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Renal Dose Dopamine Mediates the Level of Aquaporin-2 Water Channel (Aqp2) in Broiler Chickens

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ABSTRACT

Aquaporin 2 (AQP2) is a small protein located in the collecting tubules of kidneys; it plays an important role in the concentration and production of urine. The aim of this study was to determine the expression level of the AQP2 gene in the kidney of broiler chickens after the administration of renaldose dopamine. Broiler chickens (25 days-old) were randomly divided into two groups (n=20 per group): intravenous administration of saline solution (control group) or renal-dose dopamine (dopamine group). The expression and localization of the AQP2 gene were evaluated by real-time quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC), respectively. The protein level of AQP2 was analyzed by western blot analysis. The dopamine group presented no significant difference (p>0.05) in the biochemical criterion or mRNA expression of AQP2 compared with the control group. However, AQP2 protein level was significantly reduced (p<0.05) in the membrane of renal tubular epithelial cells. In contrast, protein level was significantly increased (p<0.05) in the cytoplasm of the dopamine group compared with the control group. Moreover, AQP2 protein was apparently more distributed and localized in the cytoplasmic vacuoles than in the membranes of the kidney in the renal-dose dopamine administered chickens group. In conclusion, present findings suggest that renaldose dopamine mediates the level of AQP2 protein via shuttle from the cell membrane to the cytoplasm rather than changing the expression of AQP2 gene to adjust the secretion and absorption of water in kidney.

INTRODUCTION

Aquaporins (AQP) are important integral membrane proteins for rapid and selective transport of water and some small solutes across cell membranes in mammals and plants. AQPs have strong water permeability, and water molecules across renal tubules through various channels (Preston *et al.,* 1992). In the last decade, 13 water-channel proteins were found in mammals, namely AQP0 to AQP12 (Yang, 2000; Verkman, 2002; Hatakeyama *et al.,* 2001). AQPs present an open pore that can control the channel insertion and retrieval from the cell surface for transmembrane water transport (Marinelli *et al.,* 1997; Nielsen *et al.,* 1993). AQP2 (water channel of the collecting duct, WCH-CD) is one of the best-characterized water channels located in the kidney collecting tubules and duct, distributed in cell apical plasma membrane and intracellular vesicles (Verkman, 2002). AQP2 water channel proteins are affected by hormones in the body, and also are the main target of the hormone arginine-vasopressin (AVP) for regulation of collecting duct water permeability (Deen *et al.,* 1994, Digiovanni *et al.,* 1994). Various studies have confirmed that altered expression levels and apical targeting of AQP2 play a critical role in water balance

disorders in animals (Nielsen *et al.,* 2002). Inadequate cell-surface expression of AQP2 results in nephrogenic diabetes insipidus, whereas increased AQP2 cell surface expression and excessive water reabsorption are observed in congestive heart failure and preeclampsia in animals (Marples *et al.,* 1995).

Dopamine (DA) is an important catecholamine neurotransmitter in mammals, and it is also synthesized in plants and tubule cells. DA is an important sodium homeostasis regulator, and plays a critical role in memory and behavior. Renaldose DA can induce natriuresis and diuresis via acute inhibition of most sodium reabsorption by the renal tubules, and it is widely used to improve renal function in the treatment of a variety of renal diseases (Jeon *et al.,* 2014). Based on pharmacological characteristics, DA is divided into two subtypes of receptors (D1 and D2) and signal transduction pathways. D1 dopamine receptors play an important role in diuresis via combine with dopamine. As a diuretic and natriuretic hormone, dopamine can act on renal tubules to inhibit sodium reabsorption (Nielsen & Pedersen, 1997).

AQP2 has been investigated in several animal models of kidney diseases and kidney tubule defects. In addition of maintaining and regulating water balance, AQP2 is also important for maintaining the structural and functional integrity of the kidney tubules. Identifying changes in AQP2 expression levels may allow understanding the function of DA in water-electrolyte metabolism. In this context, the reviewed literature shows that there is little research conducted on the role of dopamine on the expression of genes encoding AQP2 in the collecting ducts of broiler chickens. The objective of the present study was to explore the influence of dopamine on the AQP2 gene expression and protein level to understand the mechanism of water and salt metabolism regulation by dopamine in broiler chickens.

MATERIALS AND METHODS

Experimental design and intravenous administration methods

Forty 1-day-old AA broiler chicks were purchased from a commercial hatchery (Chia Tai Animal Husbandry co., ltd., Wuhan, China), and were reared under recommended temperature and standard hygienic conditions. Birds were fed a regular diet *ad libitum*.

