylcholine, *A* adrenalin, *PLC* phospholipase C, *NA* noradrenalin, *VIP* vasoactive intestinal peptide, *AC* adenylyl cyclase, *cAMP* cyclic adenosine monophosphate, *PKC* protein kinase C

endothelial [14, 15, 29, 30] cells, as well as in rat endothelial cells [22, 31–34].

Other AQPs have been detected in salivary glands. In human, AQP3 is located at the basolateral membrane of serous and mucous acini, but not the ducts [14, 29, 30] while only AQP4, AQP6, and AQP7 mRNAs have been detected [14, 30]. In rat, some controversy still exists concerning the expression of both AQP3 and AQP4 [21, 22, 35, 36]. In rat parotid glands, AQP6 is located to secretory granule membrane [37], while AQP8 is present in myoepithelial cells [38–40]. In mice, AQP3, AQP4, and AQP8 are expressed at the basolateral membrane of acinar and ductal cells [27]; AQP7 is located in endothelial cells; [20] AQP9 distribution remains to be determined [19, 20, 41]; AQP11 is found in ductal cells [19, 20]. Distinct patterns of AQPs expression have been found during the development of salivary glands in mouse, rat, and human [22, 42–45].

In some patients suffering from Sjögren's syndrome, an autoimmune disease characterized by lymphocytic infiltration of exocrine glands and particularly salivary and lacrimal glands, altered AQP5 localization is hypothesized to play a role in the disease pathogenesis and saliva flow reduction. However, altered AQP5 localization has not been detected in all patients suffering from Sjögren's syndrome [46–48]. These data could arise from the use of distinct patient subsets and/or antibodies. In mouse model of Sjögren's syndrome, altered AQP5 localization has indisputably been reported in several studies [49–54]. The presence of inflammatory infiltrates within salivary glands [51], cytokines [55–58], autoantibodies against muscarinic M3 receptors [59, 60] have been suggested to play a role in the modified AQP5 distribution. Even though altered expression and/or localization of AQP5 could not totally account for saliva impairment observed in Sjögren's syndrome patients, it could still play a role in the pathogenesis of the disease. Very recently, in salivary glands from patients suffering from Sjögren's syndrome, it has been shown that altered distribution of prolactin-inducible protein and ezrin, identified as new proteins partners of AQP5 in salivary glands under physiological conditions, may also account for abnormal AQP5 localization [61–63]. Anti-AQP5 antibodies have been detected in blood samples from patients suffering from Sjögren's syndrome and have been incriminated in disease manifestations. Indeed, anti-AQP5 antibodies may be directly linked to salivary gland dysfunction [64] and may represent additional useful biomarker for Sjögren's syndrome diagnosis. However, this remains to be confirmed as anti-AQP5 antibodies have not been detected in all patients with Sjögren's syndrome [65], possibly due to distinct patient subsets and methods of determination. Concerning AQP1, studies using knockout mice showed that this AQP is not involved in saliva secretion [6, 25]. However, decreased AQP1 expression in salivary gland myoepithelial cells from Sjögren's syndrome patients and reduced saliva flow [29] can be counteracted using Rituximab depleting B-cells [66]. Autoantibodies have been detected in patients with Sjögren's syndrome patients [65, 67] but were not associated with decreased saliva flow rate [67]. Therefore, further

investigation is required to better understand the role of AQP1 in salivary gland function. Abnormal distribution of AQP4 has also been described in salivary glands from patients suffering from Sjögren's syndrome [68], but its physiological significance remains to be further studied considering this AQP does not appear to be involved in saliva secretion using knockout mice [6, 25].

In patients with head and neck cancer treated with ionizing radiation therapy, decrease or loss of AQP5 expression [69, 70] and/or impaired AQP5 trafficking [71] could account for xerostomia. In mice and rats, ionizing radiation also induced decrease in AQP5 expression [72–76]. Pilocarpine, a muscarinic receptor agonist restored AQP5 expression and saliva flow in irradiated rats [77].

In diabetes, it is presently unclear whether high glucose induces [78] or not [79] an altered distribution of AQP5 and decreased AQP5 expression [80]. Distinct mouse species, experimental conditions, and analytical methods could account for these distinct results.

In salivary glands, AQPs represent new therapeutic targets or can be used as therapeutic agents to treat xerostomia. Cevimeline restored proper AQP5 trafficking [81–83]. DNA demethylation agents increased AQP5 expression [57, 84]. Treatment with cystic fibrosis transmembrane regulator (CFTR) corrector and potentiator allowing the correction of CFTR activity restored AQP5 expression and saliva secretion in mouse model of Sjögren's syndrome [85]. Furthermore, the delivery of a recombinant adenovirus vector coding for AQP1 (AdhAQP1) to irradiated glands of animals and human led to saliva flow restoration [86–90], as well as resolution of inflammation [91]. New viral vectors allowing more efficient and persistent expression of a transgene, such as, for instance, hAQP1, in salivary glands, would be useful to further study the usefulness of gene therapy to treat xerostomia. The use of CRISPR-CAS9 gene editing allowing the replacement of endogenous AQP1 gene promotor with the cytomegalovirus (CMV) promoter led to increased AQP1 expression and could open avenues to new gene therapy [92]. The gene therapy approaches described hereabove represent promising therapies for patients suffering from xerostomia consequent to head and neck irradiation therapy or Sjögren's syndrome, but the presence of autoantibodies against AQP1 may represent an obstacle to such therapeutic approach.

16.1.2 Gastric Glands

Mammalian gastric glands found in gastric pits within the gastric mucosa are composed of fundic glands (in the cardia), cardiac glands (in the fundus and body of the stomach), and pyloric glands (in the antrum of the pylorus). Gastric glands are made of distinct cell types with specific function. Indeed, foveolar cells produce mucous, parietal cells secrete gastric acid and bicarbonate ions, chief cells secrete pepsinogen, G cells secrete gastrin, and enterochromaffin-like cells release histamine [93].

Many AQPs have been localized to various areas of the stomach. The fundus express AQP1, AQP3, AQP4, AQP5, AQP7, AQP8, AQP10, and AQP11 mRNA and the antrum of the pylorus express AQP1, AQP2, AQP3, AQP5, AQP7, and AQP11 mRNAs [94–96]. Both parietal and chief cells express AQP4 protein at their basolateral membrane [36, 97–100]. AQP4 internalizes in a vesicle-recycling compartment and undergo phosphorylation upon histamine stimulation in gastric cells [101]. AQP4 is unlikely involved in acid and fluid secretion as shown using AQP4 knockdown mice [102], even though other AQPs could compensate for the lack of AQP4. However, it remains to be determined if AQP4 could still be involved in gastric cell volume maintenance. AQP5 is strictly localized to the apical and lateral membranes of pyloric glands [103].

Several AQPs promote or are involved in chronic gastritis and gastric cancer [96, 104–111]. Particularly AQP3 and AQP5 play significant roles in gastric cancer [112] and promote gastric cancer cell epithelial-mesenchymal transition [106, 113]. Lower levels of miR-877 and miR874, shown to regulate AQP3 and AQP5 expression, respectively, may account for the

increased AQP3 and AQP5 expression and epithelial mesenchymal transition [114, 115]. AQP3 and AQP5 expression has been shown to be positively correlated with gastric mucosal disease progression in gastric carcinoma and other stages of gastric diseases as well as with *Helicobacter pylori* infection [116, 117]. *Helicobacter pylori* promote AQP3 and AQP5 expression (through the activation of downstream HIF-1α or ERK1/2, MEK, respectively) that could be used as novel molecular targets for therapeutic interventions [116, 117]. Furthermore, as the expression of certain AQPs is associated with better or poor overall survival of patients with gastric cancer, it can be used as predictive prognostic gastric cancer biomarker [110, 118].

In light of the involvement of AQPs in gastric cancers, they have been considered as additional molecular targets for therapeutic intervention [119].

16.1.3 Duodenal Brunner's Gland

The role of AQPs in duodenal Brunner's gland function remains poorly understood due to the limited number of studies performed so far. Brunner's gland cells express AQP5 at their apical, lateral, and secretory granule membranes [103] and AQP1 at their apical and lateral membranes [120]. The secretion of bicarbonate and protein as well as the overall flow rate of rat Brunner's gland are increased by the vasoactive intestinal peptide (VIP) acting though a cAMP-dependent signaling pathway [121]. In addition, VIP induces the trafficking of AQP5, but not of AQP1, from secretory granules to apical plasma membrane [120, 122]. The resulting presence of AQP5 at the apical plasma membrane could account for increased water flow and fluid secretion. This hypothesis is further supported by the co-localization and co-trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) and AQP5 providing a parallel pathway for electrolyte secretion and osmotic water movement [122]. The expression of AQP5 in Brunner's gland was decreased in celiac disease and cystic fibrosis and may consequently be involved in the pathogenesis of these diseases characterized by altered duodenal secretion [122].

16.1.4 Liver, Bile Ducts, and Gallbladder

Bile is a complex fluid composed of an aqueous solution (95% of water) of organic and inorganic compounds [123]. The major organic compounds are represented by three lipids, bile acids, cholesterol, and phospholipids, and the bile pigments. Proteins and metabolites deriving from various endogenous substances (i.e., hormones) are present at low concentrations [123]. Ions Na^+, K^+, Ca^{++}, Mg^{++}, Cl^-, and HCO_3^- are the major inorganic electrolytes whose concentrations in the common duct bile are very close to those found in plasma.

Bile is the main route for the excretion of body cholesterol in the form of unesterified cholesterol or as bile acids. In turn, biliary bile acids assist the emulsification and absorption of lipids at intestinal level. Also, bile mediates the elimination of drugs and toxins from the body. In health, humans secrete about 0.8–1.0 L of hepatic bile daily at a rate of 30–40 mL per hour. Bile production is about six times higher in rats [124], a species lacking gallbladder. Human canalicular bile is remodeled into the lumen of the bile ductules and duct through secretory and absorptive processes operated by the ductal epithelial cells. Bile is stored and concentrated in the gallbladder, and released into the duodenum [125, 126]. Bile water is mostly reabsorbed in the proximal segment of the small intestine [127] while bile salts are recovered in distal ileum to be carried back to the liver by the enterohepatic circulation [128, 129]. Bile formation starts at the bile canaliculus triggered by an osmotic process that involves solutes and water and where the driving force needed to bile formation is represented by the active concentration of bile acids and other biliary constituents in the bile canaliculi [124]. Canalicular bile flow can also be found in the absence of bile acids or at low bile acid outputs, indicating the existence of two components for canalicular bile formation, the

bile acid-dependent bile flow (i.e., bile flow related to bile acid secretion) and the bile acid-independent bile flow (i.e., bile flow attributed to active secretion of osmotically active inorganic electrolytes and organic anions). Lastly, total bile flow consists of constant ductal/ductural secretion and total canalicular bile flow with a linear relation in both total bile flow and total canalicular bile flow.

The epithelial cells of the mammalian hepatobiliary tract express several AQPs variously localized among the different system sections (Table 16.1). Endothelial cells express AQP1 [34] and AQP7 [130]. AQPs are also present in Kupffer cells [130, 131] and hepatic stellate cells [132–136].

16.1.4.1 Liver

Rodent hepatocytes express AQP8, AQP9, and AQP11 [130, 137–142]. Two more homologues, AQP3 and AQP7, have been reported in human hepatocytes. The distinctive subcellular localization and transport selectivity featured by these AQP channels may explain their redundancy in hepatocytes [143]. Important roles have been ascribed to AQP8, AQP9, and AQP11 in hepatocytes whereas the function (if any) of hepatic AQP3 and AQP7 is unclear.

Table 16.1 Reported localization and suggested physiological relevance of hepatobiliary aquaporins expressed at significant levels

Hepatobiliary section	Aquaporin	Cellular location and species	Subcellular location	Suggested functional involvement
Liver parenchyma	AQP3	Hepatocytes (h)	Undefined	Unclear
	AQP7	Hepatocytes (h)	Undefined	Unclear
	AQP8	Hepatocytes (r, m, h)	APM, SAV, IMM, SER	Canalicular bile secretion; cytoplasmic osmotic homeostasis; mitochondrial ammonia detoxification and ureagenesis; mitochondrial H_2O_2 release hepatocyte cholesterol biosynthesis; regulation of metabolic signaling
	AQP9	Hepatocytes (r, m, h)	BLM	Uptake of glycerol during starvation; lipid homeostasis; import of water from sinusoidal blood; catabolic urea extrusion
	AQP11	Hepatocytes (m)	RER	RER homeostasis; liver regeneration
Intrahepatic bile ducts	AQP1	Cholangiocytes (m, r, h)	APM, SAV, BLM	Secretion and absorption of ductal bile water
	AQP4	Cholangiocytes (m, r)	BLM	Secretion and absorption of ductal bile water
Gallbladder	AQP1	Epithelial cells (m, h)	APM, BLM, SAV	Cystic bile absorption/secretion
	AQP8	Epithelial cells (m, h)	APM, SAV	Cystic bile absorption (?)
Portal sinusoids; PVP; BV	AQP1	Endothelial cells (h)	APM, BLM	Bile formation and flow
Other hepatic cell types	AQP3	Kupffer cells (h)	PM	Cell migration and proinflammatory cytokines secretion (?)
	AQP8	Kupffer cells (r)	PM	Repopulation of Kupffer cells during liver regeneration (?)
	AQP3	Stellate cells (h)	PM	Adiponectin-mediated inhibition of hepatic stellate cells activation
	AQP11	Stellate cells (r)	Undefined	Control of activated hepatic stellate cells proliferation

APM apical plasma membrane, *BLM* basolateral plasma membrane, *BV* blood vessels, *IMM* inner mitochondrial membrane, *PM* plasma membrane, *PVP* peribiliary vascular plexus, *RER* rough endoplasmic reticulum, *SAV* subapical membrane vesicles, *SER* smooth endoplasmic reticulum

Likely due to its multiple subcellular localizations [138, 139] and ability to allow transport of ammonia and hydrogen peroxide in addition to water, several functions have been suggested for AQP8 in hepatocytes such as those of facilitating the secretion of canalicular bile water [144], preserving the cytoplasm osmolarity during the synthesis and degradation of glycogen, [139] transporting ammonia in mitochondrial ammonium detoxification and ureagenesis [145–147], and mediating the release of hydrogen peroxide from mitochondria [148, 149]. Peroxiporin mitochondrial AQP8 has been suggested to intervene in the hepatocyte cholesterol biosynthesis controlled by the sterol regulatory element-binding protein (SREBP) [150–152]. The AQP8-facilitated diffusion of H_2O_2 across the hepatocyte plasma membrane has been recently reported to be involved in the differential regulation of metabolic signaling by α1- and β-adrenoceptors (ARs) and to induce Ca^{2+} mobilization. Since H_2O_2 inhibits the β-AR-mediated activation of the glycogenolytic, gluconeogenic, and ureagenic responses induced by α_1-AR this observation was suggested to be a novel NOX2-H_2O_2-AQP8-Ca^{2+} signaling cascade acting downstream of α_1-AR in hepatocytes. The inhibitory effect exerted by H_2O_2 on β-AR signaling leads to negative crosstalk between the two pathways [153]. Intense is the investigation addressed to the role exerted by AQP8 in the secretion of canalicular bile. After stimulation by choleretic agonists, such as dibutyryl cyclic adenosine monophosphate or glucagon, subapical AQP8 was suggested to translocate to the apical plasma membrane via phosphatidylinositol-3-kinase-dependent microtubule-associated trafficking [154]. This redistribution raises the hydric permeability of the canalicular plasma membrane facilitating the osmotically driven transport of water into the bile canaliculus (Fig. 16.3) [144, 155, 156]. A similar cAMP-induced redistribution to the canalicular membrane also occurs for carriers implicated in canalicular bile secretion such as the isoform 2 of the Cl^-/HCO_3^- exchanger (AE2) and the multidrug resistance-associated protein 2 (MRP2). This mechanism is in line with a work with rat primary hepatocytes where glucagon increased the expression AQP8 reducing its degradation through a process involving cAMP-PKA and PI3K signal pathways [157]. However, in another study, hepatocytes isolated from AQP8 knockout mice showed water permeability comparable to that of hepatocytes from wild type mice [26]. This apparent discrepancy may be explained by the redundancy of AQPs in hepatocytes and/or to the functional modification to which other genes may undergo in response to the disruption of the *Aqp8* gene. On the other hand, in rat hepatocytes it has been observed that a 60% decrease in AQP8 level in the apical membrane leads to a 15% decrease in the overall osmotic permeability of the canalicular membrane [158].

AQP9 is an aquaglyceroporin of broad selectivity allowing transport of a wide variety of non-charged solutes including glycerol and other polyols, hydrogen peroxide, urea, carbamides, nucleosides, monocarboxylates, purines, pyrimidines, and metalloid arsenic besides to water. It is mainly expressed in liver parenchyma, at the sinusoidal plasma membrane of hepatocytes [137]. In rodents, AQP9 is the main pathway through which glycerol is taken up from portal blood to hepatocytes during short-term fasting [159–161]. Once transported into the cells, by means of the glycerol kinase glycerol is promptly converted into glycerol-3-phosphate (G3P) to be used as substrate for gluconeogenesis. Hepatocyte AQP9 is also involved in lipid homeostasis as G3P is required for the synthesis of triacylglycerols (TAGs) [162]. AQP9 has also been suggested to contribute to rodent bile formation [163] and to the extrusion of catabolic urea [164]. In rodents, the transcriptional expression of hepatocyte AQP9 is negatively regulated by insulin [165], an observation that may explain why liver AQP9 is increased in conditions of insulin resistance [166, 167]. Functional significance for AQP9 in glucose and lipid homeostasis and energy balance is also indicated by *Aqp9* knockout mice where the ablation of AQP9 is associated to reduced liver glycerol permeability and increased levels of plasma glycerol and TAGs [164, 168]. Mouse models of obesity and obese patients with type 2 diabetes show reduced

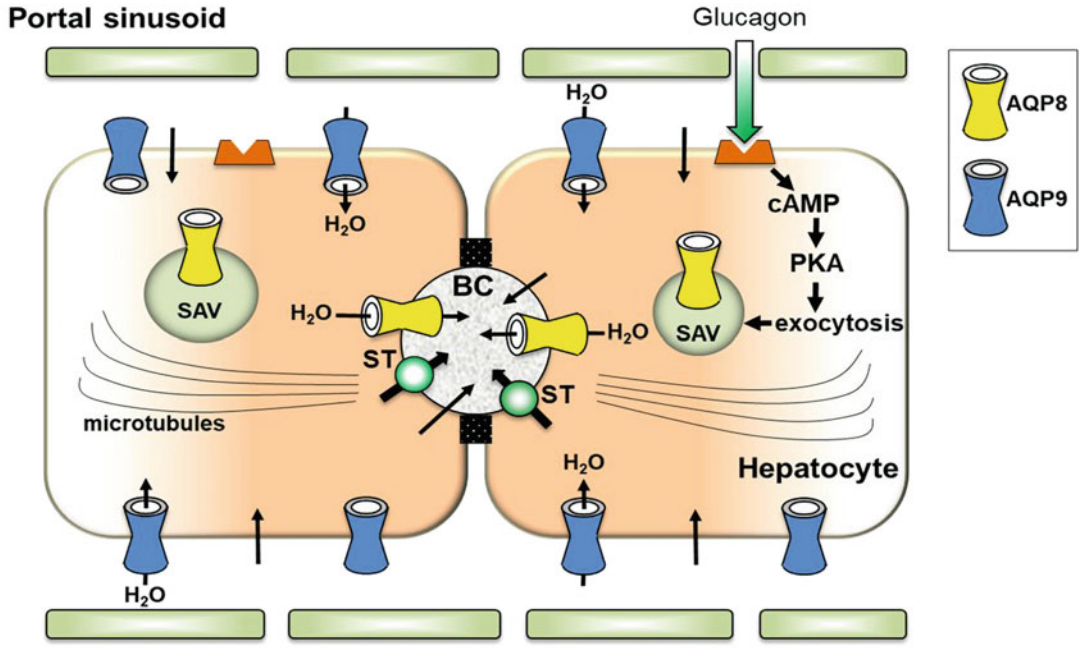

Fig. 16.3 Proposed mechanism of AQP-mediated water transport in canalicular bile formation and secretion in hepatocytes. AQP8 facilitates the osmotic secretion of water into the bile canaliculus, whereas AQP9 contributes to the diffusion of water from the sinusoidal blood into the cell. Choleretic hormones, such as glucagon, can stimulate the microtubule-dependent canalicular targeting of AQP8-containing subapical vesicles. AQP8 is also found in mitochondria and smooth endoplasmic reticulum where it is suggested to play other roles other than facilitating the canalicular secretion of bile water. AQP9 is also the main pathway through which glycerol is imported by hepatocytes (see Table 16.1). *BC* bile canaliculus, *PKA* protein kinase A, *SAV* subapical vesicles, *ST* salt transporters

levels of hepatocyte AQP9 with a significant decrease of the liver glycerol permeability [169, 170]. Liver AQP9 is also regulated by leptin [162, 171]. However, the regulation played by both insulin and leptin on the gene transcription of AQP9 seems to differ between rodents and humans [167]. Sex-specific dimorphism of hepatic AQP9 expression is found both in rodents and humans consistent with the differences with which the two genders handle glycerol [171–174].

Sex-dependent differences were also seen regarding two other aquaglyceroporins of metabolic relevance, AQP3 and AQP7, in fat tissue [171]. Hepatocyte AQP9 has been recently found to be involved in the lipid-lowering activity of the nutraceutical phytocompound silybin through modulation of autophagy and lipid droplets composition [175]. A role of liver AQP9 in the early acute phase of the inflammatory reactions triggered by TLR4 ligands has been suggested where AQP9-facilitated uptake of hydrogen peroxide would be implicated in the production of inflammatory NO and O_2^- through the involvement of the NF-kB pathway [176]. AQP11 has been found in mouse and human hepatocytes where roles are suggested in rough endoplasmic reticulum homeostasis and liver regeneration [130, 141]. The recent functional identification of AQP11 as a peroxiporin opens new horizons about the potential function of this homologue to the regulation of intracellular H_2O_2 homeostasis to prevent ER stress [177]. Further studies are expected to assess the role of AQP11 in liver.

16.1.4.2 Bile Ducts

Cholangiocytes, the epithelial cells lining the biliary tree, account for secretin-induced ductal bile secretion through a cAMP-dependent pathway [124] and activation of Cl^- efflux via cystic fibrosis transmembrane conductance regulator (CFTR) that drive the extrusion of HCO_3^- into the lumen via apical AE2 (i.e., the chloride/bicarbonate exchanger). Both HCO_3^- and Cl^- provide the main driving force for the osmotic movement of water by means of apical AQP1 into the biliary lumen [124]. AQP1 is expressed in human and rodent cholangiocytes [34, 178] where it plays a key role in the apical water secretion during both basal- and hormone-regulated ductal bile formation [179]. AQP1 is also located in subapical membrane vesicles [180] where co-expression with AE2 and CFTR was observed [181]. Secretin regulates the exocytic insertion of these vesicles into the cholangiocyte apical membrane leading to the novel concept of functional bile secretory unit [180, 181]. At their basolateral plasma membrane cholangiocytes express AQP4 and AQP1 [180, 182]. AQP-facilitated water movement would allow the relative isosmolar status of the cell to be maintained during ductal bile formation. This is consistent with the physical association between the basolateral membrane of cholangiocytes and the peribiliary vascular plexus that surrounds bile ducts and from which bile water originates explaining the relative isosmolar status seen during ductal bile formation (Fig. 16.4) [143, 183]. Surprisingly, cholangiocytes from *Aqp1*$^{-/-}$ knockout mice did not show impairment in water movement [184]. Lack of AQP1 could lead to compensatory upregulation of other AQPs expressed in mouse cholangiocytes [185, 186] such as AQP8. Intrahepatic bile ducts not only secrete but also absorb water. Osmotically induced net water absorption has been demonstrated in isolated rodent intrahepatic bile duct units [187]. Water would be absorbed osmotically following the active absorption of sodium-coupled glucose and bile salt by means of the SGLT1 and ASBT cotransporters, respectively [124]. Hormones decreasing the intracellular levels of cholangiocytes cAMP such as somatostatin, gastrin, and insulin could act by inhibiting the secretin-induced vesicular transport of AQP1, CFTR, and AE2 to the cholangiocytes apical membrane with a decrease of the ductal bile secretion. This mechanism could explain why somatostatin can cause inhibition of ductal secretion and stimulation of net ductal water absorption.

16.1.4.3 Gallbladder

The mammalian gallbladder acts as a storage compartment for bile fluid produced by hepatobiliary secretion with important roles in maintaining digestive and metabolic homeostasis. Water movement across gallbladder epithelium is driven by osmotic gradients created from active salt absorption and secretion. Human and mouse gallbladder epithelial cells express AQP1 and AQP8. Both in human and mouse AQP1 is localized at the apical and basolateral domains of the plasma membrane of the epithelial cells that line the neck of the organ [188, 189]. In mouse gallbladder, additional immunoreactivity was seen at the corpus portion with staining at level of subapical vesicles and over the plasma membrane [190]. Leptin was found to slightly upregulate AQP1 in mouse gallbladder [191]. AQP8 has been found at the plasma membrane and, at lesser extent, at intracellular level of the gallbladder epithelium of different species [34, 138]. Recently, liver X receptor β (LXRβ), an oxysterol-activated transcription factor strongly expressed in the gallbladder epithelium, was seen to regulate the expression of AQP1 and AQP8 and the cystic fibrosis transmembrane conductance regulator (CFTR) [192]. Constitutively high water permeability in mouse gallbladder epithelium involving transcellular water transport through AQP1 was found in a study using AQP1 knockout mice [193]. Subapical AQP1 was hypothesized to translocate to the apical membrane to secrete water as in the bile duct epithelium, a functional homologue of the gallbladder epithelium. Based on its pattern of

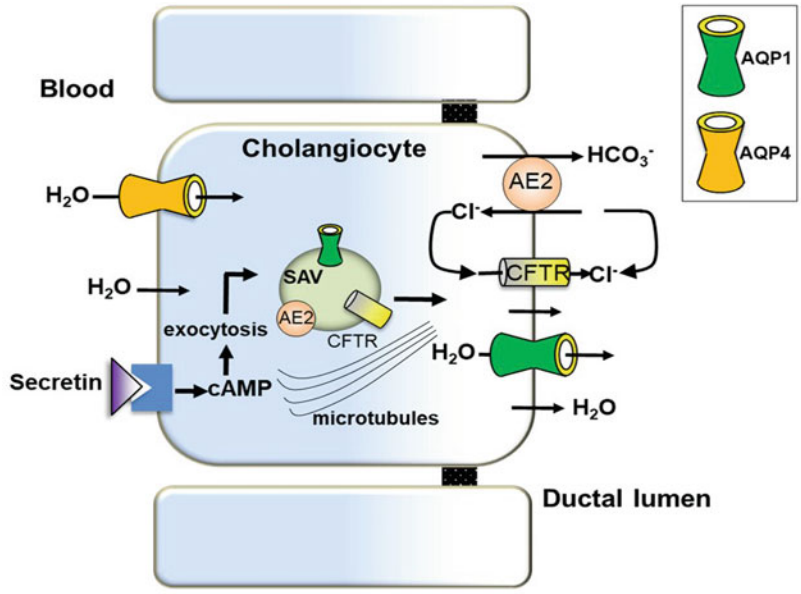

Fig. 16.4 Proposed mechanism of AQP-mediated water movement in ductal bile secretion. Intrahepatic bile ducts cholangiocytes. Secretin hormone, via cAMP, induces the microtubule-dependent apical targeting and exocytic insertion of subapical vesicles containing AQP1 and CFTR Cl^- channels, and the Cl^-/HCO_3^- exchanger AE2 into the apical membrane. The efflux of Cl^- via CFTR provides the luminal substrate to drive the extrusion of HCO_3^- into the lumen by means of AE2. HCO_3^- and Cl^- ions provide the osmotic driving force for the movement of water from blood plasma (mostly through basolateral AQP4) to biliary lumen (through apical AQP1). *AE2* anion exchanges isoform 2, *CFTR* cystic fibrosis transmembrane conductance regulator, *SAV* subapical vesicles

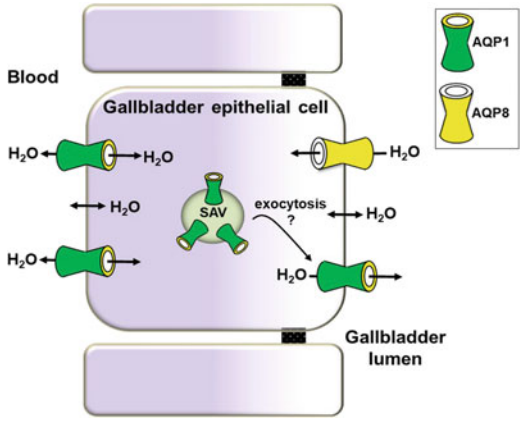

Fig. 16.5 Proposed mechanism of AQP-mediated water in cystic bile absorption/secretion. Gallbladder epithelial cells. AQP8 and AQP1 facilitate the osmotic absorption and secretion of water into and from the gallbladder lumen, respectively. Basolateral AQP1 mediates the entry/extrusion of water into/out of the epithelial cells. *SAV* subapical vesicle

subcellular localization gallbladder AQP8 was suggested to contribute to the secretion of water and to facilitate the absorption of water (Fig. 16.5) [138]. However, the physiological importance of AQP1 and AQP8 roles in gallbladder function remain debated matter due to the discrepant results reported in literature. Bile salt concentration was of similar extent in gallbladders from wild type and *Aqp1* knockout mice with AQP8 that was not appearing to functionally substitute for AQP1 [193]. This observation was not consistent with previous studies showing temporal association between decreased gallbladder concentrating function and reduced AQP1 or AQP8 expression [190], and leptin-deficient mice submitted to leptin replacement where leptin was altering the gallbladder volume likely by influencing the AQP-mediated absorption/secretion of water [194]. Additional work is needed to clarify the question.

16.1.5 Intestinal Goblet Cells

Current knowledge concerning the role of AQPs in intestinal goblets cells is very limited. So far, only AQP9 mRNA has been detected in a subset of mucus-secreting intestinal goblet cells [195]. Therefore, additional studies would be valuable to further study the expression and function of AQPs in these cells.

16.1.6 Exocrine Pancreas

The exocrine pancreas accounts for about 90% of the total pancreas and morphologically resembles salivary glands despite few differences. Indeed, it contains serous acinar cells only and centroacinar cells (extension of intercalated ducts into each acinus). In addition, the exocrine pancreatic fluid secretion drains into a main collecting duct. The major role of pancreatic fluid is to neutralize the stomach acid and the food digestion. Pancreatic fluid secretion is regulated by several neurotransmitters (i.e., acetylcholine, cholecystokinin, and secretin) that stimulate both pancreatic enzyme and fluid secretion or mainly fluid secretion, and that exert potentiated effects [196].

AQP1, AQP3, AQP4, AQP8, and AQP12 mRNAs are expressed in human exocrine pancreas. However, only few AQPs proteins have been detected, i.e., AQP1, AQP5, and AQP8 [197, 198]. Endothelial cells, centroacinar cells (apical membrane), intercalated ductal cells [197], and pancreatic zymogen granules express AQP1 [199, 200]. Intercalated ductal cells (apical membrane) express AQP5 [197]. AQP12 expression localization remains to be determined [198].

AQP1, AQP4, AQP5, AQP8, but not AQP12, mRNAs are expressed in rat exocrine pancreas [197, 198, 201]. AQP1 is localized to the apical and basolateral membranes as well as caveolae and vesicle-like structures of intralobular and intralobular ductal cells [202, 203], in acinar zymogen granules [199] and in endothelial cells [201]. AQP5 is expressed at the apical membrane of centroacinar and intercalated ductal cells [204]. AQP8 is located at the apical acinar cell membrane [198].

AQP1, AQP5, and AQP12 are expressed in mouse exocrine pancreas. Indeed, AQP1 and AQP5 are located at the apical membrane of interlobular ductal cells, and AQP5 is also expressed at the apical membrane of intercalated and intralobular ductal cells [204]. AQP12 is expressed intracellularly in acinar cells [205].

Pancreatic juice is produced by acinar cells secreting a small volume of isotonic fluid and ductal cells secreting ions and ensuring most of the water movement [4, 206]. The presence of AQP8 located at the apical acinar cell membrane, AQP1 located at both apical and basolateral ductal cell membranes, and AQP5 located at the apical ductal cell membrane ensure water movement to the acinar or ductal lumen [204]. AQP8 accounts for most water permeability (90%) in rat pancreatic acinar cells [201]. However, exocrine pancreatic function is unmodified in AQP8 knockout mice, possibly due to the fact the much contribution of acinar cells than ductal cells to the overall water movement [26]. In rat pancreatic acinar zymogen granules, AQP1 contributes to basal and GTP-mediated vesicle water movement and swelling [199, 200]. In rat interlobular ductal cells, AQP1 account for most of secretin-stimulated pancreatic juice secretion [203]. However, AQP1 knockout mice display normal exocrine pancreatic function, like the AQP5 knockout mice [197]. These data may be due to weak level of AQP1 and AQP5 expression or functional redundancy. In this context, double AQP1 and AQP5 knockout mice might be useful to assess the specific contribution of each of these AQP to the exocrine pancreatic function. In addition, further studies are necessary to shed light on the possible role of AQP12 in pancreatic juice secretion.

16.1.7 Endocrine Pancreas

Endocrine pancreatic cells account for a minor fraction of total pancreatic cells (about 10). They form the islets of Langerhans composed of insulin-producing β-cells surrounded by glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-

producing PP cells [207]. The major function of human endocrine pancreas, and in particular of the β-cells, is to secrete insulin [208, 209]. Insulin secretion by β-cells relies on the following subsequent steps: glucose entry via the glucose transporter type 2 (GLUT2), glucose metabolization, intracellular ATP concentration increase, ATP-sensitive K^+ channels inhibition, membrane depolarization, voltage-dependent Ca^{2+} channels opening, intracellular calcium elevation, and finally insulin-containing granules exocytosis [208]. Moreover, glucose induces β-cell swelling [210] that triggers subsequent volume-regulated anion channel (VRAC) activation, cell membrane depolarization, voltage-dependent Ca^{2+} channels activation, calcium entry and insulin secretion [211, 212].

Although to our knowledge the expression of AQPs in human endocrine pancreas remains to be assessed, it has been shown that rat β-cells express AQP7 [213–215] and mouse β-cells express AQP5, AQP7, and AQP8 [214]. Nevertheless, the expression of AQPs remains to be determined in the other cell types composing the rat and mouse islets of Langerhans.

Functional studies have shown the involvement AQP7 in the regulation of intracellular glycerol content, insulin production, and secretion in β-cells. Indeed, AQP7 knockout mice displayed a reduction in β-cell size and mass, insulin content and cAMP-driven glycerol release [215, 216] and an increase in basal and glucose-stimulated insulin secretion rates, glycerol and triglyceride contents and glycerol kinase activity [215]. However, genetic background influences the AQP7 knockout mouse phenotype. Indeed, according to their genetic background, AQP7 knockout mice had hyperinsulinemia [215, 216] with [216] or without [215] hyperglycemia, or had normal glycaemia with undetermined insulin levels [217]. In both β-cells and rat pancreatic β-cell line BRIN-BD, the addition of extracellular isosmotic glycerol induces sequential cell swelling, VRAC activation, membrane depolarization, electrical activity, and insulin secretion (Fig. 16.6) [213, 218, 219]. The entry of glycerol

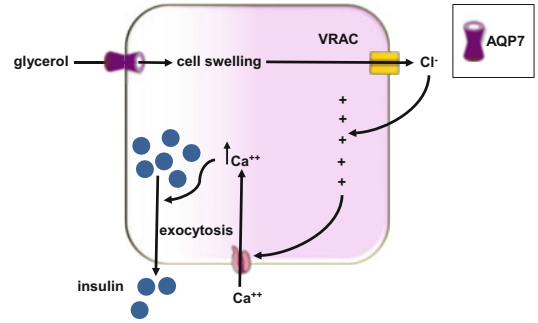

Fig. 16.6 Proposed mechanism of AQP7-mediated insulin secretion in pancreatic β-cells. Glycerol entry via AQP7 induces sequential cell swelling, VRAC activation, membrane depolarization, electrical activity, and insulin secretion. VRAC Volume-regulated anion channel

and its subsequent metabolization are likely contributing to the activation of β-cells [213]. Compared to AQP7 wildtype mice, AQP7 knockout mice had reduced insulin release in response to increased D-glucose concentration, extracellular hypotonicity or extracellular isosmotic addition of glycerol [214]. AQP7 regulates insulin release by allowing both glycerol entry and exit, and by acting directly or indirectly at a distal downstream site in the insulin exocytosis pathway [214]. So far, no clear conclusion has been drawn regarding the association between mutations or single-nucleotide polymorphisms of AQP7 and diabetes and/or obesity [220–224]. In rat pancreatic β-cell line RIN-m5F, tumor necrosis factor α decreased AQP7 expression and insulin expression but increased AQP12 expression, while lipopolysaccharides increased AQP7 and AQP12 expression but decreased insulin secretion. In addition, in cells treated by tumor necrosis factor α or lipopolysaccharides, overexpression and silencing of AQPs revealed the involvement of AQP7 in insulin secretion and of AQP12 in inflammation [225]. In rat RIN-m5F β-cells, AQP8, located in the mitochondrial and plasma membranes, has been shown to play a role in attenuating cytokine-mediated cell toxicity [226]. Further studies are required to pursue deciphering the physiological and pathophysiological role of AQPs within β-cells.

