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AQP4 KO exacerbating renal dysfunction is mediated by endoplasmic reticulum stress and p66Shc and is attenuated by apocynin and endothelin antagonist CPU0213

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ABSTRACT

Aquaporin 4 (AQP4) is essential in normal kidney. We hypothesized that AQP4 knockout (KO) may exacerbate pro-inflammatory factors in the stress induced renal insufficiency. Mechanisms underlying are likely due to activating renal oxidative stress adaptor p66Shc and endoplasmic reticulum (ER) stress that could be mediated by endothelin (ET)—NADPH oxidase (NOX) pathway. AQP4 KO and wild type (WT) mice were randomly divided into 4 groups: control, isoproterenol (1 mg/kg, s.c., 5d), and interventions in the last 3 days with either apocynin (NADPH oxidase inhibitor, 100 mg/kg, p.o.) or CPU0213 (a dual endothelin receptor antagonist 200 mg/kg, p.o.). In addition, HK_2 cells were cultured in 4 groups: control, isoproterenol (10^{-6} M), intervened with apocynin (10^{-6} M) or CPU0213 (10^{-6} M). In AQP4 KO mice elevated creatinine levels were further increased by isoproterenol compared to AQP4 KO alone. In RT-PCR, western blot and immunohistochemical assay p66Shc and PERK were significantly increased in the kidney of AQP4 KO mice, associated with pro-inflammatory factors CX40, CX43, MMP-9 and ET_A compared to the WT mice. Expression of AQP4 was escalated in isoproterenol incubated HK_2 cells, and the enhanced protein of PERK and p-PERK/PERK, and p66shc in vivo and in vitro were significantly attenuated by either apocynin or CPU0213. In conclusion, AQP4 KO deteriorates renal dysfunction due to exacerbating ER stress and p66Shc in the kidney. Either endothelin antagonism or NADPH oxidase blockade partly relieves renal dysfunction through suppressing abnormal biomarkers by APQ4 KO and isoproterenol in the kidney.

1. Introduction

Renal function is modulated by a family of aquaporin (AQP) at least 7 subtypes (AQP1, 2, 3, 4, 6, 7 and 8), and dehydration or water overload may change the expression of AQP2, 3 and 4 in the collecting ducts (Takata et al., 2004). AQP4 which is initially found in the lung and brain in rats plays a role in the heart in response to stress (Cheng et al., 2012; Moe et al., 2008). In AQP4 knockout (KO) mice brain edema following subarachnoid hemorrhage is more predominant and the urine concentrating activity is mildly compromised. (Maeda et al., 2008; Tait et al., 2010). Since stress is one of the causal factors to induce renal dysfunction, it may suggest that a loss of AQP4 could exacerbate the stress induced renal impairment.

Endoplasmic reticulum (ER) is the site of protein biosynthesis in cells, and is active in cellular stress. In the presence of oxidative stress, unfolded protein response (UPR) at the ER occurs resulting in an accumulation of misfolded proteins to initiate ER stress. Renal dysfunction caused by stress may resemble the abnormalities at some extents in diabetic kidney (Hu et al., 2011; Xu et al., 2009). ER stress is a signal of cellular inflammation where AQP4 is altered, as we found in inflammation caused by carrageenin (Cong et al., 2012b). However, it remains unclear if ER stress chaperone could be exacerbated in the absence of AQP4.

P66Shc regulates the generation of oxidants participating in cell injury by oxidative stress which adversely affects the life span (Menini et al., 2006). Lack of p66Shc becomes resistant to reactive oxygen species. An activation of p66shc results in enhancing NADPH oxidase to produce more reactive oxygen species damaging cells and is involved in inflammatory reactions in cells. It is unknown if p66Shc could be modulated by AQP4 in the kidney.

CX43 (Connexin 43) responsible for gap junctional intercellular communications is widely distributed in the kidney and is critically involved in mechanisms contributing to normal renal function those including tubuloglomerular feedback and salt and water reabsorption (Hanner et al., 2010). Abnormal Cx43 is actively involved in tissue injury and wound healing in which inflammatory reactions are present (Chew et al., 2010; Chin, 2011; Tyml, 2011). An

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activated endothelin (ET) receptors impairs vascular and cardiac activity (Cheng et al., 2009a) and is found in diseased kidney (Saleh and Pollock, 2011). Abnormal matrix metalloproteinase 9 (MMP-9) has been well described in cellular reactions to inflammation and stress responsible for extra-cellular matrix degradation process (Li et al., 2012; Mishra et al., 2012). It is uncertain if the abnormal MMP-9 and CX43 in response to stress are exacerbated in the absence of AQP4.

Endothelin receptor antagonist CPU0213 improves diabetic nephropathy by inhibiting ET-NADPH oxidase pathways (Hu et al., 2011). In diabetic kidney stressful reactions are significant due to the markedly activated NADPH oxidase (Cheng et al., 2012) and isoproterenol may damage tissue due to initiating oxidative stress and dysfunctional mitochondria.(Sankar et al., 2013).

Therefore, we hypothesized that by applying isoproterenol the renal function is impaired, and AQP4 KO may adversely affect ER stress, p66Shc, CX43 and MMP-9 exaggerating dysfunction of the kidney administrated with isoproterenol. The stress induced damage to the renal function may be partly mediated by AQP4 trafficking in association with activation of the ET-NADPH oxidase pathway. We may suggest that suppression on either ET receptors by CPU0213 or NADPH oxidase by apocynin provides protection on the kidney in AQP4 KO mice through ameliorating the activated ER stress and p66Shc in renal tissue.