After 25 days, the birds were randomly and equally divided into two groups and anesthetized. The control group (n=20) received intravenous saline solution by micro-injection (Hong zhan SK-500II, Shenzhen) at constant speed (2 mL/h) for 4 hours, whereas the dopamine group (n=20) received intravenous renal-dose dopamine (5 μg/kg/min) by micro-injection at constant speed (2 mL/h) for four hours. At the end of the injection period, all birds from both groups were sacrificed by cervical dislocation. Blood was collected. Blood samples were centrifuged at 3000×g for 20 min for the separation of serum, and stored at -20 °C until subsequent use. Kidney samples were removed, and fixed in 4% paraformaldehyde. Some of the kidney tissues were dissected out, immediately frozen in liquid N_2, and then stored at -70 °C until analyses.

Biochemical criterion determination

The concentration of sodium, potassium, chlorine, creatinine, and urea nitrogen in the serum were measured using a semi-automatic biochemical equipment (COULTER®LH 750, Guangdong).

Immunohistochemistry (IHC)

Formalin-fixed kidney tissues were embedded in paraffin, and 4-μm thick histological sections were cut and mounted on polylysine-coated slides. After de-waxing with xylene and antigen recovery, the slides were washed thrice in peroxidase blocking solution (DakoCytomation, Carpinteria, CA, USA). The slides were then incubated with rabbit anti-goat polyclonal antibodies (KPL) at 1:600 dilution at 4 °C overnight (Tuojie Biological Technology Co., Ltd, Wuhan). After washing with PBS wash buffer, sections were incubated at 37 °C for 1.5 h with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Tuojie Biological Technology Co., Ltd, Wuhan). The immunolabeled slides were examined under a microscope (Olympus CX31; Olympus, Japan). Primary antibodies were removed from the negative control samples.

RNA extraction and real-time quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from the kidney tissues collected from each group of birds using the TIANamp Virus DNA/RNA Kit (Tian Gen, China), and a final volume (20μL) of total RNA was converted into cDNA by using a first-strand reverse transcription cDNA kit (Tian Gen, China) according to manufacturer's instructions. Based on the published AQP2 gene sequences in the GenBank database, we designed a pair of primers with the aid of the software Primer Premier (version 5.0) and synthesized by Wuhan Qingke Biotechnology Co., Ltd (Wuhan, China). The RT-qPCR was performed

in quadruplicate using the Step One-Plus ™ Real-Time PCR System (Applied Biosystems) with specific forward (5'-TGCCGTTGGACATCTCCTTGG-3') and reverse (5'-CCTCTCCGAGAAGGTTTTGGA-3') primers for the AQP2 gene expression analysis. The RT-qPCR mixture contained 6.8 µL RNase-free water, 2 µL cDNA, 10 µL Tip Green qPCR SuperMix, 0.4 µL Passive Reference Dye, 0.4 µL of each forward and reverse primer (working concentration: 10 µL mol/L) in a 20 µL reaction mixture. All RT-qPCR reactions were performed with following thermal cycling parameters: 95 °C for 30 s, 40 amplification cycles at 95 °C for 8 s, 59 °C for 30 s, and 72 °C for 30 s. All reaction mixtures were normalized against the reference gene GAPDH.

Protein extraction and Western blot

Kidney samples were washed with PBS and homogenized 4-5 times every ten seconds in dissection buffer (500 µL/100mg) (Beyotime Institute of Biotechnology, Hangzhou, China), and then kept for 30 min at 4 °C. The supernatant was obtained after centrifugation at 17,000 × g for 20 min at 4 °C. Protein was separated into membrane protein (pellets) and intracellular protein (supernatant) after centrifugation. The concentration of the total proteins was determined by Coomassie Brilliant Blue g-250 method, and samples were stored at -70 °C for subsequent use.

Protein samples (40 µg) were mixed with the loading buffer and then electrophoretically separated by SDS-PAGE on 12% polyacrylamide gels at 100 mV and transferred at 130 mV for 1 h and 40 min to nitrocellulose filter membranes (Millipore, BioSharp, Anhui, China). The membranes were incubated in 5% skimmed milk at room temperature for 1 h to prevent nonspecific binding sites, and then incubated with goat anti-rabbit primary antibody IgG-FITC (1:5000) overnight at 4 °C (Tuojie Biological Technology Co., Ltd, Wuhan). The membranes were washed 3 times with TBST (tris-buffered saline containing 0.1% Tween 2.0) for 5 min each then incubated with goat anti-mouse secondary antibody (1:5000 dilution) for 30 min at room temperature (Tuojie Biological Technology Co., Ltd, Wuhan). The membranes were again washed four times with TBST. After washing, images were captured with an imaging system (UVP, Upland, CA,USA).