16.2 Airway Submucosal Glands

Airways submucosal gland are present in the human trachea and bronchial airways or in rat and mouse trachea. They are made of serous and mucous acinar cells forming secretory tubules, and ductal cells forming lateral and collecting ducts [227]. The airway submucosal glands secrete a fluid rich in water, ions, and mucins to ensure proper hydration of the airway surfaces, mucociliary transport, and reception of secreted molecules such as mucins [227]. Acetylcholine and VIP stimulate submucosal gland secretion [227]. The secretion of Cl^- and HCO_3^- creates an electrical gradient allowing paracellular movement of cations such as Na^+. This leads to the formation of an osmotic gradient driving the transcellular movement of water to the glandular lumen [227]. AQP5, located at the apical membrane of submucosal serous epithelial cells, plays a role in the transcellular water movement [228, 229] as shown in AQP5 knockout mice displaying a 50% reduction in submucosal secretion as compared to wild type mice [230]. Interestingly, in patients suffering from chronic obstructive pulmonary disease, AQP5 expression is decreased in submucosal glands and correlated to the disease's severity [231]. Submucosal glands from asthmatic patients displayed increased AQP5 expression [232]. In an animal model of asthma, AQP5 deletion decreased both mucin secretion and inflammatory cytokines levels [232]. Therefore, it is hypothesized that AQP5 is involved in the development of mucous hyperproduction and inflammation during chronic asthma [232, 233]. Further studies will contribute to a better understanding of the regulation and role of AQP5 in submucosal glands in relation to pulmonary diseases.

16.3 Lacrimal Glands

Lacrimal glands are made of multi lobules. Each lobule is made of acinar cells secreting a fluid into a network of ducts made of intralobular, interlobular, intralobar, interlobar, and ducts. Acinar cells are surrounded by myoepithelial cells. Acetylcholine and adrenalin are the major neurotransmitter controlling lacrimal glands secretion. The main function of lacrimal glands is to secrete a fluid rich in water, lipids, mucins, and antimicrobial substances to protect cornea from exogenous and environmental insults, thus facilitating the maintenance of a refractive surface necessary for clear vision [234].

Rat lacrimal glands express several AQPs. Indeed, AQP1 and AQP5 are expressed in endothelial cells express. Acinar cells express AQP3 at their basolateral membrane, AQP4 at their lateral membrane, AQP5 at their apical membrane, and AQP11 intracellularly [235]. Mouse lacrimal acinar cells express AQP3 only in fetal tissue but not in adult tissue [236], AQP4 at their basolateral membranes, and AQP5 at their apical membranes [16, 236, 237]. Mouse lacrimal ductal cells express AQP5 at their apical membrane [236, 238]. Mouse lacrimal ductal and myoepithelial cells express both AQP8 and AQP9 [236].

Lacrimal fluid secretion results from the formation of a primary isotonic fluid by acinar cells and its subsequent modification by the ductal cells [239]. However, ductal cells have been considered to also play a role in electrolytes and water secretion [240, 241]. The final lacrimal fluid composition may vary according to the flow rate and species considered [239]. AQPs expressed in both acinar and ductal cells are likely contributing to tear secretion. However, the involvement of AQPs in lacrimal fluid secretion has not been confirmed using knockout mice for AQP1, AQP3, AQP4, or AQP5 [238, 242]. However, one study showed significant in situ tear film hypertonicity in AQP5 knockout mice [243]. Recently, it was shown that AQP5 knockout mice presented primary dye eye phenotype that may result from the differential expression of circular RNA [244]. Genetic background and/or ways to generate AQP5 knockout mice could account for these phenotypic differences in terms of lacrimal fluid secretion. Therefore, further studies are necessary to address the assumption that AQPs may not be required for low rates such as in lacrimal glands [245] and to further

study the role of AQPs in lacrimal glands, and particularly AQP8 that has recently been shown to be expressed in ductal cells.

Defective AQP5 trafficking has been shown in lacrimal acinar cells from patients suffering from Sjögren's syndrome, an autoimmune disease characterized by dry eyes and dry mouth [246]. In addition, animal model of Sjögren's syndrome displayed modified AQP5 mRNA and protein levels in ductal (increased) and acinar (decreased) cells, as well as AQP4 expression in ductal cells (decreased) [247]. Altered calcium signaling and volume regulation occurring in Sjögren's syndrome may account these modifications [248]. Further experimentation is necessary to decipher the role of AQPs pathologies affecting lacrimal glands.

16.4 Mammary Glands

Mammary glands are apocrine glands made of alveoli lined with milk-secreting cuboidal acinar cells surrounded by myoepithelial cells, and lactiferous ducts (intralobular and interlobular ducts) draining milk to the openings in the nipple [249]. Milk is composed of sugars, lipids, proteins, vitamins, minerals, and water [250]. According to species and physiological status considered, milk contains variable percentage of water [251].

Rat and mouse mammary glands express AQP3 at the basolateral membrane of acinar cells and in intralobular and interlobular ductal cells, and AQP5 at the apical membrane of acinar cells [252]. They also express AQP1 at the apical and basolateral membranes of endothelial cells [253]. Bovine mammary glands express AQP3 and AQP4 respectively at the basolateral membrane of acinar cells and at the apical membrane of some ductal cells [254]. In addition, AQP7 is present at the apical membrane of some acinar cells and AQP1 is expressed in endothelial and myoepithelial cells [254].

AQP3 may be involved in both water and glycerol transport that are essential for milk synthesis and secretion [253]. Glycerol uptake via AQP3 may participate to milk triglycerides synthesis [253]. Interestingly, the expression pattern of AQP3 and AQP5 is distinctly regulated by lactogenic hormones in acinar and ductal mammary cells before and after parturition [255]. Besides, AQP5 may regulate milk osmolarity [255]. In mammary glands with mastitis, proinflammatory cytokines reduce milk production possibly by inducing decreased AQP3 expression [256]. Higher AQP3 expression induced by polyherbal formula accounts for increased milk production in rats [257]. AQPs are likely to play a role in mammary tumors and breast cancer [107, 258, 259]. However, it is unclear whether altered AQP expression is the cause or the consequence of neoplasia [258]. The use of *Aqp* knockout mice models and further studies will be valuable for a better understanding of the role of AQPs in milk secretion under physiological and pathological conditions, and to determine if AQPs could be used as therapeutic targets, diagnostic or prognostic biomarkers.

16.5 Eccrine Sweat Glands

Eccrine sweat glands are made of single tubular structure containing acinar cells and ductal cells. Mouse, rat, and human eccrine sweat gland acinar cells express AQP5 at their apical membrane [260–262]. Upon stimulation, AQP5 traffics to that location [260]. Acinar cells secrete a primary fluid rich in ions and water that undergoes salt reabsorption when reaching the ductal cells [263].

Whether AQP5 plays a role in eccrine sweat glands remains an open debate due to variable data obtained using different *Aqp5* knockout mice strains and methods to assess the secretion [261, 264]. Therefore, further studies will help precising the role of AQP5, and possibly as well other AQPs, in sweat secretion.

Various skin pathologies are characterized by modified AQP5 expression within the eccrine sweat glands [265–267]. Activin a receptor type 1 and cholinergic receptor nicotinic alpha 1 subunit are involved in the AQP5 overexpression detected in hyperhidrosis [268, 269]. In addition,

mutations of AQP5 gene are responsible for palmoplantar keratoderma [270–273].

16.6 Conclusions

A variety of exocrine and endocrine gland express AQPs that play a role in exocrine or endocrine secretory processes. Furthermore, some AQPs are involved in some secretory gland dysfunction or diseases. Despite considerable efforts made to understand the role of AQPs in the physiology and pathophysiology of secretory glands, further studies are still necessary to further advance the current knowledge in the field.

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Aquaporins in Fetal Development

17

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Abstract

Water homeostasis is essential for fetal growth, and it depends on the successful development of the placenta. Many aquaporins (AQPs) were identified from blastocyst stages to term placenta. In the last years, cytokines, hormones, second messengers, intracellular pH, and membrane proteins were found to regulate their expression and function in the human placenta and fetal membranes. Accumulated data suggest that these proteins may be involved not only in the maintenance of the amniotic fluid volume homeostasis but also in the development of the placenta and fetal organs. In this sense, dysregulation of placental AQPs is associated with gestational disorders. Thus, current evidence shows that AQPs may collaborate in cellular events including trophoblast migration and apoptosis. In addition, aquaglyceroporins are involved in energy metabolism as well as urea elimination across the placenta. In the last year, the presence of AQP9 in trophoblast mitochondria opened new hypotheses about its role in pregnancy. However, much further work is needed to understand the importance of these proteins in human pregnancies.

Keywords

Aquaporins · Placenta · Fetal

17.1 The Water Transport across the Human Placenta

The development of the placenta and the fetus is a continuous process that begins with fertilization. The placenta is a transient organ with highly specialized functions, found only in mammals. It consists of an intricately branched, fetally derived villous tree, bathed directly by maternal blood released from the uterine spiral arteries into the intervillous space. The fetal-maternal exchange takes place in these villi.

The main functions of the placenta are to allow the selective transfer of substances between the mother and the fetus and to keep the fetal fluid homeostasis [1, 2]. Fetal water requirements rise significantly during pregnancy as a result of the exponential increase in fetal weight [2, 3]. Consequently, proper fetal growth and homeostasis

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depend on the successful formation of the placenta.

Water exchange can occur directly between amniotic fluid (AF) and fetal blood. This is known as the intramembranous pathway. On the other hand, water can also move transcellular between the intervillous space and the fetal blood by a route governed by the syncytiotrophoblast, the outmost covering cell layer of the floating chorionic villi. Previous studies using isolated vesicles showed that water could permeate across the syncytiotrophoblast by lipid diffusion [4, 5]. However, this mechanism is insufficient to meet the fetal water needs during pregnancy. In subsequent experiments, AQP3 and AQP9 were identified in the apical membrane of the human syncytiotrophoblast, supporting the idea of a water-facilitated transport across the syncytium [6]. In addition, functional experiments using human placental explants confirmed that these AQPs could mediate water transfer across the placenta [7]. Therefore, the discrepancy between isolated vesicles and placental explants results may indicate that the function of these proteins is tightly controlled by several mechanisms, such as the cytoskeleton and other proteins, which may be lost during the isolation of the vesicles [8]. On the other hand, emerging physiological data reveals the presence of scarce tight junctions in syncytiotrophoblast that allows a paracellular pathway for water transfer [9]. Nowadays, it is well-accepted that water is transferred across the syncytiotrophoblast through paracellular and AQP-mediated transcellular routes [2].

17.2 Expression of AQPs in Placental and Fetal Membranes throughout Pregnancy

After fecundation, the zygote undergoes rapid cellular division to form a morula. Subsequently, compaction begins by enhancing the cell-to-cell contact. This process triggers the development of the trophectoderm that starts and regulates the formation of a fluid-filled cavity inside the morula. Thus, the morula develops into the blastocyst [10, 11].

Previously, in murine embryos from the one-cell stage up to the blastocyst stage the expression of AQP1, AQP3, AQP5, AQP6, AQP7, and AQP9 was found [12]. In addition, Barcroft and co-workers reported a differential localization of AQP3, AQP8, and AQP9 in mouse blastocysts [12]. They showed that AQP9 was detected in the apical membrane of the trophectoderm, while AQP3 and AQP8 were localized in the basolateral membrane. AQP3 was also observed in the inner cell mass [12]. The presence of these AQPs in both, the apical and basolateral membrane domains of the trophectoderm, suggests that they may promote the trans-trophectoderm fluid accumulation that takes place during cavitation [10, 11].

In preimplantation human embryos, mRNAs of AQP1, AQP2, AQP3, AQP4, AQP5, AQP7, AQP9, AQP11, and AQP12 were detected. However, only AQP3 and AQP7 expressions are sustained from the zygote through the blastocyst stage, indicating that both AQPs may have an important role in the early stages of embryogenesis [13].

Since the placenta (trophoblast) and the chorion are derived from the trophectoderm, it seems to sense that these proteins would be expressed in both the placenta and the fetal membranes. The differentiation of the trophoblast cells is required for placentation. Thus, trophoblast cells develop into two cell types: the villous mononucleated cytotrophoblast cells, which proliferate, differentiate, and establish the placental floating villi by fusing into multinucleated syncytiotrophoblast cells, and the extravillous trophoblast (EVT) cells related to the anchoring villi [14]. Villous syncytiotrophoblast cells are in immediate contact with the maternal blood and mediate the feto-maternal exchange. The syncytiotrophoblast cells also participate in the synthesis of critical hormones for the progression of gestation. On the other hand, EVT cells take part in the remodeling of the uterine spiral artery to ensure the proper blood supply to the fetal-placental unit [15].

Early in human pregnancy, the expression of AQP1, AQP3, AQP4, AQP5, AQP8, AQP9, and AQP11 mRNA was detected in chorionic villi. Interestingly, AQP3 and AQP9 showed the highest levels of expression [16, 17]. As AQP3 and AQP9 can permeate glycerol and urea, in addition to water, it was proposed that both proteins may take part in energy metabolism throughout gestation. However, the role of AQPs in the early stages of gestation remains unknown.

The expression of AQP4 was particularly studied throughout gestation. It was reported that its expression decreases in chorionic villi with the advance of gestation [18, 19].

Regarding AQP11, given its cytoplasmatic location, it could have an important role in intravesicular homeostasis and organogenesis [16, 20, 21].

In human term placenta, AQP3 and AQP9 were the first AQPs identified in the apical membranes of the villous syncytiotrophoblast [6]. AQP8 was also detected in the trophoblasts but its cell polarity distribution was not established yet [22]. AQP4 expression was nearly undetectable in the syncytiotrophoblast at term, but its expression was higher in endothelial cells and stroma of placental villi [18, 19] Table 17.1.

Conflicting evidence exists on AQP2 expression in human placenta. Although several publications have shown that AQP2 cannot be detected in human placenta, Table 17.1 [16, 26], Zhao and co-workers have found AQP2 expression in chorionic villi and fetal membranes from normal placentas, as well as an increase of this protein in placentas from preeclampsia-affected pregnancies [25].

AQPs have also been extensively explored in human fetal membranes. The expression of AQP1, AQP3, AQP8, AQP9, and AQP11 mRNAs and proteins were found in human amnion and chorion throughout gestation [17]. It was also described that the levels of AQP1, AQP3, and AQP8 were higher during the first trimester, while the expression of AQP9 and AQP11 increased in the second trimester [17]. Thus, the time-specific expression pattern of these AQPs may reflect modifications in the volume and composition of human AF throughout gestation.

Mann and co-workers observed the expression of AQP1 and AQP3 in fetal membranes at term and also localized AQP1 in placental endothelial cells [23]. Chorioamniotic membranes were also shown to express AQP8 and AQP9 [22, 24]. Moreover, cytosolic expression of AQP11 was also detected in amniotic membranes at term [17].

Interestingly, Bednar and co-workers described regional differences in the individual expression of the five AQPs found in the amnion. They observed higher AQP-mRNA levels and lower AQP-protein levels in the placental amnion (which covers the placenta) compared to the reflected amnion (which covers the membranous chorion) [21]. Based on these findings, it was assumed that each AQP may have a particular role in the human amnion.

In Table 17.1, the expression of AQPs in human placenta and fetal membranes throughout pregnancy is shown.

17.3 Expression of AQPs in Fetal Tissues

During fetal development, AQPs are expressed in fetal tissues to allow the proper fluid exchange. Park and Chon have found that AQP11 expression is enhanced during embryogenesis and proposed that this protein may participate in the development of several organs including the kidneys and salivary glands [20]. Furthermore, AQP11 gradually decreases with the advance of gestation and fetal growth, suggesting that when fetal organ functionality has reached maturity, other AQPs such as AQP1, AQP3, AQP8, or AQP9, may take over water regulation [20].

In animal models, it is well-documented that AQPs are necessary for the correct development of several organs including the lung, skin, heart, central nervous system, and so others [1, 27]. Conversely, limited data describes the expression and function of AQPs in human fetal tissues. In Table 17.2, AQPs found in fetal tissues of rats, mice, pigs, sheep, and humans are summarized. AQPs identified in human adult organs are also

Table 17.1 Expression of AQPs in the human placenta and fetal membranes throughout pregnancy

	Location	AQPs EXPRESSION	Reference
Blastocyst	Trophectoderm	AQP3, AQP7	[13]
Early placenta	Chorionic villi	AQP1, AQP3, AQP4, AQP5, AQP8, AQP9, AQP11	[16–20]
Term placenta	Fetal membranes	AQP1, AQP3, AQP8, AQP9, AQP11	[17, 22–24]
	Placental vessels	AQP1, AQP4	[18, 23]
	Villous trophoblast	AQP2, AQP3, AQP4, AQP8, AQP9	[6, 18, 19, 22, 25]

shown [28, 40, 48]. It is important to mention that there are some differences among the different species in terms of placentation, gestation length, and vital organ maturation, which can affect water transport into and out of the compartments.

The expression of AQPs in fetal lung and kidney are of particular significance because fetal urine and lung secretions are involved in AF formation.

17.3.1 Fetal Kidneys

Nephrogenesis begins during the embryonic stage but continues throughout the fetal stage. The expression of AQPs during fetal kidney development was studied in rats, mice, pigs, sheep, and humans [29–38, 53]. In human beings and sheep, the expressions of AQP1 and AQP2 can be found earlier in gestation compared with mice and rats. AQP1 is expressed in the proximal tubules before AQP2, AQP3, and AQP4 are expressed in the collecting ducts [29, 35, 39]. In human fetuses, AQP1 and AQP3 increase progressively with gestational age, whereas AQP1 expression is very intense in renal tubules [36, 37]. Studies on knockout mice lacking AQP1, AQP2, AQP3, or AQP4 revealed that these AQPs have a central role in the kidney's ability to concentrate urine [29, 54, 55]. Thus, the increase in the expression levels of these AQPs may indicate maturation of the urinary

Table 17.2 AQPs expressed in fetal tissues of rats, mice, pigs, sheep, and humans

		Fetus				
	Human adult	Rat	Mouse	Pig	Sheep	Human
Kidney	AQP1, AQP2, AQP3, AQP4, AQP5, AQP6, AQP7, AQP8, and AQP11 [28]	AQP1, AQP2, AQP3, and AQP4 [29–31]	AQP1, AQP2, AQP3, and AQP4 [29, 31, 32]	AQP1, AQP2, AQP3, and AQP4 [33]	AQP1 and AQP2 [34, 35]	AQP1, AQP2, and AQP3 [29, 36–39]
Bladder and urothelium	AQP3, AQP4, AQP7, AQP9, and AQP11 [40]	–	–	AQP1, AQP3, AQP5, AQP9, and AQP11 [41]	–	–
Lung	AQP3, AQP4, and AQP5 [28]	AQP1, AQP4 [42, 43]	AQP5 [44]	–	AQP1, AQP3, AQP4, and AQP5 [45]	–
Skin	AQP0, AQP1, AQP3, AQP4, and AQP5 [28]	AQP1, AQP3 [46]	–	–	–	–
Heart	AQP1, AQP3, AQP4, AQP5, AQP7, AQP9, AQP10, and AQP11 [28]	–	–	–	AQP1, AQP3, AQP4, and AQP8 [27, 47]	–
Brain	AQP1, AQP4, and AQP9 [28, 48]	AQP4 [49, 50]	AQP4 [51]	–	–	AQP1 and AQP4 [52]

concentrating capacity [56]. Low levels of AQP2 were also reported and correlated with the big amounts of dilute urine produced, which is essential for the maintenance of AF homeostasis [29].

17.3.2 Fetal Urinary Tract

Jakobsen and co-workers have recently reported the expression of AQP1, AQP1, AQP3, AQP5, AQP9, and AQP11 mRNA along the urinary tract in porcine fetuses [41]. They also described that expression levels of these proteins change throughout gestation. AQP11 is downregulated. In contrast, other AQPs are upregulated, such as AQP1. In addition, AQP5 mRNA transcript was detected in the urethra while AQP1 was also found in endothelial cells of vessels in the bladder wall [41]. However, the functional and developmental consequences of these findings have not been explored.

17.3.3 Fetal Lungs

Lung development during fetal life depends on water secretion into early alveolar spaces and airways. In addition, AQPs in fetal lungs may also be contributing to AF formation. Regarding AQP expression in the lungs, four AQPs (AQP1, AQP3, AQP4, and AQP5) were described in sheep before birth, with high mRNA levels of AQP1 and AQP5 [45]. In mice, a small amount of mRNA of AQP5 was found [44]. In the rat lung, Early stages of development already show AQP1 expression and its levels increase just before birth and during the first weeks after birth and adulthood [42]. On the other hand, during the prenatal period, AQP4 expression is relatively low, and it briefly rises in the first few days following delivery [43]. Finally, AQP5 expression rises throughout pregnancy and then gradually increases until maturity [42]. These perinatal changes in AQPs expression correlate with an increase in water permeability depending on increased water channel activity.

17.3.4 Fetal Heart

In sheep fetal hearts, AQP1 was found in the endocardium at a very early stage [27]. Later in gestation, this protein localizes predominantly in vascular sites [47]. Wintour and co-workers have also detected AQP1, AQP3, AQP4, and AQP8 in the late gestational ovine fetal heart [27].

17.3.5 Fetal Skin

The expression of AQPs was also explored in rat skin at different stages of fetal growth. AQP1 was localized in dermal capillaries, while AQP3 was present in the basal cells of the epidermis of fetal rats [46]. The increased expression of these AQPs in fetal skin compared to more mature skin suggests that these proteins may be involved in the control of epidermal hydration.

17.3.6 Fetal Brain

In the chick brain, the expression of AQP4 begins during prenatal life, while in rodents its expression appears just after birth [49, 50]. These differences between avians and mammals may result from species-specific characteristics and a different time course for the development of the blood–brain barrier. In the fetal mouse brain, a non-polarized AQP4 expression was also observed [51]. In humans, Gömöri showed that AQP1 and AQP4 proteins are expressed in the fetal brain since the 14th week of gestation and progressively increase with the advance of gestation [52].

17.4 Physiological and Pathological Significance of AQPs in Fetal Membranes

AF volume homeostasis is necessary for healthy fetal growth and development. AF volume regulation depends on a balance between the production and resorption of this fluid. Physiologically,

fetal water requirements progressively increase throughout gestation. Near term, the fetal membranes allow up to 400 mL of fluid per day to flow from the amniotic cavity into the fetal circulation, driven by an osmotic gradient between the amniotic compartment and the fetal blood (255 mOsm/kg versus 280 mOsm/kg, respectively) [57–59].

Many theories were postulated to clarify the mechanisms that control the intramembranous flow. According to recent research, an active unidirectional vesicular transfer of water and solutes through the amnion is the primary mechanism that regulates the intramembranous flow [60, 61]. A second mechanism is the passive bidirectional transcellular water movement in response to osmotic gradients, mediated by AQPs [62, 63]. Although it appears that AQPs have a limited impact on AF volume regulation, an abnormal expression of these proteins is associated with several disorders such as abortion, premature birth, AF volume abnormality, malformation, and fetal growth restrictions. Thus, these disorders may affect the homeostasis of the maternal-fetal fluid exchange [2].

Consequently, the relationship between AQPs and obstetrical pathologies such as polyhydramnios (high AF volume) and oligohydramnios (low AF volume) was widely studied. In idiopathic polyhydramnios, Mann and co-workers reported that AQP1 increased 33-fold mainly in the amnion [64], and hypothesized that this increase may be a compensatory response to polyhydramnios. Moreover, Zhu and colleagues showed a significant increase of AQP8 in the amnion and AQP9 in the amnion and the chorion, and a considerable decrease in the placenta [65]. Along with these findings, these authors assumed that several modulating factors trigger changes in AQP8 and AQP9 expressions, increasing the intramembranous absorption and decreasing the water flow from the mother to the fetus.

In contrast, in pregnancies complicated with oligohydramnios, Zhu and co-workers found a downregulation in AQP1 expression in the amnion. However, no significant changes were observed in the chorion and the placenta [66]. Concerning AQP3 in oligohydramnios, these authors showed a decrease in the amnion and the chorion and a significant increase in the placenta [66]. Furthermore, Jiang and co-workers reported that in this pathology, AQP8 and AQP9 decreased in amnion and increased in the placenta [67]. They also found a reduced AQP9 expression in the chorion, while AQP8 remained unchanged [67].

Even though changes in AF volume and the expression of AQPs seem to be linked, their role remains uncertain. In this regard, some AQP-knockout mice have been established, but none of them are suitable for studying changes in AF volume [68–70]. Thus, AQP1 null mice showed an increase in AF volume with a reduced osmolarity [68], but in humans, polyhydramnios is not associated with a decrease in AQP1 expression [64]. Additionally, a recent study by Luo and co-workers found that the absence of AQP1 led to the overexpression of AQP8 and the downregulation of AQP9 in fetal membranes [71].

Furthermore, Sha et al. showed in AQP8-knockout mice an AF volume increase [69]. Assuming that AQP8 and AQP1 are highly permeable to water, these results revealed a compensating mechanism between both AQPs in the regulation of AF volume. Interestingly, neither oligohydramnios nor polyhydramnios occurred in AQP3- knockout mice [70]. Indeed, the lack of AQP3 in the placenta and fetal membranes led to reduced metabolite concentrations in the AF and affected normal fetal growth, suggesting that AQP3 may facilitate the transport of glycerol from the mother to the fetus [70].

According to these findings, Di Paola and co-workers have recently investigated the participation of the amnion AQPs in the transamniotic water flux [63]. They demonstrated that the maintenance of AF volume homeostasis is more largely supported by AQP1 and cannot be substituted by any other AQPs. This fact strongly suggests the idea that AQP1 might play a key role in oligohydramnios and polyhydramnios. However, amnion AQPs are more expected to be engaged in compensatory mechanisms rather

than in the pathogenesis of these pregnancy disorders.

17.5 Physiological and Pathological Significance of AQPs in Human Trophoblast

The classical role of AQPs is to mediate water movements across cell membranes induced by osmotic gradients. Therefore, these proteins may contribute to the feto-maternal water exchange and also to the rapid movement of solutes across cell membranes, with minimal osmotic perturbation [6]. Nevertheless, the importance of placental AQPs in the water transport between the mother and the fetus is still debated.

Several obstetric complications such as preeclampsia and diabetes showed an altered expression of these proteins in the placenta with no impact on water transfer between the mother and the fetus [7, 25, 72–74]. In preeclamptic placentas, AQP3 and AQP4 decreased, whereas AQP2 and AQP9 increased [7, 25, 72, 73]. Functional experiments showed that transcellular water movement was dramatically reduced in these placentas, and it was not mediated by AQPs [7]. In addition, an unusual increase in AQP9 expression was observed in diabetic placentas [74]. None of these changes were linked to clinical evidence of altered feto-maternal water flux. Consequently, these findings support the hypothesis that these proteins may be involved in other cellular processes that require rapid changes in cell volume [75].

The most extensively studied AQP in trophoblast cells is AQP9. AQP9 is of particular relevance because, additionally to water, it is permeable to neutral solutes such as polyols, purines, and pyrimidines [76]. In this regard, in vitro experiments were performed in placental explants to explore AQP9 function. Thus, it was reported that AQP9 protein decrease mediated by insulin did not modify water uptake [77]. On the other hand, in explants exposed to hypoxia/reoxygenation, the increased expression of AQP9 did not correlate with an increase in water uptake [78]. Given that AQP9 also functions as a metabolite channel, it is plausible that this protein has a minimal impact on water transfer across the human placenta. Recently, Medina and co-workers have reported that trophoblast cells can use lactate as a glucose substitute source of energy by an AQP9-mediated transport. Furthermore, it was observed that the blocking of AQP9 in placental explants cultured in a low glucose medium supplemented with L-lactate triggered trophoblast apoptosis [79]. Consequently, AQP9 may facilitate the transfer of lactate together with monocarboxylate transporters in the human placenta.

Interestingly, this protein was also localized in the large/heavy mitochondria subpopulation [79] which is related to the apoptotic processes needed for the normal differentiation and turnover of villous trophoblast cells [80]. In this context, an abnormal AQP9 may impair mitochondrial function, giving rise to the activation of the mitochondrial pathway of apoptosis. Consequently, the presence of heavy/large mitochondria with a functional AQP9 may help the villous trophoblast cells to respond to stress more effectively [79].

Previous reports in preeclamptic placentas revealed an abnormal increase of AQP9 which was localized not only in the syncytiotrophoblast membranes but also in the cytoplasm [7]. Functional experiments also demonstrated that water and monocarboxylate uptakes were dramatically reduced and were not sensitive to $HgCl_2$ [7]. Altogether these results reveal that AQP9 permeability is altered in this pathological condition [7]. Preeclampsia is a pregnancy syndrome associated with enhanced oxidative and nitrative stress which is crucial in exacerbating villous trophoblast apoptosis [81, 82]. Thus, reactive oxygen (ROS) and nitrogen (RNS) species increase may modify the structure of many proteins such as AQPs, affecting their function [83]. It was recently found an increase of a nitrated AQP9 in placentas from pregnancies complicated with preeclampsia [84]. As a result of the reduced expression of glucose transporters [85] and the decreased aerobic glycolysis in preeclamptic placentas [86], lactate concentrations may be raised in this syndrome [87–89]. In this scenario, it was hypothesized that a dysfunctional

AQP9 may affect the ability of the placenta to use lactic acid, promoting more accumulation of ROS and negatively compromising the survival of the trophoblast cells.

Furthermore, AQP9 overexpression in the placentas of women with gestational diabetes mellitus may also strengthen the role of AQP9 in energy metabolism [74, 90].

Regarding AQP3, it was recently documented that this protein participates in the migration, invasion, and endovascular differentiation of EVT cells [91–93]. Even though the expression of AQP3 in early placentas that result in preeclampsia or fetal growth restriction at the end of pregnancy is unknown, it was observed in cell lines of EVT such as Swan 71 [91, 92] or HTR8/SVneo [93], and in rat models [94] that a reduced AQP3 expression or the blocking of its function impaired these processes. Thus, an abnormal expression of AQP3 at the early stages of placenta formation may lead to a superficial trophoblast invasion and inadequate remodeling of the maternal spiral arteries impairing fetal growth [75]. This poor transformation of the maternal spiral arteries may also produce an ischemia-reperfusion insult, enhancing the oxidative stress and the apoptosis of the villous trophoblast. Thus, the results of these changes could lead to a combination of preeclampsia and fetal growth restriction.

On the other hand, it is well-accepted that the efflux of K^+ creates an osmotic gradient that moves water out of the cell through AQPs forcing the decrease of the cell volume known as apoptotic volume decrease (AVD) [95]. After AVD, the inactivation of AQPs results in alterations in the plasma membrane, which become much less permeable to water, while the ongoing loss of ions K + reduces the ionic strength of the cytoplasm and triggers the activation of the apoptotic caspases [96]. Szpilbarg and co-workers have shown that in placental explants exposed to hypoxia/reoxygenation to induce cell death, the blocking of AQP3 abrogates the villous trophoblast apoptosis [97]. Consequently, placental AQP3 may also play a role in the physiological apoptosis required for the normal turnover of the villous trophoblast [98]. Therefore, the reduced expression of AQP3 in preeclamptic placentas could affect this physiological process increasing the cellular stress of the syncytiotrophoblast [73].

Finally, uptake experiments using placental explants have demonstrated that AQP3 and AQP9, together with urea transporters (UTs), may also facilitate urea transfer. Thus, the participation of both AQPs in the urea excretion across the placenta cannot be discarded [7].

Up to now, the significance of AQP2, AQP4, and AQP8 in human trophoblast was not explored. However, it is important to remark that the expression of AQP4 in the trophoblast progressively decreases with advancing pregnancy, proposing that this protein may have a role in placentation [19, 72]. In recent years, in autoimmune diseases associated with AQP4-IgG, it was found that these autoantibodies cause placental necrosis and inflammatory cell infiltration into the placenta [99, 100]. Moreover, in a pregnant animal model, the AQP4 antibody was reported to induce placenta insufficiency [19].

Finally, AQP11, in contrast to the other AQPs identified during gestation, is localized in the membranes of some organelles as the endoplasmic reticulum (ER) [101]. Due to its unusual location in the cell, it is still unclear if this protein can act as a water channel [102, 103]. It was suggested that AQP11 may regulate the permeability of the endoplasmic reticulum membrane to water, ensuring an adequate environment for protein translation and folding [16, 17, 101]. In this way, the increased levels of AQP11 during embryogenesis could be related to the development of fetal organs [1, 2, 20].

Recent evidence demonstrated that AQP11 may also function as a peroxiporin, expanding its role in the management of intracellular H_2O_2 homeostasis to avoid ER stress [104]. However, additional research is required to elucidate its role in the human placenta.

17.6 Regulation of AQPs in Human Fetal Membranes and Trophoblast Cells

Emerging evidence shows that cytokines, hormones, second messengers, intracellular pH, and membrane proteins may control AQPs expression and function in the human placenta and fetal membranes. The identified factors, to date, involved in the regulation of these AQPs are summarized in Table 17.3.

17.6.1 Fetal Membranes

17.6.1.1 cAMP

In human amnion epithelial cells, the effect of 3,5-cyclic adenosine monophosphate (cAMP) was widely studied. cAMP is one of the most frequently second messengers in the endocrine signaling pathway. It was reported that AQP1, AQP3, AQP8, and AQP9 mRNA expressions are stimulated by cAMP [105–107]. However, these AQPs respond differently to cAMP activation. In the case of AQP3, the rise in intracellular cAMP levels triggers a quick but transient increase in AQP3 expression through a PKA-dependent mechanism [107]. On the other hand, AQP1, AQP8, and AQP9 mRNA expressions were upregulated by cAMP via a PKA-independent pathway [105, 106]. In addition, the increase of AQP8 was rapid and sustained whereas AQP1 and AQP9 response was delayed and persistent [106]. Up to now, the link between cAMP and disorders associated with altered AF volume is unknown.

17.6.1.2 Osmotic Stress

In amnion epithelial cells, osmotic stress was also found to be a regulator of AQP8 expression. In this way, AQP8 mRNA and protein expression were significantly increased in hypotonic media while being dramatically lowered in hypertonic media [108]. These changes in AQP8 are of special interest in oligohydramnios. However, the molecular mechanism involved in this regulation is not explored yet.

17.6.1.3 Insulin

Regarding hormone regulation, it was recently documented in the amnion, but not in the chorion, that insulin considerably reduces the transcriptional expression of AQP3 and AQP9 [109]. The reduced expression of both AQPs could reduce the glycerol transcellular transport. Moreover, it was also reported that the inhibition of the phosphatidylinositol 3-kinase abrogates the AQP-downregulation mediated by insulin [109]. These results propose that in pregnancies complicated by Diabetes Mellitus type 2 and Gestational Diabetes Mellitus, commonly linked to polyhydramnios, insulin may repress AQP3 and AQP9 in the amnion, resulting in AF volume increase.

17.6.1.4 Retinoic Acid

It was documented that the all-trans-retinoic acid, the main active metabolite of vitamin A, regulates AQP3 mRNA in human amnion and epithelial amniotic cells [110]. The upregulation of AQP3 results in an increased uptake of glycerol. However, the relevance of these findings in maintaining AF homeostasis is still uncertain, but it was speculated to be linked to the premature rupture of membranes.

17.6.2 Trophoblast Cells

17.6.2.1 Oxygen

A relatively hypoxic environment is required for the human placenta development to promote proper embryonic growth, trophoblast differentiation, and placental angiogenesis. It is well-established that placentation is tightly controlled by oxygen tensions through the hypoxia-inducible factor-1 (HIF-1) [116].

Previous research showed that AQP9 protein decreases abruptly in human placental explant cells, exposed to O_2 deprivation [78]. The stabilization of HIF-1α by $CoCl_2$ treatment showed the same response. In silico analysis of the human AQP9 gene revealed 14 putative hypoxia-response elements (HRE) sites (5′-ACGTGC-3′), although none of them were in the promoter

Table 17.3 Regulation of AQPs in human placenta and fetal membranes

Fetal membranes				
	AQP1	AQP3	AQP8	AQP9
cAMP	↑ in amnion cells [105, 106]	↑ in amnion cells [107]	↑ in amnion cells [105, 106]	↑ in amnion cells [105, 106]
Osmotic stress	–	–	↑ in amnion cells exposed to hypotonic media ↓ in amnion cells exposed to hypertonic media [108]	–
Insulin	–	↓ in amnion but not in chorion [109]	–	↓ in amnion but not in chorion [109]
Retinoic acid	–	↑in amnion [110]	–	–
Trophoblast cells				
	AQP1	AQP3	AQP4	AQP9
Oxigen	–	↓ in VT [97]	↑ in VT [72]	↓ in VT [78]
hCG	–	–	–	↑ in VT [111]
Vasopressin	↑ in EVT cells [112]	–	–	
cAMP	↑ in EVT cells [112]	–	–	↑ in VT [111]
Leptin	–	–	–	↑ in VT [113]
Insulin	–	= in VT [77]	–	↓ in VT [77]
TNF-α	–	↓ in EVT cells and VT [94]	–	= in VT [77]
CFTR	CFTR is required to preserve the function of AQPs [114]			
pH	Disturbances in the pHi of the syncytiotrophoblast negatively affect the water permeability of AQPs [115]			
Caveola/ Caveolin-1	–	↓ by disruption of the caveolar structure in EVT cell [92]	–	–

EVT extravillous trophoblast, *VT* villous trophoblast

region, proposing that they were not critical to trigger AQP9 transcription upregulation [78]. Thus, HIF-1α may enhance the expression of some intermediate which promotes the downregulation of AQP9 expression.

Hypoxia also reduced AQP3 protein expression in syncytiotrophoblast cells and changed its subcellular localization, exhibiting a predominance in the cytoplasm [97].

Finally, the impact of low oxygen tension on AQP4 expression has recently been investigated. Hypoxia and HIF-1, in contrast to AQP3 and AQP9, were found to promote AQP4 mRNA and protein expression in human placental explants. In silico analysis revealed three potential HIF-1 binding sites in the AQP4-gene promoter region [72].

Oxygen modification of AQPs expression showed a reduced water uptake, which is non-sensitive to $HgCl_2$, a common blocker of AQPs, suggesting that oxygen can also change AQP-water permeability [78].

17.6.2.2 hCG

Human chorionic gonadotropin (hCG) is released by villous trophoblast cells. This hormone increases the molecular expression and functionality of AQP9 in a concentration-dependent manner [111]. Experimental evidence also showed that the stimulatory effect of hCG on AQP9 takes place via cAMP.

