

## Altered Renal Expression of Aquaporin-2 Water Channels in Rats with Experimental Two-Kidney, One Clip Hypertension

The present study was aimed at examining the regulation of aquaporin (AQP)-2 water channels in the kidney in two-kidney, one clip (2K1C) hypertension. Rats were made 2K1C hypertensive for 6 weeks, and their expression of AQP2 channel proteins was determined in the clipped and contralateral kidneys. To examine the upstream affecting AQP2 channels, adenylyl cyclase activity was also determined. Along with the hypertension, in the clipped kidney, the abundance of AQP2 proteins was significantly decreased in the cortex, outer and inner medulla, while their trafficking remained unaltered. Concomitantly with the reversal of the blood pressure at 24 hours following removal of the clip, the AQP2 abundance also returned to the control level. The arginine vasopressin-evoked generation of cAMP was decreased in the clipped kidney, which again was reversed to the control level following removal of the clip. In contrast, the expression of AQP2 channels as well as the activity of adenylyl cyclase remained unaltered in the contralateral kidney. These results indicate an altered regulation of AQP2 water channels in the clipped kidney in 2K1C hypertension.

**Key Words :** Two-Kidney, One Clip Hypertension; Aquaporins; Adenylyl Cyclase

JongUn Lee, YoonWha Oh, Soo Wan Kim

Department of Physiology and Research Institute of  
Medical Sciences, Chonnam National University  
Medical School, Kwangju, Korea

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### Address for correspondence

JongUn Lee, M.D.  
Department of Physiology, Chonnam National  
University Medical School, 5 Hak-dong, Dong-gu  
Kwangju 501-746, Korea  
Tel : +82.62-220-4262, Fax : +82.62-232-1242  
E-mail : julee@chonnam.ac.kr

### INTRODUCTION

Two-kidney, one clip (2K1C) hypertension has been primarily attributed to an enhanced activity of the renin-angiotensin system, in which the increased circulating level of angiotensin II results in increased vasoconstriction and total vascular resistance. However, the urinary excretion of sodium and water is also altered in the clipped and contralateral kidneys (1, 2), and the total urine volume is increased in this model of hypertension (3). Mechanisms underlying the altered renal handling of sodium and water have not been established.

Recent advances in molecular biology have shown existence of aquaporin (AQP) channels that allow rapid movement of water across the permeable epithelia. They have a unique tissue distribution with only rare overlap among the multiple isoforms. In the kidney, at least six isoforms of AQP (AQP1, AQP2, AQP3, AQP4, AQP6, and AQP7) are expressed (4-6). Among them, AQP2 is mainly expressed in the collecting duct principal cells, not only in the apical domain but also in the subapical vesicles. Its physiological function is short-term and long-term regulated by arginine vasopressin (AVP) via V2 vasopressinergic receptors that are coupled to cAMP second-messenger system (7, 8).

Certain pathophysiological states associated with an altered urinary concentration have been causally related to an altered regulation of AQP channels in the kidney (9-13). The present study was aimed at examining the hypothesis that the altered urinary excretion is related to an altered regulation of AQP channels in 2K1C hypertension. Specifically, the expression and trafficking of AQP2 water channels in the kidney were determined in rats experimentally made 2K1C hypertensive. To further examine the upstream affecting AQP2 water channels, adenylyl cyclase activity was also determined.

### MATERIALS AND METHODS

#### Animal preparation

Male Sprague-Dawley rats weighing 160-190 g were used. The experimental procedure was approved by Institutional Review Board. To induce 2K1C hypertension, they were clipped at the left renal artery with a silver clip having an inner gap of 0.25 mm under anesthesia with ketamine (50 mg/kg, i.p.). Sham-clipped rats served as control. Six weeks after the clipping, systolic blood pressure was mea-

sured using an automated tail-cuff method. The arterial clip was then removed under ketamine anesthesia. Twenty-four hours after the unclipping, systolic blood pressure was again measured to confirm that the blood pressure returned to the control level. The rats were then killed by decapitation under a conscious state. The kidneys were rapidly removed and stored at  $-70^{\circ}\text{C}$  until assayed.