Statistical analysis

Data were submitted to one-way analysis of variance. The Student's t-test was applied to compare the mean differences between control and renal-dose dopamine groups. Differences were considered statistically significant when $p<0.05$. Values are presented as means ± standard error of mean (SEM). All statistical analyses were performed by using the SPSS 19.0 software.

RESULTS

Serum biochemical criterion analysis

Serum concentrations of sodium, potassium, chlorine, creatinine, and urea nitrogen were not statistically different ($p>0.05$) between the control and the dopamine groups (Table 1).

Table 1 – Biochemical criterion analysis of serum between control and renal-dose dopamine group.

Items	Control group	Renaldose dopamine
Sodium (mmol/L)	144.60±2.35	144.80±2.65
Serum potassium (mmol/L)	6.04±0.29	6.07±0.33
Serum chlorine (mmol/L)	107.60±3.47	106.95±3.56
Urea nitrogen (mmol/L)	0.85±0.04	0.85±0.04
Creatinine (µmol/L)	58.838±0.732	58.785±0.733

Immunohistochemical (IHC) evaluation of the kidney

In order to confirm localization of AQP2 gene, immunohistochemical analysis was performed in the kidneys of the control and the dopamine groups. The results revealed that AQP2 was expressed in the collecting-duct epithelial cells and intracytoplasmic vacuoles of the dopamine-injected broilers (Fig. 1B), but not in the control broilers (Fig. 1A).

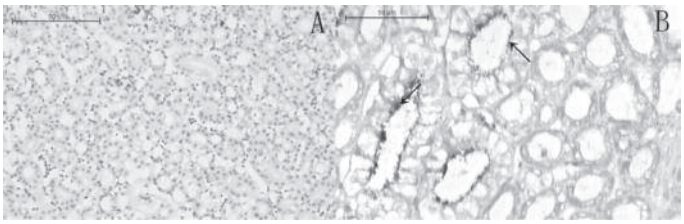

Figure 1 – Expression pattern of AQP2 by immunohistochemistry (IHC) analysis of membrane protein and intracellular protein from broiler chicken kidney tissue. Panel A: negative control; Panel B: AQP2 in collecting duct epithelial cells of medullary or intracytoplasmic vacuoles as compared to renaldose dopamine groups, the antibodies mainly distribute in kidney collecting duct epithelial cells (Arrow) of medullary. (magnification = 40x).

Western blot analysis

Total protein level was analyzed in the kidney of the control and renaldose dopamine groups broilers. The results showed significantly lower ($p< 0.05$) AQP2 protein levels in the membrane of the collecting duct epithelial cells of the kidney of the dopamine group compared with the control group (Fig. 2A). On the other hand, AQP2 levels in intracellular protein were higher ($p<0.05$) in the dopamine group compared with the control group (Fig. 2B).

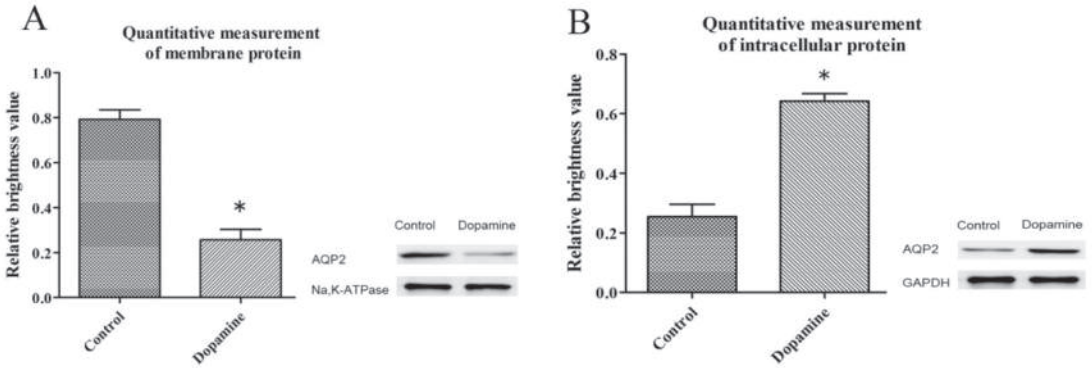

Figure 2 – Western blot analysis and quantitative RT-PCR analysis of AQP2 in membrane (A) and intracellular (B) after injecting the renaldose dopamine compared with control group. *Indicates a significant statistical difference ($p<0.05$). AU=arbitrary units.

AQP2 Expression levels in renal-dose and control group

The mRNA expression of the AQP2 gene in kidney tissue was investigated in control and dopamine groups. The results indicated that AQP2 gene expression was not significantly different between these two groups ($p>0.05$) (Fig. 3).

