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Expression of V2R and AQPs in the Renal Medulla of High-fat Nutritional Obesity of Rat

Expresión de V2R y AQPs en la Médula Renal de Ratas con Obesidad Nutricional Alta en Grasas

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SUMMARY: Recent evidence has indicated that adipose tissue produces bioactive substances that contribute to obesity-related kidney disease, altering the renal function and structure. Eight of the AQPs are expressed in the kidney, where several of them contribute to water absorption and maintenance of body water balance. In the study, we mainly examined the localization of AQP2, AQP3 and V2R in renal medulla of Normal Diet (ND) and High-fat Diet (HFD) of rats, respectively. In renal medulla of HFD, immunolight microscopy revealed weak expression of AQP2 at the apical plasma membrane and intracellular vesicles of principal cells of the IMCD and OMCD. AQP3 and V2R expression also observed a decrease in immunolabelling in the IMCD and OMCD. It was suggested that excess lipid accumulation may lead to lipotoxicity and may be the major driver of organ dysfunction such as water reabsorption dysfunction, which may be resulted from abnormal response of rphan G-protein-coupled receptors in kidney.

KEY WORDS: Obesity; Kidney ; AQPs; V2R; Kidney disease.

INTRODUCTION

Aquaporins (AQPs) are a family of small, approximately 30 kDa integral membrane proteins that act as water channels. In mammals, 13 AQP subtypes, named AQP0–12, have been identified and these are expressed in various tissues such as the kidney, brain, liver, lung and salivary glands (Ishibashi *et al*., 2009). Eight isoforms of AQPs (1–4, 6–8, 11) are expressed in the kidney, five of which have been proved to play a role in body water balance. AQP1, AQP2, AQP3, AQP4 and AQP7 are distributed differentially and strategically along the nephron to facilitate water reabsorption and urine concentration (Kortenoeven & Fenton, 2014).

The cortical collecting duct (CCD), the outer medullary collecting duct (OMCD) and the inner medullary collecting duct (IMCD) are very important portion of final water reabsorbtion in the animals through AQP2, 3 and 4 (Terris *et al*., 1995, 1996). It has been reported that AQP2 is regulated both in trafficking (short-term regulation) and in abundance (long-term regulation) via renal V2 Vasopressin Receptor (V2R), and arginine vasopressin (AVP) (Terris *et al*., 1996). AVP is a peptide hormone synthesized in the supraoptic and paraventricular nuclei of hypothalamus and regulated body water homeostasis (Bisset & Chowdrey, 1988). Interestingly, both AQP3 and AQP4 are regulated in abundance by AVP in long-term (Poulsen *et al*., 2013).

In this century particularly, the growing epidemic of obesity is a major factor in reducing expected life expectancy and is an added serious health burden. Obesity-related kidney disease is associated with renal hemodynamic abnormalities, endothelial and podocyte dysfunction, glomerular basement membrane thickening and mesangial expansion, tubular atrophy, interstitial fibrosis and a progressive decrease in renal function leading to end-stage renal disease (Marcussen *et al*., 1992; Taft *et al*., 1994; Gilbert & Cooper, 1999; Chagnac *et al*., 2000). Moreover, central obesity is a major risk factor for diabetes and hypertension which together account for about 70 % of all cases of end-stage renal disease (Collins *et al*., 2011; Declèves & Sharma, 2015). Obesity led to a progressive decrease in renal function, which inevitably affected on renal water reabsorption capacity. Diabetes insipidus (DI) is characterized by an impaired renal water reabsorption, leading to polyuria and consequently,

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polydipsia. DI can present with hypernatremia, but patients most often have a normal plasma osmolality, provided the thirst mechanism is normal and there is adequate access to fluid (Kortenoeven & Fenton). However, a full understanding of mechanisms involved to the progressive renal disease is still unclear, in particular V2R and AQPs in the kidney of obesity-induced adipose tissue. Therefore, here, we will to copy the mode of high-fat nutritional obesity, and determine V2R and AQPs (AQP2 and AQP3) expression and related functions in the obesity, thereby discussing the potential mechanisms between the impaired water balance and obesity-related kidney disease during obesity.