#### Protein preparation and Western blot analysis

The cortex, outer medulla, and inner medulla of the kidney were dissected and homogenized at 3,000 rpm in a solution containing 250 mM sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 mM Tris-HCl buffer, at pH 7.6. Large tissue debris and nuclear fragments were removed by two low speed spins in succession (1,000 *g*, 10 min; 10,000 *g*, 10 min). Protein concentrations were measured by bicinchonic acid assay kit (BioRad; Hercules, CA, U.S.A.). Protein samples (cortex, 100  $\mu\text{g}$ ; outer medulla, 12  $\mu\text{g}$ ; inner medulla 6  $\mu\text{g}$ ) were loaded and electrophoretically size-separated with a discontinuous system consisting of 12.5% polyacrylamide resolving gel and 5% polyacrylamide stacking gel. The proteins were then electrophoretically transferred to a nitrocellulose membrane at 20 V overnight. The membranes were washed in Tris-based saline buffer (pH 7.4) containing 0.1% Tween-20 (TBST), blocked with 5% nonfat milk in TBST for 1 hr, and incubated with affinity-purified anti-rabbit polyclonal AQP2 (1:750) antibodies (Alomone Lab; Jerusalem, Israel) in 2% nonfat milk/TBST for 1 hr at room temperature. The membranes were then incubated with a horseradish peroxidase-labeled goat anti-rabbit IgG (1:1,200) in 2% nonfat milk in TBST for 2 hr. The bound antibody was detected by enhanced chemiluminescence (Amersham; Little Chalfont, Buckinghamshire, UK) on hyperfilm. The relative protein levels were determined by analyzing the signals of autoradiograms using the transmitter scanning videodensitometer.

#### Differential centrifugation

To separate the apical membrane-enriched and subapical vesicle-enriched fractions, a differential centrifugation was done as described by previous investigators (14). The homogenates of the cortex, outer medulla, and inner medulla were separately centrifuged at low-speed spins (1,000 *g* for 10 min) to remove cell debris and nuclear fragments. The supernatant was centrifuged at 17,000 *g* for 20 min to yield membrane-enriched pellets (high-density fraction, HD). The supernatant was centrifuged again at 100,000 *g* for 1 hr to obtain a vesicle-enriched pellet (low-density fraction, LD). Total protein of 10  $\mu\text{g}$  was loaded per lane in HD and LD fractions, respectively. Comparing the magnitude of their expression in the two fractions assessed the trafficking

of AQP2, in which a decrease in the ratio of HD/LD represents an inhibited trafficking.

#### Membrane preparation and adenylyl cyclase activity

The membrane preparation was obtained as described previously (10). The renal medulla was separated. They were homogenized in ice-cold homogenizing buffer (50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 0.2 mM PMSF, and 250 mM sucrose), and centrifuged at 1,000 *g* and 100,000 *g* in succession. The resulting pellet was used as membrane preparation. Protein concentrations were measured by bicinchonic acid assay kit (BioRad; Hercules, CA, U.S.A.).

The catalytic activity of adenylyl cyclase was assayed as described by previous investigators (15), with a slight modification. Adenylyl cyclase activity was examined using AVP, which was used to provoke the catalytic unit of adenylyl cyclase via V2 receptors. The reaction was started by adding the membrane fraction, of which protein contents were 20, 10, and 10  $\mu\text{g}$  for the cortex, outer medulla, and inner medulla, respectively, in 100  $\mu\text{L}$  working solution (50 mM Tris-HCl, pH 7.6, containing 1 mM ATP, 20 mM phosphocreatine, 0.2 mg/mL creatine phosphokinase, 6.4 mM  $\text{MgCl}_2$ , 1 mM 3-isobutyl-1-methylxanthine, 0.02 mM GTP). After 15 min, the reaction was terminated by application of cold solution consisting of 50 mM sodium acetate, pH 5.0, and centrifuged at 1,000 *g* for 10 min at  $4^{\circ}\text{C}$ .