In concordance with its effect on AQP9 expression, hCG may also increase the transcellular water flux mediated by AQPs [111].

17.6.2.3 Vasopressin

In EVT cells, vasopressin may upregulate AQP1 expression [112] by a cAMP-dependent pathway.

Thus, vasopressin action on AQP1 may promote EVT cell migration and the following transformation of maternal spiral arteries. Furthermore, increased levels of vasopressin were found in the AF of fetuses with oligohydramnios [117]. Taking into account that AQP1 detected in human fetal membranes responds to cAMP [106], it is possible that vasopressin also may have a role in the upregulation of AQP1 in the amnion.

17.6.2.4 Leptin

Leptin is a peptide hormone that has a significant role in the regulation of trophoblast development and metabolism. In Gestational Diabetes Mellitus it was observed an increase in leptin serum levels which correlates with an increase in placental AQP9 expression [74]. In subsequent experiments, it was demonstrated that leptin enhanced AQP9 transcriptional and protein levels [113]. These results may hypothesize that AQP9 might be involved in the glycerol transfer to the fetus, to meet the increased energy intake needs in the macrosomic fetus of diabetic pregnancies [90].

17.6.2.5 Insulin

Insulin repressed the expression of placental AQP9 through a negative insulin response element (IRE) in the promoter region of the AQP9 gene [77]. On the other hand, insulin did not modify AQP3 expression in trophoblast cells [62].

Interestingly, in vitro experiments showed that water uptake mediated by AQPs was not perturbed after insulin treatment despite the AQP9 decrease, suggesting a non-classical role for this protein in the human placenta [77].

17.6.2.6 TNF-α

It was recently reported that the tumor necrosis factor-alpha (TNF-α) downregulates AQP3 expression in EVT cells, and negatively affects cell migration [94].

On the contrary, TNF-α has no direct effect on placental AQP9 expression [77]. However, it was proposed that this cytokine may impair insulin signaling. It was found that TNF-α may enhance the serine phosphorylation in the Ser(307) residue of the insulin receptor substrate-1 (IRS-1) [118]. Consequently, the increased serine phosphorylation impedes tyrosine phosphorylation of IRS-1 induced by insulin, reducing the action of the hormone. Along with this, in vitro experiments using normal placental explants, showed that the previous treatment with TNF-α, avoided the insulin-mediated decrease of AQP9 expression [77].

17.6.2.7 CFTR

Cystic fibrosis transmembrane conductance regulator (CFTR) co-localizes with AQP9 in the apical microvillous membranes of syncytiotrophoblast cells [114]. Although CFTR did not modify AQP9 expression in the human placenta, water uptake experiments demonstrated that the AQP-facilitated transcellular water transport was dramatically reduced after the inhibition of CFTR. These findings highlight that both proteins work synergistically and propose that CFTR protein is necessary to maintain the functionality of AQPs.

17.6.2.8 pH

Changes in the pH microenvironment can affect many cellular processes, including water transport and cell volume regulation [83, 119–121]. Na+/H+ exchangers (NHEs) may control the intracellular pH (pHi) of syncytiotrophoblast cells [122]. As a result of the presence of functional NHEs, AQP-mediated transcellular water movement was unaffected in normal placental explants after cytosolic acidification [115]. However, the blocking of these exchangers leads to disturbances in the pHi of the syncytiotrophoblast negatively altering the water permeability of AQPs [115].

17.6.2.9 Caveola/Caveolin-1

The fluidity of the lipid bilayer strongly affects transmembrane transport activities. Caveolae are a particular type of lipid rafts membrane subdomains enriched in sphingomyelin and cholesterol. Caveolin-1 (Cav-1) is an integral membrane protein and the main component required for the formation of caveolae [123].

Several proteins that interact with Cav-1 have cytoplasmic accessible sequences that are assembled to the caveolin scaffolding domain [124]. A caveolin-binding motif was recently identified in the protein sequence of human AQP3 [92]. In addition, it was demonstrated that both proteins, AQP3 and Cav-1, co-localized in the plasma membrane of EVT cells. Consequently, the disruption of the caveolar structure increases Cav-1 degradation, losing its interaction with AQP3 [92]. Since the proper assembly of caveolae needs a particular lipid composition, changes in sphingomyelin and cholesterol content may affect the caveola structure. Altogether, these alterations may impair cell migration and endovascular differentiation of EVTs resulting in preeclampsia or fetal growth restriction. Previous works have shown that the content of sphingomyelin increased in the apical membranes of the syncytiotrophoblast from preeclamptic placentas, leading to a reduced number of caveolae and a decreased Cav-1 protein expression [125]. In this scenario, the lack of Cav-1 may negatively affect AQP3 expression, which is markedly decreased in these pathological placentas [73].

17.7 Conclusion

Accumulating evidence suggests that AQPs are necessary for the regulation of fetal water homeostasis and the proper formation of the placenta. Although data in humans is limited, several reports in animal models highlight the role of these proteins in organogenesis.

The identification of AQP9 in trophoblast mitochondria has led to new speculations about its role in pregnancy.

Nonetheless, much remains still to do to elucidate their significance in healthy and pathological pregnancies.

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Aquaporins in Diabetes Insipidus

18

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Abstract

Disruption of water and electrolyte balance is frequently encountered in clinical medicine. Regulating water metabolism is critically important. Diabetes insipidus (DI) presented with excessive water loss from the kidney is a major disorder of water metabolism. To understanding the molecular and cellular mechanisms and pathophysiology of DI and rationales of clinical management of DI is important for both research and clinical practice. This chapter will first review various forms of DI focusing on central diabetes insipidus (CDI) and nephrogenic diabetes insipidus (NDI). This is followed by a discussion of regulatory mechanisms underlying CDI and NDI, with a focus on the regulatory axis of vasopressin, vasopressin receptor 2 (V2R) and the water channel molecule, aquaporin 2 (AQP2). The clinical manifestation, diagnosis, and management of various forms of DI will also be discussed with highlights of some of the latest therapeutic strategies that are developed from in vitro experiments and animal studies.

Keywords

Diabetes insipidus · nephrogenic diabetes insipidus · aquaporin 2 · V2R

18.1 Diabetes Insipidus

Maintaining water homeostasis is essential for mammalian life. Proper water metabolism is responsible for the balance between water intake and secretion. Each side of this balance is important for fluid homeostasis. Diabetes insipidus (DI) is characterized by excessive water loss/polyuria. DI is classified as central diabetes insipidus (CDI) and nephrogenic diabetes insipidus (NDI). CDI is due to impaired production and/or secretion of the antidiuretic hormone, ADH, also called vasopressin (VP) from the central nervous system. NDI is caused by lack of response of the target tissue, the collecting ducts of the kidney, to circulating ADH/VP. In both cases, the kidney fails to concentrate urine and results in polyuria. DI patients may produce up to 18 L of urine a day and exhibit a constant need for water intake. Both CDI and NDI can be either inherited or acquired. If undiagnosed or improperly managed, DI is associated with a range of clinical symptoms due to severe volume depletion and electrolyte abnormalities.

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In this chapter we will review the regulatory function of VP for central DI, and the critical role of vasopressin receptor 2 (V2R) and aquaporin 2 (AQP2), in modulating water reabsorption in the collecting duct principal cell in NDI. Finally, we will discuss the clinical manifestation, diagnosis and treatment for DI, and review some of the most recent progress in developing novel strategies for treating nephrogenic diabetes insipidus.

18.2 Central Diabetes Insipidus and Vasopressin

The antidiuretic hormone, ADH, later known as vasopressin (VP), is the primary determinant of free water excretion or absorption in mammals. Central diabetes insipidus is usually caused by inadequate production and/or secretion of VP from the post-pituitary gland in response to osmotic stimulation. Central DI is rarely hereditary in humans. Most frequently it is caused by traumatic or pathological destruction of the neurohypophysis that leads to complete or partial absence of circulating VP.

Vasopressin is a nine-amino acid peptide in most mammals. It is synthesized in the hypothalamus. Substitution of lysine for arginine at position 8 yields lysine vasopressin which is found in pigs. Substitution of isoleucine for phenylalanine at position 3 and leucine for arginine at position 8 yields oxytocin (OT), a hormone with weak antidiuretic activity but a potent smooth muscle constrictor in the uterus. Arginine vasopressin (AVP) and lysine vasopressin (LVP) are the major antidiuretic hormones for mammals. In addition to regulating water reabsorption by the kidney, they also cause vasoconstriction, an effect that occurs at concentrations many times higher than those required for antidiuresis. AVP and OT are produced by the posterior pituitary gland [1, 2]. Even though secretion of AVP can be influenced by many factors, the most important stimulus under physiological conditions is the plasma osmolality [3–8]. Cells located in the anterior hypothalamus are able to sense small changes in plasma osmolality and stimulate AVP secretion [2, 8, 9]. Most studies support the existence of a possible osmotic threshold of VP secretion and there is a linear relationship between plasma osmolality and circulating AVP concentration indicating a sensitive regulation of water excretion by vasopressin [5, 6, 8, 10, 11].

The osmolality threshold or set point of vasopressin secretion varies from person to person. Normally, in adults, it ranges from 275 to 295 mOsm/kg H_2O, with an average of 280 to 285 mOsm/kg H_2O [10]. Many factors are known to affect the set point of the osmoregulation for AVP secretion [4]. For example, aging increases the sensitivity of osmoregulation, pregnancy dramatically reduces the set point of osmoregulation and exercise could increase plasma osmolality [4, 12, 13]. Observations have suggested that osmoregulation by AVP secretion is subjected to both stimulatory and inhibitory inputs to the neurohypophysis [6]. Therefore, osmolality sensing is a highly regulated and sensitive process [7, 11]. In addition to the central regulation of production and secretion, VP has a short half-life in circulation from 10 to 20 minutes, allowing the kidneys to respond to changes in plasma osmolality on a minute-to-minute scale. Therefore, from the VP secretion and its action on the kidney, this system enables a fine-tuned osmoregulation that adjusts the rate of water excretion acutely and accurately to the plasma osmolality [11].

In addition to the primary stimulation of the plasma osmolality, hypovolemia is also a potent stimulus for AVP secretion in humans [14–16]. In rats, plasma AVP increases as an exponential function of the degree of hypovolemia and hypertension [17, 18]. This hemodynamic influence of AVP secretion is thought to be mediated, at least in part, by neural pathways that originate in the stretch-responsive receptor, the baroreceptor in the central nervous system. This osmolality-independent regulation of VP production was found to be associated with many pathological

conditions, such as sodium depletion, hypotension, congestive heart failure, cirrhosis, and nephrosis [16, 18–20]. In addition, mutations that affect VP prohormone processing such as Sel1L-Hrd1 ER-related degradation also modulate VP production and function [21].

18.3 Gestational Diabetes Insipidus

This type of diabetes insipidus is caused by a relative deficiency of circulating VP during pregnancy [22]. It is only observed in pregnant women and therefore is termed as gestational diabetes insipidus [23, 24]. It is due to the elevation of a circulating enzyme, cysteine aminopeptidase, also called vasopressinase or oxytosinase that degrades plasma vasopressin and oxytoxin [25, 26]. The vasopressinase or oxytoxinase is normally produced by the placenta to prevent premature uterine contractions induced by oxytocin. Vasopressinase causes accelerated metabolic clearance of circulating VP, and overwhelms the VP-generating capacity of the neurohypophysis, leading to VP deficiency. In addition, the activity of vasopressinase may be abnormally elevated in pathological conditions that associate with pregnancy, such as preeclampsia, acute fatty liver and **HELLP** syndrome (**he**molysis, **e**levated **l**iver enzymes and **l**ow **p**latelet count). This is due to a decreased metabolism of vasopressinase by the liver [27, 28].

Gestational DI can become overtly symptomatic and poses a serious threat of dehydration and electrolyte imbalance in pregnant women, and therefore should be readily recognized and managed [29]. The pathophysiology of gestational diabetes insipidus is similar to that of central diabetes insipidus, except that gestational diabetes insipidus is resistant to AVP treatment. Like endogenous vasopressin, AVP can be rapidly degraded and cleared from the circulation. However, the synthetic vasopressin receptor V2R agonist, desmopressin is resistant to vasopressinase degradation, and has been used to diagnose and correct gestational diabetes insipidus [24, 30, 31]. Whether there is also a component of nephrogenic diabetes insipidus (due to lack of response to circulating vasopressin) during pregnancy is unclear.

18.4 Nephrogenic Diabetes Insipidus

Nephrogenic diabetes insipidus (NDI) is defined as diabetes insipidus caused by resistance of vasopressin action by the kidney [32, 33]. In contrast to central diabetes insipidus (CDI), in NDI, the plasma VP level is usually normal or elevated. NDI can be either acquired or congenital in origin [33].

18.4.1 Congenital Nephrogenic Diabetes Insipidus

Congenital NDI was first recognized in the 1950s in several male patients presented with the familial, sex-linked form of the diabetes insipidus (Cannon 1955). Subsequently, it was found that the disorder was due to defects in arginine vasopressin receptor 2 gene (*V2R*) located on the X chromosome (Xq28) [34, 35]. In congenital NDI, patients frequently present with polyuria from birth. The disease manifestation in congenital forms of NDI varies from partial NDI to complete NDI. It affects mostly males, and is usually mild or absent in female carriers. Genetic analysis revealed that more than 90% of cases of congenital NDI are caused by mutations in the arginine vasopressin receptor 2 (*V2R*) [36, 37]. To date, over 225 mutations that result in congenital NDI have been identified in the V2R [38–41]. Most of these mutations are missense mutations [38].

The remaining 10% of congenital NDI cases are due to genetic defects in the water channel AQP2 gene that is located on chromosome 12 (12q13) [40]. AQP2 is the major aquaporin that mediates water transport in principal cells of the collecting ducts (CDs) of the kidney. While congenital NDI due to AQP2 mutations is mostly inherited in an autosomal recessive mode, a few cases have also reported autosomal dominant inheritance [38, 42]. Since the first report of a compound heterozygote of two missense

mutations of AQP2 genes (R187C and S217P) in a male NDI patient [43–45], approximately 49 putative disease-causing AQP2 mutations have been described [38, 40, 43, 46–50]. These mutations are roughly grouped into two categories, based on the outcome. The first category of mutations affect the formation of the functional channel "pore" structure that allows the translocation of water molecules across the plasma membrane. Most of the autosomal recessive forms of NDI are thought to be due to mutations that fail to form the tetramer with wild type AQP2, therefore causing a defect in "pore" formation. The second category of AQP2 mutations affect the routing or trafficking of AQP2 to the plasma membrane and/or inside the cells. For example, AQP2 is retained in the Golgi apparatus, or sorted to late endosomes, lysosomes or the basolateral plasma membrane instead of the apical membrane [49, 51–58]. This defect is frequently due to mutations that occur in the C-terminal tail of AQP2, which is essential for the correct intracellular routing in response to multiple signaling pathways [51, 52, 54, 56–59].

18.4.2 Acquired Nephrogenic Diabetes Insipidus

Compared to congenital NDI, the acquired form of NDI is much more common. It can be caused by multiple factors, including lithium toxicity, urinary obstruction, hypokalemia, hypercalcemia, etc. [60–62]. Sometimes, the etiology may not be well defined in the clinical situation. Despite complicating factors that are involved in the pathophysiology of acquired NDI, direct or indirect interruption of VP-V2R and AQP2 signaling and trafficking is evident. Therefore, the VP-V2R/AQP2 regulatory axis is the central component of both congenital and acquired NDI. Indeed, genetic studies of congenital NDI have greatly facilitated the discovery of the vasopressin/ vasopressin V2 receptor and AQP2 signaling pathway that is known to be the major regulatory pathway for water transport in the mammalian kidney.

18.4.3 VP-V2R/AQP2 Axis in Regulating Water Transport

AQP2 is a major water channel expressed in the principal cells (PCs) of the collecting ducts of the kidney [63, 64] (Fig. 18.1). It mediates water transport across the plasma membrane in response to vasopressin. Circulating vasopressin binds to V2R located in the basolateral membrane of the PCs, activates the adenylyl cyclase (AC), and thus causes the elevation of intracellular cyclic AMP. Elevation of cAMP activates the protein kinase A (PKA), leading to the phosphorylation of AQP2. Phosphorylation of AQP2, mainly at the serine 256 residue results in accumulation of AQP2 on the apical membrane. Within the plasma membrane, AQP2 forms tetramers containing a functional "pore" facilitating the passage of water molecules through the channel. Water absorbed from the apical membrane via AQP2 is transported into the interstitium through basolaterally located AQP3 and AQP4 channels. In addition to cAMP, the cGMP and calcium/calmodulin pathways are also involved in regulating AQP2 traffic [47]. Further details of AQP2 trafficking mechanisms are elucidated in Chap. 1.

In addition to regulated trafficking, AQP2 is also constitutively recycling [41, 64]. Blocking AQP2 endocytosis by a cholesterol-chelating reagent, methyl-beta cyclodextrin or by expressing dominant negative dynamin causes a rapid and dramatic membrane accumulation of AQP2 in cultured cells [65, 66]. It suggests that a significant amount of AQP2 is recycling under baseline conditions, and this recycling does not require any phosphorylation, since the phosphorylation "dead" mutation of AQP2, AQP2-S256A recycles as well as the wild type AQP2 [65, 67]. The discovery of the presence of a significant pool of AQP2 that is able to recycle independently of VP stimulation prompted a burst of studies searching for molecular mechanisms and alternative approaches that cause membrane accumulation of AQP2 in the absence of VP-V2R regulation. Many novel targets have been identified to regulate AQP2 trafficking while

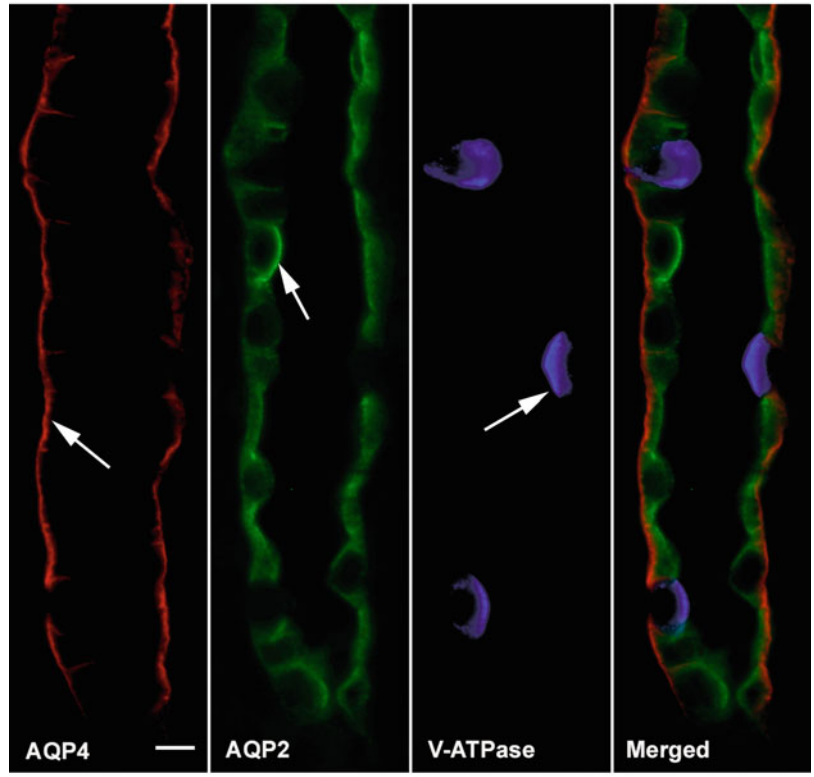

Fig. 18.1 Cellular composition of a kidney collecting duct (CD) from the medulla of a mouse kidney. The CD is immunostained for AQP4 (red), AQP2 (green), and V-ATPase (blue). The merged image shown in the right panel revealed the presence of principal cells (PCs) that are positive for AQP2 and AQP4 staining, and the intercalated cells (ICs) that are positive for V-ATPase staining. AQP4 staining is located on the basolateral membrane of the PCs. AQP2 staining is present mainly in the subapical and basolateral region inside cells. AQP2 signal is occasionally seen on the apical membrane without VP stimulation (indicated by arrow). V-ATPase signal is clearly present on the apical membrane of intercalated cells

bypassing the VP/V2R signaling, the commonly defective pathway in most congenital DI patients [47]. This novel strategy has led to several important discoveries that have proven to be effective in treating NDI in animal models [65, 68, 69]. This will be further detailed in the treatment of NDI section in this chapter. More recent discovery of the existence of clathrin-dependent transcytosis of AQP2 adds more complexity of the VP-independent AQP2 trafficking and its physiological significance is unknown [70].

18.5 Clinical Manifestation, Diagnosis, and Treatment of Diabetes Insipidus

18.5.1 Clinical Manifestation of Diabetes Insipidus

The primary clinical symptoms that are characteristic for diabetes insipidus are polyuria and polydipsia resulted from the impairment of urinary concentrating mechanisms. Patients with DI frequently describe a strong sensation of thirst (if their thirst sensation is intact) and are craving for water, especially cold water [71]. Under normal circumstances, DI patients have an intact thirst mechanism, and therefore they are able to maintain normal serum osmolality and volume status without clinical symptoms other than polyuria and polydipsia. However, when water deficits occur due to inadequate water intake to compensate for polyuria, symptoms of dehydration and electrolyte abnormalities such as hypernatremia develop. Volume depletion leads to hypotension, acute kidney injury, liver injury, muscle injury, and shock. Hyperosmolality and dehydration also cause a series of neurological symptoms ranging from irritability, cognitive decline, disorientation, and confusion to decreased levels of consciousness, seizure, and coma. These signs are suggestive of hypertonic encephalopathy [72]. Various focal neurological

deficits may also develop in this context. Increased incidence of subarachnoid hemorrhage, cerebral infarction, and deep venous thrombosis (DVT) are also reported in patients with hyperosmolality. The severity of symptoms is roughly correlated with the degree of hyperosmolality, however, the individual variability is marked, and therefore the serum sodium level cannot accurately predict the clinical presentation of a DI patient. The chronicity of the hyperosmolality is important for the development and degree of clinical manifestation. Acute and severe hyperosmolality are frequently associated with marked neurological presentation compared to generally milder symptoms in patients with subacute and chronic hyperosmolality [72].

18.5.2 Differential Diagnosis of Diabetes Insipidus

Clinical differentiation of central DI and nephrogenic DI, and sometimes primary polydipsia are important for the management [62, 73]. The presence of truly hypotonic polyuria should be established by measuring urine osmolality and volume from a 24-hour urine collection. The generally accepted diagnostic criteria of DI is that a 24-hour urine volume exceeds 50 ml/kg and urine osmolality is less than 300 mOsm/kg H_2O [62, 74, 75]. Meanwhile the presence of hyperglycemia from diabetes mellitus and kidney failure should be ruled out. In patients with hyperosmolality, polyuria and suboptimal urinary concentration (urine osmolality less than 800 mOsm/kg H_2O) define the diagnosis of DI; primary polydipsia can be ruled out with normal or low serum osmolality and concomitant low urine osmolality. Once the DI is diagnosed, the central DI can be distinguished from NDI by its response to exogenously administrated AVP (1-deamino-8-D-arginine vasopressin), or DDAVP (1–2 g subcutaneously or intravenously). A significant increase in urine osmolality of more than 50% within 2 h after administration of AVP or DDAVP supports the diagnosis of central DI. In contrast, an increase of less than 10% in urine osmolality indicates NDI. Partial responders (in between these values) are undetermined and need to be further assessed by measuring serum AVP level to aid the diagnosis. Although it has been a subject of debate in the literature, the water deprivation test has been proposed to better distinguish the different types of DI and aid with diagnosis and management [76]. Measurement of serum VP level has been difficult and time consuming, therefore it is rarely used clinically. We need to keep in mind that clinical diagnosis of various forms of DI can be complex and confusing. Firstly, measuring serum AVP is difficult and most available assays are not sensitive enough. Recently sandwich immunoassay has been developed to measure serum copeptin level. Copeptin is the C-terminal segment of the arginine vasopressin prohormone, and is used as a surrogate marker for circulating VP. However copeptin assay has not yet been widely used in the clinical setting for the diagnosis of DI [77]. Secondly, many disorders of DI can overlap and co-exist. We need to be mindful while interpretate laboratory data and clinic presentation [74].

18.5.3 Treatment of Diabetes Insipidus

The principles for treating all forms of diabetes insipidus are a correction of water deficit and a reduction in the ongoing water loss from the kidney. Theoretically, with an intact thirst mechanism and ability to access water, most DI patients should be able to drink a sufficient amount of water and attain a relatively normal fluid balance [71]. However, polydipsia and polyuria can significantly affect the quality of life of a DI patient. DI treatment becomes necessary in order to manage the symptoms of DI. The specific treatment varies based on the type of DI and the specific clinical situation.

18.5.4 Treatment of Central Diabetes Insipidus

The synthetic form of human AVP, pitressin, has been used for the treatment of acute central DI. It

is given intravenously with a short half-life (2–4 h). Desmopressin/DDAVP, a synthetic AVP V2R agonist, has been commonly used for treating both acute and chronic central DI. It has a long half-life (8–20 h), and can be administered intranasally, orally, or by injection based on the clinical situation and the patient's preference [78, 79]. Because it is specific to vasopressin signaling through the V2R, it normally does not affect the blood pressure as AVP does. Although the central DI can be easily managed by DDAVP, one needs to be aware of and closely monitor a critical complication, which is hyponatremia. Hyponatremia is a rare complication of desmopressin therapy, which can cause severe, even fatal sequelae [80]. It is reported in children who are treated with desmopressin for hemophilia and von Willibrand's factor disorders and in children treated for primary enuresis [76]. Therefore, serum electrolytes need to be monitored closely in patients during the initiation of desmopressin therapy. The dose and intervals of administrated desmopressin need to be adjusted to control the symptoms of polyuria and polydipsia while maintaining a safe serum sodium level [39].

Although not classified as central DI, the treatment is the same for gestational diabetes insipidus, which is with desmopressin. The AVP is rapidly degraded by the high level of circulating oxytoxinase or vasopressinase, while desmopressin is resistant to the enzymatic degradation and has been used successfully for the treatment of gestational DI [30]. The dose of desmopressin should be titrated to the individual patient, and fluid administration should be performed with caution. Serum electrolytes should be closely monitored at the time of delivery.

18.5.5 Treatment for Nephrogenic DI

In contrast to the relatively intact vasopressin-V2R and AQP2 pathway in central DI, NDI has a defective VP-V2R and AQP2 axis. Therefore, patients with congenital NDI are resistant to the water concentrating effect medicated by vasopressin. Clinical therapy for treating congenital NDI is limited to restricting sodium intake, administrating a thiazide diuretic alone or in combination with a non-steroid anti-inflammatory drug (NSAID) or amiloride [39, 81]. Dieticians play a pivotal role in managing NDI patient in their first year of life [82]. The thiazide class of diuretics is considered the mainstay for treating NDI. They block the sodium reabsorption in the cortical diluting segment. In combination with restricted sodium intake, it causes modest hypovolemia. Hypovolemia stimulates isotonic solute absorption in the proximal tubule and reduces solute delivery to the distal diluting segment. Thiazide also enhances water reabsorption in the inner medullary collecting ducts independently of vasopressin. However, care must be excised when treating with thiazide diuretics to correct hypokalemia, and to avoid severe volume depletion and resultant kidney injury, especially in combination with NSAIDs. Commonly used NSAIDs to treat NDI are indomethacin and ibuprofen. Administrating high doses of NSAIDs or long-term NSAID use increases the risk of developing AKI and chronic kidney disease (CKD) [83, 84]. Therefore, renal function needs to be monitored with chronic use of NSAIDs in DI patients.

Treatment for acquired NDI is focusing on correcting insulting factors if possible. It includes withholding lithium in lithium-induced NDI at the early stage, correcting hypokalemia in hypokalemia-induced NDI, correcting hypercalcemia if it is the cause, relieving urinary obstruction, and managing congestive heart failure with medication or cardiac intervention. However, under many circumstances, management of underlying etiology for acquired NDI can be clinically challenging.

One important consideration for treating diabetes insipidus in general is avoiding over correction of hyperosmolality/hypernatremia. The theory is that under a state of hyperosmolality, the brain counteracts osmotic shrinkage by increasing the intracellular content of solutes, including electrolytes such as potassium and many organic osmolytes. The net effect of this process is to protect the brain against excessive shrinkage during sustained hyperosmolality.

However, once the brain has adapted to this new hyperosmolality state, rapid correction of hyperosmolality can cause brain edema since it takes time to re-equilibrate the previously accumulated solutes. Similar to correction of hyponatremia, cautious correction of chronic hyperosmolality or hypernatremia needs to be practiced. Even though severe sequelae from rapid correction of hyperosmolality and hypernatremia are rarely reported, they can occur [85].

Despite that, the clinically proven treatment for congenital NDI is limited. Very excitingly, in recent years, with greater understanding of AQP2 trafficking mechanisms, multiple novel targets have been identified and provide promising new strategies for treating NDI.

18.5.6 Novel Therapeutic Strategy for NDI

Fundamental research on kidney physiology has provided important insights into the development of novel therapeutic targets and strategies to treat human diseases. It is especially true for NDI [86]. As mentioned previously, over 90% of congenital NDI is due to mutations in the *V2R* gene and less than 10% is due to mutations in *AQP2*. The water channel AQP2 remains intact in the majority of congenital NDI patients. Similarly, in many forms of acquired NDI, such as lithium-induced NDI, AQP2 is also intact. Therefore, it is possible to develop a strategy to induce membrane accumulation of AQP2 independent of vasopressin stimulation, therefore bypassing the V2R-VP signaling regulation [68]. This has led to great discoveries of multiple new targets for treating NDI [87–91]. More recently, an exocytosis assay was established and used for high-throughput chemical screening, and identified, a several new compounds, such as AG-490 that modulates AQP2 trafficking to the plasma membrane [84]. With continuous discovery, many reagents were proved to be effective in NDI animal models, and a few reagents are subsequently tested in X-linked NDI patients. The results are promising.

18.5.6.1 Phosphodiesterase Inhibitors

It is well known that increasing intracellular cyclic AMP in principal cells leads to membrane accumulation of AQP2. Increasing cyclic guanosine monophosphate (cGMP) by sodium nitroprusside, L-arginine and atrial natriuretic peptide (ANP) also causes an increased AQP2 abundance on the apical membrane. The selective cGMP phosphodiesterase (PDE5) inhibitor sildenafil citrate (Viagra) was shown to cause elevation of cGMP and subsequent membrane accumulation of AQP2 in cells and in Brattleboro rat kidney [92]. Sildenafil citrate was also reported to reduce polyuria in rats with lithium-induced NDI [93]. More recently, a case study showed that sildenafil improves polyuria and increases urinary osmolality in an X-linked NDI patient [94].

18.5.6.2 Statins

The statin family is a family of 3-hydroxy-3methyglutaryl-coenzyme A reductases. They inhibit the synthesis of cholesterol and are used for treating hyperlipidemia. Simvastatin was shown to increase membrane accumulation of AQP2 in cultured kidney epithelial cells. In addition, simvastatin treatment in Brattleboro rats causes apical membrane redistribution of AQP2 in CDs in parallel with increased urinary concentration in a VP-independent manner (Fig. 18.2) [89]. A later study has also demonstrated that atorvastatin, another member in the statin family, significantly improves urinary concentration in polyuria caused by urinary obstruction in animals [95]. The specific mechanism underlying the effect of statins is not fully understood, but has been attributed to changes in prenylation of RhoA family proteins that are involved in regulating cytoskeleton and AQP2 trafficking [89].

18.5.6.3 Prostaglandins

Prostaglandin E2 is known to increase water permeability in the absence of vasopressin possibly through activating prostanoid receptor EP2 and/or EP4. Both Butaprost, an EP2 agonist, and CAY10580, an EP4 agonist, stimulate AQP2 trafficking to the apical membrane in cultured MDCK cells [91, 96, 97]. Another EP4 agonist,

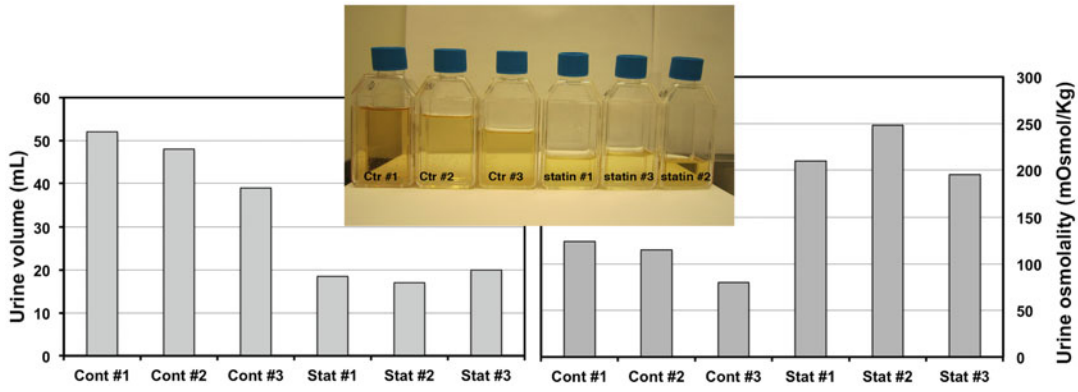

Fig. 18.2 Simvastatin increases urine concentration, reduce urine volume in vasopressin-deficient Brattleboro rats. After 6 h treatment with simvastatin, urine volume (left panel and inset image) was significantly reduced and urine osmolality was significantly increased in simvastatin treated Brattleboro rats

ONO-AE1-329, increases AQP2 membrane expression, improves polyuria and increases urine osmolality in V2R knock animals. Similarly, Butaprost was shown to reduce urinary volume and increase urine osmolality in rats treated with a V2R antagonist [91]. More interestingly, long-term treatment with ONO-AE1–329 increases AQP2 abundance in V2R knock-out animals. These studies suggest that activating the prostaglandin pathway through EP2 and EP4 holds promise for treating NDI independently of VP-V2R signaling.

18.5.6.4 Metformin

Metformin is an oral antidiuretic drug that stimulates the 5′ AMP-activated protein kinase or AMPK, an enzyme that plays a role in cellular energy homeostasis. A recent study has shown that metformin stimulates AQP2 membrane accumulation in rat inner medullary collecting duct cells and increases urine concentrating ability in two rodent models of NDI, V2R knock-out mice and rats treated with Tolvaptan, the V2R antagonist [98]. Metformin was shown to increase protein abundance of inner medullary urea transporter UT-A1 and AQP2, and membrane accumulation of AQP2 possibly through phosphorylating AQP2 at serine 256. Metformin is able to produce a sustained urinary concentrating effect for up to 10 days in Tolvaptan treated animals. This study suggests that through stimulating AMPK to phosphorylate and activate AQP2 and UT-A1, metformin increases urine concentrating ability, and therefore, is a promising treatment for congenital NDI [98].

Besides the above listed reagents, other studies have uncovered more and more novel targets for therapeutic intervention for NDI. Many of them were proved to be effective in vivo using various NDI animal models. For example, calcitonin, a 32-amino acid peptide produced by the follicular cells of the thyroid, causes an increase of intracellular cAMP and membrane expression of AQP2 in principal cells. More importantly, calcitonin was shown to improve urine concentration in Brattleboro rats [90]. A heat shock protein 90 (HSP90) inhibitor, 17-Allylamino-17-demethoxygeldanamycin, was shown to partially correct NDI in a model of autosomal recessive form of NDI in which the AQP2 mutation AQP2-T126M is retained in the ER [99]. More recently, Erlotinib, a receptor tyrosine kinase inhibitor that acts on the epidermal growth factor receptor (EGFR), was shown to cause membrane accumulation of AQP2 in a cAMP-independent manner and to alleviate polyuria in lithium-induced NDI animals [100].

In summary, research on water transport disorders including NDI is a fast evolving and

exciting field. More and more novel reagents and small molecules will continuously be discovered and will provide more and possibly better therapeutic targets for treating NDI. Clinical trials are urgently needed to examine and/or confirm the efficacy and validity of many of the novel targets that are identified by in vitro systems and animal models.

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Aquaporins in Edema

19

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Abstract

One of the most prevalent indications of water–electrolyte imbalance is edema. Aquaporins (AQPs) are a protein family that can function as water channels. Osmoregulation and body water homeostasis are dependent on the regulation of AQPs. Human kidneys contain nine AQPs, five of which have been demonstrated to have a role in body water balance: AQP1, AQP2, AQP3, AQP4, and AQP7. Water imbalance is connected with AQP dysfunction. Hyponatremia with elevated AQP levels can accompany edema, which can be caused by disorders with low effective circulating blood volume and systemic vasodilation, such as congestive heart failure (CHF), hepatic cirrhosis, or the syndrome of incorrect antidiuretic hormone secretion (SIADH). In CHF, upregulation of AQP2 expression and targeting is critical for water retention. AQP2 is also involved in aberrant water retention and the formation of ascites in cirrhosis of the liver. Furthermore, water retention and hyponatremia in SIADH are caused by increased expression of AQP2 in the collecting duct. Fluid restriction, demeclocycline, and vasopressin type-2 receptor antagonists are widely utilized to treat edema. The relationship between AQPs and edema is discussed in this chapter.

Keywords

Water channel · Edema · Hyponatremia · Water balance

19.1 Introduction

Edema is one of the most typical symptoms of water–electrolyte imbalance. It is caused by a buildup of fluid in the gaps between the body's tissues and organs. The capillary hydrostatic pressure gradient and the oncotic pressure gradient across the capillary regulate the fluid exchange between the interstitial and intravascular compartments. The accumulation of fluid occurs when local or systemic conditions disrupt the equilibrium, resulting in increased capillary hydrostatic pressure, increased plasma volume, decreased plasma oncotic pressure (hypoalbuminemia), increased capillary permeability, or lymphatic blockage [1].