MATERIAL AND METHOD

All experimental procedures and protocols in the study were approved by Animal Ethics Committee of the Ningxia Hui Autonomous Region, China.

Sprague Dawley (SD) rats were obtained from the Laboratory Animal Center, Ningxia Medical University and Ningxia Hui Autonomous Region, and bred in the Laboratory Centre. To avoid the impact of gender on this experiment, healthy and male SD aged about 8 weeks and weighting about 240 g were used at the beginning of the experiment (n=60). All animals were bred in-house and had free access to clean water and rat chow for the duration of the study. After 1 week of adaptive feeding, the rats were randomly divided into two groups, Normal Diet (ND, n=30) and High-fat Diet (HFD, n=30), respectively. ND was fed with normal rat chow, the fat of which accounted for 10 % of the total calories. Meanwhile, HFD was fed with High-fat Diet, where the fat accounted for 66 % of the total calories (Carbohydrate: 15.48 %; Protein: 18.08 %; Fat: 66.43 %, lard-saturated fatty acid mainly). Animals were housed in steel cages in a controlled temperature room at 23±2 °C, exposed to a daily 12-hour light–dark cycle (lights on at 07:00 a.m. and off at 07:00 p.m.). Hereditary obesity (ND) and obesity-resistant (HFD) were artificially eliminated during the study.

After half-year feeding, the body weight of ND and HFD were recorded. A blood sample was collected at end of the experimental period. AST, ALT, T-BIL, TP, ALB, GLB, ALP, TG, TCHO, HDL-C and LDL-C were measured by standard methods using an autoanalyzer (JCA-BM6010/C, Sysmex CA-620, Japan). Meanwhile, kidney samples were collected and immediately fixed with 4 % paraformaldehyde solution (PH7.4), and shortly afterward, blocks were cut from various parts of the kidneys for immunolight microscopy.

For immunolight microscopy, specimens were dehydrated through a graded series of alcohol, cleared and embedded in paraffin wax, and sectioned at 5mm used for immunohistochemical staining. The sections were dewaxed and rehydrated, an antigen retrieval procedure was performed to unmask antigens by treating the samples three times in a microwave oven at medium-high fire for 5 min each time in 10 mM citrate buffer, pH 6. After cooling to room temperature, the sections were treated with 3 % hydrogen peroxide (H_2O_2) in distilled water for 30 min, then washed three times with PBS for 10 min each. The sections were incubated in blocking reagent for 1 h and incubated overnight at 4 °C with anti-AQP2/AQP3/V2R diluted 1:200 (Boster, Wuhan, China). The primary antibody was diluted in 10 mM PBS supplemented with 0.1 % BSA and 0.3 % Triton X-100 [10 mM PBS (7mM Na_2HPO_4, 3mM NaH_2PO_4, 150 mM NaCl, pH 7.4)]. Subsequently, the sections were rinsed with 10 mM PBS for 3x10 min and incubated with biotinylated anti-rabbit IgG (ZSGB-BIO, Beijing, China, dilution: 1:200) secondary antibody for 2 h at room temperature. Then, the sections were rinsed with 10 mM PBS for 3x10 min and incubated with horseradish peroxidase streptavidin (ZSGB-BIO, Beijing, China, dilution: 1:200) tertiary antibody for 2h at room temperature followed by coloration with diaminobenzidine (DAB; ZSGB-BIO, Beijing, China) for 30s to visualize positive reaction. Counterstaining was with hematoxylin staining. The sections were carried out using bright microscopy (NI-V, Nikon, Japan).

