Immunohistochemical identification of aquaporin 2 in the kidneys of wild boars (Sus scrofa)

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Abstract: Mammalian aquaporins (AQPs) are a family of at least 13 small, integral membrane proteins expressed in various fluid-transporting epithelial cells. Aquaporin 2 (AQP2) is mainly expressed in the kidneys, where it plays an important role in the reabsorption of water from the renal tubular fluid to concentrate urine. Studies of AQP2 expression in the kidneys have been performed in humans, monkeys, sheep, dogs, rabbits, rats, mice, and cattle. We analyzed the expression of AQP2 in the kidneys of wild boars (Sus scrofa). AQP2 was localized in the principal cells of collecting ducts in medullary rays penetrating the renal cortex and in the collecting duct of the renal medulla. The presence of AQP2 was confirmed mainly in the apical plasma membrane but also in the intracellular components and basolateral membrane of the principal cells of the collecting ducts. Due to the key role of AQP2 in the regulation of water balance in mammals and the lack of studies in the literature concerning analysis of this protein in wild boars, this work completes the present state of knowledge by providing new information on immunolocalization and immunoexpression of aquaporin 2 in Sus scrofa kidneys.

Key words: Aquaporin 2, wild boars, immunohistochemistry, kidney

1. Introduction

Aquaporin 2 (AQP2) belongs to a large family of water channel proteins (WCPs), small tetrameric transmembrane proteins with a molecular mass of about 30 kDa. WCPs are better known as aquaporins (AQPs), as they have a specific three-dimensional structure with a pore that can be permeable to water and other small molecules, such as glycerol and urea (Nielsen et al., 2000; Fenton and Knepper, 2007; Benga, 2009; Gomes et al., 2009). The discovery of the first aquaporin in the human erythrocyte membrane by Peter Agre and coworkers in the 1980s revealed the previously unknown mechanism of rapid water flow across cell membranes (Agre et al., 1993). To date, at least 300 aquaporins have been discovered, and their presence has been confirmed in virtually all organisms examined. At least thirteen different aquaporin isoforms have been identified in mammals, and they are expressed in various fluid-transporting epithelial cells (Holmes, 2012). Aquaporins include three subfamilies: (1) aquaporins that are permeable only to water molecules (AQP0, AQP1, AQP2, AQP4, AQP5, and AQP6); (2) aquaglyceroporins, permeable to water and other small molecules (AQP3, AQP7, AQP8, AQP9, and AQP10); and (3) “unorthodox” aquaporins or “superaquaporins”, which either have a low homology with conventional aquaporins or differ functionally from both aquaporins and aquaglyceroporins (AQP11 and AQP12) (Hub and Groot, 2008; Benga, 2009; Kortenoeven and Fenton, 2014).

The first aquaporin discovered, AQP2, was found in 1993 by Fushimi and coworkers in rat renal collecting tubules (Fushimi et al., 1993). In subsequent years, the presence of this protein was also confirmed in human and mouse kidneys. In the different sections of renal tubules 7 additional isoforms of AQPs (AQP1, AQP3, AQP4, AQP6, AQP7, AQP8, and AQP11) have been identified, but AQP2 is of particular importance for the regulation of water balance according to body needs. It is this protein that is fused to the apical plasma membrane in response to stimulation by antidiuretic hormone vasopressin (AVP) in the collecting duct principal cells, thereby increasing membrane permeability to water. As a result, low amounts of concentrated urine are excreted (Noda and Sasaki, 2005).

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The great importance of AQP2 for renal water resorption and maintaining proper water balance in the body can be seen in patients suffering from nephrogenic diabetes insipidus (NDI). In the course of the first or second type of NDI resulting from mutations in the V2R gene (the second type of receptor for vasopressin) or mutation in a gene encoding AQP2, a loss of water retention in the renal collecting tubules occurs, which leads to increased excretion of large volumes of unconcentrated urine (Nielsen et al., 2002; Boone and Deen, 2008; Holmes, 2012).

The essential role played by AQP2 in fluid balance and urine concentration as well as its significant involvement in cell migration and epithelial morphogenesis has caused increased interest in this protein. The studies conducted in humans and laboratory animals have been extended to other species. The presence of this protein in the kidney and its potential role in renal water retention has been confirmed in animals such as monkeys, dogs, rabbits, cattle, horses, and mini pigs (Lofving et al., 2000; Floyd et al., 2007; Bauchet et al., 2011; Mobasheri et al., 2011; Michalek et al., 2014; Lou et al., 2014). To date, however, this type of study has not been performed in free-living animals, including wild boars.