The kidney is the key organ involved in maintaining body water and electrolyte balance. There are nine aquaporins (AQPs) in human kidney, including AQP1–8 and AQP11. AQP1 is highly expressed in proximal tubules, descending thin limbs and endothelial cells of the descending vasa recta [2], where it controls water

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reabsorption. The critical role of AQP1 is constitutive absorption of 70% of water in the glomerular filtrate. It was reported that deletion of AOP1 exhibits polyuria and impaired urinary concentration [3].

AQP2 is expressed in the apical and subapical vesicles of the luminal plasma membrane in the principal cells of the collecting duct, where it allows water to enter the cells [4]. AQP2 is one of the most important channel proteins involved in urine concentration regulation. The water reabsorption function of AQP2 is mainly regulated by arginine vasopressin (AVP) [5–7]. Although AVP has been shown to have a role in AQP2 transcription and trafficking via AVP-dependent and -independent pathways, other variables, such as inflammation and insulin/aldosterone/prostaglandin levels, can also have an impact [8]. Along with this, the expression of AQP2 can be regulated by several transcription factors, such as activator protein-1 (AP-1), nuclear factor kappa-B (NF-κB), and nuclear factor of activated T-cells (NFAT) [9].

AQP3 is localized to the basolateral membrane of the connecting tubule and collecting duct principal cells [10, 11]. AQP3 is thought to represent an exit pathway for AQP2-mediated water reabsorption. AQP4 is present in the basolateral membrane of the principal cells in the kidney connecting tubule and collecting duct [12]. Despite the fact that AQP3 and AQP4 are both basolateral water channels, they are distributed differently along the collecting duct system, with AQP3 being more abundant in cortical and AQP4 being more abundant in inner medullary collecting ducts [13].

AQP5 is the closest homolog to AQP2, with 66% sequence identity. It is not detectable by immunoblotting in normal mouse and human kidneys [14, 15]. A few years ago, AQP5 was firstly discovered to be expressed in the renal cortex at the apical membrane of type-B intercalated cells [16]. AQP6 is located in the intracellular membranes in renal epithelia and colocalized with H^+-ATPase [17, 18]. AQP7 is expressed in the brush border of proximal tubule [19]. AQP8 is expressed at low levels in intracellular domains of the proximal tubule and the collecting duct cells [20] as well as in the inner mitochondrial membrane of rat kidney [21]. AQP11 is expressed in whole segments of the proximal tubules [22] and localized in the membrane of endoplasmic reticulum (ER). The proximal tubules are important for water and glucose reabsorption. AQP11 is regulated by glucose and may be involved in maintaining ER homeostasis and osmoregulation of the proximal tubule [23]. Dysfunction of AQPs, especially AQP1-4, can lead to various clinical conditions associated with water–electrolyte imbalance [24], such as edema.

19.2 AQPs and Edema

19.2.1 Congestive Heart Failure

Retention of sodium and water is a common and clinically important complication of congestive heart failure (CHF). CHF is characterized by elevation of AVP leading to hyponatremia and increased extracellular volume [25]. Stroke volume in patients with heart failure is reduced, and cardiac output is subsequently reduced, resulting in a decrease in effective circulating blood volume, and a decrease in the sensitivity of baroreceptors located in the carotid artery, aortic arch, left ventricle, and afferent arteriole of kidney, which drives the non-osmotic release of AVP. Although the osmotic pressure falls, the osmotic pressure reception is extremely sensitive, the AVP release is lowered. Due to the positive adjustment of the AVP is insufficient, the AVP level in the body is raised [25]. When V2R is triggered, AQP2 expression is increased, and water reabsorption of the collecting duct rises as well is increased. In addition, AVP combines with V1aR in vascular smooth muscle, which causes the minor arterial vascular contraction, increasing cardiac overloads. Activated V1aR in myocardial cells, causing coronary artery contraction, directly leads to myocardial ischemia [26]. The amount of cardiac output is reduced, and the sympathetic nervous system (SNS) of

kidney is activated. On the one hand, the renal vasocontraction (the degree of intraoperative arterial contraction is more than the spherical veneer), the blood flow velocity of glomerular capillary is reduced, and the glomerular filtration rate is lowered. The small tubule solution decreases. To a large extent, the volume of solution in the distal tubule determines the level of urine concentration, thus limiting the rate of renal water excretion [27]. On the other hand, the activation of the SNS will also promote AVP secretion and AQP2 expression, which increases water reabsorption [28] (Fig. 19.1).

In 1997, Nielsen S [29] examined the changes in renal AQP2 expression in rats with CHF induced by ligation of the left coronary artery to test if upregulation of AQP2 expression and targeting may play a role in the edema in CHF. The study found rats with severe CHF had significantly elevated left ventricular end-diastolic pressures (LVEDP) and lower plasma sodium concentrations. Besides, the results demonstrated that renal water retention in severe CHF in rats is associated with dysregulation of AQP2 in the renal collecting duct principal cells involving both an increase in the AQP2 expression and a marked redistribution of AQP2 to the apical plasma membrane [29], whereas AQP3 expression slightly decreased. Acute exacerbation of CHF stimulates the pituitary, leads to the activation of renin–angiotensin–aldosterone system (RAAS) and increases the release of adrenocorticotropin (ACTH) and AVP. AVP increases the water permeability of the renal collecting duct cells, allowing more water to be reabsorbed from collecting duct urine to blood. In addition, AVP acts on V2 receptors in the renal collecting duct, which regulates the expression and trafficking of AQP2. The selective increase in AQP2 expression and enhanced plasma membrane targeting provide an explanation for the development of water retention and hyponatremia in severe CHF.

The other study showed upregulation of both AQP2 protein and AQP2 mRNA levels in kidney inner medulla and cortex in rats with CHF [30]. These severe CHF rats had significantly decreased cardiac output and increased plasma vasopressin levels. In comparison to untreated rats with CHF, the V2 antagonist OPC-31260 dramatically increased urine output, decreased urine osmolality, raised plasma osmolality, and decreased AQP2 expression [30]. The large-scale event-driven Phase III trial—EVEREST—was designed to explore both the short- and long-term effects of the V2 antagonist tolvaptan when added to standard therapy in patients hospitalized with worsening HF and with symptoms of fluid overload [31, 32]. Taken together, the evidence from these trials do not justify continuation of tolvaptan beyond the time of improvement in fluid balance and clinical status in patients hospitalized with worsening HF.

19.2.2 AQPs and Hepatic Cirrhosis

Hepatic cirrhosis is another chronic condition associated with edema, hyponatremia, and increased AVP levels [25]. The most important reason for the development of hyponatremia in patients with cirrhosis is suggested to be systemic vasodilation, which causes increased AVP secretion. Renal sodium retention leads to increase of excellular fluid volume and development of edema (Fig. 19.1). Unlike CHF, the changes in expression of AQP2 protein levels vary considerably between different experimental models of hepatic cirrhosis. Several studies have explored the variations in renal AQP2 expression in rats with carbon tetrachloride-induced cirrhosis [33, 34]. A strong correlation was seen between AQP2 level and ascites volume, suggesting that AQP2 is involved in the abnormal water retention that leads ascites in cirrhosis [35]. Another study showed that total AQP2 levels were not changed, but the AQP2 increased in plasma membrane, accompanied by the increased AQP3 expression [36]. In contrast, rats with cirrhosis induced by common bile duct ligation (CBDL) [35, 37–39] exhibited impaired vasopressin-regulated water reabsorption despite normal plasma vasopressin levels. Furthermore, AQP2, AQP3, and AQP4 expressions were decreased, and AQP1 level was unchanged.

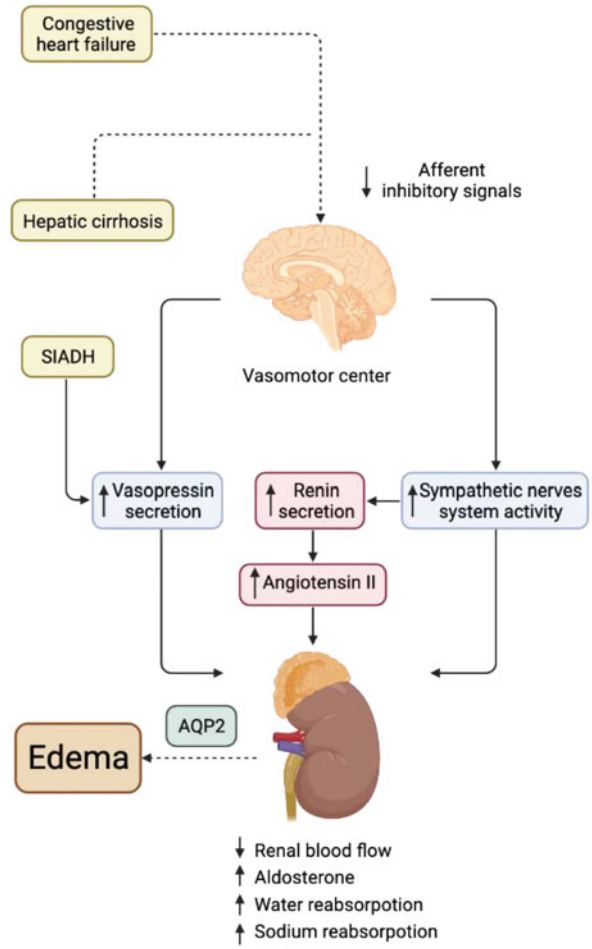

Fig. 19.1 AQPs and edema. In the disorders of edema, such as CHF, hepatic cirrhosis, and SIADH, the secretion of AVP is promoted, which increases the expression of AQP2 that mediates water reabsorption in kidney

There are studies on cirrhosis patients [40, 41]. The results demonstrated a higher abundance of AQP2 in the urine compared to control group. Patients with ascites had the highest AQP2 level, which increased with the clinical severity of cirrhosis, and levels were highest in patients with ascites, suggesting the important role of AQP2 in cirrhosis-induced water retention and edema. Conversely, other studies have shown no significant change, or even a decrease, in urinary AQP2 level in patients with cirrhosis [42, 43].

A study showed that urinary AQP2/creatinine ratios were significantly higher in cirrhotic patients with ascites than in healthy controls. After administration of tolvaptan, urinary AQP2/creatinine ratios decreased by 45.0% at 4 h and 77.0% at 8 h. Similarly, urinary osmolarity decreased [44]. The results indicate that the vasopressin-AQP2 system plays a major role in water retention in cirrhosis.

19.2.3 AQPs and Syndrome of Inappropriate Secretion of Antidiuretic Hormone

Syndrome of inappropriate secretion of antidiuretic hormone (SIADH) is one of the disorders of disturbed osmoregulation. SIADH occurs most frequently in relation to neoplastic abnormalities in the lung or central nervous system, neurological diseases, lung diseases, and a wide kinds of drugs, particularly psychoactive drugs and chemotherapy [45]. In SIADH, the

levels of AVP are abnormally increased, and result in excessive renal water reabsorption, which can lead to severe hyponatremia [46]. It was discovered that AQP2 expression increased in the collecting duct of an experimental rat model of SIADH [33]. The AQP2 increase can be locked by a V2R antagonist, which correlates closely with a marked diuresis and a normalization of serum sodium levels, indicating that AQP2 plays an important role in water retention and development of hyponatremia in SIADH (Fig. 19.1).

The degree of hyponatremia is limited by the process of counteracting the water retention of vasopressin, that is, vasopressin escape. Vasopressin escape is characterized by a sudden rise in urine volume and drop in urine osmotic pressure that is not related to high circulating vasopressin levels. Renal AQP2 expression decreased significantly at the same time as the commencement of escape [47]. In contrast to AQP2, the expression of AQP1, AQP3, or AQP4 in renal was not decreased. These results suggest that escape from vasopressin-induced antidiuresis is attributable, at least in part, to a selective vasopressin-independent decrease in AQP2 expression in the renal collecting duct.

19.3 Conclusion

In the last 20 years, the molecular and cell biology mechanisms of AQP regulation have grown dramatically. The regulation of AQPs is critical to osmoregulation and maintaining bodily water homeostasis. It is not surprised that a variety of AQPs, particularly AQP2, are involved in edema-related illnesses such as CHF, hepatic cirrhosis, and SIADH. The research revealed that AQP2 dysregulation is associated with edema. The upregulation of AQP2 expression is critical in CHF water retention. AQP2 is also involved in the development of ascites in hepatic cirrhosis. Furthermore, hyponatremia in SIADH is caused by increased expression of AQP2 in the collecting duct. All these studies suggest that AQP2 could be employed as a biomarker or prognostic marker in the treatment of edema.

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Aquaporins in Obesity

20

Inês V. da Silva and Graça Soveral

Abstract

Obesity is one of the most important metabolic disorders of this century and is associated with a cluster of the most dangerous cardiovascular disease risk factors, such as insulin resistance and diabetes, dyslipidemia, and hypertension, collectively named Metabolic Syndrome. The role of aquaporins (AQP) in glycerol metabolism facilitating glycerol release from the adipose tissue and distribution to various tissues and organs unveils these membrane channels as important players in lipid balance and energy homeostasis and points to their involvement in a variety of pathophysiological mechanisms including insulin resistance, obesity, and diabetes. This review summarizes the physiologic role of aquaglyceroporins in glycerol metabolism and lipid homeostasis, describing their specific tissue distribution, involvement in glycerol balance, and implication in obesity and fat-related metabolic complications. The development of specify pharmacologic modulators able to regulate aquaglyceroporins expression and function, in particular AQP7 in adipose tissue, might constitute a novel approach for controlling obesity and other metabolic disorders.

Keywords

Aquaporins · Water transport · Obesity

20.1 Introduction

Obesity can be defined as the enlargement and inflammation of the adipose tissue and is the most significant metabolic disorder of this century, reaching epidemic proportions [1]. Accumulation of fat in visceral and subcutaneous abdominal tissue and its deposition in internal organs is a major risk for the development of numerous disorders, including insulin resistance and diabetes, dyslipidemia, hypertension, and cardiovascular and neurodegenerative diseases among others. Some of these metabolic complications appearing as a cluster are termed as metabolic syndrome and have been associated with the most dangerous cardiovascular risk factors. In this way, abdominal obesity, the most prevalent manifestation of this syndrome and a marker of adipose tissue dysfunction is now recognized as the predominant contributor to type 2 diabetes and cardiovascular risk [2].

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20.2 Adipose Biology and Pathophysiology

Adipose tissue is categorized into two major types according to its physiological and endocrine functions: the white adipose tissue (WAT), mostly anabolic and involved in energy storage in the triacylglycerol form, and the brown adipose tissue (BAT), highly catabolic and involved in thermogenesis [3]. The accumulation of fat in WAT with subsequent dysfunction and peripheral lipotoxicity is the main etiological factor in obesity.

The WAT is composed by adipocytes, vascular tissue, and immune cells, surrounded by an extracellular matrix formed by proteins, mostly collagen. Preadipocytes are the mature adipocyte precursors that undergo differentiation to become mature and fully differentiated cells. This process of differentiation has been widely studied in a variety of models [4]. In situations of positive energy balance (increased food uptake or decreased energy expenditure), mature adipocytes increase in number (hyperplasia) and size (hypertrophy) to accommodate excess lipid and their morphology changes due to increased free fatty acids (FFA) uptake and triacylglycerols (TAG) synthesis. To allow adipocyte enlargement, the extracellular matrix must be adjusted by the action of proteases that hydrolyze the excess of collagen to allow adipose hypertrophy. In addition, the formation of new blood vessels (angiogenesis) is also essential for adipose tissue growth and is a duality between a response to signals emanating from proliferating and enlarging adipocytes and a response to developmental and metabolic signals, preceding the adipocyte proliferation and enlargement [5]. Adipocytes descend from adipose stem cells localized close to the microvasculature of adipose tissue but not in the vasculature of other tissues [6]. These stem cells have already committed either prenatally or early in postnatal life, differentiate into adipocytes probably by signals coming from the adipose vasculature that may function as an adipocyte niche [6]. By secreting signaling proteins collectively known as adipokines, adipose tissue is an important endocrine and paracrine organ that communicates with many other organs in the body contributing to the maintenance of energy, lipid, and glucose homeostasis and mediating multiple biological processes such as inflammation, immunity, and metabolism.

While it is common to link abdominal obesity with insulin resistance based on population studies, the pathogenicity of obesity and related metabolic complications such as insulin resistance and type 2 diabetes is still not clear. Several hypotheses have been advanced to explain the development of adipose tissue dysfunction and obesity. One of the most accepted that emerged from corroboration of clinical and experimental data, the adipose tissue expandability hypothesis, is based on the limitation of the adipose tissue to expand above a given threshold for a specific individual [7]. When an individual gains weight and increases in fat mass, the adipose tissue enlarges till a point where it may exceed the limit capacity of storage and is no longer able to efficiently accumulate more fat. At this point, bloodstream lipids start depositing ectopically in other non-adipose tissues such as liver, muscle, and heart, leading to lipid-induced toxicity (lipotoxicity) and resulting in inflammation and insulin resistance (Fig. 20.1) [7]. Importantly, the maximal capacity of adipose tissue expansion is dependent on the type of fat depot, subcutaneous or visceral, the first being more adipogenic and with greater expansion capacity and the latter metabolically more active. It is well accepted that in humans, increased visceral fat is associated with increased metabolic complications, whereas subcutaneous adiposity is not so harmful and may even be protective [7]. While the reason is not clear, the fact that visceral fat is more closely related with liver through the portal vein than subcutaneous adipose tissue, together with its diminished expansion capability, supports the increased risk of metabolic syndrome strongly associated with visceral obesity. Moreover, evidences that the individual adipose expandability threshold is determined by genetic and environmental factors may explain why both apparently lean and obese people may develop insulin resistance [8].

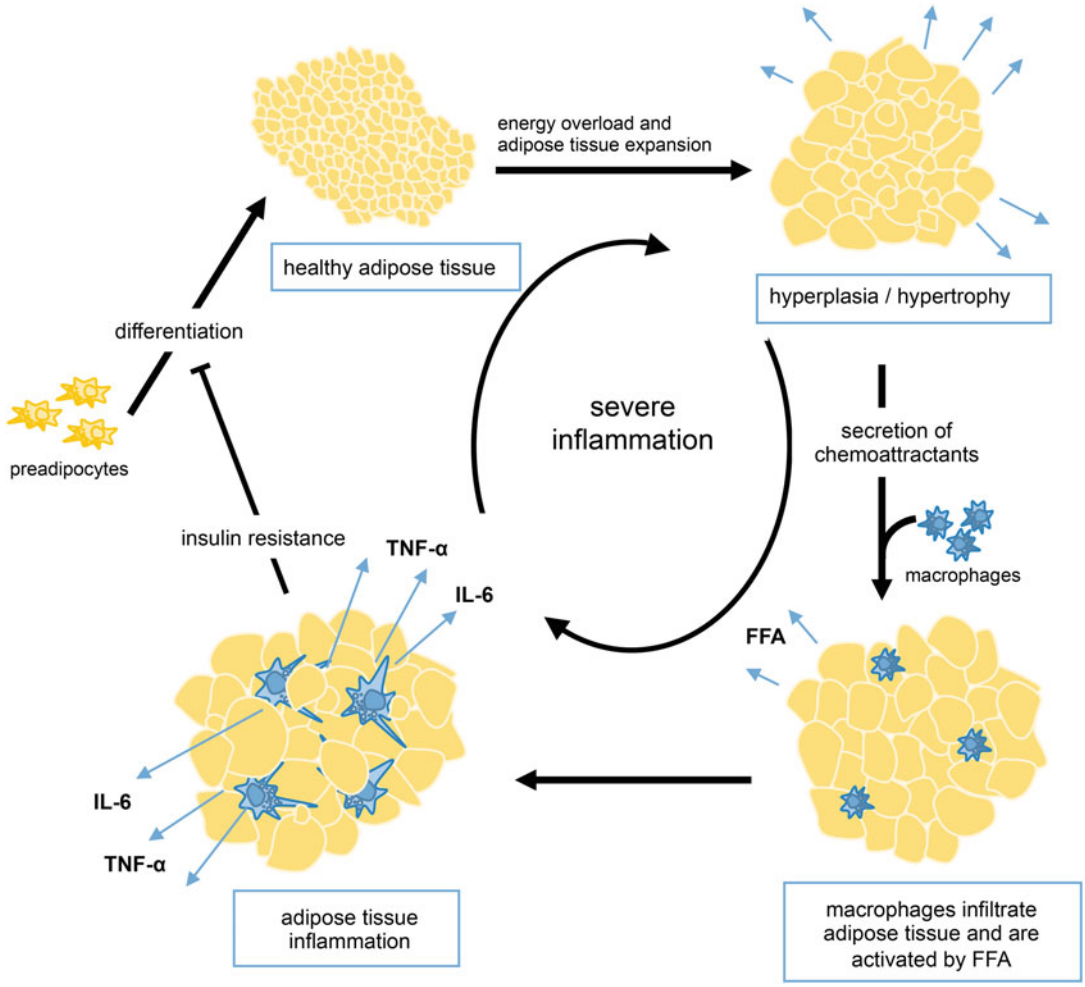

Fig. 20.1 Illustration of cyclic mechanism of adipose tissue inflammation linking to insulin resistance and obesity. When the uptake of nutrients overcomes the energy expenditure, TAG accumulation in adipocytes induces adipose hyperplasia and hypertrophy, secretion of chemoattractants leading to macrophage recruitment. Large adipocytes are induced to secrete more cytokines and FFA, which in turn activate macrophages. Macrophages secrete anti-adipogenic cytokines (TNF-α and IL-6) that inhibit insulin action and lead to adipose tissue inflammation. These cytokines also block the differentiation of preadipocytes into new adipocytes, thus inducing the enlargement of insulin resistant-adipocytes that continue secreting more cytokines and FFA, recruiting macrophages, and leading to severe inflammation

In addition to the expandability hypothesis, adipose tissue inflammation mediated by overproduction of pro-inflammatory adipokines and anti-adipogenic cytokines such as TNF-α and IL-6 is another recognized mechanism linking obesity to insulin resistance. Large adipocytes express and secrete high levels of chemoattractants, thus inducing macrophage infiltration in the adipose tissue and activation by FFA release. These macrophages secrete anti-adipogenic cytokines that inhibit insulin action. Insulin-resistant adipocytes continue releasing FFA, thus activating macrophages that surround adipocytes to destroy compromised cells and secrete more anti-adipogenic cytokines, increasing insulin resistance in mature adipocytes and blocking maturation of preadipocytes [7]. The cyclic

mechanism of adipose tissue inflammation linking to insulin resistance is depicted in Fig. 20.1.

20.3 Aquaporins in Obesity

A number of recent studies evidenced aquaporins (AQPs) as key players in adipose tissue biology and involved in obesity onset. AQPs are transmembrane proteins that facilitate the permeation of water and small solutes across membranes, driven by osmotic or solute gradients [9]. In mammals, the 13 aquaporin isoforms identified so far (AQP0–12) are expressed in a wide range of tissues and are involved in many biological functions, including transepithelial fluid transport, cell migration, proliferation and adipocyte metabolism [10, 11]. AQPs are composed of around 320 amino acid residues with approximately 28 kDa, architected in membranes as tetramers. Each monomer is formed by six transmembrane domains and behaves as an independent pore [12].

Based on their primary sequences and permeation specificities, AQPs are divided into three subfamilies: orthodox aquaporins, considered strict water channels (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8); aquaglyceroporins, permeable to water and small uncharged solutes like glycerol (AQP3, AQP7, AQP9, and AQP10); and unorthodox aquaporins, found mostly intracellularly, with lower sequence homology and permeability still unclear (AQP11 and AQP12) [13, 14]. A subgroup of aquaporins that additionally permeate hydrogen peroxide was recently identified (AQP0, AQP1, AQP3, AQP5, AQP8, AQP9, and AQP11) and named peroxiporins [15–17].

Among the three subfamilies, aquaglyceroporins are emerging as important players in adipose tissue homeostasis and insulin response with possible implications in metabolic disorders such as obesity and metabolic syndrome [18]. In fact, their roles in glycerol metabolism, mediation of glycerol release from adipose tissue, and uptake in liver and heart reveal that these membrane channels are crucial for glycerol balance and energy homeostasis and may be used for obesity therapy.

20.4 Glycerol Metabolism and Energy Homeostasis

The ability of aquaglyceroporins to facilitate glycerol permeation through adipocyte membranes and its impact in metabolic disorders have raised attention to the involvement of glycerol in metabolism and in a variety of pathophysiological mechanisms.

Glycerol (1,2,3-propanetriol) is a polyalcohol that can be produced intracellularly from various metabolic sources such as glucose, protein, and glycerolipid (endogenous glycerol) as well as taken up from dietary fats released during digestion (exogenous glycerol). Glycerol is the basis of TAG backbone and a precursor for phospholipids synthesis and is also an important intermediate in both carbohydrate and lipid metabolism. In addition, glycerol-3-phosphate (G3P) is a key molecule in the regeneration of NAD^+ from NADH resultant from glycolysis, acting as a shuttle of electrons from the cytosol into the mitochondria [19, 20].

Dietary TAG are digested by lipases in the digestive tract and converted to mono and diacylglycerols by pancreatic lipases in the small intestine that are then absorbed by the duodenum mucosa. In the enterocytes, monoacylglycerols and FFA are reconverted into TAG that are then secreted through the basolateral membrane into the lymphatic system as low-density lipoproteins known as chylomicrons. Chylomicrons are released from the lymph to the bloodstream where they circulate till reaching adipose, cardiac, and skeletal muscle tissues. Lipoprotein lipases, attached to the surface of endothelial cells of capillaries, hydrolyze TAG components of chylomicrons in FFA, absorbed actively by the tissues, and glycerol, taken up by the liver and other organs. In the liver, glycerol is used in glycolysis or gluconeogenesis but not before being converted in the intermediate G3P by the enzyme glycerol kinase (GK), which is mainly present in the liver and kidney, but also, in low

Table 20.1 Expression of aquaporins by organ/tissue and their implication in glycerol balance

Organ/tissue	Aquaglyceroporin	Glycerol balance
Small intestine	AQP3 [26]	Glycerol secretion and enterocyte proliferation [27]
	AQP7 [28]	Rapid glycerol absorption in villus epithelium [28, 29]
	AQP10 [30–32]	Carrier and channel for glycerol and other solutes transport [29]
White adipose tissue	AQP3 [20]	Glycerol metabolism [20]
	AQP7 [33–35]	Main glycerol transporter; control glycerol uptake and release [35–37]
	AQP9 [20]	Glycerol influx [20]
	AQP10 [35]	Maintain normal glycerol levels [35]
	AQP11 [38]	Mediate intracellular glycerol movements [38]
Brown adipose tissue	AQP3, AQP7, AQP9 [39, 40]	Glycerol permeation [39, 40]
Cardiac and skeletal muscle	AQP3 [21, 41, 42]	Glycerol transport for energy production in skeletal muscle [41]
	AQP7 [21, 41, 42]	Glycerol transport for energy production mainly in cardiac muscle [21, 41, 43]
Liver	AQP9 [44]	Uptake of glycerol for glucose production [44]
Endocrine pancreas	AQP7 [45]	Regulation of insulin production/secretion [45, 46]
Kidney	AQP7 [47]	Involved in glycerol reabsorption [48–50]
Endothelia	AQP3 [51]	Endothelium glycerol permeation [51]
	AQP7 [48]	Involved in glycerol permeation in capillary endothelia of adipose tissue [48]

concentrations, in muscle and brain. G3P is the more important form of glycerol for the cell physiology. In addition, glycerol is an energy substrate via the G3P shuttle, which has a key role in oxidizing glucose rapidly and generating adenosine triphosphate (ATP) in the mitochondria through the oxidation of G3P [21].

In humans, gluconeogenesis occurs mainly in the liver and kidney, sites of greatest measured GK activity. Under normal feeding conditions, glycerol contribution to gluconeogenesis is reduced, but it increases considerably during starvation where it becomes the primary source for gluconeogenesis along with lactate, pyruvate, alanine, and glutamine [22]. In prolonged fasting, glycerol can be used as the only source for gluconeogenesis, since glycogen reserves are depleted within two fasting days [19].

In situations of negative energy balance, such as fasting or exercise, lipolysis of TAG stored in white adipose tissue yields glycerol and FFA that are released in the bloodstream to be used by other organs as energy source. Lipolysis rates are finely regulated by hormones and by biochemical signals that modulate lipolytic enzymes, allowing the finest response from adipose tissue to changes in energy requirements and availability [23, 24].

Besides the intake of dietary glycerol absorbed in the small intestine, the amount of glycerol circulating in the bloodstream is as well dependent on the amount reabsorbed in kidney microtubules; but its main source results from lipolysis in adipose tissue.

All the above-described metabolic pathways occur exclusively in intracellular compartments forcing glycerol molecules to move across the different tissues. Glycerol permeation through membranes is facilitated by aquaglyceroporins, and thus regulation of glycerol transport by AQPs is crucial to control fat accumulation, lipolysis, gluconeogenesis, and energy homeostasis [20, 25]. Table 20.1 lists the tissue expression and implication in glycerol balance described for aquaglyceroporins, anticipating its possible implication in fat fate and associated metabolic alterations.

Figure 20.2 depicts a schematic model showing the involvement of aquaglyceroporins in glycerol metabolism and energy balance. The expression and localization of the various aquaglyceroporins in multiple organs are

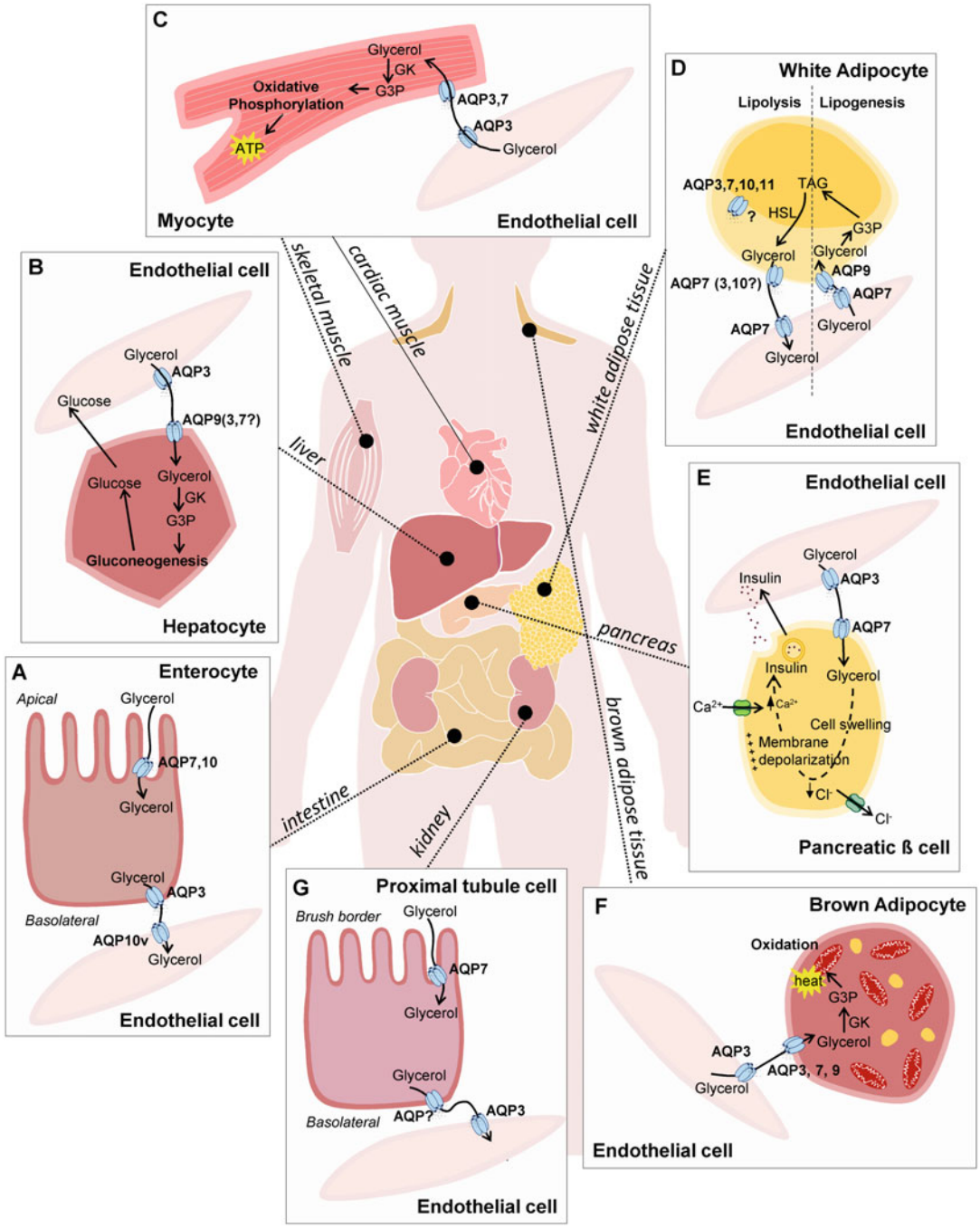

Fig. 20.2 Involvement of aquaglyceroporins in glycerol metabolism and energy homeostasis. (**a**) Glycerol from dietary fat is absorbed via AQP7 and AQP10 in the apical membrane of small intestine epithelial cells and exits via AQP3 located in the basolateral membrane. Endothelial cells from adipose vasculature express AQP10 that allows glycerol entry to the bloodstream to be used by other organs as energy source. (**b**) Glycerol is taken up to the liver by AQP9 (and possibly also AQP3 and AQP7) expressed at the basolateral sinusoidal membrane of hepatocytes. In the hepatocyte, glycerol is converted to G3P by GK to be used in gluconeogenesis. (**c**) Glycerol is used in cardiac and skeletal muscle as an energy source. It permeates the membrane by AQP3 and 7 and, after conversion in G3P by GK, is used for ATP production. (**d**) In fasting conditions when lipolysis occur in the white

important to assure glycerol fluxes across tissues and are crucial for glycerol metabolism and homeostasis. A detailed description can be found in Fig. 20.2 legend.

20.5 Aquaporins in Adipose Tissue

20.5.1 AQP7 Expression and Role in Fat Accumulation

Among the various mammalian aquaglyceroporins, AQP7 is the most representative glycerol channel and the first to be detected in human and mouse adipose tissue [47, 52, 53] and adipocytes [33, 34]. Despite claimed by other researchers that AQP7 was localized in the vascular tissue surrounding fat rather that in adipose tissue membranes [48, 54], a double localization in adipocytes and endothelial cells was also described [35, 50] and AQP7 expression in both human subcutaneous and visceral adipose tissue was reported [20, 34].

The fundamental role of AQP7 in glycerol release from adipocytes was attained when it was found to function as a glycerol channel [33]. Several experiments were performed to demonstrate AQP7 function. Obese insulin-resistant db/db mice showed higher AQP7 expression compared to control mice [33], and a similar increase in AQP7 mRNA was also observed in adipose tissue of a rodent model of type 2 diabetes with obesity when compared with normal rats [55]. These experiments suggested that the dysregulation of AQP7 could lead to an augmented input of glycerol for hepatic gluconeogenesis and to an increase of glucose in type 2 diabetes [47]. Studies using AQP7 knockout mice showed development of adipocyte hypertrophy and early obesity onset were due to an accumulation of glycerol and TAG [36, 37]. In addition, aged AQP7 knockout mice developed insulin resistance, compromising the whole body metabolism. Hibuse et al. [37] proposed a mechanism to explain adipocyte hypertrophy, where an increased accumulation of glycerol in adipocytes stimulated glycerol kinase activity and led to increased TAG levels in adipose tissue, indirectly favoring the development of obesity and insulin resistance [37]. Yet, susceptibility to develop obesity was not confirmed by other AQP7 null mouse lines [48, 56]. Nevertheless, although the different phenotypes reported in distinct AQP7 knockout mouse studies, all confirmed the involvement of AQP7 in glycerol metabolism.

Correlation of adipose AQP7 expression with glycerol metabolism and related metabolic complications were not so obvious in human studies [57]. Albeit there was a link between adipocyte AQP7 expression and insulin resistance, genome-wide analysis found *AQP7* gene linked to type 2 diabetes [58] and metabolic syndrome [59] as well as associated with obesity but only for the female participants [60]. Gender differences in the role of AQP7 in adipose tissue metabolism were supported by higher fasting circulating levels of glycerol in women than in men, probably due to higher percentage of subcutaneous fat in females, higher lipolytic rates and higher AQP7 expression levels [54, 61].

Three AQP7 missense mutations (R12C, V59L, and G264V) and two silent mutations (A103A and G250G) were described in humans. G264V mutation, held by a male homozygous

Fig. 20.2 (continued) adipocytes, TAG hydrolysis yields FFA and glycerol that is released to the blood via AQP7 (and possibly also 3 and 10) expressed in both white adipocyte and endothelial cell membranes. In feeding conditions when plasma glycerol reaches high concentrations, glycerol is taken up by white adipocytes possibly via AQP9 being converted to TAG and stored in the lipid droplets. (**e**) In response to high plasma nutrient levels, glycerol enters pancreatic β-cells via AQP7 and participates in a cascade of events that culminates with insulin exocytosis. (**f**) During exercise or under cold exposure, glycerol is taken up by the brown adipocyte via AQP3, 7, and 9 and is oxidized in the mitochondria producing heat. (**g**) Glycerol filtered in the kidney is reabsorbed to the blood via AQP7 expressed in the brush border membrane of proximal tubule cells, thus preventing its excretion in the urine

patient, was the only one demonstrating water and glycerol permeability loss; however, it did not correlate to obesity nor diabetes, and a lack of increase in plasma glycerol was observed only when stressed by exercise [57, 62]. Three other patients with the same mutation were also diagnosed as neither obese nor diabetic, but they presented increased glycerol excretion in the urine [63]. Moreover, recently identified variations in the *AQP7* promoter associated with *AQP7* downregulation and high serum glycerol levels might contribute to child obese phenotype and were correlated with familial predisposition to type 2 diabetes [64].