Measurements results of general physiological parameters were performed by unpaired t-test (two tailed). All data are presented as the mean±SEM (standard error of the mean), *P<0.05, **P<0.01, ***P<0.001.

RESULTS

General physiological parameters. General physiological parameters of normal diet (ND) and high-fat diet (HFD) in SD rats were measured by standard methods using an autoanalyzer. Table I shows the results of general physiological parameters. AST, ALT, T-BIL, GLB, ALP, TG, TCHO and LDL-C were increased in the HFD as compared with the ND of rats (P<0.05 or P<0.01 or P<0.001). Whereas TP and ALB were no difference between the ND and HFD (P>0.05). In addition, HDL-C was decreased in the HFD as compared with the ND of rats (P<0.05).

Protein examination. We examined the localization of AQP2, AQP3 and V2R in renal medulla of Normal Diet (ND) and High-fat Diet (HFD) of rats, respectively. Counterstaining with hematoxylin staining was used to

Table I. General physiological parameters of normal diet (ND) and high-fat diet (HFD) in SD rats (n=15).

Items	Groups_Mean±SD_		*P*-value
	ND	HFD	
AST (U/L)	102.42±25.67	135.89±25.73	<0.05
ALT (U/L)	50.65±16.26	77.05±18.50	<0.05
T-BIL (umol/L)	0.33±0.11	0.79±0.19	<0.01
TP (g/L)	51.40±1.94	56.29±1.71	>0.05
ALB (g/L)	27.39±0.67	25.70±1.22	>0.05
GLB (g/L)	24.01±0.66	30.59±1.21	<0.05
ALP (U/L)	138.85±7.78	173.19±6.25	<0.05
TG (mmol/L)	1.00±0.15	1.68±0.12	<0.05
TCHO (mmol/L)	2.47±0.35	4.53±0.69	<0.01
HDL-C (mmol/L)	2.13±0.47	1.61±0.32	<0.05
LDL-C (mmol/L)	0.63±0.23	2.73±0.51	<0.001

*P<0.05, **P<0.01, ***P<0.001. Data represent mean±SD.