2. Materials and methods

2.1. Animals and chemicals

Specific pathogen free (SPF) AQP4 KO and WT mice either sex 30 ± 2 g, 4 months old were used, offered as a gift from Prof. Gang Hu, the Nanjing Medical University. The AQP4 KO mice were characterized in the appearance with white belly against grey one in WT mice (Fan et al., 2005). Mice were maintained under room temperature about 22 °C, and free access to food and water. All experiments were carried out by personnel who performed animals handling procedures in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All the performances were also in consistent with the Guidelines for the Care and Use of Laboratory Animal in Jiangsu Province and the approval was granted by Animal Ethics Committee of Jiangsu Province, China [NO.SYXK2007-0025].

Drugs: Isoproterenol injection was purchased from the Shanghai Hefeng Pharmaceutical Co., Ltd, Shanghai, China, and apocynin (APO, a blocker for NADPH oxidase) was from Sigma-Aldrich Corporate, St. Louis, MO, Lot S43156-327. CPU0213, purity $>98\%$, a dual endothelin receptor antagonist was synthesized and provided by the Department of Medicinal Chemistry of China Pharmaceutical University.

2.2. Experiment design

The AQP4 KO and WT mice were randomly divided into 3 groups with 3–6 in each: control, isoproterenol stimulation (1 mg/kg, sc, 5d), and interventions conducted during the last 3 days with either apocynin (NADPH oxidase inhibitor 100 mg/kg, p.o.), or CPU0213 (CPU, endothelin receptor A and B blocker, 200 mg/kg, p.o.). Mice in control or isoproterenol group were given an equal amount of 0.5% carboxymethyl cellulose–Na (CMC-Na).

On day 6, the mice were anesthetized with urethane (1.5 g/kg, i.p.). Blood samplings were collected through bleeding from a cut on the neck and kidneys were harvested and dissected for the procedures of Reverse transcriptase polymerase chain reaction (RT-PCR), western blot and immunochemical assay.

2.3. Malondialdehyde (MDA) and creatinine (Cr) in plasma

Blood samples were collected and centrifuged. An aliquot of plasma was applied for measuring MDA and creatinine according to the instructions provided by the kit manufacturer, as the previous practice (Hu et al., 2011).

2.4. Histological examination

The kidney, fixed with neutral 10% buffered formalin, was embedded in paraffin and sliced into 4-μm-thick pieces for histo-pathological examination. Then the slides were deparaffinized in xylene, dehydrated by decreased concentration of ethyl alcohol and stained with haematoxylin–eosin. All pictures were viewed and compared under an inverted microscope (Nikon TE 2000-U, Japan) by pathologists blinded to the design of experiment.

2.5. RT-PCR

RT-PCR was conducted according to previous literature (Cheng et al., 2009a). Briefly, Trizol reagent was added to homogenate of the renal samples (100 mg/ml), and the integrity of mRNA was reserved due to the presence of RNase inhibitors. Extraction of mRNA was conducted by chloroform–isopropanol −75% ethanol method. An amount of 2 μg mRNA was employed to generate cDNA by reverse transcriptase by using the reverse transcriptase kit from Promega Corporation, USA. A template of cDNA was applied in the following PCR reactions (Eppendorf Mastercycler, Germany). GAPDH was used in parallel as internal standard to compare and calculate density of the deposit. The nucleotide sequences of primers and reaction conditions are listed in Table 1.

2.6. Western blot

Western blot procedure was conducted according to the previous report (Cheng et al., 2009a). Take 100 mg fresh renal tissue and add 1 ml RIPA lysis buffer, then, homogenized on ice. The homogenate liquid were transferred to 1.5 ml centrifuge tube and centrifuged at 4 °C, 10,000*g* for 20 min. Protein concentration of the supernatant was determined by a Coomassie brilliant blue

Table 1
A list of RT-PCR primers for AQP4, CX40, CX43, ETA, P66Shc, PERK, MMP9, AQP4, PERK and GAPDH.

Genes	Primers
AQP4 (mice)	Sense 5′-CTGGAGCCAGCATGAATCCAG-3′ Antisense 5′-TTCTTCTCTTCTCCACGGTCA-3′
CX40 (mice)	Sense 5′-TTGTCTTTATGATGGCTGTG-3′ Antisense 5′-TTTGGTCCTCGTCTAAGG
CX43 (mice)	Sense 5′-GATCGCGTGAAGGGAAGAAG-3′ Antisense 5′-CAGCCATTGAAGTAAGCATATTTTG-3′
ET_A (mice)	Sense 5′-TGACCTCCCCATCAACGTG-3′ Antisense 5′-TCCAAAATCATTGTGGTCGAAA-3′
P66Shc (mice)	Sense 5′-CCAAGCCGAAGTACAACC-3′ Antisense 5′-CGAACCAAGTAGGAAACCC-3′
PERK (mice)	Sense 5′-AGTTTCCGTTGCTGATTG-3′ Antisense 5′-TAGTTTACCTTCGGGAAT-3′
MMP9 (mice)	Sense 5′-AAACCTCCAACCTCACGG-3′ Antisense 5′-ACTTCTGAACGGCGCTCT-3′
GAPDH (mice)	Sense 5′-AGGCCGGTGCTGAGTATGTC-3′ Antisense 5′-TGCCTGCTTCACCACCTTCT-3′
AQP4 (HK2 cells)	Sense 5′-GGAATTTCTGGCCATGCTTA-3′ Antisense 5′-AGACTTGGCGATGCTGATCT-3′
PERK (HK2 cells)	Sense 5′-TTGTCGCCAATGGGATAG-3′ Antisense 5′-CCAAAGCCAACGACTGAC3′
GAPDH (HK2 cells)	Sense 5′-AGAAGGCTGGGGCTCATTTG-3′ Antisense 5′-AGGGGCCATCCACAGTCTTC-3′