*Sus scrofa*, the wild boar, is a species of large land mammal of the order Artiodactyla. In Europe it is the only suid species living in the wild. The current natural range of the wild boar is primarily the northern areas of Africa and central and southern Eurasia. Wild boars are omnivorous animals, but 90% of their food comes from plants, and approximately 10% is of animal origin (Pilarczyk et al., 2010). The suids (*Suidae*) have multipyramidal (multipapillary) kidneys but without the external lobation typically found in the bovine species. Some pyramids are simple, while others, as a result of fusion of two or more primitive separate pyramids, are compound. The apical portion of the pyramid, called the papilla, projects into the renal pelvis or its ramifications, the latter referred to as calyces. Papillae of simple pyramids are narrow and conical, whereas the compound pyramids, often located in the area of the renal poles, are broad. The suids have approximately 8–12 papillae per kidney. Porcine kidneys contain well over 1 million nephrons. Collecting ducts of the kidney have their openings at the tips of the papillae (Drolet, 2012). The daily urine output of *Sus scrofa* depends on many factors, among which diet, fluid intake, ambient temperature and humidity, and size and weight of the animal should be listed. The mean specific gravity of urine in adult swine is about 1.020, one of the lowest in comparison to farm animals (Ruckebusch et al., 1991), while the maximum urine concentration is about 1.1 osmol/L (Ketz, 1960). We think that, as in humans and other animal species, AQP2 plays a key role in water reabsorption in wild boar kidneys. In the literature there are no data concerning this protein in free-living *Sus scrofa* and so we conducted a study aimed at: (i) identification and (ii) localization of AQP2 in the kidneys of this animal species.

### 2. Materials and methods

We analyzed six kidneys obtained from 2- to 3-year-old male wild boars from northwestern Poland. Wild boars were shot during the 2013–2014 hunting season. Immediately after shooting, the kidneys were removed, cut into blocks, fixed in 4% buffered formalin, embedded in paraffin blocks, and sectioned at 2 µm on a rotary microtome. To conduct morphological and histochemical studies, sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). Sections were examined using an Olympus BX41 light microscope coupled to an Olympus digital camera (Olympus Optical Co., Tokyo, Japan).

For immunohistological studies, sections were deparaffinized using xylene, then rehydrated through graded ethanol solutions. Sections were boiled in a Target Retrieval Solution (DAKO, Denmark) with pH 9.0. The activity of endogenous peroxidase was blocked by peroxidase blocking solution (Dako, Denmark) for 10 min in a humid chamber. Subsequently, the sections were incubated in a humid chamber with rabbit polyclonal primary antibody anti-AQP2 H7661 (Department of Biomedicine, Aarhus University, Denmark) diluted 1:900 in PBS with 0.1% BSA and 0.3% Triton X-100 overnight at 4 °C. Next, the sections were incubated with complex-containing secondary antibody conjugated with horseradish peroxidase (Dako REAL EnVision detection system peroxidase/DAB+, rabbit/mouse; Dako, Denmark). For the visualization immunohistochemical reaction DAB (Dako, Denmark) was used. The sections were counterstained with hematoxylin, dehydrated, and coverslipped. After each step in the above procedure, the sections were rinsed with PBS. They were examined using an Olympus BX41 light microscope coupled to an Olympus digital camera (Olympus Optical Co., Tokyo, Japan). Specificity of immunostaining was confirmed by following the above procedures in the absence of the primary antibody (negative control).

### 3. Results

Figures 1A–1J present the results obtained. In the preparations stained with H&E and PAS, normal and typical structures of the renal cortex and medulla were found in the animals examined (Figures 1A–1E). Glomeruli, proximal tubules with the brush border directed into the lumen, and distal tubules devoid of the brush border were observed in the renal cortex (Figures 1A, 1B, and 1D).
In addition, medullary rays were also visible penetrating through the renal cortex (Figure 1B). Collecting ducts with high epithelium and wide lumen were found in the medulla of the wild boar kidneys (Figures 1C and 1E).

Figures 1F–1I present immunoperoxidase labeling for aquaporin 2 in the sections of paraffin-embedded kidneys from wild boars. The presence of AQP2 was found both in the collecting ducts of medullary rays penetrating the renal cortex (Figures 1F and 1G) and in the collecting ducts of renal medulla (Figures 1H and 1I) of the kidneys. Strong staining of aquaporin 2 is mainly visible in the apical membrane of the collecting duct principal cells. Weak immunohistochemical staining was also present in the intracellular vesicles and the basolateral membrane (Figure 1I).

4. Discussion
The results of the current study clearly confirmed the presence of AQP2 in the principal cells of kidney collecting ducts in wild boards. Comparable results have been reported in the kidneys of rats, mice, rabbits, dogs, monkeys, horses, and mini pigs (Nielsen et al., 1993; Kishore et al., 1996; Loffing et al., 2000; Rojek et al., 2006; Eskild-Jedsen et al., 2007; Floyd et al., 2007; Bauchet et al., 2011). To our knowledge, the present study is the first report of AQP2 localization and expression in the kidneys of Sus scrofa.