20.5.2 Regulation of AQP7 Expression

In mice and humans, *AQP7* gene expression is upregulated by fasting or exercise, leading to glycerol production from endogenous TAG, whereas during the feeding state it is downregulated. AQP7 abundance is inversely related with plasma insulin levels [33]. Transcription of *AQP7* gene is inhibited by the increase of insulin levels in the bloodstream through a negative insulin response element (IRE) identified in the promoter region of *AQP7* gene in mice and humans [62, 65] and by blockage of the phosphatidylinositol-3 kinase (PI3K) pathway [20, 65]. Upregulation of AQP7 by peroxisome proliferator-activated receptor gamma (PPARγ) is also demonstrated in mouse and human adipocytes [57, 66, 67]. PPARγ regulates adipocyte differentiation through the regulation of several adipose genes, and, in differentiating adipocytes, simultaneous increase in glycerol release to the media and AQP7 mRNA levels were observed [33], suggesting a common regulatory pathway dependent of cell differentiation [68]. In line with this, thiazolidinediones (synthetic PPARɣ) and insulin sensitizers were reported to upregulate AQP7 [55, 62, 65], whereas leptin [20, 69], TNF-a, adrenergic agonists and steroids, being insulin resistance inducers, downregulate AQP7 expression [70]. Ghrelin, a lipogenic hormone, also interacts in *AQP7* regulation, stimulating TAG accumulation in parallel with a decrease in AQP7 expression [71].

In the fasting state, concomitant with lipolysis, low plasma insulin levels and catecholamine stimulation induce *AQP7* gene transcription and intracellular AQP7 translocation to the plasma membrane, thus potentiating glycerol release from adipocytes [33]. A recent study demonstrated that AQP7 was bound to the lipid droplet protein perilipin 1 (PLIN1), suggesting that PLIN1 was involved in the coordination of the subcellular translocation of aquaglyceroporins in human adipocytes [72].

In humans, studies with obese subjects showed a different regulation of AQP7 depending on the type of adipose tissue, subcutaneous or visceral. Obese individuals show low AQP7 expression in subcutaneous fat reflecting fat accumulation and adipocyte hypertrophy but, conversely, show increased AQP7 levels in visceral fat, which can be correlated with increased lipolysis [20, 25]. Since subcutaneous adipose tissue is more insulin sensitive than the visceral, AQP7 downregulation may represent a feedback mechanism attempting to prevent lipid depletion and consequent lipotoxicity and associated disorders [25]. Moreover, gonadal steroids are important factors in the determination of sex-dependent fat distribution and accumulation and have been reported to modulate *AQP7* expression [73]. In fact, estrogen response elements in the promoter of the *AQP7* gene, resulting in fat catabolism in adipocyte, might explain the development of menopausal obesity [74].

20.5.3 Other Aquaporins in Adipose Tissue

The fact that obese AQP7 null mice still show measurable glycerol secretion and AQP7 altered expression or dysfunction could not be clearly correlated with obesity and type 2 diabetes, suggesting the existence of alternative glycerol pathways in adipose tissue. In fact, the aquaglyceroporins AQP3 and AQP9 were detected in human subcutaneous and visceral adipose tissue, with AQP3 being even more

expressed in visceral tissue than AQP7 [20], although other authors did not confirm these same results [33, 34, 75].

AQP3 was found both intracellularly and in the plasma membrane of adipocytes in subcutaneous and visceral adipose tissue, but with a stronger expression in the stromal vascular tissue adjacent to adipose [20, 34, 75].

AQP5 was found expressed in mouse adipose cells and with a crucial role in adipocyte differentiation [67], and *AQP5*-KO mice have lower body weight than wild-type animals [76]. Interestingly, high-fat diet induces AQP5 expression in subcutaneous adipose tissue [40], suggesting a role for AQP5 on adipose biology and cellular adaptation to fat accumulation.

AQP9 was also detected in the plasma membrane of adipocytes [20]. Described as mostly expressed in the liver, AQP9 is responsible for hepatic glycerol uptake for gluconeogenesis in close coordination with adipose glycerol efflux through AQP7 during fasting [77, 78] and is strongly associated with adipose tissue metabolism and fat accumulation. In addition, non-alcoholic fatty liver disease (NAFLD) is associated with altered hepatic AQP9 and glycerol permeability [69, 79] that could be reverted by leptin administration [69].

AQP10 was detected in the cytoplasm and in the plasma membrane of adipocytes, in human subcutaneous adipose tissue [35]. Both AQP3 and AQP10 were shown to translocate to the plasma membrane in response to β-adrenergic stimuli [35, 80]. More recently, the unorthodox AQP11 was detected in both subcutaneous and visceral adipocytes being localized in the vicinity of the lipid droplets [38] and associated with the endoplasmic reticulum (ER) in human visceral adipose tissue [81]. AQP11 water and glycerol transport was demonstrated in an adipose cell model [38] possibly unraveling a facilitated glycerol gateway from the intracellular lipid droplets [25]. Moreover, since AQP11 facilitates H_2O_2 diffusion, its overexpression along adipocyte differentiation was suggested to constitute a compensatory mechanism to alleviate endoplasmic reticulum stress in obesity [81].

Although most studies were focused in AQP expression in WAT, their role in BAT has been highlighted in the last years. Since brown adipocytes are more prone to expend energy as heat, browning of the white resulting in beige adipocytes might reveal a useful approach to treat obesity. When the beige phenotype is induced in a murine adipocyte cell line, the most expressed glycerol channels AQP7 and AQP9 are downregulated along with upregulation of several brown adipocyte markers such as the mitochondrial UCP1 [82], suggesting a relation between AQP expression and the metabolic shift from anabolic to catabolic thermogenic metabolism.

20.6 Aquaporins as Drug Targets of Obesity

The implication of aquaglyceroporins in fat metabolism and obesity indicates that from a pathophysiological point of view these proteins are promising drug targets. The possibility of regulating the expression of aquaglyceroporins, in particular AQP7 in adipose tissue, offers a potential therapeutic approach for the regulation of fat accumulation and treatment of obesity. Altered AQP7 expression by hormones (insulin, catecholamines), cytokines, and adipokines [20, 69–71] is described in the previous section. As for AQP7 channel activity, the only specific modulator described so far is the gold compound auphen that showed to inhibit glycerol permeability in an adipocyte cell line [83]. However, auphen may have a possible application in diagnosis to uncover AQP7 activity and gating [84, 85], strategies to treat obesity point toward activation of AQP7 function rather that inhibition. Thus, the design of small molecule upregulators of AQP7 expression and function is of utmost interest and would undoubtedly have therapeutic applications.

A recent study reported that supplementation of high-fat diets with apple polyphenols impaired adipocyte hypertrophy and prevented adiposity increase by a mechanism that included increased AQP7 and leptin mRNA levels in rat visceral adipocytes [86]. However promising, the

complex mixture of polyphenols included in this functional food may hinder the identification of structure–activity relationships of the phenolic substances. Another study reported the anti-obesogenic effect of resveratrol and pterostilbene, reducing epididymal and subcutaneous adipose tissue, respectively, in rats fed high-fat diet. Further epigenetic studies are needed to gain more insight concerning the regulation of AQPs by these polyphenols [87]. In a similar animal model, raspberry ketone administration ameliorated adiposity via upregulation of AQP7 expression, but their clinical efficacy and safety data to treat obesity need clarification [88].

Recent studies revealed the usefulness of biologics, such as monoclonal antibodies [89] and micro-RNAs [90], to modulate AQPs, overcoming the toxic side effects of numerous reported chemical compounds and opening new strategies for AQP-based therapies. Hence, further investigations to untangle aquaporins interplay with other adipose regulatory molecules, regulation by hormones, and possibility of channel gating are needed to better establish the mechanistic basis of AQPs involvement in pathogenesis of obesity, which is crucial for the identification of novel modulators design of new compounds.

20.7 Final Considerations

Notwithstanding the importance of glycerol as key energy source in multiple vital metabolic processes and in the physiopathology of several disorders, the role of aquaporins on glycerol membrane permeation has only recently been recognized. Given the involvement of aquaglyceroporins in energy and metabolic homeostasis serving as mediators of glycerol delivery and bridging tissues and organs, their targeting might constitute a novel approach for controlling several metabolic disorders. Adipocyte glycerol permeability is a regulator of adipocyte enlargement and weight gain, and thus, upregulation of AQP7 expression or its functional activation may provide a novel therapeutic approach to prevent or treat obesity. However, potent and selective pharmacologic modulators are still missing. Identification or design of new molecules targeting adipose aquaporins might be seed for drug development and open new perspectives of obesity treatment.

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Aquaporins in Tumor

21

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Abstract

Recent researches have demonstrated that aquaporins (AQPs), including water-selective channels, aquaglyceroporins and superaquaporins, are generally expressed in various tumors, such as lung, colorectal, liver, brain, breast tumors, etc. Therefore, it is imperative to study the accurate relationship between AQPs and tumor, which may provide innovative approaches to treat and prevent tumor development. In this chapter, we mainly reviewed the expression and pathophysiological function of AQPs in tumor, and summarize recent work on AQPs in tumor. Although, the underlying mechanism of AQP in tumor is not very clear, growing evidences suggest that cell migration, adhesion, angiogenesis, and division contribute to tumor development, in which AQPs might be involved. Therefore, it is still necessary to conduct further studies to determine the specific roles of AQPs in the tumor.

Keywords

Aquaporins · Tumor · Pathophysiology

21.1 Introduction

Aquaporins (AQPs) are membrane proteins that allow the penetration of water, glycerol, and hydrogen peroxide across bio-membranes and play a pivotal role in the homeostasis of water in various tissues and organs [1]. AQPs are expressed in numerous endothelia, epithelia, and other types of cells. Thirteen members (AQP0–AQP12), having been identified in humans and mammals, are segmented into three groups based on their sequence homology and permeability profile, such as water-selective channels (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8), which are only permeable to water, aquaglyceroporins (AQP3, AQP7, AQP9, and AQP10), which are permeable to water and some physiological solutes, e.g., glycerol, urea and gas, and superaquaporins (AQP11 and AQP12) [2–4]. Recently, a variety of studies have showed that AQPs are found in more than 20 cell types of tumor and deeply related to tumor biopathological functions [1, 5]. The expression of AQP is positively linked with diagnostic and therapeutic targets like migration, proliferation,

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angiogenesis, grades, and edema formation [1, 2, 6, 7]. In this chapter, we mostly discussed the essential role of AQPs in various specific tumors.

21.2 AQPs in Tumor

AQPs are found in numerous human normal tissues and exert pivotal functions in erythrocytes, epithelium cells, astrocytes, endothelium, adipocytes, and skeletal muscle [8, 9]. However, a growing body of studies suggest that AQPs are dysregulated in several tumor tissues and closely involved in the progression of tumor. The role of AQPs in breast, brain, lung, liver, and colorectal tumors has been mostly reported as shown in Fig. 21.1 [6].

21.2.1 Water-Selective Aquaporins

21.2.1.1 AQP1

Normally, AQP1 is mainly found in kidney, choroid plexus, vascular endothelial cells, and corneal endothelium [10, 11]. Recently, it is reported that AQP1 is involved in the neovessels, which is important for the survival of tumor cells [12]. Some preclinical studies suggest a correlation between AQP1 and the development of melanoma [13, 14] and highlight AQP1 as a pivotal key indicator of tumor dissemination by accelerating tumor cell extravasation and metastatic formation. Inhibition of AQP1 reduced metastatic formation and increased survival through regulating VEGF and MMP2 in mice bearing melanoma tumor [15]. The main reason maybe that AQP1 promotes tumor angiogenesis by allowing faster endothelial cell migration [16]. It is known that vascular permeability is associated with endothelial cell migration, and some researchers reported that vessel permeability can be accelerated by AQP1, which augment the transportation of cellular water, and angiogenic cascade is promoted by plasma protein extravasation as a scaffold for endothelial cell migration [17].

In addition to tumor angiogenesis and cell migration, AQP1 also promoted the proliferation of tumor cells. For example, researchers found cell proliferation induced by AQP1 in mouse embryo fibroblast cell line [18]. In lung tumor cells, increased cell volume and migration speed are associated with AQP1 overexpression that is regulated by ERK1/2 and caveolin-1 signaling [19]. In gastric tumor, one of the most common malignancies of the digestive tract, AQP-1 promoted the invasion and proliferation of gastric tumor cells through GRB7-induced ERK and Ras activation, suggesting that AQP1/RAS/ERK signaling is a potential pathway in gastric tumor aggression [20]. What is more, AQP1 knockout not only apparently modified the expression of some key cell cycle proteins associated with the enhanced cell proliferation [21] but also had the effect on the migration and proliferation of tumor cell along with the downregulation of matrix metalloprotein 9 (MMP-9) [22], focal adhesion kinase (FAK) expression, which can induce multiple intracellular signaling cascades for tumor cell adhesion, growth, migration, survival, and invasion [23], and thrombospondin type-1 domain containing 7A (THSD7A) in glioblastoma [24].

With the increased cellular invasion and proliferation, hypoxia is regarded as a common characteristic to most tumors and conduces to tumor resistance and progression [25]. Some researchers reported that hypoxia-induced upregulation of AQP1 in spongioblastoma cells is associated with the glycolysis, which suggests that expression of AQP1 is regulated by hypoxia-induced glycolysis [26]. It is reported that the expression of AQP1 in PC-3M cells is regulated by low oxygen tension, MAPK, protein kinase C (PKC), and calcium [27]. Collectively, AQP1 is upregulated in many human tumors, such as breast, prostate, cervix, nasopharynx, bladder, biliary duct, brain, and lung tumors [28], and contributes to tumor development by enhancing angiogenesis, migration, invasion, proliferation, and hypoxia.

21.2.1.2 AQP2

AQP2 was initially discovered in the renal collecting ducts and acts as a vasopressin-sensitive water channel [29]. AQP2 plays an

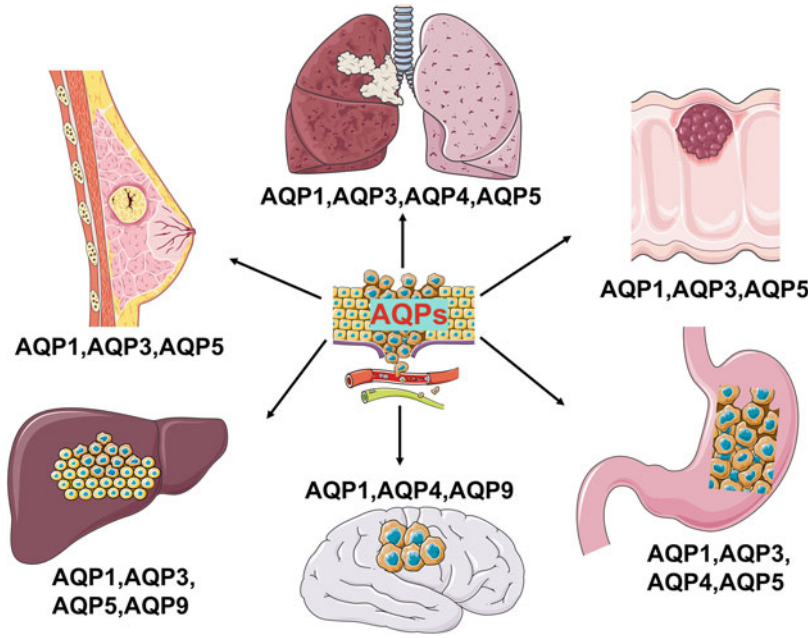

Fig. 21.1 The expression of AQPs most studied in specific tumor tissues[6]

important role in water homeostasis [30]. Recently, it is reported that AQP2 may not only serve as a potential indicator for the diagnosis of adrenal tumors, such as pheochromocytoma and/or paraganglioma [31], but also is capable of inhibiting cell invasion in glioma cell lines [32] and endometrial carcinoma [33]. Recently, it is found that Micropeptide Inhibiting Actin Cytoskeleton (MIAC) bounds directly with AQP2 to prohibit the actin cytoskeleton through regulating Septin 2/Integrin Beta 4I, and finally inhibiting the metastasis and tumor growth of head and neck squamous cell carcinoma (HNSCC) [34].

21.2.1.3 AQP4

AQP4 is one of the most common brain water channel protein and is mainly regulated by astrocytes [35], which participate in forming specialized microdomains at the interfaces between cerebrospinal fluid (CSF) (CSF–brain barrier) and blood (blood–brain barrier) [36]. Several researchers reported that compared to healthy brain, AQP4 is overexpressed in brain tumors. Upregulation of AQP4 is correlated with AQP4-OAP mislocalization in human glioma [37]. Some authors disclaimed that upregulation of AQP4 induced the invasion and migration of glioma cells [38, 39]. Decreased expression of AQP4 could lead to apoptosis in glioblastoma cell [40], indicating that regulation of AQP4 may be the key treatment target for glioblastoma. However, there are also some disagreements for the function of AQP4 in brain tumors. Ding et al. reported that AQP4 knockdown could lead to impaired migration and invasion in glioma cells [38].

In migrating cells, AQP4 polarizes to the lamellipodia and results in the augment of size or number of lamellipodia, where there is fast transmembrane water movement [12, 41]. It is reported that knockout of AQP4 in mouse and human cells may be related to actin depolymerization and morphological dramatic changes. In astrocyte from AQP4 deficiency animal, the rearrangement of F-actin cytoskeleton in the cerebral cortex is thoroughly taken place of fibers with a star-like organization [42]. Ding et al. [38] reported that the decreased expression of MMP-2 in LNLN229 cells by the reduction of AQP4 coincided with fewer cell invasive ability and increased of glioblastoma cell to cell adhesion ability through β-catenin and connexin 43, indicating that AQP4 is participate in the

regulation of glioblastoma cell migration and invasion and may be an effective therapeutic target for it.

Hui et al. [43] found a linear positive correlation between the cell migrating speed and the expression of AQP4 through studying more than 20 various types of tumor cells. AQP4 knockdown in invasive repopulated tumor stem cells decreased their migration ability. Beside the brain tumor, AQP4 also displayed essential role in other tumor. For instance, long noncoding RNA LINC00629 competitively bound to miR-196b-5p to increase the expression of AQP4 and retarding gastric tumor invasion [44]. AQP4 downregulation prohibited breast tumor cell migration, proliferation, and invasion via extracellular regulated protein kinases (ERK)/Ecadherin pathway [45].

21.2.1.4 AQP5

AQP5 encodes a protein of 265 amino acids containing five connecting loops and six transmembrane domain [46]. The AQP5 expression has been reported in the renal, digestive, integumentary, respiratory, reproductive systems, and sense organs [47]. It is demonstrated that AQP5 was distributed on the various cell membranes like acinar cells, pulmonary epithelial cells, and corneal epithelium cells in the lacrimal gland [48]. Some researchers reported that promoter region of AQP5 gene including activator protein-1 (AP-1) binding sequences and NF-κB responsive elements [49], estrogen response elements [50], which suggests that the above elements could directly control the expression of AQP5. Recently, some researches demonstrated that upregulation of AQP5 has been correlated with different tumors/cancers, such as cervical, colorectal, breast, liver, epithelial ovarian tumor, and lung cancer, and suggest to be an important therapeutic biomarker [6, 51, 52].

Some researchers demonstrated that the expression of AQP5 and AQP3 was obviously higher in triple-negative breast tumor (TNBC) than normal tissue, and overexpression of AQP5 was correlated with high expression of Ki67, the key marker proliferation, in TNBC samples [53], and associates with worse outcomes regardless of tumor stage and type, suggesting AQP5 as an independent prognostic marker of survival [54]. In response to oxidative stress, AQP5 promotes transmembrane diffusion of H_2O_2 and regulates cell growth of AQP5-transformed yeast cells, affecting tumor cell migration [55]. Moreover, the cyclic adenosine monophosphate (cAMP)-dependent phosphorylation of AQP5 on Ser156 by protein kinase A (PKA) modulates the RAS/MAPK signaling pathway which participates in cell survival and proliferation in different tumors [56–58].

He et al. [59] indicated that highly expressed AQP5 was found in hepatocellular carcinoma (HCC) cell lines and its inhibition suppressed tumor metastasis and HCC cell invasion in vitro and in vivo. What is more, decreased expression of AQP5 inhibited the epithelial–mesenchymal transition (EMT) process in HCC cells by regulating EMT-related proteins, such as α-catenin, N-cadherin, E-cadherin and vimentin, and suppressed HCC metastasis and EMT via inhibiting the NF-κB signaling pathway. In line with this research, inhibition of AQP5 retarded the migration and proliferation of different tumor cells. During tumorigenesis, interfering of AQP5 could significantly reduce the tumor growth in mice [51] and remarkably inhibited the ERK1/2 pathway and the phosphorylation level of epidermal growth factor receptor in NSCLC [60] and also increase the sensitivity of 5-fluorouracil to colorectal tumor cells through inhibiting the Wnt-β-catenin pathway [61]. Researchers also found that AQP5 regulating miRNAs (miR-19b-3p, miR-19a-3p, miR-1226-3p, and mir-185-3p) reduced breast tumor and colorectal tumor cell invasion and migration through decreasing the AQP5, suggesting that it can be a possible therapeutic target of tumor [62, 63].

21.2.1.5 AQP6

AQP6 was originally exclusively found in the intracellular vesicle membranes of acid-secreting intercalated cells in the collecting duct of kidney [64]. Recently, studies showed that AQP6 was also distributed on gastrointestinal epithelium, cerebellum, support cells of inner ear, and the follicular cells of parotid gland [65–67]. Some

researchers also demonstrated that APQ6 was detected in ovarian tumors, and the expression of AQP6 was remarkably reduced in serous ovarian tumors when compared to benign tumors tissues and normal healthy ovarian [68]. Zhu et al. [69] reported that the AQP6 was positively associated with relapse-free survival in breast tumor patients but negatively associated with post progression survival in grade II breast tumor and lymph node-positive tumor. Moreover, the gene expression of AQP0, AQP1, AQP4, AQP5, AQP6, AQP8, and AQP10 was correlated with worse overall survival in the prognosis of gastric tumor [70].

21.2.1.6 AQP8

AQP8 is demonstrated to work as oncoprotein in human cervical tumor and esophageal tumor, but as a potential inhibitor in colorectal tumor. Some studies showed that AQP8 inhibits apoptosis, increases tumor cell viability, and promotes metastasis in SiHa cells, suggesting AQP8 may regulate EMT-related markers [71]. In accordance with this study, others also reported that epidermal growth factor (EGF) leads to upregulation of AQP8 which involved in cellular migration in human cervical tumor cells through the EGFR/Erk1/2 pathway [72, 73]. The knockdown of mtAQP8 in HuH-7 and HepG2 cells contributes to necrotic but not apoptotic death, suggesting AQP8 might be a good strategy against liver tumor [74]. However, a recent study indicated AQP8 was inhibited in colorectal tumors and upregulation of AQP8 was positively correlated with better survival in colorectal cancers. Upregulation of AQP8 retarded the invasion, proliferation, migration abilities of colorectal cancer cells in vitro. Overexpression of AQP8 also suppressed CRC cell growth and metastasis in vivo [75] via inhibiting protocadherin7 (PCDH7) and PI3K/AKT signaling [76] and modulating EGFR-Erk1/2 pathway [77].

21.2.2 Aquaglyceroporins

21.2.2.1 AQP3

AQP3 facilitates glycerol transport in addition to water. AQP3 is not only distributed in the human urinary tract transitional epithelium, renal collecting ducts, and respiratory epithelium but also expressed in stratified squamous epithelial cells of the esophagus, oral cavity, and skin [78]. AQP3 is significantly dysregulated in different kinds of tumors, including breast, gastric, prostate, pancreas, lung, bladder, skin, cervical, squamous cell carcinoma, colon, adenoid cystic carcinoma, ovarian, colorectal, and liver tumors [52].

Researchers found that expression of AQP3 is upregulated in skin cancer, promotes glycerol transport into the cell, and leads to the generation of ATP and cell proliferation. AQP3 knockout inhibited skin tumor formation in mice via reducing ATP and glycerol content of cells for biosynthesis [79, 80]. Glycerol is a humectant to retard the evaporation of water, and maintaining the barrier action of the skin [81]. Glycerol may lead to cancer cell proliferation and tumor growth via building block in phospholipid synthesis and/or regulator of ATP production [82]. It is known that these pathways are pivotal to fast proliferating tumor cells [1]. What is more, it was reported that AQP3 knockdown downregulated some lipid synthases via PI3K/Akt pathway in gastric tumor cells, which was related to the ATP production and impaired lipid metabolism. It is reported that it is not only the results of reduced glycerol uptake but also the consequence of impaired lipid synthesis [83]. On the one hand, AQP3 overexpression provides the carcinoma with good glycerol permeability, then producing more ATP content [84]. On the other hand, glycerol converted into triglyceride (TAG), which is crucial for cell survival and proliferation. Tumor cells metabolized triglyceride into free fatty acid through lipolytic processes. Fatty acid oxidation (FAO) takes part in the generation of ATP to support the tumor development [1, 83]. Therefore, lipid synthesis suppression with inhibition of AQP3 may lead to energy supply defects and retards tumor development.

There are also a lot of studies related to the contribution of AQP3 in tumor. For example, human epidermal growth factor (hEGF) induces the expression of AQP3, which regulates the migration capacity of human CRC cells in a

time- and dose-dependent manner. The reinforced migration capacity of HCT116 cells was retarded by CuSO, the AQP3 inhibitor. hEGF-mediated AQP3 overexpression was retarded by a novel PI3K/AKT inhibitor, LY294002, suggesting that AQP3 can promote CRC cell migration and regarded as a therapeutic key indicator and target for colorectal carcinoma prognosis and metastasis [85]. It is also reported that AQP3 increased the expression and secretion of matrix metalloproteinase-3 (MMP-3) in prostate tumor cells through the positive regulation of the ERK signaling pathway. Silencing of AQP3 inhibited the motility and invasion of these tumor cells. Inhibition of the ERK pathway blocked AQP3-induced invasion and motility in prostate tumor cells [86]. AQP3 overexpression increased lymphovascular invasion, lymph node metastasis, and Lauren classification. Blocking Wnt/β-catenin pathway with XAV939 inhibited the overexpression of AQP3, suggesting that AQP3 contributes to stem-like properties of gastric carcinoma cells through mediating Wnt/GSK-3β/β-catenin pathway [87]. Simultaneously, AQP3-mediated H_2O_2 was necessary to activate the phosphorylation of Akt and regulates subsequent directional cell migration of chemokine (C-X-C motif) ligand 2 (CXCL2)-dependent breast tumor cells in vitro [88], implicating that AQP3 is an important driving factor of tumor motility. Moreover, some researches have also demonstrated that upregulation of AQP3 can induce tumor growth, cell proliferation, cell invasion in solid tumors, such as esophageal squamous cell carcinomas (SCCs), hepatocellular carcinoma (HCC), gastric adenocarcinoma (GC) [86, 89–92], and pancreatic tumor cells through stimulating the mTOR pathway [93], suggesting that downregulation of AQP3 may be a key therapeutic target for some oncotherapy. However, there are some discrepancies that AQP3 is low expressed in urothelial carcinomas (UCs) and breast tumor, with the potential mechanism being unclear [69, 94, 95].

21.2.2.2 AQP7

As a member of aquaglyceroporin family, AQP7 also conveys glycerol, ammonia, arsenite, urea, and hydrogen peroxide in adipocytes [96–100] and plays a vital role in glucolipid metabolism [101, 102]. Accumulating evidence revealed that AQP7 may contribute to tumor pathogenesis and development [103–105]. For instance, researchers found that the expression of AQP7 was significantly higher in borderline and malignant tumors than in normal healthy ovarian tissue and benign tumor, suggesting that high level expression of aquaglyceroporin could be crucial for ovarian cancer [105]. Since AQP7 acts as a channel for glycerol, a feasible interpretation is that AQP7 is a main factor for glycerol availability. Knockout of AQP7 in adipocytes results in the accumulation of triglycerides and glycerol [106]. During high energy demands, the exported glycerol is absorbed by other cells and used for energy supply, then promotes tumor development [107]. Moreover, downregulation of AQP7 inhibited lung metastasis and tumor burden in mice bearing breast tumor. Complex lipid profile and metabolomics in tumors and cancer cells with decreased expression of *Aqp7* remarkably altered the glutathione, lipid, and arginine/urea metabolism compared to control mice. To respond to stress and tumor nutrition, AQP7 regulates tumor metabolism through p38 MAPK and PI3K/AKT/mTOR pathways. These studies suggest AQP7 as an essential metabolic regulating factor in breast carcinomas, highlighting it as a pivotal therapeutic treatment target for tumor [107]. Recently, it was reported that inhibitor of AQP7 can induce cellular stress to eliminate the cancer cells and to reduce tumor bearing in combination with mTOR inhibitors [108].

21.2.2.3 AQP9

It is known that AQP9 is a channel protein that transports water, glycerol, and urea and promotes glycerol uptake of hepatocyte. The protein expression of AQP9 in murine and human has been mainly found in epididymis, liver, skeletal muscle, urothelium, skin, epidermis, adipose tissues, and neuronal cells [100, 109, 110]. Previous researches mainly investigate the expression of AQP9 in hepatocellular carcinomas, because AQP9 is widely distributed in the hepatocytes basolateral membrane. AQP9 was remarkably

downregulated in the tissues and cells of hepatocellular carcinoma and correlated with 5-year survival rate, tumor size, tumor lymph node metastasis stage, lymphatic and distal metastasis of the patients, and stimulation of AQP9 retarded the invasion, migration and proliferation of HCC cells via Wnt/β-catenin signaling pathway [111], which is crucial for differentiation, proliferation, and apoptosis. A research reported that the expression of AQP9 is deregulated in liver cancer cells. AQP9 upregulation inhibited liver cancer invasion via prohibiting epithelial-to-mesenchymal transition [112] and PI3K/Akt/forkhead box O1 (FOXO1) pathway [113]. Zheng et al. [114] found that AQP9 overexpression led to the accumulation of ROS, which suppressed β-catenin through inhibiting the interaction of β-catenin with TCF4 while concurrently enhancing the interaction of β-catenin with FOXO3a, at the end, attenuating LCSCs stemness, implying that the regulation of AQP9 signaling may be a promising therapeutic target and approach for retarding liver tumor stem cells (LCSCs).

Recently, a study demonstrated that overexpression of AQP9 was remarkably associated with bad prognosis in some types of tumor tissues, such as colon, breast, and lung, but associated with better prognosis in gastric tumor. Furthermore, AQP9 is correlated with different immune infiltrating cells, such as macrophages, $CD4^+$ and $CD8^+$ T cells, dendritic cells (DCs) and neutrophils, and some other immune-related genes in breast invasive carcinoma, colon adenocarcinoma, lung adenocarcinoma, stomach adenocarcinoma, and lung squamous cell carcinoma, implying that AQP9 can be an essential biomarker to determine the levels of immune infiltrating and the prognosis in some tumors [115]. Moreover, overexpression of AQP9 was remarkably associated with poor survival, immune infiltrations and aggressive progression through inflammatory response, IL6/JAK–STAT3, and TNF-alpha signaling pathways in ccRCC patients [116]. The level of AQP9 could be useful for estimating the prognosis with kidney renal clear cell carcinoma (KIRC) patients, especially to the TME state transition via JAK/STAT, P53, and lipid metabolism-related pathways that regulate M2 polarization [117], suggesting that AQP9 may be a potential prognostic target for kidney carcinoma.

The upregulation of AQP9 inhibited the glioma-related lactic acidosis through clearance of lactate and glycerol from the extracellular space through energy metabolism of the glioma and/or surrounding neuronal cells [118]. Some researchers give the evidence that AQP9 can be a promising predictive indicator for adjuvant chemotherapy in colorectal cancer. The higher expression of AQP9 had the better rate of disease-free survival (DFS) when compared to those patients with low expression of it. Moreover, upregulation of AQP9 increased the antitumor sensitivity of 5-fluorouracil (5-FU) both in vivo and in vitro. Induction of AQP9 expression increased intracellular level of 5-FU in colorectal cancer cells, contributing to more apoptosis rates after 5-FU treatment via cell cycle arrest through RAS activation [119].

21.2.3 Super Aquaporins

Superaquaporins, including AQP11 and AQP12, are a novel subfamily of AQPs and mainly distributed in the cytoplasm to regulate the water transport or intra-vesicular physiological states [8, 120, 121]. AQP11 is mainly distributed in testis and, to a lesser extent, in the adipose tissue, liver, brain, and kidney [122, 123]. AQP12 is located on the intracellular organelle membrane and found in pancreatic acinar cells [124]. The role of superaquaporins in tumor is relatively less reported than other aquaporins, and there is no study about the relationship between the AQP12 and tumor. Recently, some researchers found that higher expression of AQP11 had better OS in ovarian tumor [125] and gastric tumor patients [70]. Furthermore, survival analyses demonstrated that overexpression of AQP11 was significantly correlated with better relapse-free survival in breast cancers [69], indicating that AQP11 might play an important role to estimate prognosis and be a promising therapeutic strategy for tumor treatment.

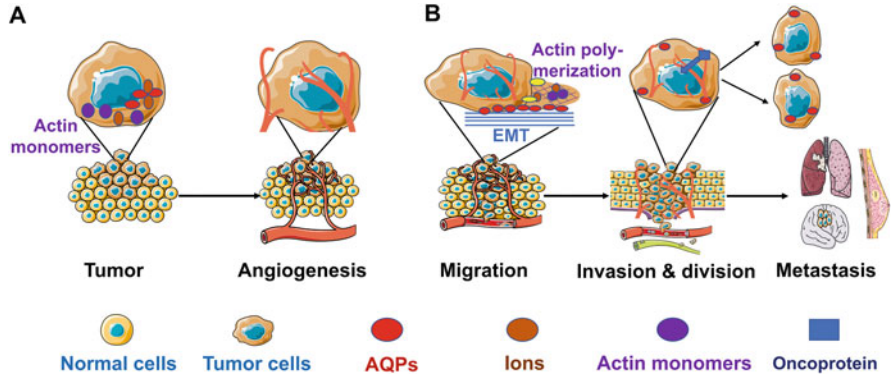

Fig. 21.2 Roles of AQPs in tumor. (**a**) AQPs in primary tumor cells may induce angiogenesis for vascular exchange of some substance, like nutrients. (**b**) AQPs promote lamellipodium formation and stabilization by actin polymerization. Then, cancer cells lead to loss of cell–cell adhesions that capacitate cancer cells to migrate and dissociation, then invade the neighboring ECM, and spill over into blood circulation, and finally extravasate to corresponding organs or sites like brain, breast, and lung, resulting in metastasis. Simultaneously, AQPs could bound to some oncoproteins, and stimulate the related intracellular signaling pathways that induce tumor cell invasion, division, and proliferation

21.3 Conclusion and Prospect

Aquaporin has been extensively reported in tumor tissues and cancer cells. Consistent investigations revealed that almost all AQPs are upregulated, but some of them, such as AQP8, AQP9, and AQP11, downregulated in tumors, suggesting that dysregulation of AQPs closely related to cancer incidence and plays a different role in different organs and tissues. Although the mechanisms by which AQPs interfere with tumorigenesis are not completely clear, an amount of studies indicated that AQPs are involved in angiogenesis, cell migration, adhesion, invasion, and division in tumors (Fig. 21.2), which are essential to tumor development. Therefore, it is necessary to underlying the accurate correlation between AQPs and tumor, which can provide new treatment strategies for cancer.

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Aquaporin Inhibitors

22

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Abstract

Aquaporins (AQP) working as membrane channels facilitated water transport, play vital roles in various physiological progress including cell migration, energy metabolism, inflammation, etc. They are quite important drug targets, but elusive for discovery due to their undruggable properties. In this chapter, we summarized most fluently used methods for screening AQP inhibitors, including cell swelling assay, cell shrinking assay, and stopped-flow assay. And three classes of AQP inhibitors have been discussed, including metal-related inhibitors, quaternary ammonium salts, and small molecule inhibitors which further divided into four parts, sulfanilamide analogies, TGN-020, antiepileptic drugs, and others. It has been suggested that although they showed inhibition effects on AQP1, AQP3, AQP4, AQP7, or AQP9 in some researches, none of them could be asserted as AQP inhibitors to some extent. Discovering AQP inhibitors is a big challenge, but if successful, it will be a great contribution for human health.

Keywords

Aquaporins · Inhibitors · Water permeability · Chemical molecules · Screening

22.1 Introduction

As reviewed in elsewhere in this book, in mammalian, there are 13 subfamilies of aquaporins (AQP), vary from AQP0 to AQP12. From them, some channels (AQP3, 7, 9, 10) also facilitate the transport of glycerol and other small neutral solutes such as urea, carbon dioxide, and ammonia, namely aquaglyceroporins. All these aquaporins are assembled by four monomers with ~30-kDa molecular size, each monomer has a narrow aqueous pore (contained Asn-Pro-Ala (NPA) motif) flanking a narrowing (~2.8 Å in diameter for AQP1) allowing a single-file water transport driving by an osmotic gradient which participate in the regulation of physiological functions including cell migration, energy metabolism, inflammation, etc. The narrowest segment of the water channel is within the transmembrane region of the pore, which is 2.8 Å for AQP1, which is similar to the size of a single water molecule [1]. Although each channel is

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functionally independent, which means an AQP protein can be bound with four inhibitors, the narrow pore and the small molecular size bring a great challenge for identifying drugs targeting AQPs. AQPs play a crucial role in the development of diseases as previous chapters described. But up to now, most molecules under investigation target AQP1, AQP3, AQP4, AQP7, and AQP9.

In kidney, AQP1 is expressed in the epithelium of the renal proximal tubule, the thin descending limb of the loop of Henle, and the descending vasa recta. In an AQP1 knockout mouse model, severe urine concentration deficiency is observed, with increased urine output and reduced urine osmolality. AQP1 deficiency impairs the renal counter-current multiplication by reducing the permeability in the descending limb of the loop of Henle and the descending vasa recta. Besides, AQP1 has been found that overexpressed in breast cancer [2]. In an AQP1-knockout mouse model, impaired tumor growth was observed, including a reduced tumor vascularity and extensive tumor necrosis, and an enhanced survival of tumor-bearing mice was presented [3]. These studies suggest AQP1 inhibitors may have clinical indications as diuretics for the treatment of the glaucoma, cerebral edema, elevated intraocular pressure which are directly or indirectly related to abnormal fluid homeostasis and as antitumor agents [4].