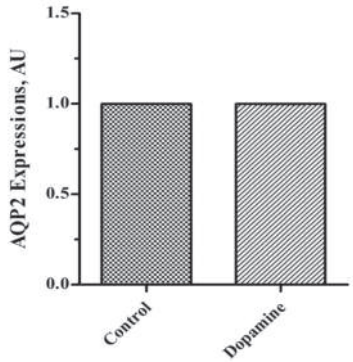

Figure 3 – Effect of renal dose dopamine on AQP2 mRNA expression in control and dopamine administered group. Results are expressed in arbitrary unit (AU).

DISCUSSION

AQP2 is a channel located in the apical membrane of the collecting ducts of the kidneys that responds to vasopressin (VP) regulation both *in vitro* and *in vivo* (Deen *et al.,* 1994; Deen *et al.,* 2002). However, it has also been observed that AQP2 has a bipolarized distribution in the kidney collecting ducts, with both basolateral and apical/subapical expression. However, the physiological function of basolaterally-located AQP2 is not yet understood (Coleman *et al.,* 2000). AQP2 plays an important role in the urine concentration mechanism, as approximately 10% of glomerular filtration liquid is resorbed under the participation of AQP2 (Nielsen *et al.,* 2002; Yang, 2000; Verkman, 2002; Yang *et al.,* 2001). The level of antidiuretic hormone (ADH) is positively correlated with the ratio between collecting tube cell membrane AQP2 and intracellular AQP2. When ADH levels are reduced, membrane AQP2 is transferred to the intracellular milieu, with consequent reduction of membrane AQP2 content, which results in reduced water absorption, and ultimately urine dilution (Frokiser *et al.,* 1998).

Two main types regulate the expression and localization of the sodium transport protein, long-term adjustment and instantaneous adjustment functions. Long-term adjustment mainly consists of adjusting the content and shuttle of these proteins, whereas instantaneous adjustment includes post-translational modifications, which may enhance endocytosis (Yang, 2000). In the present study, mainly concentrated in the content changed of water channel proteins, but the instantaneous adjustment of protein modification and shuttle is rarely reported.

Urea nitrogen is the end product of protein metabolism, and is an indicator of general glomerular function. Serum creatinine is a sensitive indicator of glomerular filtration rate, and directly represent the filtration function of the kidney (Digiovanni *et al.,* 1994). Our study did not find any significant changes in serum sodium, potassium, chlorine, creatinine, or urea nitrogen levels, indicating that glomerular filtration rate did not increase. This suggests that the effect of renaldose dopamine on natriuresis and diuresis did not occur through changes in the glomerular filtration rate (Verkman, 2002). Therefore, it is speculated that renaldose dopamine influences natriuresis and diuresis mainly through changes in the renal tubular reabsorption function in broilers.

In order to confirm the natriuretic function mechanism of renaldose dopamine in the proximal convoluted tubules, this experiment was conducted to evaluate the expression of AQP2 in the membrane and cytoplasm of collecting duct epithelial cells. The findings showed that renaldose dopamine reduced the

expression of AQP2 in the membrane, while increased its expression in the cytoplasm. Consequently, water absorption was reduced in collecting duct epithelial cells and intracytoplasmic. In addition, no changes in the expression of AQP2 mRNA was found by RT-qPCR analysis, indicating that the continuous perfusion of renaldose dopamine has no influence on the expression of the AQP2 gene. The results indicate that renaldose dopamine does not affect the update of AQP2 protein. During this experiment, the chickens' feces change from soft to clear water as urine volume increased after the injection of renaldose dopamine. Thus, it may be speculated that renaldose dopamine can mediate the shuttle of AQP2 from membrane to the cytoplasm.

Previous studies reported that AQP2 localization in the membrane can be rapidly determined by ubiquitination and dephosphorylation, AQP2 finished endocytosis after dephosphorylation by the proteasome pathway, and returned to the cell membrane via phosphorylation (Deen *et al.,* 1994, Deen *et al.,* 2002). Thus, the rapid internalization of the water channel protein can reduce renal tubular reabsorption of water and increase the velocity of urine formation (Kamsteeg *et al.,* 2006). Therefore, this study indicates that the changes in natriuresis and diuresis promoted by renaldose dopamine are not influenced by the glomerular filtration rate, but by the inhibition of water reabsorption in the renal tubular epithelial cells of broiler chickens.

In conclusion, our study indicates that the effect of dopamine on natriuretic diuresis occurs mainly by the instantaneous adjustment rather than by the long-term adjustment of the AQP2 shuttle in broiler chickens.

ETHICS APPROVAL

All the experimental procedures were conducted according to the guidelines of the Animal Welfare and Research Ethics Committee of Huazhong Agricultural University (Wuhan, China).

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