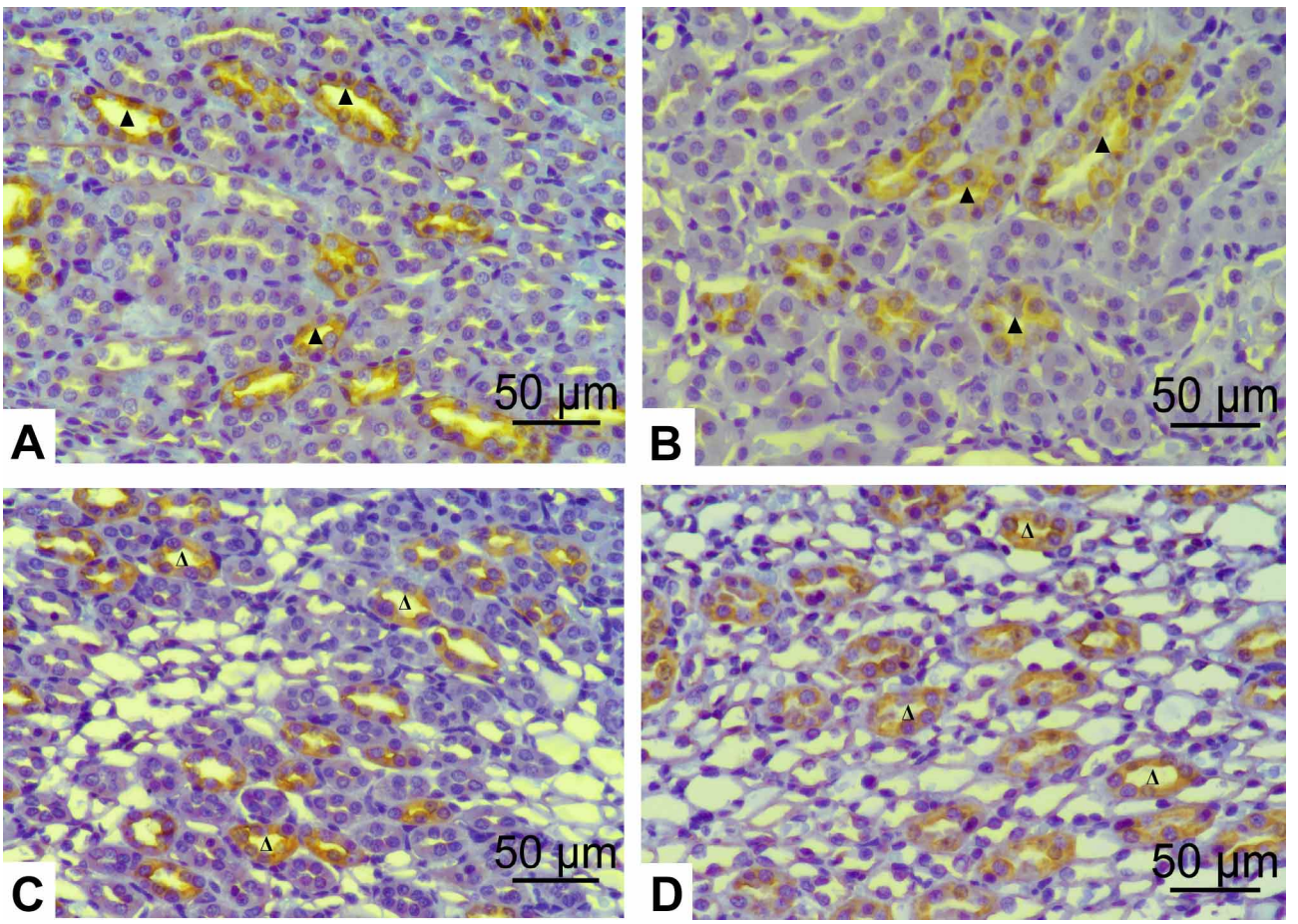

Fig. 1. Immunohistochemical examination of AQP2 protein abundance in the renal medulla in tissue section from ND (A&C) and HFD (B & D) rat. s, Expression in the outer medullary collecting duct (IMCD); D, Expression in the inner medullary collecting duct (OMCD).

identify negative segments. In this paper, renal medulla was divided into the outer and inner medulla, for collecting duct, thereby forming the outer medullary collecting duct (OMCD) and the inner medullary collecting duct (IMCD).

In the IMCD and OMCD, immunolight microscopy revealed strong expression of AQP2 at the apical plasma membrane and intracellular vesicles of principal cells of ND, respectively (Figs. 1A,C), whereas weak expression of AQP2

was observed at the apical plasma membrane and intracellular vesicles of HFD compared with the ND, respectively (Figs. 1B,D).

We investigated whether the expression of AQP3 in abundance was parallel with AQP2 at the apical plasma membrane of the IMCD and the OMCD of HFD compared with the ND. However, in the OMCD, strong AQP3 expression was observed at the basolateral plasma membrane of ND compared with the HFD (Figs. 2A,C). Compared with the ND, weak expression of AQP3 was observed at the basolateral plasma membrane of the IMCD of HFD (Figs. 2B,D).

To investigate whether V2R expression in abundance was similar to the AQP2 and AQP3 in the IMCD and OMCD of HFD, respectively. We examined V2R expression in the IMCD and the OMCD of ND and HFD, simultaneously. It was observed different V2R expression in medulla between the ND and HFD of rats. Strong V2R expression was observed at the apical plasma membrane and intracellular vesicles in the OMCD for ND compared with the HFD (Figs. 3A,C). However, for V2R expression in abundance in the IMCD, there was apparently no difference between the ND and HFD (Figs. 3B,D).