AQP3 is a water- and glycerol-transporting membrane protein, expressed in the collecting duct of the kidney [5], airway epithelia, secretory glands, and skin [6, 7], that is involved in cell proliferation and migration. Previous studies show that inhibitors of AQP3 glycerol or H_2O_2 transport are thought to prevent or retard skin tumor growth [8] or chronic inflammatory skin diseases including psoriasis [9]. In addition, AQP3 has a significant role in the parasite replication by inducing in human hepatocytes in response to parasite infection [10]. And glycerol mediated by AQP3 contributes to the replication of the parasite during the asexual intraerythrocytic stages [11]. So the inhibitors of AQP3 might be regarded as antiparasitic drugs.

AQP4 is expressed in perivascular end feet of astroglia in the central nervous system, which is proposed to serve physiologically as a route for the net movement of water out of the brain but in pathological conditions create vulnerability to cerebral edema, for example, after acute brain injury or stroke [12, 13]. AQP4 inhibitors are predicted to reduce brain swelling in ischemic stroke. And the glymphatic system, that is aquaporin 4 (AQP4) facilitated exchange of CSF with interstitial fluid (ISF), may provide a clearance pathway for protein species such as amyloid-β and tau, which accumulate in the brain in Alzheimer's disease [14]. AQP4 plays an important role in the glymphatic clearance of tau from the brain, suggesting AQP4 might be a target for the treatment of Alzheimer's disease.

AQP7 expressed in adipose tissue and AQP9 expressed in liver tissue are members of aquaglyceroporins, which are involved in adipose metabolism and insulin resistance in liver [15, 16]. AQP7 deficiency leads to the decreasing of the permeability of the plasma membrane to glycerol, which causes cellular accumulation of glycerol and triglyceride as well as upregulation of glycerol kinase expression [17]. It might increase the accumulation of adipose and lead to obesity. In addition, AQP7 is now considered as a β-cell protein and critical regulator of intraislet glycerol content as well as insulin production and secretion [18]. There is evidence that AQP9 is related with the uptake of hepatocyte glycerol that AQP7 and AQP9 might be target for obesity or diabetes [17].

22.2 Methods for Screening AQP Inhibitors

In this chapter, we are about to list main methods used for screening the AQP inhibitors. All these methods based on the measurement of the kinetics of the cell volume in response to an osmotic gradient presented as fluorescent quenching or scattering time-course or directly measurement using cell imaging.

22.2.1 Cell Swelling Assay

Cell swelling assay is the earliest functional assay for water permeability. Tetraethyl ammonium (TEA) [19], acetazolamide [20] (AZA), and some small molecules [21, 22] were identified by this method, although it is proved to be fraught with artifact because there are many determinants of oocyte swelling later researches [17, 23, 24]. *Xenopus laevis* oocytes expressing AQPs were used as tool cells. Oocytes are so big that could be easily measured by image analysis (Fig. 22.1). Water permeability and aquaporin function in *X. laevis* oocytes should only be calculated from initial osmotically induced volume changes [25].

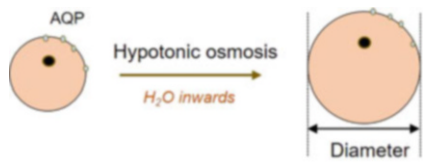


Fig. 22.1 Cell swelling assay. AQP-overexpressed oocytes are used for imaging analysis which records the diameter representing relative increases of the cell volume induced by hypotonic osmosis by video microscopy

22.2.2 Cell Shrinking Assay

Cell shrinking assay is also regarded as fluorescence quenching assay. It is on the basis of the self-quenching of certain fluorophores such as calcein at high concentrations. It correlates cell volume with fluorescence intensity where cell shrinking should have increased the fluorescence concentration and thereby self-quenching occurs, decreased fluorescence intensity could be observed by Fluostar Optima plate reader [26]. Calcein AM is the most useful fluorescent material, which is a membrane permeable substance, releasing calcein intracellular as fluorescent material to indicate the viable cells.

The main steps could be divided into three parts (Fig. 22.2). The first part is cell culture. Cells expressing AQPs are grown as monolayer in 96-well plate or other solid supports. Cell lines

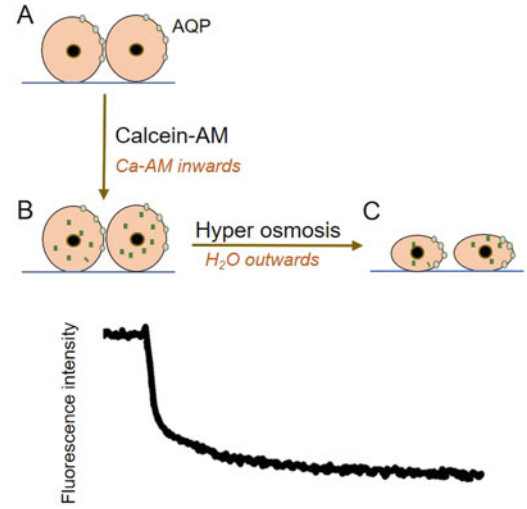


Fig. 22.2 Cell shrinking assay. (**a**) Monolayer cells are formed. (**b**) Fluorescent materials (Ca-AM) are loaded. Initial fluorescence intensity is recorded. (**c**) Cells shrink under hypertonic buffer and the intensity decrease

cultured could be CHO cells [27, 28], MDCK [29], and other adherent cell lines that are used for transfection such as RPE cells [26]. The second part is fluorescent loaded. A fresh medium containing probenecid (an anion transporter inhibitor, to reduce the leakage of dye indicators) and Calcein AM is added, and the cells are cultured for at least 30 min. At the same time, tested compounds could be added. The last part is self-quenching. High osmotic gradients are applied, a linear dependence of fluorescence intensity is recorded, the kinetic curve is presented as Fig. 22.2. A similar method, which does not require dye loading, uses the genetically encoded, cytoplasmically expressed yellow fluorescent protein YFP-H148Q-V163S, whose fluorescence is quenched by chloride [30].

22.2.3 Stopped-Flow Assay

Stopped-flow assay is carried out using an apparatus in which two solutions are mixed together rapidly (in <1 ms) and have an optical read-out [17]. Stopped-flow measurements can be made in plasma membrane vesicles from AQP-expressing cells, in reconstituted proteoliposomes or in small

cells such as erythrocytes. Their suspensions are prepared and then mixed with hyper osmosis medium rapidly, and cell volume reduces, thereby scattering intensity changes. Water permeability should be calculated from initial slope of scattering intensity changes induced by osmotic pressure. Besides it could assess the water outflow ability by mixed suspensions which is incubated with hyper osmosis medium previously with isotonic medium. And this technique is widely used for screening AQPs inhibitors [23, 24] and other channel inhibitors such as urea transporters [31], and it could quantitate the water permeability, but it requires specialized instruments.

22.3 AQPs Inhibitors

Due to the narrow Asn-Pro-Ala (NPA) motif in AQPs, the drug discovery of AQP inhibitors seems unexpectedly challenging. Here we discussed the proposed direct inhibitors of AQPs rather than molecules which affect AQP in indirect ways. Three classes of AQP inhibitors have been described: metal related inhibitors, quaternary ammonium salts, and small molecule inhibitors.

22.3.1 Metal-Related Inhibitors

Metal ions exist in body fluids, and these will form different bonds with biomolecules, involving in vital cellular processes. Hemoglobin is an indispensable protein in mammals and plays a role in transporting oxygen. It consists of four chains, two α-chains and two β-chains, of which contained a cyclic heme with a ferrous atom, that would bond with oxygen. In addition, exogenous metal-containing small molecules can be effect on proteins or biomolecules targeting their metal moiety [32]. In clinic, cisplatin is widely used for the treatment of solid cancer [33], by damaging DNA of vigorously proliferating cells by forming intrastrand diadduct [34, 35] with bases in DNA chain, especially, purines.

Some metal-related compounds also showed AQP inhibition effect with lowering water permeability. The first reported metal-related inhibitor was pCMBS (*p*-chloromercuribenzene sulfonate), which has been found that has inhibition of water permeation into erythrocytes. And $HgCl_2$, which has a covalent interaction with Cys189 of AQP1 in the vicinity of the conserved NPA motif in loop E, restrain the water permeability sterically [17], too. Cys189 residue is essential for the inhibition by mercury and in a mutation in the Cys189 residue of AQP1 prevents the inhibition by mercury [36]. Ag is one of the transition elements, and the diameter of Ag^+ ion (2.5 Å) matches the predicted AQP channel diameter of 2.8 Å, whereas the Hg^{2+} ion is 2.2 Å which might also build interaction with sulfhydryl groups. Indeed, it has been reported that Ag as $AgNO_3$ or silver sulfadiazine (Fig. 22.3) inhibited the water permeability of human red cell (AQP1) with high potency (EC_{50} = 3.9 μM or 1.24 μM, respectively) [37]. However, these inhibitions are non-reversible, while mercury-based inhibition is reversible in the presence of mercaptoethanol, suggesting there is a different mechanism, but it is still not clear. Another common metal element, Au^{3+} with the diameter of 2.7 Å also has been reported that it has inhibitory effect on AQPs. AuPhen [32, 38] ([Au(III)(phen)Cl_2] Cl, (phen = 1,10-phenanthroline)) is a specific inhibitor against AQP3, which has inhibition effect on glycerol transport in human red blood cells (hRBC) with an IC_{50} value of 0.08 mM, while has no inhibitory effect on AQP1-mediated water permeability. In addition, Audien [39] also showed inhibition on glycerol permeability with an IC_{50} value of 16.62 ± 1.61 μM in hRBC. It is reported that the mechanism of Au is not the same as mercury, but does have effect on pore closure, due to protein conformational channel upon metal binding with Cys40 which was confirmed by computational modeling [39].

Although there are no direct evidences to support this assumption, it can be assumed that metal-related inhibitors might have strong toxicity due to its easily covalent bonding with Cys residues which are abundant in proteins in vitro. In addition, few researches have been done to assess these novel metal-related inhibitors as diuretics in animal models, not mention to

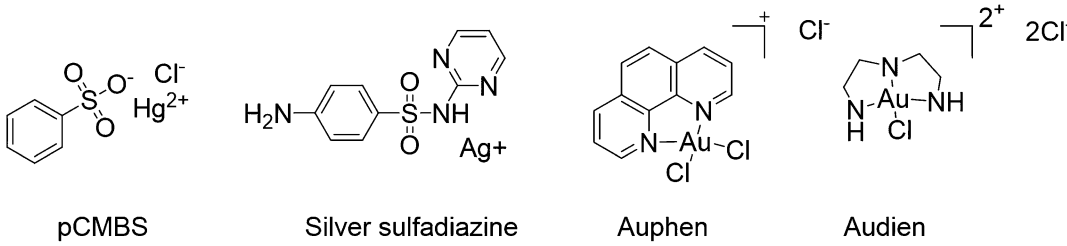


Fig. 22.3 Structures of metal-related compounds

human, except for $HgCl_2$ that was used historically only before the discovery of thiazides and loop diuretics. However, these might be applied to treat some uncurable diseases such as cancer if these could be proved effective for clinic.

22.3.2 Proposed Quaternary Ammonium Salts

Tetraethylammonium (TEA) chloride, which is known as a blocker of voltage-gated potassium channels, was reported to reduce the water permeability of human AQP1 channels expressed in Xenopus oocytes reversibly [22]. TEA also inhibits water permeation through AQP2 and AQP4, whereas the water permeabilities of oocytes expressing AQP3 or AQP5 were not affected [19]. However, this effect could not be reproduced at a concentration of up to 10 mM by stopped-flow light scattering in erythrocytes, which natively express AQP1, and in epithelial cells that were stably transfected with AQP [23]. The different results occurred are due to the lower-sensitivity techniques used in earlier studies, which might have been susceptible to the secondary effects related to the distribution of ions across the plasma membrane [17].

22.3.3 Small Molecules

Although AQPs show as elusive drug targets, many efforts have been made.

22.3.3.1 Sulfanilamide Analogies

Sulfanilamide analogies (Fig. 22.4) which were reported as AQP inhibitors could be divided into two types, namely arylsulfonamide which derived from CA (carbonic anhydrase, CA) inhibitors and sulfamoyl benzoic analogies which developed from bumetanide as loop diuretics.

AZA is used as a carbonic anhydrase inhibitor. Previous studies showed that there was a big similarity between AQP1 and some carbonic anhydrase isoenzymes in the tissue distribution and even the subcellular localization, suggesting that the potential relationship between the two proteins in structures or functions [40]. In an AQP1-cRNA inject oocyte model [40] and HEK293 cells transfected with pEGFP/AQP1 model, AZA inhibited the osmotic water permeability, and surface plasmon resonance (SPR) study proved this inhibition might function through direct binding between AZA and AQP1 [20]. However, other conflicting data from the same group showed acetazolamide-inhibited AQP1 protein expression [41]. What is more, in erythrocytes or AQP1-expressing epithelial cells, no inhibition of AQP1 water transport at concentrations of AZA up to 2 mM was observed [23]. AZA might be an AQP1 downregulator.

Later, due to the sequence homology between AQP1 and AQP4, AZA was tested using Xenopus oocytes expressing AQP4 (hAQP4b), and it was found to have an IC_{50} against AQP4 of 0.9 μM with a maximum inhibition of 85% [42]. Besides, they explored additional pan-CA inhibitors, namely *N*-(4-sulfamoylphenyl) acetamide and ethoxyzolamide (EZA), which showed potential inhibition against AQP4 in different extent in Xenopus oocyte model [42]. Also, the

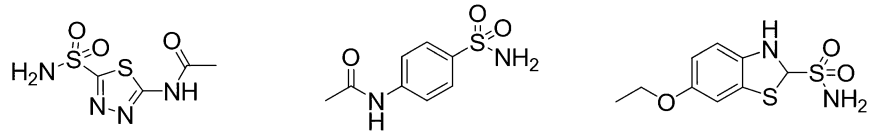


Fig. 22.4 Structures of sulfonamides

virtual docking studies showed that the sulfonamide interacts with the guanidyl group of Arg216 as well as with the carbonyl group of Gly209. It remains that the sulfonamide moiety might be essential for AQP4 "inhibition," but these might be artifacts that compounds might affect cell size or shape, cell volume regulation, nonaquaporin ion, or solute transporters [24]. It can not be concluded the inhibition only rely on the Xenopus oocyte expression system.

AQPs are membrane channels, which have similarities with other ion channels. With this opinion, various channels and transporter blockers have been screened for AQP inhibition. From them, some loop diuretics, mainly, bumetanide and furosemide [43] showed modest inhibition effect on AQP-mediated osmotic swelling in Xenopus oocytes. And though computational docking and structure–function relationship (SAR) study, sulfamoyl benzoic scaffold was supposed to be an important pharmacophore element, so based on the core structure, series of compounds were developed, AqB013 showed block effect on water permeability facilitated by AQP1 and AQP4 with IC50 values of approximately 20 μM and 50 μM, respectively [43].

Besides, bumetanide derivatives AqB007 and AqB011 (Fig. 22.5) were proved as selective blockers to inhibit AQP1 ion conductance with no effect on water channel activity, and AqB011 was the most potent blocker with an IC_{50} value of 14 μM by two-electrode voltage clamp and optical osmotic swelling assays [21]. Except for AqB007 and AqB011, AqB050 (the chemical structure was not found) was regarded as a selective inhibitor of AQP1 by effecting ion conductance (A. Yool et al. manuscript in preparation), and it only showed significant decrease in cell proliferation in AQP1-high cells, while no statistically difference in AQP1-low cells [44]. Although the data in vitro presented antimalignant mesothelioma potential, but in a xenograft mouse model, AqB050 had no biologically significant effect on growth of established tumor [44]. It needs to be clarified why "AQP1 inhibitor," which derived from loop diuretics, has the same bioactivity with that caused by AQP1 knockdown, but showed no antitumor effect in vivo.

And the inhibition on AQP1 needs to be confirmed using alternative functional assays that are less prone to artifacts [17]. In addition, Verkman's group has retested the inhibition against AQP1 of AqB013 by stopped-flow light scattering in human and rat erythrocytes that natively express AQP1, in hemoglobin-free membrane vesicles from rat and human erythrocytes, and in plasma membrane vesicles isolated from AQP1-transfected Chinese hamster ovary cell cultures, and it showed no significant inhibition on AQP1 water permeability [24], which is more convincible. And in a MCAO mouse model [45] and a spinal cord injury rat model [46], bumetanide-treated group had a significant attenuation of AQP4 protein expression, which reminds bumetanide might be an AQP1 downregulator, too.

22.3.3.2 TGN-020

Eighteen compounds were identified based on conserved physicochemical features of previously discovered compounds in silico, and more than half (10 compounds) of the compounds (structures are showed in Fig. 22.6) showed AQP4 inhibition in Xenopus oocytes transfected to express AQP4 model [47, 48]. From them, three compounds including TGN-020

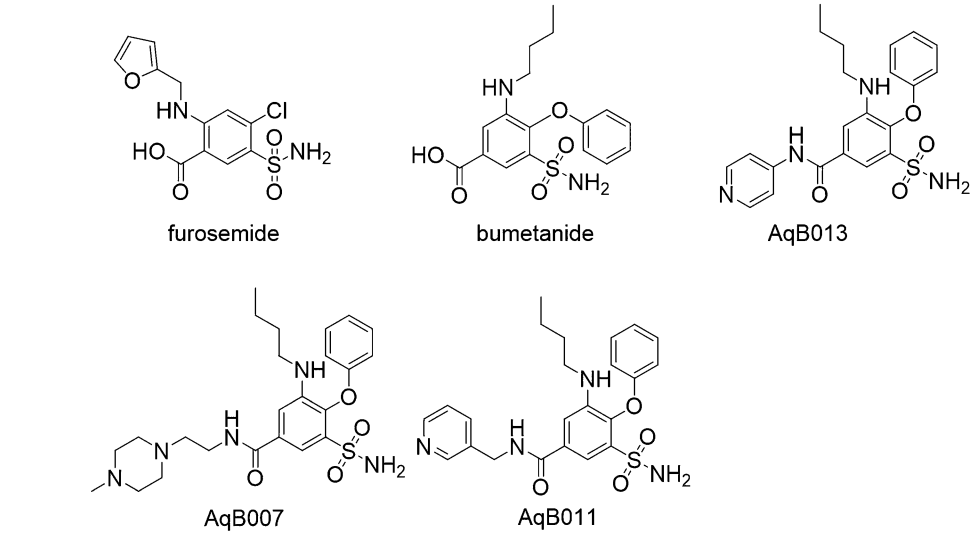


Fig. 22.5 Structures of bumetanide derivatives

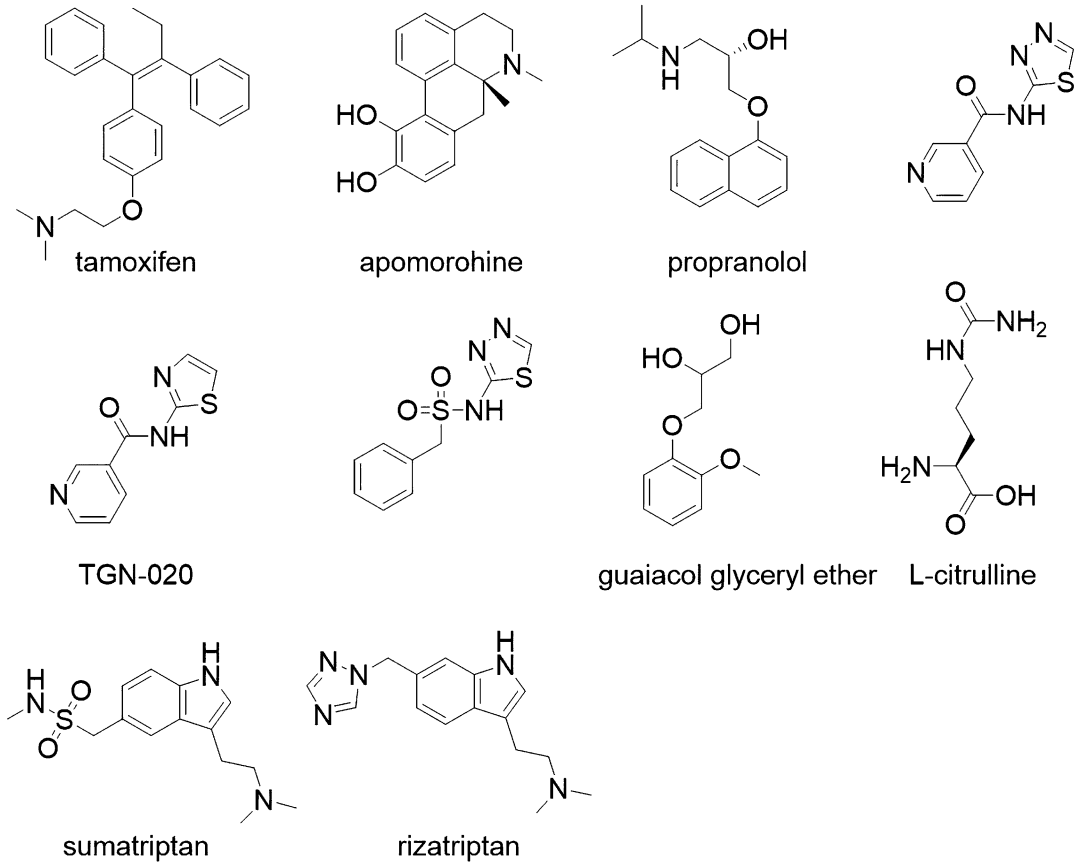


Fig. 22.6 Structures of 10 compounds which might have AQP4 inhibition

(2-(nicotinamoyl)-1,3,4-thiadiazole), sumatriptan (5-HT1B/1D agonist), and rizatriptan (5-HT1B/1D agonist) had strong AQP4 inhibition with IC50 values of 3, 11, and 2 μM, respectively [47]. And docking model showed TGN-020 directly blocked the pore of water transport.

The effects of TGN-020 on regional cerebral blood flow (rCBF) were examined in wild-type (WT) and AQP-4 knockout (KO) mice in vivo [49]. And TGN-020 increased regional cerebral blood flow but showed no effect on KO mice, suggesting that the TGN-020 worked on AQP4. In the diabetic retina model, TGN-020 suppressed the expression of AQP4 and GFAP [50]. And in another unilateral middle cerebral artery occlusion (MCAO) model, TGN-020 also showed downregulating effect on AQP4 in the SON [51]. So it is more exactly to define TGN-020 as an AQP4 modulator rather than an AQP4 inhibitor.

22.3.3.3 Antiepileptic Drugs

Cause of their pan-CA isozyme inhibitions and similarity in physiochemical properties with AZA and EZA, 14 antiepileptic drugs (AEDs), such as topiramate (TPM) and zonisamide (ZNS) were tested using virtual docking experiments in silico, and nine of them were investigated functionally in vitro in Xenopus oocyte expressing system [52]. Seven of the candidates were found to inhibit AQP4 function, then four compounds including topiramate (TPM), zonisamide (ZNS), phenytoin (PHT), and lamotrigine (LTG) (Fig. 22.7) were then selected for a dose-dependent study. The IC50 values were 10, 3.3, 9.8, and 8.1 μM, respectively. And the correlation studies suggested that AEDS with a docking energy >50 kcal/mol might have inhibitory effect on AQP4. However, due to the use of a nonstandard algorithm and no computational details (such as search space and energy minimization criteria), it is difficult to assess the merit of the reported binding computations.

Despite its association with elevated seizure threshold following chemical convulsants, it is predicted that AQP4 deficiency could reduce sound- and light-evoked potentials and increased threshold and prolonged duration of induced seizures [53]. In short, AQP4 inhibition would likely worsen rather than prevent seizures. With this doubt, Yang et al. [54] retested reported AEDs with AQP4 inhibition in FRT cell plasma membrane vesicles measured by stopped-flow light scattering, in AQP4-expressing FRT cell monolayers and in brain glial cells, none of these showed inhibitory effect on AQP4-mediated water permeability. None of them have AQP1 inhibitions, too.

22.3.3.4 Other Compounds

A total of 3575 compounds including 418 FDA-approved drugs were screened by calcein-loaded cells using an automated fluorescence microplate reader-based assay [55]. Four molecules of National Cancer Institute's chemical library (NSC164914, NSC670229, NSC168597, NSC301460, Fig. 22.8) were identified that affected both AQP4- and AQP1-mediated water permeability with IC50 values varying from 20 to 50 μM. Nevertheless, in another report [24], these 4 compounds showed no AQP1 or AQP4 inhibition by stopped-flow scattering analysis. Interestingly, these two literatures came to different conclusions although they adopted the same assessment method by stopped-flow light-scattering measurement in erythrocytes from adult Wistar rats. Artificial or objective factors might be affected, but some points could be confirmed that NSC 168597 and NSC 164914 as organolead and organotin molecule, respectively, were reported to be neurotoxins [56] and would cause erythrocyte lysis [57]. As to NSC 301460 which belongs to aminolipopeptide antibiotics [58] is isolated from a marine sponge-derived fungus *Trichoderma* sp., whose mechanism is to damage bacterial cell membranes by forming pores [24, 59]. Large volume of NSC 301460 chemical structure makes it impossible to block the water pore in AQPs directly. Another report [60] identified two more compounds from NSC derived from NSC 670229 as novel hAQP1 inhibitors by yeast freeze-thaw assay and stopped-flow water permeability assay. However, yeast freeze-thaw assay could not exclude high toxicity compounds affecting yeast viability and stopped-flow spectrometer. And in this chapter

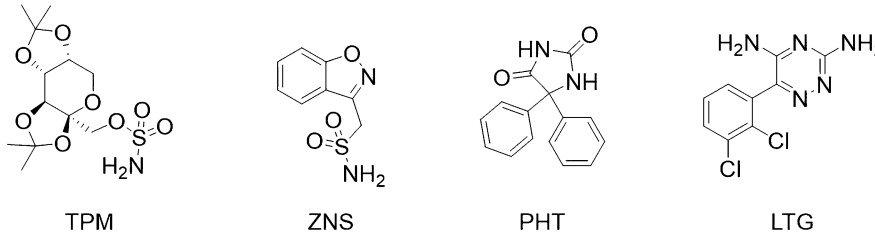


Fig. 22.7 Structures of four compounds of AEDs

somehow, interpretation of possible inhibitory effects was confounded by the multiexponential kinetics of the light-scattering data [24].

Novartis Co. also participated in drug discovery of AQP1 inhibitors on account for the function of AQP1 by mediating water permeability into the lens. Approximately 6000 drug-like molecules from AICON's collection were selected for screen using a high-throughput assay based on fluorescence quenching assay in CHO cells overexpressed AQP1 [61]. Two classes of compounds belonging to aromatic sulfonamide (ASQ) and dihydrobenzofuran (DHBH) (Fig. 22.9) showed IC50 values of hAQP1 in the range of 3–30 μM in primary screening assay. In addition, two lead compounds have AQP1

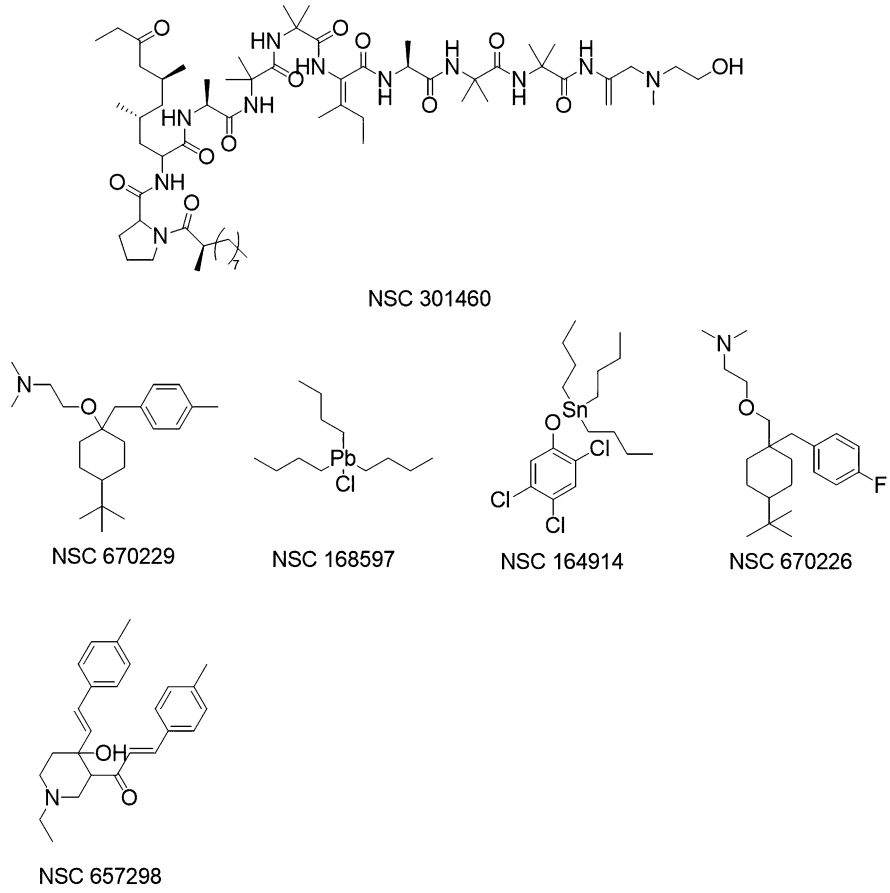


Fig. 22.8 Structures of NSC compounds

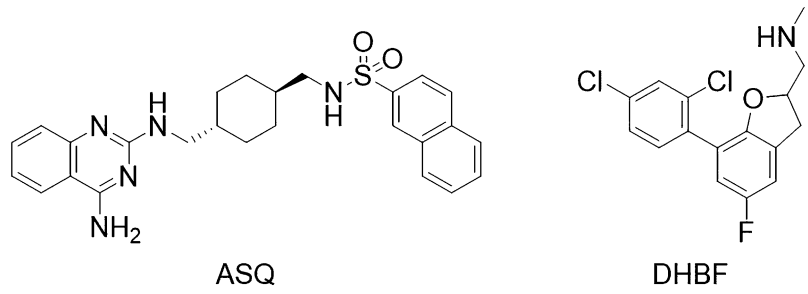


Fig. 22.9 Structures of ASQ and DHBH

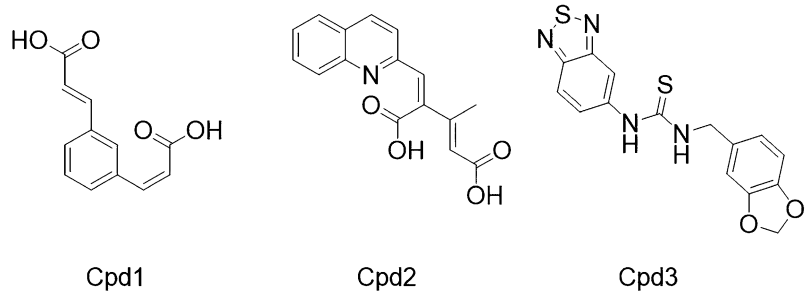
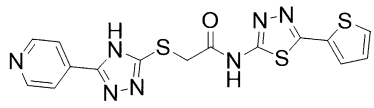


Fig. 22.10 Structures of Cpds identified by molecular docking

inhibition in Xenopus oocyte-swelling assay and stopped-flow assay [61]. Nevertheless, in another research [24], lead compounds showed variable activities in Xenopus oocyte, erythrocyte ghost, and AQP1 proteoliposome assays due to the erythrocyte crenation and aggregation which is the main reason to induce the potential artifacts in light-scattering assays.

Molecular docking is the efficient method for discovering compounds which has interactions with the target protein. Scientists have tried to apply this method into primary screening for AQP inhibitors followed by functional assays. AEDs were discovered as AQP4 inhibitors by the computational method [52], even though they showed less AQP inhibition in a follow-up research [23]. But we could not deny the computational method and efforts have been made to optimize the algorithm to improve the precision. The novel compounds (Fig. 22.10) were identified by molecular docking against the hAQP1 and experimentally tested the activity on AQP1 inhibition in a Xenopus oocyte swelling assay [62]. Subsequent molecular dynamics simulations suggested a new binding mode that strongly involves the ar/R selectivity filter and Lys36, a residue that is not conserved among the hAQP family. Although none of the molecules showed an inhibitory effect in a red blood cell assay, the inhibition of oocyte swelling of these compounds could be abolished by mutating Lys36 to alanine. It suggested that the observed reduction of water flux is hAQP1-dependent and not triggered by an indirect effect, but there is an obvious discrepancy between results obtained from Xenopus oocyte and erythrocyte.

Besides the continuous efforts made in discovering AQP1 inhibitor and AQP4 inhibitors, it is also worthy to mention that a compound, namely HTS13286 from a 1920 small molecules library stands out by its stronger selective inhibition of AQP9 with an IC_{50} value of 0.15 μM by a CHO mAQP9 cells shrinking assay [27]. And HTS13286 also affected mAQP9 solute permeability, including glycerol and urea, which has the same effect with *AQP9* gene deletion on glycerol gluconeogenesis in perfused mouse livers. In a word, a glycerol-specific increase in glucose output in wild-type livers was suppressed by HTS13286 and absent in $AQP9^{-/-}$ livers. However, due to its low solubility, it is not suitable for



HTS13286

Fig. 22.11 Structure of HTS13286

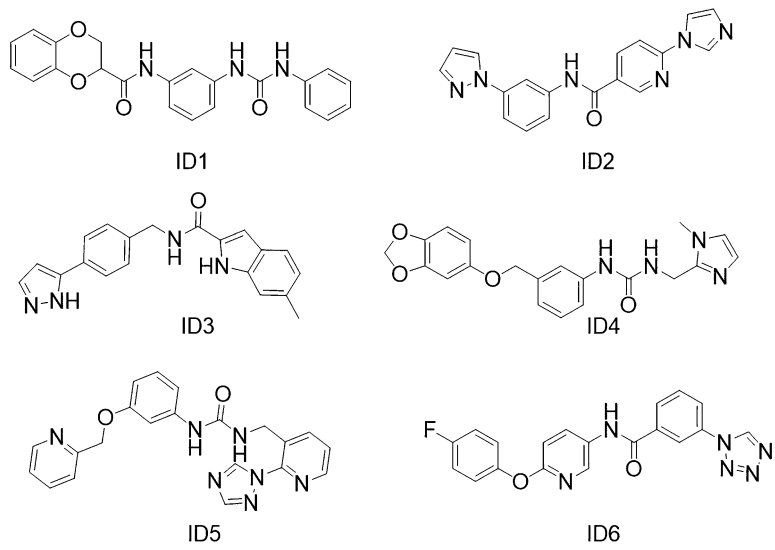
experiment in vivo, yet. For 10 years, it has been still no new developments for AQP9 inhibitors and no further progress for this series of HTS 13286 (Fig. 22.11), which remind us there is a long way and more exactly a tough way to go.

An optimization was applied in molecular docking for screening both channel-binding compounds and channel-blocking compounds [63]. Thirty active compounds with the 105 compounds that were top-ranked by virtual screen were identified by CHO-hAQP9 cell shrinking assay. Nine of the 30 compounds produced an IC_{50} values of less than 50 μM (the structures of best six compounds were presented in Fig. 22.12). It is worth noting that they found hAQP9 F180V mutant cells presented reduced water permeability. We hope drug discovery in AQP inhibitors could be benefitted from advanced technology in silico.

AQP3 as an aquaglyceroporin is known to conduct water, glycerol, as well as H_2O_2. Its inhibitors have potential for treating disorders of water retention. DFP00176 was defied as hit compound by CHO cells expressing mAQP3 shrinking assays from a library of 7360 drug-like small molecules [28]. Then SAR study was operated among 12 commercially available structurally compounds, DFP00173 that possesses a urea linker, 2,6-dichlorophenyl in the right-hand site, and Z433927330 that possesses a methylurea linker were selected for specificity test for AQPs (Fig. 22.13). It was found that DFP00173 has inhibition against mouse and human AQP3 with IC_{50} of 0.1–0.4 μM, but low efficacy toward mouse AQP7 and AQP9 while Z433927330, a partial AQP3 inhibitor (IC_{50}, 0.7–0.9 μM), also has potent and efficacious inhibition against mouse AQP7 water permeability (IC_{50}, 0.2 μM) [28]. These two compounds could be tools for investigating the functions of aquaglyceroporins, and we are looking forward to the further research in vivo.

22.4 Summary and Prospect

From the point of the view, it is still no effective AQP inhibitors except for metal-related compounds whose toxicity could not be denied. AQPs play important roles in various physiological activities, including proliferation and



ID1 ID2 ID3 ID4 ID5 ID6

Fig. 22.12 Structures of the best six compounds screened by CHO-hAQP9 cell assay

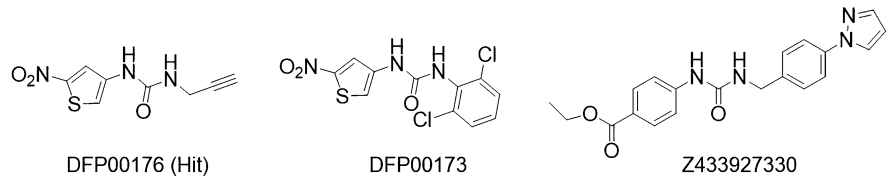


Fig. 22.13 Structures of DFP00173, DFP00176, and Z433927330

migration in tumor disease, brain edema, and other water-retention disorders, which makes them important drug targets. With the rapid development of artificial intelligence and computer-aided drug discovery, new approach might be applied, and we believe novel AQP inhibitors could be expected. In addition, further study on DFP00173 is worth waiting for.

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Non-Aquaporin Water Channels

23

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Abstract

Water transport through membrane is so intricate that there are still some debates. AQPs are entirely accepted to allow water transmembrane movement depending on osmotic gradient. Cotransporters and uniporters, however, are also concerned in water homeostasis. UT-B has a single-channel water permeability that is similar to AQP1. CFTR was initially thought as a water channel but now not believed to transport water directly. By cotransporters, such as KCC4, NKCC1, SGLT1, GAT1, EAAT1, and MCT1, water is transported by water osmosis coupling with substrates, which explains how water is transported across the isolated small intestine. This chapter provides information about water transport mediated by other membrane proteins except AQPs.