cAMP was measured in the supernatant by equilibrated radioimmunoassay. Iodinated 2-*o*-monosuccinyl-adenosine 3', 5'-cyclic monophosphate tyrosyl methyl ester ( $^{125}\text{I}$ -ScAMP-TME) was prepared as described by previous investigators (16). Standards or samples were taken up in a final volume of 100  $\mu\text{L}$  of 50 mM sodium acetate buffer (pH 4.8). Dilute cAMP antiserum (Calbiochem-Novabiochem; San Diego, CA, U.S.A.) and  $^{125}\text{I}$ -ScAMP-TME (10,000 cpm/100  $\mu\text{L}$ ), 100  $\mu\text{L}$  each, were added and incubated for 15 hr at  $4^{\circ}\text{C}$ . The bound form was separated from the free form by charcoal suspension, and the supernatant was counted in gamma counter (Packard Instrument; Meriden, CT, U.S.A.). All samples in one experiment were analyzed in a single assay. Nonspecific binding was  $<2.0\%$ . The 50% intercept was at  $16.5 \pm 0.8$  fmol/tube ( $n=10$ ). Intra- and inter-assay coefficients of variation were  $5.0 \pm 1.2$  ( $n=10$ ) and  $9.6 \pm 1.9\%$  ( $n=10$ ), respectively. Results were expressed as moles of cAMP generated per mg protein per minute.

#### Drugs and statistics

Drugs were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.), unless stated otherwise. Results are expressed as mean  $\pm$  SEM. The statistical significance of differences between the groups was determined using unpaired *t*-test.

## RESULTS

### Systolic blood pressure

Systolic blood pressure measured indirectly at the tail artery in a conscious state was significantly higher in the experimental group than in the control ( $178 \pm 8$  and  $124 \pm 12$  mmHg,  $n=7$  each,  $p<0.01$ ). The blood pressure at 24 hr after removal of the clip fell to  $128 \pm 4$  mmHg ( $n=7$ ).

### Expression of AQP2 proteins

Fig. 1 and Fig. 2 show the protein expression of AQP2 in the cortex, outer medulla, and inner medulla of the clipped and contralateral kidneys, respectively. The anti-AQP2 antibody recognized 29-kDa and 35- to 50-kDa bands, corresponding to nonglycosylated and glycosylated AQP2, respectively. The AQP2 expression was significantly decreased in the clipped kidney, while remained unaltered in the contralateral kidney. The expression in the clipped kidney was normalized to the control level following removal of the clip.

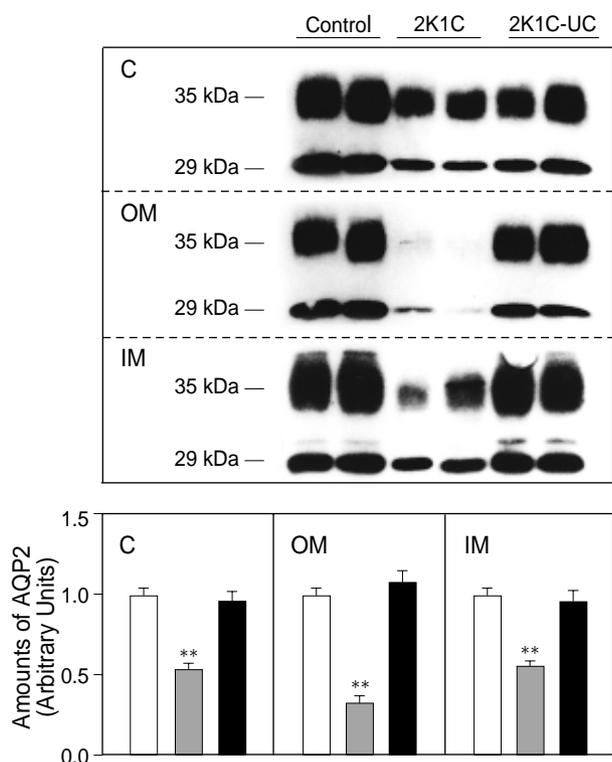


Fig. 1. The expression of AQP2 in the cortex (C), outer medulla (OM) and inner medulla (IM) of the clipped kidney. 2K1C and 2K1C-UC represent the experimental groups with their renal arterial clips remained intact and removed, respectively. The open column represents the control, the hatched column 2K1C, and the double-hatched column 2K1C-UC (mean  $\pm$  SEM of 6 rats each). \*\* $p<0.01$ , compared with control.

Fig. 3 represents the trafficking of AQP2 channels in the clipped kidney. The AQP2 expression in the membrane fraction decreased in proportion to that in the cytoplasmic fraction, suggesting a preserved trafficking.

### Adenylyl cyclase activity

Fig. 4 shows cAMP generation in response to graded doses of AVP in the medulla. The AVP-evoked cAMP generation was decreased in the clipped kidney, which was normalized to the control level after removal of the clip.