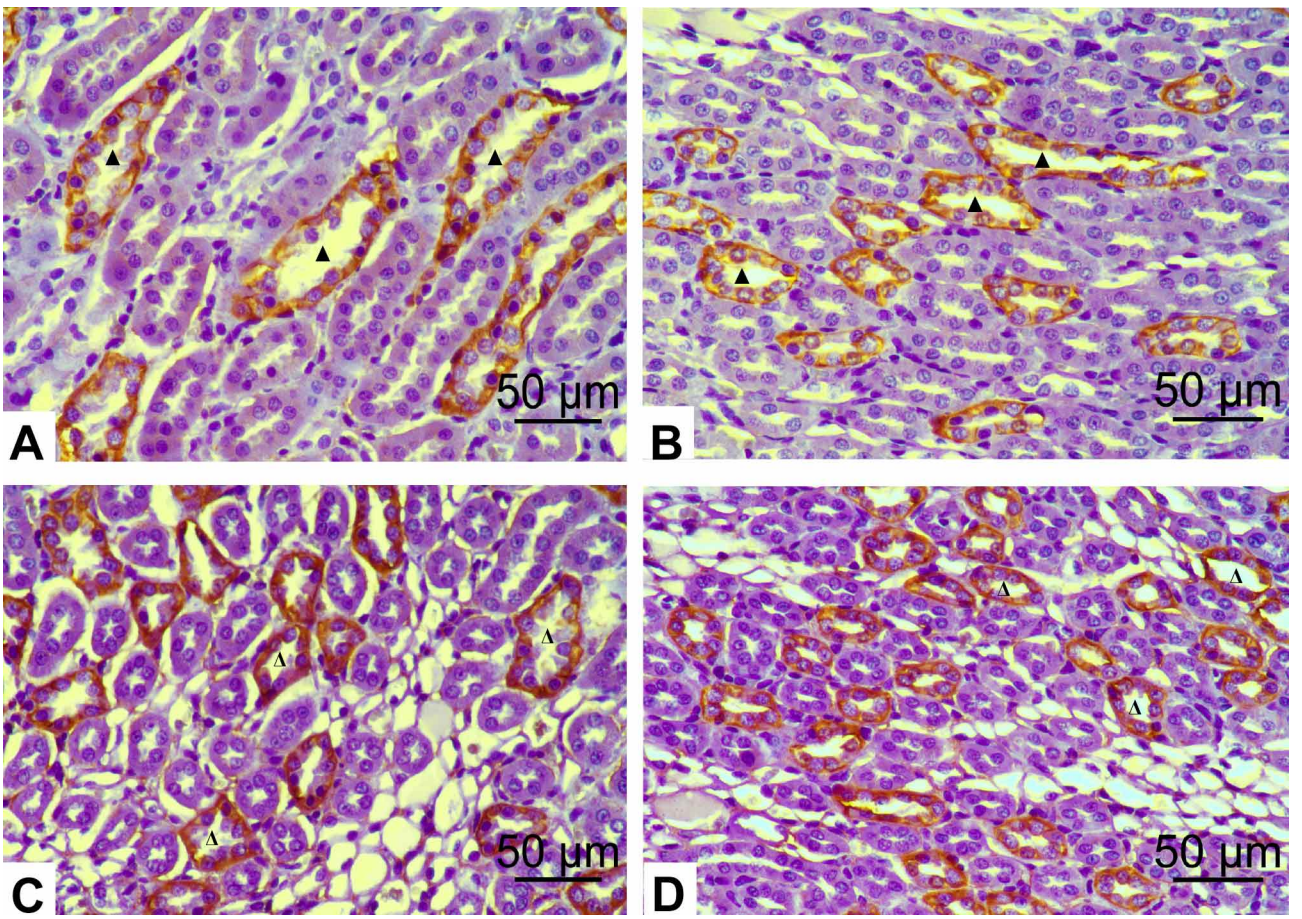

Fig. 2. Immunohistochemical examination of AQP3 protein abundance in the renal medulla in tissue section from ND (A&C) and HFD (B&D) rat. s, Expression in the outer medullary collecting duct (IMCD); D, Expression in the inner medullary collecting duct (OMCD).