Keywords

Urea transporter B · CFTR · Cotransporter · Water transport

23.1 Introduction

Although the aquaporin (AQP) family has been identified to allow water transmembrane movement depending on osmotic gradient, there is water transport mediated by proteins exclusive of AQPs. Water is either transported through AQPs driven by osmotic gradient or cotransported with other substrates. Water is also uphill transported by water pump (Fig. 23.1). The most representative non-AQP water channel is urea transporter B. Moreover, some cotransporters not only transport specific solutes or organic molecules across the cell membrane but also act as water transporters. In this chapter, various modes of water transport and their physiological roles are reviewed.

23.2 Urea Transporter B

The urea transporter B (UT-B) is widely expressed in many tissues, such as kidney, brain, liver, colon, small intestine, pancreas,

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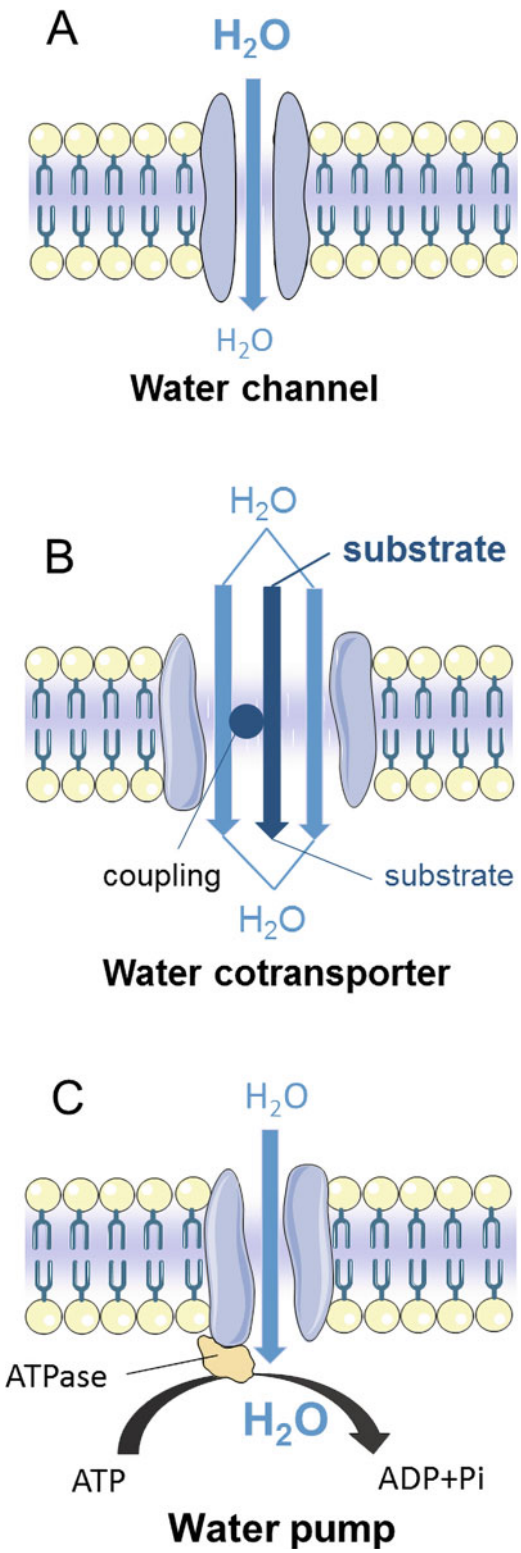

Fig. 23.1 Three ways of water transport. (**a**) Water is transported through a simple channel, driven by osmotic

testis, prostate, bone marrow, spleen, thymus, heart, skeletal muscle, lung, bladder, and cochlea [1]. UT-B transports urea and several chemical analogues of urea, such as methylurea, formamide, acetamide, acrylamide, methylformamide, and ammonium carbamate. Several studies suggested that UT-B functions as an efficient water channel [2–4].

In 1998, Yang et al. found that UT-B was permeable to water when they measured osmotic water permeability in *Xenopus* oocytes expressing UT-B (originally called UT3 or UT11) (Fig. 23.2a) [4]. Quantitative measurement of single-channel osmotic water permeability (P_f) of UT-B gave a value of 1.4 $cm^3 \cdot s^{-1}$. UT-B medicating water and urea transport were weakly temperature-dependent, and mostly inhibited by the urea transport inhibitor [2, 4, 5], but not inhibited by the AQP inhibitor $HgCl_2$ [2].

The most direct evidence for a common water/solute pathway is the low solute reflection coefficient. In the induced osmosis method, oocytes were briefly swollen in 100 mM Barth's buffer, and then the external solution was switched to 50 mM Barth's buffer containing different concentrations of urea [4]. As seen in Fig. 23.2a (*top*), oocytes expressing UT-B are initially swollen for external 200 and 400 mM urea and shrunk for 600 and 800 mM, suggesting σ_{urea} (the urea reflection coefficient) $<<$ 1. The measurements were simulated numerically using the Kedem–Katchalsky equations of coupled water and solute transport for different values of σ_{urea} (Fig. 23.2b). There was good agreement between the simulated and experimental data set for $\sigma_{urea} \sim 0.3$. An important control study was

Fig. 23.1 (continued) driving force, such as AQPs and UT-B. (**b**) Water is cotransported with another substrate through a cotransporter that is bimodal, a passive component transport and a secondary active component transport. Some cotransporters function as both water channel and water pump, such as NKCC1, KCC, and SGLT1. (**c**) Water is transported by a pump that actively transports water across membranes relying on ATP hydrolysis. (A darker and larger font indicates less osmolality or more substrates)

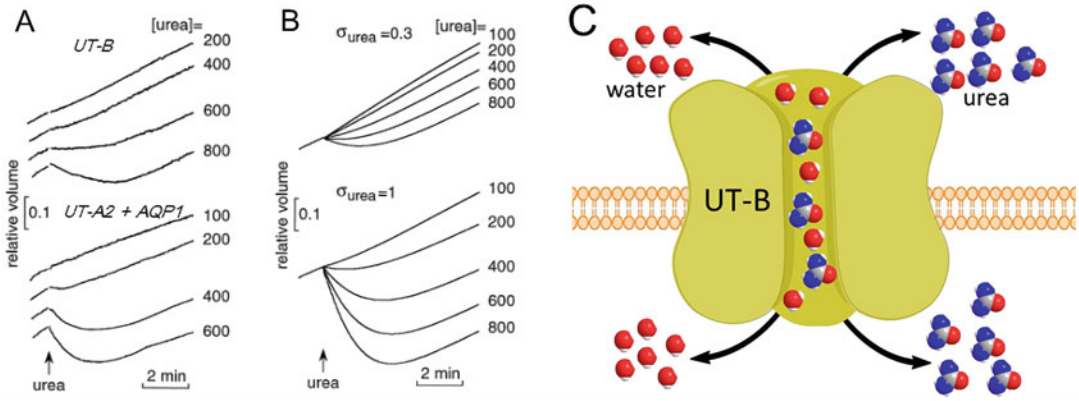

Fig. 23.2 Urea transporter UT-B functions as a water channel. (**a**) Urea reflection coefficient of the UT-B pathway determined in *Xenopus laevis* oocytes. (**b**) Predicted urea reflection coefficient of the UT-B pathway in mathematical model. Derived from ref. [4]. (**c**) Schematic diagram of UT-B as a urea/water channel utilizing a common aqueous pathway. (Derived from [5])

done with oocytes coexpressing water channel AQP1 that is permeable to water but not urea, and UT-A2 (originally called UT2) that is permeable to urea but not water. Figure 23.2a (*bottom*) shows little initial oocyte swelling or shrinking for external 200 mM urea, suggesting that $\sigma_{urea} \sim 1$, which was confirmed by the simulated curves in Fig. 23.2b (*bottom*). These results suggested that the UT-B is an aqueous channel that transports water and urea in a coupled manner (Fig. 23.2c).

Sidoux-Walter et al. confirmed increased water permeability in *Xenopus* oocytes expressing UT-B. However, they concluded that UT-B-facilitated water transport did not occur under physiological conditions. They proposed that UT-B-associated water permeability occurs only when UT-B expressed at non-physiologically high levels [6].

To quantify UT-B-mediated water transport in physiological conditions, double-knockout mice lacking both UT-B and the major erythrocyte water channel AQP1 were generated [4]. Osmotic water permeability in erythrocytes from mice lacking both AQP1 and UT-B is 4.2-fold lower than in erythrocytes from mice lacking AQP1 alone. Similar low water permeability was found in erythrocytes from AQP1 null mice after UT-B inhibition by phloretin and in erythrocytes from UT-B null mice after inhibition of AQP1 by $HgCl_2$. The single-channel (per molecule) water permeability of UT-B in erythrocytes is very similar to that of AQP1 (7.5×10^{-14} cm^3 s^{-1}) [2].

In 2013, Slim Azouzi et al. suggested that UT-B should be considered as a new member of water channel family, on the basis of the results that osmotic water unit permeability of UT-B (pf_{unit}) is similar to that of AQP1. Five water molecules were found inside the UT-B pore to form a single file, which moved rapidly along a channel by hydrogen bond exchange involving two critical threonines [7]. UT-B is a homotrimer, and each protomer contains a urea conduction pore with a narrow selectivity filter [8]. The selectivity filter is divided into three regions: So, Si, and Sm sites. When the water molecules cross the region Sm, the water–water (W-W) hydrogen bonds decrease remarkably, and the number of hydrogen bonds with the residues lining the pore increases concomitantly [7]. The fact that urea and water share the same pathway through the pore of UT-B also indicates that UT-B acts as a water channel.

A recent study reported that mouse UT-A2 and UT-A3 also transport water. Experimenters injected cRNAs encoding c-myc-tagged mouse UT-B, UT-A2, or UT-A3 into Lithobates oocytes and found that UTs successfully expressed

oocytes and showed significantly increased, phloretin-sensitive urea uptake, and water permeability [9].

23.3 Cystic Fibrosis Transmembrane Conductance Regulator

Cystic fibrosis transmembrane conductance regulator (CFTR) is a membrane protein and chloride channel in vertebrates [10]. CFTR is expressed in the apical membrane of epithelial cells in the airway, pancreas, and intestine [11]. It conducts bicarbonate [12], interacting with other Cl^-/HCO_3^- exchangers to provide a recycling pathway [13]. CFTR is different from other Cl^- channels. As a unique member of ABC transporter family, it is a primary active transporter that relies on ATP hydrolysis to actively pump the substrates across membranes [10]. It contains five domains: two membrane-spanning domains (MSDs) forming chloride ion channel, and two nucleotide-binding domains (NBDs) regulating channel to open or to close, and one regulatory domain regulating channel activity [14].

CFTR has been shown to be a regulator of Na^+, K^+, and Cl^- channel, and it also enhances osmotic water permeability when activated by cAMP [15]. Because of the CFTR-dependent activation of a water permeable membrane conductance, the osmotic water permeability is activated through stimulation of CFTR in *Xenopus* oocytes. CFTR has a calculated single-channel water conductance of 9×10^{-13} cm^3 s^{-1}, suggesting a pore-like aqueous pathway [16].

In 2000, Schreiber et al. demonstrated functional coupling between Cl^- transport as performed by the CFTR Cl^- channel and water channel performed by AQP3 [17]. Besides, other members of AQP family interact with CFTR to regulate osmotic water permeability in various cellular systems, including in the epididymis [18]. Considering that AQP7 and AQP8 are expressed in tar testis with a remarkable similar distribution of CFTR [19, 20], several studies attempted to describe their molecular interaction. The interaction between AQP4 and CFTR occurs in vivo on condition of a fully intact blood–testis barrier [21, 22]. The same mechanism was found between AQP9 and CFTR as well [23]. Pietrement et al. found water secretion maybe driven by a CFTR-dependent mechanism in the distal regions of the epididymis [24]. These studies showed that CFTR controls the seminiferous tubular fluid in close association with AQPs, providing new sights of counteract male subfertility/infertility. CFTR also acts as a regulator of other membrane transporters. However, the regulation of AQP-mediated water transport is poorly understood, we think that CFTR cannot transport water directly but through the establishment of cAMP-stimulated aqueous pore or by interaction with AQPs [15].

23.4 Cotransporters

Some cotransporters not only transport their specific substrates but also water (Table 23.1). Because of the large number of cotransporters per cell and the considerable unit water permeability, the water transport medicated by cotransporters may well be significant. The process and mechanism of water cotransport has been studied in cultured mammalian cells, native tissue, and by heterologous expression in *Xenopus laevis* oocytes. Transport of water and substrates proceed in parallel in a strict stoichiometric coupling ratio, with no change when the cotransport of water is altered abruptly by abrupt changes of substrates. A variety of techniques have been used in this area and added the new vitality to it, for example, fluorescence reporters, immunoprecipitation, electrophysiology, ion-selective micro-electrodes, and other sensitive optical methods for volume measurements.

23.4.1 K-Cl Cotransporter

The K-Cl cotransporter (KCC) has been proposed to play a role in the maintenance and regulation of cell volume [25] and the movement of chloride and water in erythrocytes, endothelium, trout hepatocytes, ascites tumour cells, and kidney epithelial cells [26]. KCC is exclusively colocalized

Table 23.1 Number of water molecules cotransported per transport cycle

Cotransporters	Coupling ratio	Passive water permeability per transporter (10^{-14} cm^3 s^{-1})	Reference
KCC4	500	0.01	[31]
NKCC1	59	4	[41]
SGLT1 (human)	230	–	[58]
GAT1 (human)	330	0.7	[52, 53]
EAAT1	425	0.2	[57]
NaDC-1	176	1.5	[77]
MCT1	500	0.3	[46]

in the same membrane regions as the Na^+/K^+ ATPase is. Four isoforms of KCC (KCC1–4) have been found and shown different expression pattern depending on tissue types and stages of ontogenesis with no cell-specific expression [27]. KCC1, as the primary choroidal KCC isoform, is localized at the basolateral membrane [28]. KCC4 is weakly expressed in the mammalian cerebral cortex, hippocapus and cerebellum, but abundant in the apical membrane of choroid plexus and peripheral neurons, the water transport medicated by KCC4 was mostly studied under circumstances [29].

Thomas et al. studied the interaction between the K^+, Cl^-, and H_2O fluxes in the membrane of the choroid plexus epithelium from *Nectyrus maculosus* [30]. They hypothesized there is cotransport of K^+, Cl^-, and H_2O. Hydration of the binding of K^+ and Cl^- induces a conformational change in the KCC, which causes the permeability barrier to shift from one side of the membrane to the other [31]. The external osmolality is higher than the intracellular osmolality by 100 mOsm; the intracellular concentrations of K^+ and Cl^- changed only a few millimolar during the exposure to KCC. When the KCC was blocked by furosemide, the cell shrank osmotically in response to the addition of 50 mM KCl [31]. Water transport by KCC is abolished with the absence of the Cl^-, and the passive water permeability is lower than other cotransporters (of the order of 10^{-16} cm^3 s^{-1}).

However, KCC1 from kidney inner medulla does not serve as a secondary active transport, with the evidence no water transport occurred in the absence of osmotic pressure [32], contrary to the water flux 1:500 stoichiometry of K^+:H_2O ratio in the choroid plexus [30].

These findings of KCC clarified some unexplained findings for water transport and questioned the simple osmotic models. As a water pump, the function of KCC well explains the ability of the epithelia, such as small intestine and gallbladder absorb water against osmotic gradients of up to 200 mOsm [33]. What surprises us is that at physiological osmolality, the KCC contributes to half of the capacity for water transport across the exit membrane.

23.4.2 Na-K-Cl Cotransporters

The Na-K-Cl cotransporters (NKCC) are a class of membrane proteins that transport Na^+, K^+, and Cl^- ions into and out of a wide variety of epithelium and other cells [34]. So far, two distinct Na-K-Cl cotransporter isoforms have been identified, i.e., NKCC1 and NKCC2. NKCC1 is present in basolateral and the apical membrane of choroid plexus such as the small intestine and the kidney proximal tubule [35–37]. NKCC2 is expressed only in the kidney epithelial cells in the thick ascending limb. Interestingly, NKCC1 is specifically distributed in the smooth muscle cell layer that penetrates the small arteries and veins of the brain and spinal cord, as well as in the endothelium of capillaries and small veins, confined to the vascular system of the subarachnoid space [38], which is asymmetrical and most residing into the luminal membrane [39].

NKCC1 transports both ions and water, but NKCC2 only transports ions. The water transport by NKCC1 proceeds uphill against osmotic gradients. In the pigmented epithelium, NKCC1 contributes to half of the passive water permeability through basolateral membrane at normal

properties [40]. Different from the conventional channel-mediated osmotic transport, the activation energy is higher than that of aqueous pores (21 kcal mol^{-1}) [41]. Water permeability per protein is rather high about 4×10^{-14} cm^3 s^{-1}. The NKCC1-dependent influx of water is voltage insensitive, temperature dependence, and independent of a functional Na^+, K^+-ATPase. Around 115 water molecules are transported per turnover cycle in NKCC1 with one Na^+, one K^+, and two Cl^- ions [42].

As a water pump, the Cl^--dependent influx proceeds inwards against the osmotic gradient of 50 mOsm imposed by the mannitol, which indicates that in NKCC1, ion fluxes are tightly coupled to water influxes [41]. The selective NKCC1 inhibitor bumetanide reduces the cytotoxic brain oedema during middle cerebral artery occlusion. NKCC1, as an important molecule in the water permeability of the blood–brain barrier, contributes to the formation of cerebral oedema after ischemia [39]. NKCC1-mediated water cotransport is important for cerebrospinal fluid (CSF) formation [43, 44], allowing CSF production independent of the osmotic gradient in a manner determined by the prevailing ionic gradient, produced and maintained by the synergistic action of Na^+/K^+-ATPase and a large number of other ion transporters and channels expressed in the choroid plexus [28]. Under basal conditions, apical NKCC1 is continuously active and works in the net inward flow manner to maintain the Cl^- concentration and cellular water volume required for CSF secretion [43]. The function that NKCC1 controls Cl^- and water transport also has clinical relevance about disorders of chloride transport and fluid absorption that mainly causes blindness.

Not all isoforms of NKCC1 transport water. The NKCC1 in renal medullary thick ascending limb cells has no capacity to cotransport water due to its low hydraulic conductance [40].

23.4.3 Moncarboxylate Transporter

Moncarboxylate transporter (MCT) catalyse the facilitated of lactate with a proton and transport other metabolically important monocarboxylates such as pyruvates, the branched-chain oxo acids derived from leucine, valine and isoleucine, and the ketone bodies acetoacetate, β-hydroxybutyrate, and acetate [45]. There are nine MCTs, and they distribute in different tissues. MCT1 and MCT4 are expressed ubiquitously in most tissue but MCT2 restrictively distributes in testis; MCT3 is exclusively expressed in the retinal pigment epithelium (RPE) [45]. Little consideration for MCT5–MCT9 made it unclear for the function of them.

It has been proposed that MCT1 on the apical surface of the retinal pigment epithelium plays an additional role in regulating the volume of the subretinal space, since lactate-H^+ transport is accompanied by water transport [46, 47]. The water cotransport properties of MCT1 have also been found in the human fetus, and the water permeability is gated by lactate. The interdependence of the fluxes of MCT cotransport had a fixed ratio of about 109 mmol of lactic acid per liter of water, that is to say MCT1 cotransports 500 water molecules with each lactate molecule, and exhibited saturation for increasing driving forces [48].

It is worth raising that NKCC1 co-localized with the MCT1 contributes to the uphill transport of water against the osmotic gradient resembles in the apical membrane [49]. The ability of MCT1 to transport rapidly both lactic acid and water across the RPE and into the blood will prevent an accumulation of lactate, which would cause osmotic swelling and the retina detaching from the RPE. It suggests the physiological significance of MCT1.

Neuronal activity induced fluctuations in extracellular lactate concentration and may have decreased transiently during the first few seconds of neuronal activation [50], such fluctuations are similar to activity-evoked K^+ transients, pH transients and extracellular space volume dynamics. Inhibition of MCT1 reduces activity-induced extracellular gap contraction in rat hippocampal slices [51]. The transient decrease in extracellular lactate concentration induced by initial activity is due to MCT-mediated lactate uptake resulting in contraction of the extracellular space, possibly through the ability of the MCT to cotransport water and the consequent cell swelling.

23.4.4 GABA Transporter

GABA is removed from the synaptic cleft by means of Na^+ -Cl^- coupled reuptake. Four different GABA transporter (GAT) subtypes have been described (GAT-1, GAT-2, GAT-3, and the betaine–GABA transporter-1 (BGT-1)). GAT1 behaved as an SKF89976A-sensitive water channel [52]. Using the *Xenopus laevis* oocyte expression system, the water permeability of the GAT-1 in the oocyte was about 3×10^{-6} cm·(s·osmol·l)$^{-1}$ or 1.6×10^{-4} cm·s^{-1}. Data showed the strict proportionality between the GABA transport and the instant influx of water. The coupling ratio was 330 water molecules per cycle. Cotransport of water was composed of two parts: the cotransport component and the osmotic component. It was constant and independent of external osmotic gradients.

The GAT-1 also worked as a Li^+ channel in the absence of GABA and Na^+. However, the water permeability was reduced by 40% when Na^+ was replaced by Li^+ in the bathing solution [53]. Linked with the debates with SGLT, which also supports the water cotransport, is dependent on Na^+-medicated cotransporter and not by the accumulation of ions in an unstirred layer. Otherwise, when Li^+ replaces Na^+ in the bathing solution, cotransport of water was also observed.

23.4.5 Na^+-Coupled Glutamate Transporter EAAT

The five Na^+-coupled glutamate transporter isoforms (EAAT1–5) have distinct expression. The human EAAT1 was primarily found in glial cells [54] and also locate in peripheral tissue [55]. EAAT1 is highly expressed in brain capillary endothelium [56], which provides a transendothelial channel for glutamate and may play a role in the endothelial contribution to CSF and ISF secretion through the ability to cotransport water. There are two modes of water transport in human EAAT1, which are separated and proceed in parallel. Every unit charge with about 436 water molecules was cotransported along with glutamate and Na^+ by a mechanism within the protein. The transporter also sustained passive water transport in response to osmotic challenges [57]. Cotransport of water proceed uphill against the water chemical potential difference. Glutamate increases the osmotic water permeability of the EAAT1 irrespective of the rate of cotransport. Unlike the high external hyperosmolarity of Na^+-glucose cotransporter SGLT1 (15 mosmol l^{-1}), in ETAA1, it took a lower external hyperosmolarity (5 mosmol l^{-1}) to match osmosis with cotransport [58].

23.4.6 Sodium Glucose Transporters (SGLT)

Twelve members are found in the human sodium glucose transporters (SGLT) family. Except for SGLT1–5 cotransporting for sugars, they include Na^+ cotransporters for myo-inositol, iodide, short-chain fatty acids, and choline. SGLT6 is also known as Na^+/inositol cotransporter 2 (SMIT, sodium myo-inositol [59]; CHT, choline; SMVT, sodium multivitamin [60]; SMCT, sodium monocarboxylic acid; NIS, sodium iodide cotransporters) [61].

As a multifunctional protein, SGLT1 works as a water channel and transporter coupled water and glucose. The passive osmotic permeability of the hSGLT1 plays an important role in the final achievement of isotonic transport. And the water cotransport (4 l of water with 1 M of glucose) in the human small intestine plays a vital important role in reuptake (total 9 l per day) [62].

Molecular dynamics studies of SGLTs have shown that water flows through the sugar transport pathway [63]. SGLT1 has three modes in isotonic water transport. First, water influx is directly correlated with Na^+ and glucose in the ratio of 260 H_2O/2 Na^+/1 glucose with no delay in human [64]. Second, it acts as a water channel [58]. Last, it generates an osmotic driving force that is employed by other pathways. Water permeability was increased more than tenfold in the circumstances of co-expression of AQP1 with SGLT1 [65]. The initial rate of water transport varied with the membrane potential, temperature. Arrhenius plots of Na^+/glucose cotransport is as

high as water flow (26 kcal·mol^{-1}) [64]. The cotransport of water was independent of the osmotic gradient and even occurred in the presence of adverse osmotic gradients.

However, Charron et al. agreed with that the water transport mediated by SGLT1 was osmotic and proposed to arise as an unstirred layer effect [66]. The cotransport hypothesis and the osmotic hypothesis explain some numerical analysis at the same time, but the cotransport hypothesis gave a better fit to the volume changes [67].

Study of the Na^+-coupled iodide transporter (NIS), in which thiocyanate (SCN^-), substituting for iodide (I^-), was conducted in the cotransporters expressed in *Xenopus* oocytes. Less water was cotransported along with the larger substrates [68]. For example, the coupling of rabbit SGLT1, human SGLT1, NIS, and a plant H^+/amino acid cotransporter (AAP5) ranged from 50 to 425 water molecules per turnover [69].

23.4.7 Sodium Borate Cotransporter

As a member of the Slc4 family, sodium borate cotransporter is an extremely important protein for both yeast and plant. Because borate plays a significant role to cross-link vicinal diols to stabilize the structure of cell walls in bacteria, plants, and fungi [70]. However, it is still confused about what role biochemical serves for borate in mammals. Sodium borate cotransporter is abundantly expressed in the renal descending loop of Henle [71] and localizes basolaterally in the corneal endothelium. Also, it is broadly expressed in salivary glands, thyroid gland, and testis [72].

Sodium borate cotransporter mediated water flux driven exclusively by an osmotic gradient when expressed in *Xenopus laevis* oocytes and HEK293 cells. Water-flux through Slc4a11 is 10^3-fold faster than water movement reported for SGLT1 [73].

Sodium borate cotransporter localizes on the opposite surface from apical AQP1, functions the basolateral pathway for the water transport from the corneal stroma into the endothelium, and AQP1 mediates water transport out of the corneal stroma into the aqueous humour. The studies suggest that AQP1 and sodium borate cotransporter are coefficient in mediating trans-endothelial water reabsorption [73].

23.4.8 Na^+-Dicarboxylate Cotransporter

Na^+-dicarboxylate cotransporter (NaDC-1) belongs to the Slc13 family of anion transporters [74]. Na^+-dependent anion transporters contain the Na^+-dependent dicarboxylate transporter and the renal Na^+-sulfate cotransporter [75]. Na^+-dicarboxylate cotransporter was found in the apical membrane of the kidney proximal tubules and contributed to the reabsorption of tricarboxylic acid cycle intermediates [76].

NaDC-1 mediates both passive and solute-coupled water transport and contributes to fluid reabsorption across the proximal tubule. Many studies suggest that SGLT1 and NaDC-1 share a common mechanism for passive water transport. Water transport medicated by NaDC-1 occurs in the absence or even against an osmotic gradient. The ratio between Na^+, citrate (or succinate), and water is 3:1:176 per transport cycle [77].

NaDC1 plays an important role in regulating succinate and citrate concentrations in the urine. Single-nucleotide polymorphisms in the human Na^+-dicarboxylate cotransporter affect transport activity and protein expression, which contributes to human diseases such as kidney stones [78].

23.4.9 Glucose Transporter (GLUT)

Twelve isoforms of glucose transporters (GLUT) have been found in mammalian cells. GLUT1 was the first cloned and most extensively studied. It is abundantly expressed in erythrocytes and endothelium of the blood–brain barrier [79]. GLUT1, GLUT2, and GLUT4 have been shown to support osmotic water fluxes in addition to its own role as glucose transporters.

In 1989, by investigating the effects of inhibitors of glucose transport on membrane osmotic water permeability, Fischbarg et al. concluded that glucose transporter serves as a

water channel in some cells and is sensitive to the specific inhibitor phloretin [80]. *Xenopus laevis* oocytes injected with mRNA encoding the glucose transporters exhibited an average of 4.8-fold of the osmotic water permeability [81]. However, GLUTs serve as water channels in brain, skeletal muscles, and liver but not in kidney or intestine epithelium [82].

Water transport in the GLUT1 and GLUT2 has been demonstrated to be bimodal. They act as a water channel, and water is cotransported together with the glucose. The water permeability of GLUT2 in oocytes is 0.11×10^{-5} cm·(s·osmol·l)$^{-1}$, equivalent to 6.1×10^{-5} cm·s^{-1}. GLUT2 cotransports less water in the inward than in the outward. Compared to the Na^+-coupled glucose transporter, the coupling ratio of GLUT2 is six times smaller [83].

Water is transported through GLUT1 by two main mechanisms. First, GLUT1 has a small but well-defined passive osmotic water permeability that is effective both in the absence and in the presence of glucose. Second, the inward flow of glucose begins when glucose is added to the external solution. Under this condition, a total of 40 water molecules were cotransported for each glucose molecule [84]. Conformational changes occur when glucose is applied to the GLUTs. Glucose together with a number of water molecules is occluded, and an aqueous cavity opens to trans side with the glucose exited [68]. The other three-compartment model for transepithelial water transport suggests the coupling space is associated with a static aqueous cavity with substrate binding site [85].

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Methods to Measure Water Permeability 24

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Abstract

Water permeability is a key feature of the cell plasma membranes, and it has seminal importance for several cell functions such as cell volume regulation, cell proliferation, cell migration, and angiogenesis to name a few. The transport of water occurs mainly through plasma membrane water channels, aquaporins. Aquaporins have very important function in physiological and pathophysiological states. Due to the above, the experimental assessment of the water permeability of cells and tissues is necessary. The development of new methodologies of measuring water permeability is a vibrant scientific field that constantly develops during the last three decades along with the advances in imaging mainly. In this chapter we describe and critically assess several methods that have been developed for the measurement of water permeability both in living cells and in tissues with a focus in the first category.

Keywords

Aquaporin · Cell volume · Osmotic water permeability · Plasma membrane

24.1 Introduction

Water transport in cell and tissue systems is a necessary property for maintaining their homeostasis [1]. Water is the most abundant component of the human body; therefore, mechanisms that regulate its transport are essential for life. Water permeability is a key feature of the cell membranes that is of critical importance for several cell functions that among others include cell fluid secretion and absorption, cell volume regulation, cell proliferation, cell migration, angiogenesis, and other processes [2, 3]. Several different pathways are involved in the transport of water across the cell plasma membrane that involve water transport by simple diffusion, transport of water molecules through water channels by facilitated diffusion, and water transport with hydrated solutes. Facilitated diffusion of water through plasma membrane channels is mediated

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through aquaporins (AQPs), a class of membrane water channels whose primary function is to facilitate the passive transport of water across the cell plasma membrane [4]. AQPs have attracted huge research interest during the past 25 years, and currently, there is enough evidence that they have important roles in nervous, respiratory, renal, cardiovascular, gastrointestinal, reproductive, and sensory system physiology [5–15]. Furthermore, various studies have shown that AQPs are involved in several pathologies such as cancer, brain edema, brain injury, epilepsy, obesity, glaucoma, and others [4]. Finally, it is important to note that in humans there is a class of diseases recently termed aquaporinopathies and refer to rare cases of loss-of-function mutations in human AQPs. More specifically, loss-of-function of AQP0 causes congenital cataract and of AQP2 causes nephrogenic diabetes insipidous (NDI) [16, 17]. Serum autoantibodies against AQP4 are a hallmark of neuromyelitis optica (NMO), a rare autoimmune disease [17]. Due to the involvement of the channels that mainly mediate the transport of water through cell plasma membranes in so many physiological and pathophysiological states, researchers need to have the appropriate tools to assess and study water permeability. The fact that a lot of focus is placed in identifying modulators of AQPs for the treatment of several diseases demonstrates the importance of having precise and reliable experimental techniques for water permeability measurements [18].

Cell volume is the most sensitive parameter to the changes of the total water current across the cell plasma membrane [19]. In general water permeability is measured from the kinetics of the cell volume changes in response to an osmotic gradient. A variety of experimental approaches have been developed to subject cells to an osmotic gradient and subsequently record the resultant kinetics of cell volume from which water permeability is deduced. The problem of water permeability determination is a problem of volume-dependent physical parameters measurement.

The methods used to measure osmotic water permeability are technically challenging, subject to various mixing/flow-related artifacts, and not easily applied to highly water-permeable cells. A very critical characteristic of an experimental setup is the time needed for the establishment of the desired osmotic gradient. All the modern experimental approaches for measuring cell water permeability underestimate this parameter, and the degree of the underestimation is dependent on the lag time of the establishment of the osmotic gradient during the experiment. Many types of cells are highly permeable to water because of the abundant expression of AQPs in their plasma membrane [5, 20]. Studies of the water permeability conducted in highly water permeable cells require methods that have very short osmotic gradient formation time. Failure in the incorporation of such a parameter in water permeability measurement studies compromises the validity of the results and subsequently their translation to clinically useful therapies. Indeed, although AQPs are considered to be important drug targets for a variety of diseases, and great effort is put in the identification of appropriate AQP modulators; there is poor progress in targeted therapeutics, partially due to challenges and artifacts in cell membrane water permeability measurements [18].

The purpose of this review is to survey the modern experimental approaches for the measurement of water permeability of living cells and multicellular tissues. We will discuss the principles of each methodology, technical details, and limitations. Strategies for the measurement of the plasma membrane osmotic water permeability (P_f) in living cells that are described involve image analysis, light scattering, total internal reflection fluorescence microscopy, confocal microscopy, interferometry, spatial filtering microscopy, scanning probe microscopy, bioelectrical impedance methods, and volume-sensitive fluorescent indicators. The fundamental physical principles of the cell membrane osmotic water permeability were reviewed in detail earlier [21]. In short, the osmotic volume flow (J_v, cm^3/s) across a selectively permeant membrane is described by the following equation and Fig. 24.1:

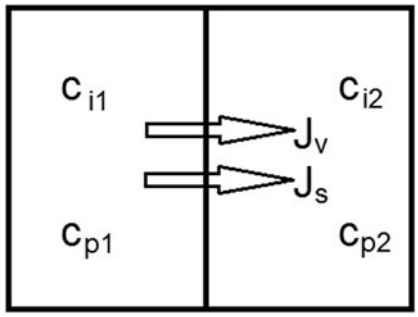

Fig. 24.1 Volume flow across a single barrier separating compartments 1 and 2. *ci*1 and *ci*2 are the osmolalities of impermeant solutes on sides 1 and 2, and *cp*1 and *cp*2 the osmolalities of permeant solutes, V_w the partial molar volume of water, S the membrane surface area, σ_p the solute reflection coefficient, P the hydrostatic pressure. Barrier permeability properties include the osmotic water permeability coefficient P_f, the solute permeability coefficient P_s, and the solute reflection coefficient σ_p. Water flux (J_v) and solute flux (J_s) are defined as positive in the left-to-right direction as indicated

$$J_v = P_f S V_w \left[(c_{i2} - c_{i1}) + p \left(c_{p2} - c_{p1} \right) + (P_1 - P_2)/RT \right] \quad (24.1)$$

The interpretation of the measured P_f assumes accurate definition of the cell membrane surface area S, the absence of significant unstirred layer effects and types of transporters, which carry water with or without hydrated solutes. As defined by Eq. (24.1), P_f is measured from the volume flux produced by a defined osmotic gradient or hydrostatic driving force. In animal cells that have just a soft plasma membrane, the hydrostatic driving force is considered insignificant.

Molecular transporters that contribute to the total water flow could be temperature dependent according to their Arrhenius activation energy. The Arrhenius activation energy (E_a, kcal/mol) is defined by the relation $\boldsymbol{ln}\ P_f = E_a/RT + A$, where R is the gas constant, T is absolute temperature, and A is an entropic term. E_a provides a measure of the energy barrier to the water movement though the molecular transporter. For water movement through aqueous channels, E_a is generally found to be low (3–6 kcal/mol). The low E_a associated with water pores is assumed to be related to the weak temperature dependence of the water self-diffusion [21]. In the case that water is transported along with hydrated solvents and contributes significantly to the cell volume changes, the E_a of the specific solute transporters could produce temperature dependence of the P_f of the living cell plasma membrane. In such a case, the P_f values should be obtained at the temperature that is normal for the cells under study to be adequate for the purpose of a physiological study.

24.2 Water Permeability Methods Based on Cell Volume Measurements

24.2.1 Water Permeability Measurement by 2D Image Analysis

In cells observed under wide field microscopy, the changes in their cell volume are often accompanied by small changes in their size that can be measured in the images captured during the experiment. To estimate the cell volume, the area of a cell is measured by tracing its outline on the video images. This method of cell volume measurement is based on the analysis of two-dimensional (2D) images and on the assumption that the cells change its sizes to the same extent in all three dimensions. Polarizing, phase contrast, and various forms of interference microscopy are the most frequently used microscopic methods for observing living cells and simultaneously recording images continuously using a video camera [22–26]. The relative volume of the cell was estimated as $V/V_0 = (A/A_0)^{3/2}$, where V is the volume, A is the area, and the subscript 0 indicates the control value. This method using differential interference contrast microscopy and digital imaging was created for the measurement of the cell volume and to examine the effects of muscarinic stimulation on single rat salivary gland acinar cells [27]. The advantage of this approach is the use of unlabeled cells and the possibility to measure the reaction of cells in small tissue structures [28]. The disadvantages of the several variations of this method are that they

are mainly limited by: (a) the resolution capacity of the lens system used, (b) the contrast that the specimen generates in the image, and (c) the time required for an image to be recorded. As mentioned above to estimate the cell volume, the area of a cell is measured by tracing its outline on the video images; therefore, this method is not adequate for cells that have high water permeability and thus fast kinetics of cell volume changes [25].

Based on the principles of this methodology in the recent years, image-based cytometry systems have been developed that use bright-field and fluorescent imaging. These image-based systems have been demonstrated to perform numerous assays, such as quantitative cell size and morphology analysis and potentially can be used for measurements of cell water permeability but no such experiments are currently published [29, 30].