AQP2 was immunolocalized in transverse sections of the renal cortex and medulla only in the cuboidal epithelium cells of the collecting ducts in the medullary rays and medullary layer of the wild boar kidneys. Particularly strong immunoreactions were observed in the apical membrane of the collecting duct cells. It is generally known that in mammals AVP is the main factor inducing the increase in AQP2 expression in the apical plasma membrane of the collecting duct cells. Vasopressin is released from the posterior pituitary under the increase of plasma osmolality (Sasaki, 2012; Takata et al., 2008). In the absence of vasopressin stimulation, aquaporin 2 is mainly localized in the endosomal vesicles of the trans-Golgi network (Moeller et al., 2012). Binding of AVP to vasopressin type 2 receptor (V2R), located on the basolateral membrane of the collecting duct principal cells, activates two type III and IV adenylate cyclases (AC).

**Figure 1.** Renal histology and immunohistochemical localization of AQP2 in the kidneys of wild boars (Sus scrofa). A, B, C – H&E staining. A – Renal cortex; glomeruli (black arrow), proximal tubules (P), distal tubules (D). B – Renal cortex; medullary rays (black arrow). C – Renal medulla; collecting ducts (CD). D, E – PAS staining. D – Renal cortex; glomeruli (black arrow), proximal tubules (P), distal tubules (D). E – Renal medulla; collecting ducts (CD). F, G, H, I – Immunolocalization and immunexpression of AQP2. F, G – Renal cortex; expression of AQP2 in the medullary rays (black arrow). H – Renal medulla; expression of AQP2 in the collecting ducts (CD). I – Renal medulla; strong expression of AQP2 in the apical plasma membrane of the collecting duct principal cells (black arrow head). Weak expression of AQP2 in the intracellular vesicles (red arrow) and basolateral membrane (black arrow). J – Renal medulla; in the absence of the primary antibody no reaction product is observed (negative control).
The active form of PKA phosphorylates Ser256, which is located in the cytoplasmic C-terminal region of AQP2 monomers. However, this site is also a substrate for other basophilic protein kinases (Wilson et al., 2013). Hoffert et al. (2008) stated that phosphorylation of at least three AQP2 monomers is necessary to start its translocation from vesicles and the consequent increase in the expression of this protein in the apical plasma membrane. The latest research in laboratory animals showed that the following serine sites were also phosphorylated: Ser261, Ser264, and Ser269 (Fenton et al., 2008; Hoffert et al., 2008). The level of serine phosphorylation in response to vasopressin increased at sites 256, 264, and 269, while it decreased at Ser261. The level of phosphorylated Ser261 is high without AVP stimulation (Moeller and Fenton, 2012). The function of phosphorylation at these sites has not been fully explained. However, it has been proved that phosphorylation of Ser256 is essential for the trafficking and fusion of AQP2 with the apical plasma membrane. It has also been found that different AQP2 phosphoforms are located in various intracellular organelles. AQP2 phosphorylated at Ser269 (AQP2pS269) was only detected in the apical membrane and was not observed in any intracellular organelles (Moeller et al., 2009). AQP2pS256 was found both in the apical membrane and intracellular vesicles (Christensen et al., 2000). Phosphorylated Ser261 (AQP2pS261) was mainly observed in the intracellular components of the Golgi apparatus and lysosomes (Hoffert et al., 2007). AQP2 phosphorylated at Ser264 is present in the apical and basal cellular membranes (Fenton et al., 2008).

We have found a weak but very distinct staining of AQP2 along the basolateral membrane of the principal collecting duct cells, especially in the inner medullary collecting duct principal cells. Weak, positive staining for AQP2 was also observed in the basolateral membrane of collecting duct cells was also reported by Fushimi et al. (1993), Marples et al. (1995), and Michałek et al. (2014). According to these authors, the location of AQP2 in the basolateral membrane supports an exit pathway for the water across this membrane where abundant expression of AQP3 and AQP4 is also observed. Similar results were obtained in our research. We have found a weak but very distinct staining of AQP2 on the basolateral membrane of the principal collecting ducts of the wild boar kidneys. The results of the present study clearly indicate that in this species AQP2 plays an important role not only in the transport of water from the luminal fluid into the cell but also from the cell into the surrounding interstitial tissue. However, to fully explain the participation of AQP2 in the regulation of water balance in Sus scrofa, more detailed analyses are required. Unfortunately, conducting this type of research is difficult due to the wild nature of these animals. Nevertheless, we hope that the results of our work will be a contribution to the development of knowledge in this field.

In summary, AQP2 in wild boars (Sus scrofa) is located in the kidney principal collecting duct cells. The presence of AQP2 was confirmed mainly in the apical plasma membrane but also in the intracellular components and basolateral membrane of the principal cells of the collecting ducts. Due to the key role of AQP2 in the regulation of water balance in mammals and the lack of studies in the literature concerning the analysis of this protein in wild boars, this work completes the present state of knowledge by providing new information on immunolocalization and immunoexpression of aquaporin 2 in Sus scrofa kidneys.
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