DISCUSSION

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 (Declèves & Sharma).

In pathological conditions like obesity, namely high-fat nutritional obesity in this study was copied. It was found that general physiological parameters of HFD in SD rats had significantly change, as compared with the ND, especially T-BIL, TCHO and LDL-C. More importantly, the physiological parameters of HDL-C, LDL-C and TCHO in

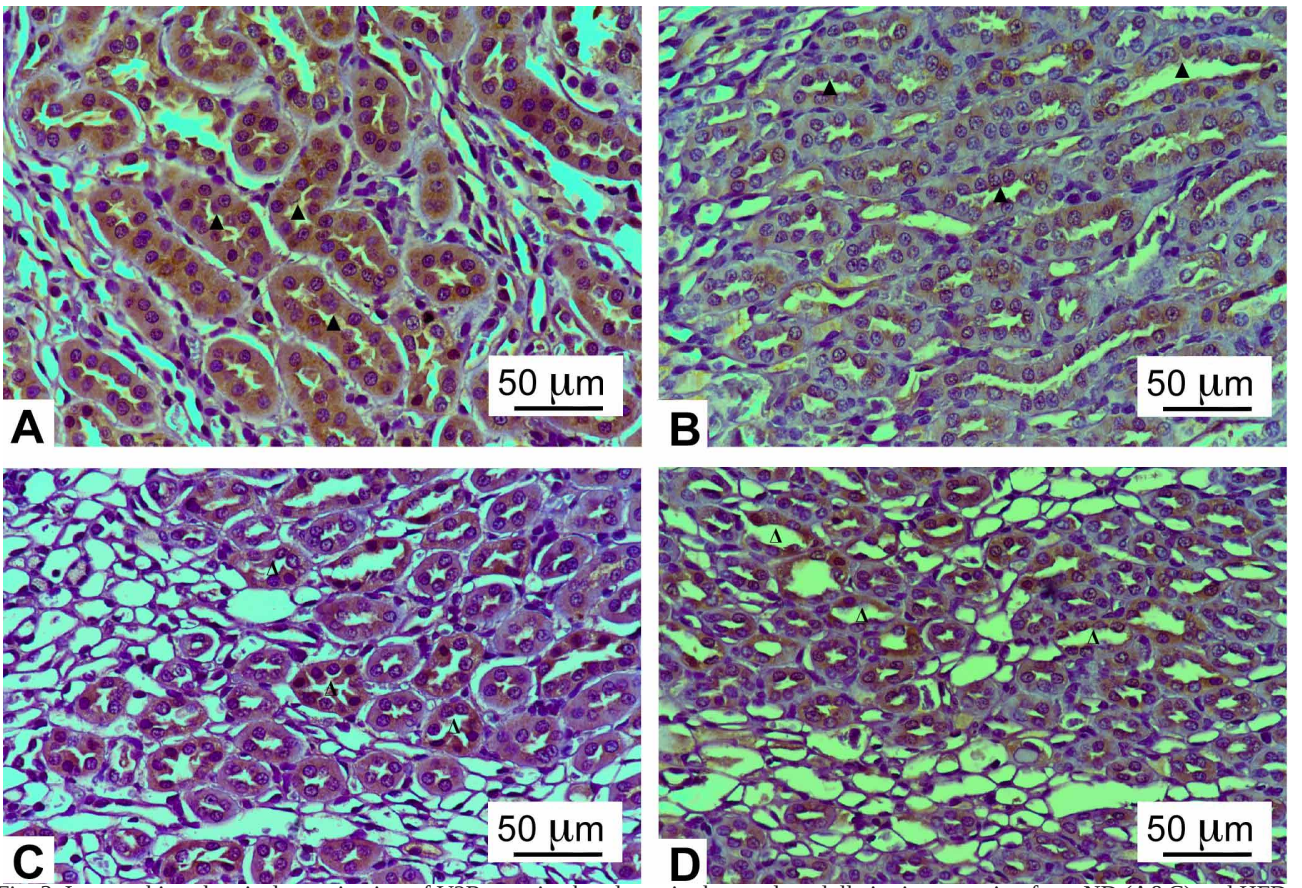

Fig. 3. Immunohistochemical examination of V2R protein abundance in the renal medulla in tissue section from ND (A&C) and HFD (B&D) rat. s, Expression in the outer medullary collecting duct (IMCD); D, Expression in the inner medullary collecting duct (OMCD).

the HFD rats showed pathological status, e.g. atherosclerosis and coronary heart disease (Chagnac *et al*.). Adipose tissue is now considered as an endocrine organ producing biologically active molecules that contribute to the onset of obesity-related disease (Declèves & Sharma). Excess lipid accumulation may lead to lipotoxicity and may be the major driver of organ dysfunction such as kidney injury (Declèves & Sharma).

Body water homeostasis is tightly controlled by regulating both water intake and urinary water excretion. In the kidney of human, 180 L of plasma is filtered by the glomeruli each day (Stanton & Koeppen, 2004; Kortenoeven & Fenton). Less than 1 % of this volume is finally excreted in the urine. Approximately 67 % of the filtered water is reabsorbed in the proximal tubule and 15 % in the descending thin limb of Henle, which both are constitutive processes (Kortenoeven & Fenton).

Depending on the body's needs, the remaining fluid can be reabsorbed in the renal tubule including the proximal convoluted tubule, the thin and thick limbs of Henle's loop, and the distal convoluted tubule, thereby resulting in the final urine concentration. As previously mentioned, this process is tightly regulated, mainly by AVP/V2R, which allows the body to adapt to periods of water load or water restriction (Stanton & Koeppen; Kortenoeven & Fenton).