24.2.2 Water Permeability Measurement by Scanning Probe Microscopy

The most accurate measurements of cell volume are achieved by the direct scanning of living cells and the use of scanning probe microscopy (SPM), and in this context both atomic force microscopy (AFM) and scanning electrochemical microscopy (SECM) have been used to image live cells [31]. In these studies, three-dimensional reconstruction of the cell shape is achieved by scanning confocal microscopy and different modifications of SPM such as real-time AFM [32, 33]. One of the drawbacks of AFM is the fact that an external mechanical force is applied to the cell because the detection of the cell surface is performed with the deflection of a cantilever. Such an external force is not acceptable in physiologically relevant experiments with mechanosensitive live eukaryotic cells. Scanning ion conductance microscopy (SICM) is a form of SPM that overcomes this problem by allowing the imaging of the cell surface under physiological conditions without any physical contact and with a resolution of 3–6 nm [34, 35]. Even SICM though like the other SPM methods is more fit for relatively flat surfaces given that in cases of convoluted surfaces there is a collision of the probe that interferes with cell integrity again. This problem is not an issue in hopping probe ion conductance microscopy (HPICM) where the probe never touches the surface of the cell. In this case the probe that senses current fluctuations and translates it in sample height even at a reduction of 1% is at a Z-position that still does not interfere with the cell membrane at the imaging point. Using HPICM non-contact imaging of the three-dimensional surfaces of live cells can be achieved with resolution better than 20 nm [36]. These methods are quantitative and provide the best spatial resolution of living cells, but scanning is a relatively slow process and like all other SPM techniques their use is restricted to imaging relatively flat surfaces with the exception of HPICM. These limitations make SPM probably not practical for studying the kinetics of cell volume fluctuations in living cells.

24.2.3 Water Permeability Measurement by Light Scattering and Spatial Filtering Microscopy

Cell volume changes lead to changes of the elastically scattered light (Rayleigh scattering), and this effect is the basis of the method of measurement of the cell water permeability by light scattering. The method is simple to apply and requires very small sample quantity; however, there is no practical theory that reflects the relationship of scattered light intensity and cell volume because of complexities in the cell optical configuration and interference phenomena. Therefore, assessment of light scattering provides a semiquantitative index of the cell volume changes in relatively large and adherent cells such as the macrophages [37]. Quantitative data interpretation for this method is efficient for a limited kind of objects that have a relatively simple shape such as vesicles or erythrocytes [38, 39]. Nevertheless, the value of P_f of the cells can be calculated from the time course of the light scattering, the cell surface-to-volume ratio, and an empirical

calibration of the cell volume and the intensity of the light scattering [40]. An advantage of this method is that it has satisfactory temporal resolution, and the main drawbacks are that a calibration step is required in order to evaluate the cell volume and the values that are obtained are relative values.

The angular dependency of the intensity of light scattering by a moving individual particle was the basis for the flying light scattering indicatrix method (FLSI) using a scanning flow cytometer (SFC) [29]. The FLSI method was used for the measurement of individual particle characteristics from light-scattering data, to determine the particle size of polystyrene, latex, milk fat, and spores of *Penicillium levitum, Aspergillus pseudoglaucus*. Measuring cell volume in a flow cytometer could potentially be a prospective approach for high-throughput screening studies of the distribution of P_f values in populations of cells in suspension.

Methods based on the measurement of scattered light intensity are suitable for investigations of homogeneous suspensions of uniform objects like cells or vesicles. They have been used for suspensions of erythrocytes [41], in flow cytometers [29, 42, 43], in suspensions of membrane vesicles and liposomes reconstituted with water channels [40, 44, 45]. Due to the sufficient temporal resolution, light scattering was used to determine the P_f of lung alveolar epithelial cells that have very high cell membrane water permeability [46]. The method was also applied to calculate the plasma membrane P_f of cells in micro-dissected fragments of mouse kidney collecting duct. More specifically, the time course of the light scattering intensity measured in dark field microscope on individual fragments positioned in a thermo-stabilized flow chamber was used to study the regulation of P_f by vasopressin [47, 48].

A theory relating the signal intensity to the relative cell volume was developed based on the spatial filtering and the changes in the optical path length associated with cell volume changes. It was found that the integrated intensity of monochromatic light in a phase contrast or dark field microscope was dependent on the relative cell volume. The method was applied to characterize transfected cells and tissues that natively express water channels, and the results established light microscopy with spatial filtering as a technically simple and quantitative method to measure water permeability in cell layers (Fig. 24.2) [49]. The method is simple, accurate, and robust if used in cell layers or uniform cells; however, it may work in systems with heterogeneous cells.

Other approaches to follow osmotically induced changes in the cell volume include tracking of immobilized fluorescent beads at the cell surface and laser reflection microscopy [50, 51]. In another method, the measurement of cell volume and osmotic water permeability in Madin Darby Canine Kidney (MDCK) cell layers has been performed by interferometry based on the cell volume dependence of the optical path length (OPL) of a light beam passing through cells. The time course of relative cell volume in response to an osmotic gradient was computed from serial interference images. To relate the interference signal to cell volume, a mathematical model was developed for this purpose [52]. However, these methods are less practical since they are experimentally challenging, require complex instrumentation while at the same time may not work in many systems.

24.2.4 Water Permeability Measurement by Bioelectrical Impedance Analysis

Impedance data obtained from microfluidic impedance flow cytometry enable the characterization of cellular sizes, membrane capacitance, and cytoplasm resistance in a high-throughput manner [53]. Bioelectrical impedance analysis is used in flow cytometry, for example, a Coulter counter measures the changing of DC resistance between two electrically isolated fluid-filled chambers when microparticles act as an insulating layer as DC passes through a small connecting orifice [54]. Microfluidics is the technology of the processing and manipulation of small amounts of fluids (10^{-9} to 10^{-18} liters) in channels with dimensions of tens of micrometers. These

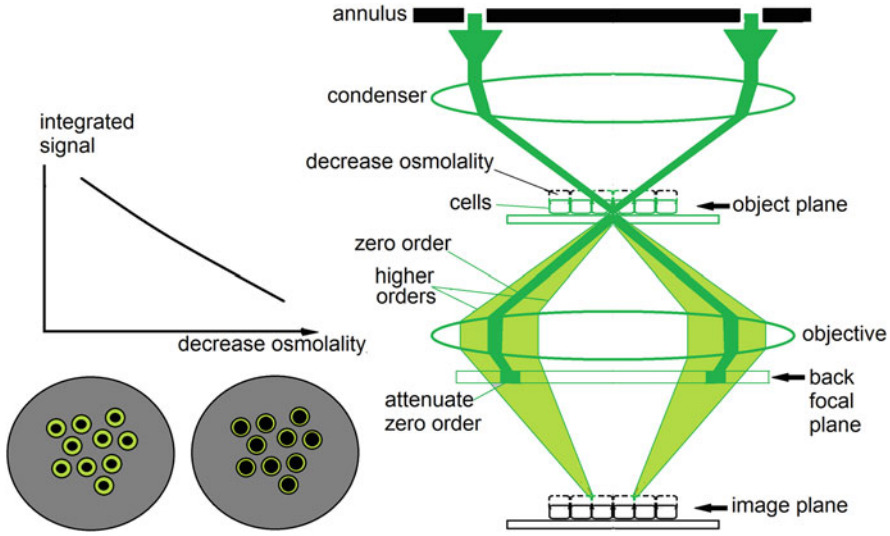

Fig. 24.2 Schematic of the principles of plasma membrane water permeability measurement by means of spatial filtering microscopy. A cell layer under phase contrast or dark field microscopy is shown to change its cell volume under corresponding changes in the medium osmolality that lead to changes in the relative intensity of the higher and zero order beams due to optical path length changes. Attenuation of the zero-order beam at the back focal plane of the objective results in light intensity dependency from the cell volume fluctuations

advantageous features of microfluidic technologies have been used for characterizing the biochemical and/or biophysical properties (mechanical and electrical) of cells at the single-cell level [55]. This technology is prospective for high-throughput single-cell characterization. Electrical impedance-based noninvasive cell and tissue-characterizing techniques have become more and more popular in several fields of application [53, 56, 57]. Microfluidic technology includes manipulation with laser tweezers to drag a single cell to a certain position for the measurement of cell characteristics in experiments of electrorotation (ROT) [58]. ROT operates at a single-organism level and does not require extensive cell preparations while the technique is a noninvasive and allows for sequential investigations. A significant disadvantage of the ROT technique for analyzing single cells is that it takes approximately 30 min per test and also requires a skilled operator to position a single cell in the middle of a rotating electric field. Therefore, the temporal resolution that it can offer now is far from being suitable for measurements of cell volume changes kinetics. On the other hand, methods with high temporal resolution based on light scattering, bioelectrical impedance changes, and cell labeling with fluorescent dyes are the most adequate to study the cells with high water permeability.

Several optical approaches are applied to measure the fluorescence intensity of an aqueous-phase fluorophore in the cytoplasm. Cell-loadable fluorescent dyes are available with various properties and wavelengths. Semiquantitative information about the cytoplasmic fluorophore concentration can be obtained using partial confocal optics in which the z-point spread function of a wide-field optical system is increased using a high numerical aperture objective and a limiting aperture in the back focal plane of the emission path [21]. The confocal methods have limited utility in making quantitative P_f measurements in polarized cell sheets. The z-point spread function of a wide-field optical system could not be increased inside most of the flat cells where thickness of the edges could be about 0.5 μm. This means that the volume where the fluorescent signal is registered from is both undefined and unstable. The ratio signal/noise in such a system could

be low and unstable. This limitation has been removed using laser confocal systems with z-size of registering the volume inside the cell less than 0.5 μm [59]. This method is extremely sensitive to proper focusing and requires high-quality optics. This limitation is the reason why it has been scarcely used for the measurement of cell water permeability.

A more robust method is based on the effect of total internal reflection (TIR) that could be applied using a conventional microscope. However, a more sophisticated approach to measure the concentration of an aqueous-phase fluorophore in the cytoplasm is the total internal reflection fluorescence microscopy (TIRFM). TIRFM involves the excitation of fluorophores in a cell membrane area in great proximity with the adjacent cytosol near a high-to-low refractive index interface [60]. Fluorescence excitation is usually accomplished using a laser source and a glass prism to illuminate the sample at a subcritical angle at a glass–aqueous interface (Fig. 24.3). It is not difficult and costly to equip a conventional epifluorescence microscope with a laser source and a prism to perform TIRFM measurements. The procedure involves loading cells with an aqueous-phase dye and the cell swelling in response to an osmotic gradient result in the cytosolic fluorophore concentration dilution and subsequently to decreased fluorescence signal [61].

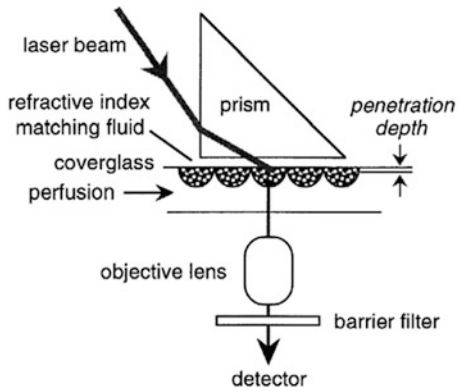

Fig. 24.3 Measurement of osmotic water permeability in adherent cells by total internal reflection fluorescence microscopy. Cells are loaded with a membrane impermeant volume marker. A thin (50–200 nm) layer of cytosol (labeled "penetration depth") is illuminated by a laser beam directed through a glass prism at a subcritical illumination angle. As the cell shrinks in response to an osmotic gradient, fluorophore concentration in the illuminated region increases, producing an increase in detected signal. (Reprinted from Ref. [21])

Optical near fields have been successfully used to confine observation volumes of surface-confined and solution fluorescence correlation spectroscopy (FCS). The standard confocal FCS has the immanent problem that the ellipsoidal observation volume has a low axial confinement. TIR-FCS uses objective-type TIRF illumination to restrict the excitation to a thin section less than 200 nm above the interface in combination with standard confocal detection to improve the lateral confinement of the detection volume. The quantitative study of cellular dynamics even in the level of cellular compartments may be useful for the study of processes close to a surface/solution interface. Potentially, it can give access to local fluorophore concentrations or kinetic rate constants for reversible association of fluorophores with specific substrates in interface [62].

The methods for the measurement of rapid osmosis in cultured cell monolayers using confocal and spatial filtering microscopy may not produce acceptable signal changes in cells that have a low water permeability profile and complex shape. Hamann and coworkers evaluated a calcein self-quenching method for water transport measurements [63]. In this method, high concentrations of calcein are loaded into the cells to produce volume-dependent changes in the total cell fluorescence in response to changes in cytoplasmic calcein concentration due to cell swelling or shrinkage. A more practical modification of the calcein fluorescence quenching method was created to measure osmotic water permeability in highly differentiated cultures of primary brain astrocytes from wild-type and aquaporin-4 (AQP-4)-deficient mice [64]. Cell swelling resulted in a reversible increase in the calcein fluorescence, which was independent of cytosolic calcein concentration at levels well below where calcein self-quenching occurs. The method is based on the quenching of calcein fluorescence by cytosolic proteins. The

fluorescent signal in cells is sensitive to osmotic challenge because of changes in the cytosolic protein concentration that alter calcein quenching, explaining the increased fluorescence with cell expansion after exposure hypotonic medium. A significant advantage of this method is that it is simple experimentally without expensive instrumentation requirements. A conventional fluorescent microscope equipped with a sensitive light detector, such as a photo multiplier tube is sufficient. However, the method provides relative results since it is not quantitative and therefore needs calibration as far as the adaptation of calcein loading protocol and the fluorescent signal is concerned for every kind of cells of tissue specimens [65].

Modern microfluidic technologies promote the improvement of the methods for measuring P_f in cells and membrane vesicles. As mentioned previously, the water permeability is generally measured from the kinetics of the cell volume changes in response to osmotic gradients. To diminish potential mixing artifacts, the temporal aspect of osmotic gradient establishment should be minimized. This is an area where microfluidic technologies can provide new potential for the rapid creation of a gradient. Indeed, recently it was demonstrated that a very fast development of an osmotic gradient was reached in a microfluidics platform in which cells labeled with a cytoplasmic, volume-sensing fluorescent dye were rapidly subjected inside a ~0.1 nL droplet surrounded by oil with a solution mixing time of $<$10 ms. The osmotic water permeability was deduced from a single, time-integrated fluorescence image of an observation area in which time after mixing is determined by spatial position. Water permeability was accurately measured in aquaporin-expressing erythrocytes with half-times for osmotic equilibration down to $<$50 ms [66]. A similar approach was used for measuring the quantitative volume changes of immobilized intestinal enteroids in a microfluidics platform. The enteroids were trapped in a "pinball machine-like" array of polydimethylsiloxane posts for measurement of the volume changes in unlabeled enteroids by imaging of an extracellular, high-molecular weight long-wavelength fluorescent dye. The enteroids volume was deduced quantitatively from area-integrated fluorescence of an excluded extracellular dye. Changes in the enteroid volume altered the total amount of dye in the enteroid-containing area [67].

Finally, the method of light sheet fluorescence microscopy (LSFM) uses a plane of light to optically section and view tissues with subcellular resolution (Fig. 24.4) as compared to confocal and two-photon microscopy. This method is well suited for imaging deep (1 cm) within transparent tissues or within whole organisms. The development of the technology and existing LSFM devices are described in the review of P.A. Santi (2011) [68]. Uncoupling of the illumination and detection axes of the microscope, so that only the part of the specimen that is imaged gets illuminated and provides the ability to image biological systems for extended periods of time with minimal phototoxicity and photobleaching of the specimens and the fluorophores, respectively. LSFM produces optical sections that are suitable for three-dimensional image reconstruction. Stability is a critical issue in light-sheet microscopy, but due to the low phototoxicity, one can image a sample for hours or days. Photobleaching is not a significant problem for repeated imaging as compared to wide-field fluorescent microscopy. Only a 13% reduction in the fluorescence over 475 s occurs [69].

This advantage on the other hand necessitates thinking about the optimal "physiological" conditions that need to be established during the imaging. The detection of fluorescent signals with a wide-field detection device such as a charge-coupled device (CCD) camera allows high-speed imaging [70]. The LSFM technology may be used in a variety of applications spanning all scales of biological systems from molecules to organisms while the high speed of image recording is an advantageous feature for P_f measuring. Current developments in LSFM by diagonal scanning have improved even further the temporal resolution and the potential of the method [71]. As far as the disadvantages of LSFM are concerned, the light-sheet microscopes are complex, require expensive hardware, and typically produce

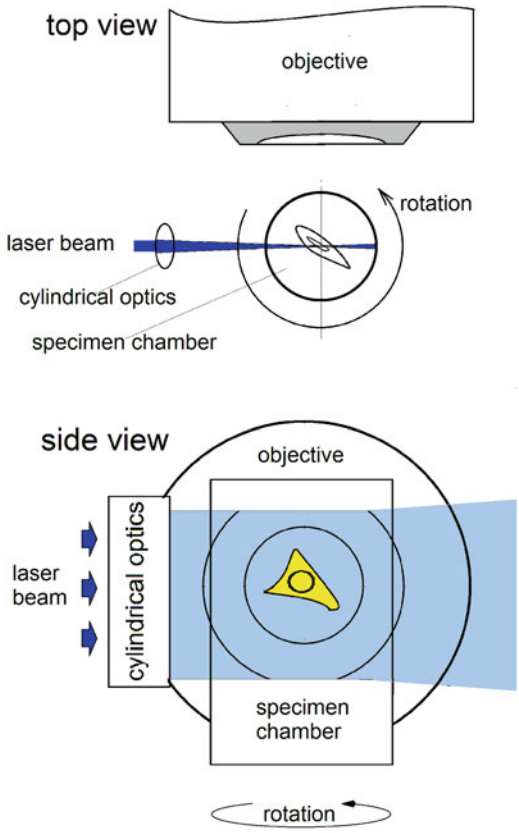

Fig. 24.4 Schematic of the principles of a light sheet fluorescence microscope (LFSM) arrangement demonstrating the top and side views. A light sheet is formed by a laser, collimated and expanded by a beam expander, and projected through an illuminative objective through a cylindrical lens. The focal point of the light sheet is centrally positioned on the specimen chamber made out of clear glass walls with an opening on the topside for specimen insertion purposes. The containing fluid in the specimens chamber depends on the experimental modality (live cell imaging or fixed tissue) therefore contains either physiological solution or clearing fluid, respectively. The specimen chamber is attached to rotating and translating stages while the specimen is intersected by the light sheet and a fluorescent plane collected by a horizontally positioned microscope

enormous amounts of data (in the range of terabytes) streamed from digital cameras to computer hard discs when the experiment lasts for hours or even days. These properties render it a powerful tool from one hand but not one that can be widely used from the other. Despite their potential utility in making quantitative P_f measurements in heterogeneous cell populations and tissue specimens, LSFM technologies have been used little to measure cell water permeability nowadays. Still so far, one can see how far we have gone methodologically since the review of J. J. McGrath (1997) on measuring membrane transport properties of water for individual cells [72].

24.2.5 Water Permeability Measurement by Digital Holographic Microscopy

Digital holography is a phase contrast technique that offers an approach to obtain both qualitative and quantitative phase information from the hologram. It gives the possibility to focus on multiple focal planes from a single digital hologram since CCD cameras allow for the recording of a digital hologram and its processing with specialized software [73–75]. Absolute cell volume measurements have been successfully achieved by confocal fluorescence imaging, staining of membrane surfaces with fluorescent beads, confocal fluorescence imaging, techniques that are limited by their low temporal resolution as they involve optical scanning [76, 77]. A digital holographic microscope (DHM) method is proposed to measure the absolute volume of living cells. The approach is based on phase retardation contrast, proportional to the thickness of the observed specimen, that is a result of the difference in refractive indices between the specimen and the surrounding medium [78]. From a digital hologram, it is possible to retrieve numerically the wavefront information of a field scattered by an object. One can recover plane by plane the three-dimensional (3D) information of an object by using a Fresnel back propagation algorithm. All these concepts can be applied, also, to recover the amplitude and phase of 3D microscope samples [75, 79, 80]. The frequency content of each object wave is then filtered in the Fourier space of the hologram, and phase images are reconstructed individually with the standard Fourier reconstruction algorithm in DHM [73]. The measurement precision of the cell parameters cellular thickness, and integral refractive index, depends strongly on

the precision of the optical path length difference (OPD) signals. When adding the absorbing dye as a dispersive agent to the extracellular medium, cellular thickness can be univocally determined. In addition to the absolute cell volume, the method can be applied to derive important biophysical parameters of living cells including osmotic membrane water permeability coefficient (Pf) while retaining the cell functionality. This method has been applied to cultured human embryonic kidney cells (HEK), Chinese hamster ovary cells (CHO), human red blood cells (RBC), mouse cortical astrocytes, and neurons [78].

DHM as a method to study cellular water transport by deriving osmotic membrane water permeability while retaining the cell functionality has sub-micron, diffraction-limited resolution of the images, and the most significant limitation of this approach is the relatively low temporal resolution. According to the current knowledge, the fastest changes of the cell volume can be accurately tracked with a reconstruction rate of 300 ms for each hologram. Nowadays the low temporal resolution makes DHM applicable only for cells with relatively low osmotic water permeability [81].

24.2.6 Water Permeability Measurement by Microfluidic-Based Methods

Microfluidics have an important role in numerous biological and engineering applications [82–84]. These applications include also the organ-on-a-chip technology (OOAC) that focuses on the biomimetic emulation of tissue characteristics in a microfluidic device [85–90]. Fabrication of microfluidic devices is currently dominated by molding approaches based on poly(dimethylsiloxane) (PDMS) and thermoplastics [83, 91]. Three major challenges have so far hindered the creation of PDMS chips: (1) PDMS molding (including PDMS curing, assembly, bonding, and inlet punching) is a largely manual process; (2) for many years, the user interfaces (inlets/outlets) of PDMS chips were challenging to connect and prone to leakage—as opposed to the leak-free, intuitive connectors such as the industry-standard Luer-lock; and (3) the control systems required to run microfluidic valves involve engineering expertise and equipment not available in most laboratories [90]. Microfluidic systems fabricated in poly(dimethylsiloxane) (PDMS) using soft lithography techniques have advantages over conventional systems [82, 92–95] and have been employed in the study of the osmotic behavior of cells [96–98]. Cells whose diameter is larger than the height of the slit may be trapped in microfluidic perfusion chambers and monitored through microscope (Fig. 24.5).

This PDMS-based microfluidic device has time for medium replacement which is around 1.7 s or shorter from 0% to near 100% when the flow speed is controlled at 400 μm/s (or 1.8 nL/s). Cell volume was measured by counting the number of pixels of cell image [99, 100].

Microfluidics provide an ideal platform for microscale cell manipulation and precise description of the cell osmotic behavior. Such applications of microfluidic approaches adopted for quantification of cell membrane permeability are reviewed by Gang Zhao and Jianping Fu (2017). Microfluidic devices were classified in two categories according to the materials from which they were made: polydimethylsiloxane (PDMS) and non-PDMS materials. The review focuses on methods for suspended cells and

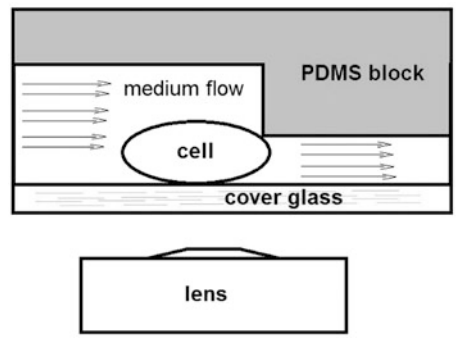

Fig. 24.5 Schematic of microfluidic perfusion chamber and trapped cell. The PDMS-based microfluidic device has medium replacement time of around 1.7 s or shorter from 0% to near 100% when the flow speed is controlled at 400 μm/s (or 1.8 nL/s). The cell volume was measured by counting the number of pixels of cell image

describes applications of microfluidic tools in cell manipulation [101].

Water movement across the plasma membrane of cells is one of the fundamental processes in cell physiology, it is largely enhanced by the presence of water channels, called aquaporins (AQPs). Malfunctioning of AQP can cause clinical diseases or disorder found in kidney [102, 103], respiratory tract [104], brain [105], and eyes [106]. It is important to understand the role of AQPs and their regulation mechanisms and search for small molecules that can selectively modulate AQP activities. Water permeability of AQPs has been indirectly measured by tracing time-dependent cell volume change when subjected to an osmotic challenge. Most of the techniques described above to measure the water permeability of the cell membrane are not suitable for high-throughput screening.

The microfluidic cell volume sensors have been developed to study the water permeability of AQPs and to screen AQP-specific drugs. Different physical principles are used in microfluidic volume sensors to measure the cell volume change in real time [96]. The first measurement of cell volume is based on the cell ability to change electrical resistance at low frequencies in a channel of fixed cross-section aimed at studying the P_f of AQP3 and AQP4, in human embryonic kidney-293 (HEK-293) cells after they were transiently overexpressed. The sensing chamber was 17 μm deep and 1.5 mm wide. A glass slide containing the tissue cells was inverted over the sensor chip. The chamber resistance was measured using a four-electrode array providing sinusoidal current of 100 Hz, 200 nA. The time constants of the cell swelling and the solution exchange by the sensor were 60.3 and 3.2 s, respectively [107]. A microfluidic device resembling the capillary flow with time constant of solution exchange sufficient to measure the rate of water exchange between erythrocytes and the surrounding medium (containing a fluorescent reporter) was constructed reaching steady state in ~60 ms. To get the appropriate parameters of solution exchange, the hydrostatic pressure was adjusted to achieve a flow rate within the main channel of 1 mm/s. This experimental setup gives water permeability of ~1.8E-2 cm/s in erythrocytes [108, 109]. It is accepted that initial volume changes are only due to water movements; however, in living cells osmotic changes are not necessarily abrupt but develop gradually. Water flux might not be the only relevant driving force shaping the vacuole volume response. A perfusion system for monitoring through time volume dynamics of isolated *Beta vulgaris* root vacuole was used to study volume response, and a mathematical model based on the work of Kedem and Katchalsky (1958) [110] was proposed to study the water transport processes across cell membranes [111].

The functional synergy between TRPV4 and AQP4 during cell swelling was studied in the heterologous expressing *Xenopus* oocyte model measured by calcein fluorescence [112]. Permeability is a fundamental characteristic of the cell membrane. Osmotic water permeability can be determined by the volumetric measurements of cells following an osmotic challenge. Microfluidic single-cell trapping devices to determine the membrane permeability of rat hepatocytes and patient-derived circulating tumor cells (Brx-142) have been recently developed. The equilibrium cell morphology in the hypertonic solution was analyzed on the basis of the Boyle-van't Hoff equation [113]. Quadriwave lateral shearing interferometry (QWLSI), a quantitative phase imaging technique based on the measurement of the light wave shift when passing through a living sample was used to study water transport in human airway epithelial CFBE and CHO cells. Phase variations during osmotic challenges reflect cellular volume changes and water fluxes [114]. An open microfluidic platform was created based on aqueous droplets, dispersed in a lipid oil solution, onto a plate with cavities, used to mimic of the cellular plasma membrane. The method allows for the study of permeation of fluorescently tagged compounds from a donor droplet to an acceptor droplet. A mathematical model was used to analyze the kinetics and determine the permeation coefficient [115]. Poly (dimethylsiloxane)-type microfluidic device integrating size-sorting and trapping modules was developed to study the chemical models of

contemporary living cells, the so-called water-in-oil emulsion transfer (WOET) method, that is one of the protocols for cell-sized liposomes encapsulating macromolecules [116]. The method does not require any added probes and can be applied to any osmotically sensitive system to probe the concentration changes at the single-cell level based on the effect of osmotically driven water flux between a droplet containing a living cell toward the surrounding empty droplets, within a concentrated inverse emulsion [117].

Intestinal enteroids are ex vivo primary cultured single-layer epithelial cell spheroids. Measurement of enteroid swelling in response to secretagogues has been applied to testing of drug candidates for cystic fibrosis and secretory diarrheas. Special microfluidics were developed for the immobilization of enteroids and the measurement of volume changes. In these settings, enteroids are trapped in a "pinball machine-like" array of polydimethylsiloxane slots. Volume changes of unlabeled enteroids were studied by imaging of an extracellular, high-molecular weight fluorescent dye [67]. For building of high-fidelity organoids, a droplet microfluidic system was developed for fabrication of massive 3D culture and formation of functional organoids derived from human-induced pluripotent stem cells (hiPSCs) [118].

A microfluidics platform that allows for the study of fast kinetics of the cell volume during osmotic challenge has been developed. Cells expressing a cytoplasmic, volume-sensing fluorescent dye were rapidly subjected to an osmotic gradient by solution mixing inside a ~0.1 nL droplet surrounded by oil. Solution mixing time was <10 ms. Osmotic water permeability was deduced from a single, time-integrated fluorescence image of an observation area in which time after mixing was determined by spatial position. Water permeability was accurately measured in aquaporin-expressing erythrocytes with half-times for osmotic equilibration down to <50 ms [66]. Also, microfluidic system where cells were immobilized on poly-D-lysine-coated cover glass was used to study membrane transport properties of the human UT-7/TPO megakaryocytic cell line [119]. Development of microfluidics platform to advancing technology of microflow cytometry faces challenges in focusing on the particles to be analyzed in the microfluidic channel. By utilizing the properties of a laminar fluid flow in microfluidic devices, fluids can be used to unsheathe and focus other fluids without mixing, and thus to create confined streams within a channel. Currently, it is important to demonstrate that the microflow systems provide data of the same quality as laboratory systems [120].

Using a microfluidic device, the water permeability (Lp) of vaginal $CD3^+$ T cells and $CD14^+$ macrophages was determined relative to the initial cell volume. The channel height of the microfluidic perfusion chamber was 15 μm to accommodate the monolayer expected cell size of 8–12 μm, and at the edge of the channel the height was 3 μm to trap cells while allowing fluid flow. To evaluate cell volume a cell image was recorded by a CCD camera at 24 frames/second until osmotic equilibrium was obtained, (within 2 min) [121].

A microfluidics platform for the measurement of water transport across a conventionally cultured epithelial monolayer on a porous filter requires a single image from a standard laboratory fluorescence microscope [122]. Osmotic water permeability was calculated from the steady-state concentration profile along the length of the channel of a membrane-impermeant fluorescent dye in the perfusate, in which an osmotic gradient was imposed by an anisosmolar solution overlying the epithelial monolayer (Fig. 24.6). A comprehensive overview of static transwell cultures and how they could be used to evaluate

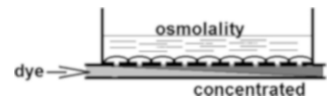

Fig. 24.6 Schematic of microfluidic perfusion chamber for static transwell culture. Concentration profile of a cell-impermeant dye along the length of the channel could be used for the measurement of water transport across a cultured epithelial monolayer on a porous filter to evaluate the epithelial water permeability. (Presented in the paper of S. Youhanna and V. M. Lauschke [123])

the epithelial permeability is presented in the paper of Youhanna and Lauschke (2021) [123].

24.3 Water Permeability Methods for In Vivo and Ex Vivo Studies

Experimental data on fluid-absorbing epithelia are obtained in in vivo and ex vivo experiments. An example of water permeability measurements of more complex systems than living cells involve in vivo studies of bodily cavities that contain certain physiological amount such as the pleural or peritoneal cavity. The balance of fluid turnover of the pleural compartment occurs in normal chest physiology and is changed in pathophysiological conditions associated with pleural effusions, thus excess accumulation of water, solutes, proteins, and cells within the cavity. Comparative physiological studies were performed on wild-type vs. AQP1 null mice. Osmotically driven water transport was measured in anesthetized, mechanically ventilated mice from the kinetics of pleural fluid osmolality after instillation of hypertonic or hypotonic fluid into the pleural space. Water permeability is determined assuming that the pleural barrier is a single barrier separating the pleural cavity and the blood compartment. The volume flux across the pleural mesothelium (J_v) is given by $J_v = P_f V_w S[C_o - C_i(t)]$, where V_w is the partial molar volume of water (18 cm^3/mol), S is pleural surface area (cm^2), C_o is plasma osmolality (320 mosmol/kgH_2O), $C_i(t)$ is pleural fluid osmolality, and P_f is the osmotic water permeability. The same experimental model used for measurement of isosmolar pleural fluid clearance and modeling of hydrostatically driven pleural effusions. Hydrostatically driven pleural fluid accumulation was induced by bilateral renal artery ligation and infusion of saline in the peritoneal cavity (40% body wt) to induce acute volume overload. The pleural fluid was collected in all cases and analyzed accordingly reaching the conclusion that AQP1-mediated water permeability is critical only in cases of aniso-osmolality [124]. This type of experimental approach involving or not AQP knockout animals and cavitary effusion formation is useful in a range of medical disciplines for the elucidation of water permeability kinetics of water transporting membranes in the whole body level. The rate of cavitary fluid absorption can be used for identifying therapeutic drugs for effusion resolution [124–128].

Regarding the ex vivo assessment of water permeability in isolated tissues the application of the Ussing System has provided a lot of insight to such processes. Initially several isolated tissues from frog have been studied for their water transporting capacities, such as frog skin as well as frog and toad urinary bladder [129–133]. The activation energy (E_a) for the diffusion of water across the epithelial cell layer of the toad bladder was determined in the absence and presence of vasopressin. It was shown that the hormone did not influence the activation energy, thus the conclusion that vasopressin was not changing the molecular structure of water channels was made [130]. More recently the transport properties of pleural, peritoneal, pericardial, and leptomeningeal membranes in human and sheep models in ex vivo studies are investigated using the Ussing system [134–139]. Ussing Chamber experiments using human and animal transporting epithelia have established many of the biophysical processes involved in the regulation of liquid homeostasis. For understanding the physiology that regulates biophysical processes involved in epithelial liquid homeostasis, a biophysical model for water and ion transport to quantify the permeabilities of all pathways apical, basolateral, and paracellular was created using Ussing Chamber data reported in the literature [140].

Another imaging method that was developed to map thickness changes in viable spinal cord and brain slices was applied to measure osmotically induced water transport in spinal cord slices from wild-type and aquaporin knockout mice. Changes in the slice thickness were mapped from the amount of light passing through a thin (~100 μm) layer of perfusate bathing the slice, in which hemoglobin (6 mg/ml) was added as an inert absorbing chromophore. In response to osmotic challenges imposed by changing perfusate osmolality by steps of 100 mOsm, the

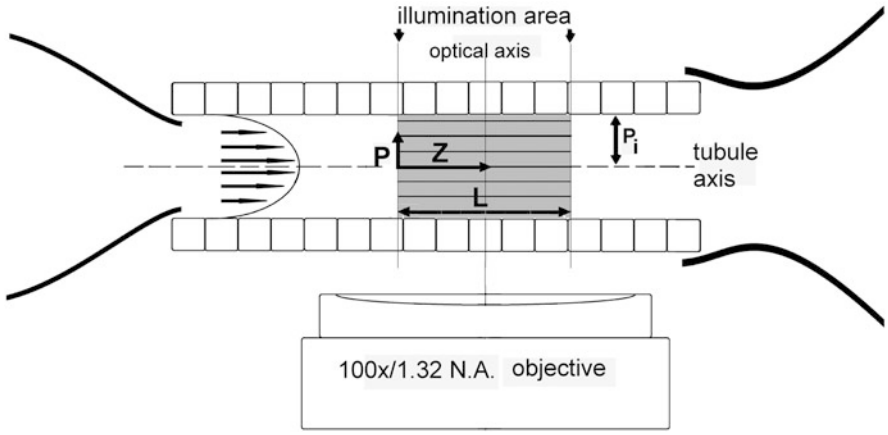

Fig. 24.7 Schematic of a cross-section of an isolated perfused tubule demonstrating the illumination and fluorescence collection geometries. The gray represents the volume of the illuminating fluorophore while P and Z are the radial and axial coordinates, L is the length of the illuminated area (defined by the illuminating window) while Pi is the inner radius of the tubule. The horizontal lines in the illuminating volume represent the stack of optical sections used to assess the spatial distribution of the light intensity and photobleaching the tubule

transmitted light intensity changed reversibly with approximately mono-exponential kinetics whose initial rate depended upon the position in the slice [141].

Understanding the mechanisms of fluid absorption and secretion by the kidney epithelia has a long story of intense studies on water transport in isolated perfused tubules [142]. In this type of experiments, the tubule segments were perfused in vitro in a modified version of the technique described by Burg et al. [143]. The tubules were placed into a temperature-controlled flow chamber with a glass bottom and were observed with an inverted compound microscope. The upstream portion of the tubule was drawn into a holding pipette, which contained a concentric pipette. The downstream end of the tubule was drawn into a holding pipette that had inside a tip of collecting pipette. The theory and experimental design of this class of studies were described by Schafer et al. [144]. The osmotic P_f was calculated from the net transepithelial fluid flux (J_v) determined in the presence of an imposed osmolality gradient. Measurements were conducted by collecting the perfusate, which contained an impermeant volume marker. The marker could be a radioisotope [145] or a fluorescent probe using continuous fluorescence measurement and photo bleaching as shown in Fig. 24.7 [146]. The fluorescein sulfonate concentration was measured in the perfusate and the collected fluid using a continuous flow fluorometer (coefficient of variation, 2%) [147]. The theoretical basis for P_f calculation in these experimental approaches is the equation formulated by Al-Zahid et al. [148].

24.4 Conclusion

In conclusion a wide variety of methods for measuring osmotic water permeability have been developed, each one of which takes advantage of distinct biophysical principles of the process or the instrumentation. The selection of the most suitable method for water permeability measurement depends on the purpose of physiological study that will be designed. When measuring accuracy and high resolution of water transporting pathways across cell membranes or in contralateral membranes of polarized cells, the experimental approach needs to be carefully

planned based on the most suitable method and instrumentation. However, fundamental limitations are imposed by the unstirred layer effects and the complex composition of tissues where the absolute water permeability coefficients and the activation energies cannot be even defined. Still, comparative measurements may be informative for understanding the physiological and pathophysiological mechanisms involved. An important application of the methods for water permeability measurement is in high-throughput screening assays for the discovery of modulators of water and electrolyte balance in range from a cell to a whole organism.

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