## DISCUSSION

The blood pressure was increased following unilateral clipping of the renal artery. Accordingly, the expression of AQP2 proteins was decreased in the clipped kidney, while it remained unaltered in the contralateral kidney. AQP2 is primarily located in the collecting duct that comprises the final step modifying the urinary excretion to normally reabsorb 10-20% of the tubular load not reabsorbed by the proximal nephron. An altered regulation of AQP2 may then result in an altered urinary concentration. In this context, the reduced expression of AQP2 channels may in part account for the increased urinary volume in 2K1C hypertension (3).

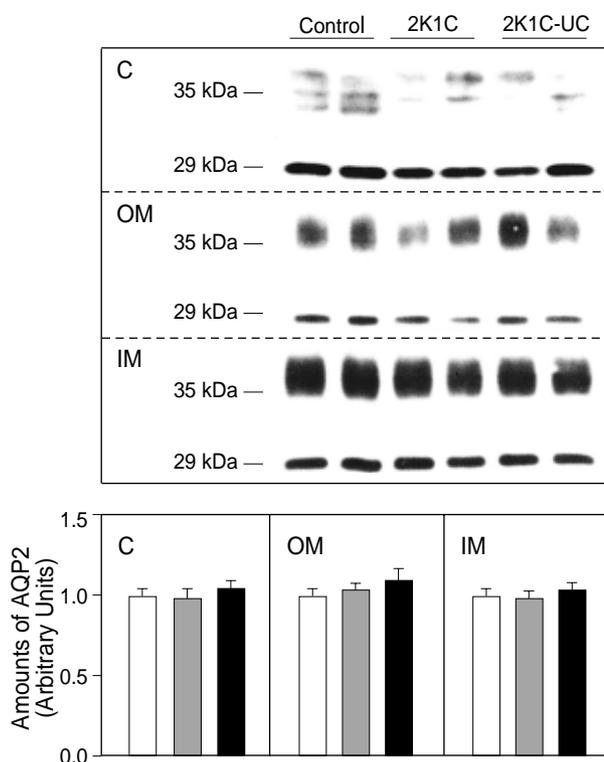


Fig. 2. The expression of AQP2 in the cortex, outer medulla and inner medulla of the contralateral kidney. Legends as in Fig. 1.

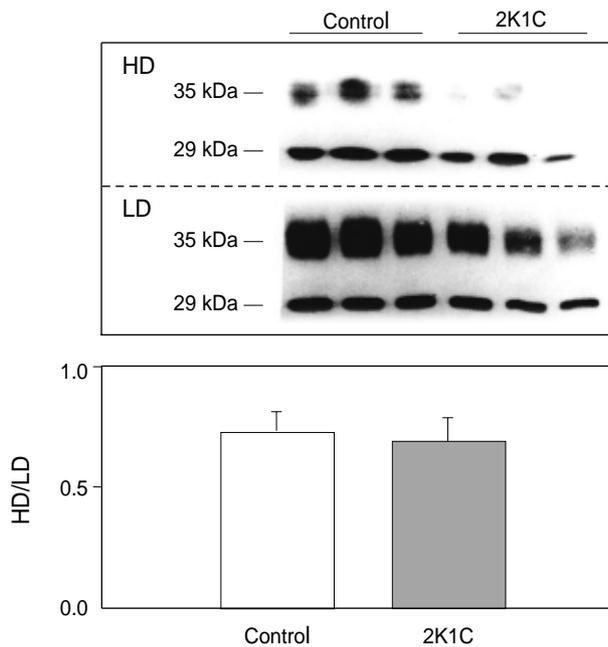


Fig. 3. The expression of AQP2 in the high-density membrane fraction (HD) and low-density cytoplasmic fraction (LD) of the clipped kidney. Legends as in Fig. 1.

AQP2 is short-term and long-term regulated by AVP/cAMP system. The long-term regulation of AQP2 exerted by cAMP is to increase the expression of its proteins (8). cAMP mediates phosphorylation and subsequent binding to cAMP response element (CRE) of CRE-binding protein in the promoter region of *AQP2* gene (17). Therefore, a diminished expression of AQP2 can be attributed to a decreased cAMP. Indeed, the cAMP generation provoked by AVP was diminished in the clipped kidney. The decreased AQP2 expression may then be attributed to a reduced cAMP generation.