AQP2 has been reported to occur in the principal cells along connecting tubule and collecting duct in mammalian kidneys, where it is localized to the apical plasma membranes and intracellular membrane vesicles (Nielsen *et al*., 1993; Fushimi *et al*., 1993). AVP regulates the body's retention of water, increasing both the osmotic driving force for water reabsorption and the transcellular route for water transport, causing the kidneys to concentrate the urine (Bisset & Chowdrey; Terris *et al*., 1996). The transcellular route of regulation mainly occurs via modulating cell surface expression of AQP2. AVP binds to V2R, present in the basolateral membrane of renal collecting duct principal cells and connecting tubule cells (Fenton *et al*., 2007; Mutig *et al*., 2007), inducing a signaling cascade, involving Gs protein mediated activation of adenylate cyclase, a rise in intracellular cAMP, activation of protein kinases, and

redistribution of AQP2 water channels from intracellular vesicles to the apical membrane (Kuwahara *et al*., 1995; Katsura *et al*., 1997). The overall process results in a concentration of the urine. In addition, AQP3 has also been shown to be important for the urine concentrating mechanism, as AQP3 knockout mice have an increased urine volume, lower urine osmolality and a reduced osmotic water permeability of the basolateral membrane of the collecting duct (Ma *et al*., 2000; Kortenoeven & Fenton). It has been suggested that AQP3 is localized in the basolateral membrane of principal cells of the collecting duct and will normally produce a net flow of water into the interstitium (Calamita, 2000; Holmes, 2012). Moreover, AQP3 is thought to represent an additional exit pathway for water reabsorbed via AQP2 (Holmes; Poulsen *et al*.).

In high-fat nutritional obesity of rat, immunolight microscopy revealed weak expression of AQP2 at the apical plasma membrane and intracellular vesicles of principal cells of the IMCD and OMCD. AQP3 and V2R expression also observed a decrease in immunolabelling in the IMCD and OMCD. Real down-regulation of AQP2, AQP3 and V2R expression may be a hint for kidney injury or disease. As previously mentioned, adipose tissue, namely excess lipid accumulation may contribute to the onset of obesity-related disease via lipotoxicity (Declèves & Sharma). It was essential for maintaining body water balance by controlling urinary water excretion in the kidney, and this process is tightly regulated mainly by the AVP/V2R pathways mediating water reabsorption as mentioned (Kortenoeven & Fenton). It was suggested that down-expression of AQP2, AQP3 and V2R in the kidney of obesity inevitably led to disorder water homeostasis. Water balance disorders was usually associated with decreased aquaporin levels, such as diabetes insipidus, which is characterized by an impaired renal water reabsorption, leading to polyuria and consequently, polydipsia (Kortenoeven & Fenton; Moeller *et al*., 2016). Then, what was the relationship between lipotoxicity and water balance disorders? Obviously, it was a key question in this study.

More recently, orphan G-protein-coupled receptors (GPCRs) have been pointed as playing key role in the progression of obesity-related disease. These receptors function as receptors for short chain fatty acids (Declèves & Sharma). The GPCRs are expressed in many tissues such as in pancreas, adipose tissue and kidney (Bartoov-Shifman *et al*., 2007). All members of AVP receptor family belong to the superfamily of rhodopsin-like GPCRs. In mammals V1 receptors (V1aR and V1bR) are involed in blood pressure regulation and central feedback mechanism whereas the V2R maintains the water balance and electrolyte homeostasis (Böselt *et al*., 2009). In this study, it is thus easy to figure out that excess food intake, and especially fat food,lead to an increase of free fatty acids, and that in turn promote abnormal response in their orphan GPCRs, promoting renal dysfunction.

In kidney, the evidence for a role of GPCR has been reported. Indeed, GPCR were detected in distal tubular cells and in macula densa, suggesting sensory role in the regulation of the tubuloglomerular feedback by regulating the renin release (Pluznick *et al*., 2009; Declèves & Sharma). A more recent study reported opposite effects of the GPCR functions (Pluznick, 2013). Therefore, here there was a decrease in immunolabelling of AQP2, AQP3 and V2R protein in obesity based on fat food intake, which may be resulted from abnormal response of rphan GPCRs in kidney. Our findings may improve our understanding of somewhat mechanism, however, the mechanisms underlying were still unclear.

In conclusion, increased knowledge of the link between adipose tissue and kidney in a context of obesity will allow for novel therapeutic approaches to prevent obesity-induced kidney injury.

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RESUMEN: La evidencia reciente ha indicado que el tejido adiposo produce sustancias bioactivas que contribuyen a la enfermedad renal relacionada con la obesidad, alterando la función y la estructura renal. Ocho de los AQP se expresan en el riñón, donde varios de ellos contribuyen a la absorción de agua y al mantenimiento del equilibrio hídrico corporal. En el estudio, examinamos principalmente la localización de AQP2, AQP3 y V2R en la médula renal de ratas con dieta normal (ND) y ratas con dieta alta en grasas (HFD). En la médula renal del grupo HFD, la microscopía electrónica de barrido reveló una expresión débil de AQP2 en la membrana plasmática apical y las vesículas intracelulares de las células principales de IMCD y OMCD. La expresión de AQP3 y V2R también observó una disminución en el inmunomarcador en IMCD y OMCD. Se sugiere que el exceso de acumulación de lípidos puede conducir a lipotoxicidad y ser el principal impulsor de la disfunción orgánica, como la disfunción de reabsorción de agua, que puede ser el resultado de la respuesta anormal de los receptores acoplados a proteína rphan G en el riñón.

PALABRAS CLAVE: Obesidad; Riñon AQPs; V2R; Enfermedad renal.

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