It is of interest that the abundance of AQP2 in the clipped kidney was rapidly resumed after removing the clip, which was also in association with a recovery of adenylyl cyclase activity. A rapid regulation of AQP2 protein has been shown, in which it took 12 hr to reverse the diuresis associated with AQP2 down-regulation induced by an administration of AVP antagonists (18).

We have recently shown an increased renal expression of AQP2 proteins in DOCA-salt hypertension (19) of which maintenance has been in part attributed to AVP (20). On the contrary, the decreased AQP2 expression in the present study may be in line with the previous notion that AVP plays no significant role in maintaining the high blood pressure in renovascular hypertension (21). Whether the decreased expression of AQP2 is a mechanism counteracting the high blood pressure in 2K1C hypertension remains to be determined.

On the other hand, the short-term regulation of AQP2

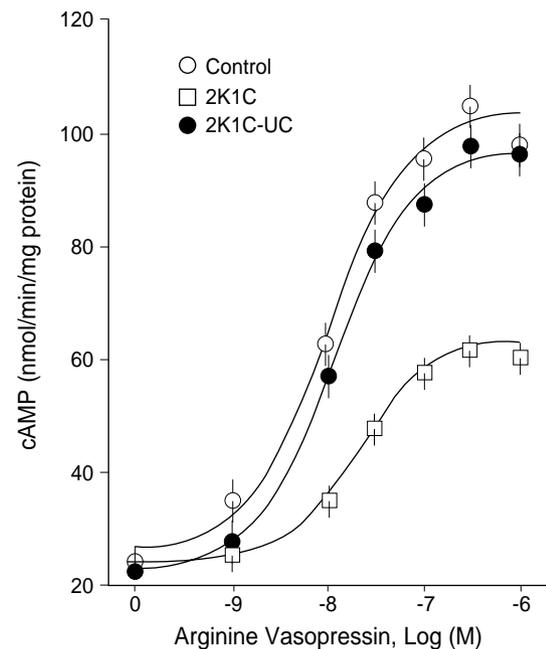


Fig. 4. The cAMP generation in response to arginine vasopressin (AVP) in the medulla. Legends as in Fig. 1. Each point represents mean  $\pm$  SEM of 6 experiments.

exerted by cAMP is an exocytic insertion of AQP2 to the apical membrane, which is rapidly reversible upon dissociation of AVP from its receptor (7). In the present study, the reduction of AQP2 channels was in parallel between the membrane-enriched and cytoplasmic fractions, suggesting a preserved targeting. Similar findings have been noted in several acquired nephrogenic diabetes insipidus such as urinary tract obstruction (9), chronic renal failure induced by surgical renal mass reduction (11), and cisplatin-induced nephropathy (12). However, it is uncertain whether there may have been a transient impairment of AQP2 targeting following clipping of the renal artery, and a reduced total abundance may prevail in the long run.

Mechanisms underlying the decreased cAMP generation and AQP2 expression in the clipped kidney are not clear. We have recently observed that an ischemic insult in the kidney causes an attenuated cAMP generation and reduction of the expression of AQP2 proteins (10). In addition, it has been known that angiotensin II causes a preferential constriction of efferent arterioles to reduce the distal oxygen delivery (22). In 2K1C hypertension, not only the constriction of the renal artery but also the accompanying increase of angiotensin II concentrations may be detrimental to the medullary oxygen balance. The decreased expression may be related to the ischemia due to clipping of the renal artery.

Indeed, during renal hypoperfusion, isothermia is one of the most sensitive measures of intrinsic renal damage (23). Medullary hypoxia is an inevitable accompaniment of efficient urinary concentration. If excessive, the medullary

blood flow disrupts the osmolality gradients built up by countercurrent exchange; if too slow, the resultant hypoxia may result in susceptibility to acute and chronic renal injury (24). Therefore, the loss of urinary concentrating ability may reflect an injury to the renal medulla. Activated protective mechanisms such as reduced medullary tubular transport may further blunt the capacity to concentrate the urine.

In summary, the present study showed an altered regulation of AQP2 water channels in the clipped kidney in 2K1C hypertension. The altered expression of AQP2 may be causally related with the altered urinary volume excretion in this model of hypertension.

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