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Low functional programming of renal AT_2R mediates the developmental origin of glomerulosclerosis in adult offspring induced by prenatal caffeine exposure

Ying Ao [a,b], Zhaoxia Sun [a], Shuangshuang Hu [a], Na Zuo [a], Bin Li [c], Shuailong Yang [a], Liping Xia [a], Yong Wu [a], Linlong Wang [c], Zheng He [a], Hui Wang [a,b,*]

[a] Department of Pharmacology, School of Basic Medical Science of Wuhan University, Wuhan 430071, China
[b] Hubei Provincial Key Laboratory of Developmentally Originated Disorder, Wuhan 430071, China
[c] Department of Orthopedic Surgery, Zhongnan Hospital of Wuhan University, Wuhan 430071, China

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ABSTRACT

Our previous study has indicated that prenatal caffeine exposure (PCE) could induce intrauterine growth retardation (IUGR) of offspring. Recent research suggested that IUGR is a risk factor for glomerulosclerosis. However, whether PCE could induce glomerulosclerosis and its underlying mechanisms remain unknown. This study aimed to demonstrate the induction to glomerulosclerosis in adult offspring by PCE and its intrauterine programming mechanisms. A rat model of IUGR was established by PCE, male fetuses and adult offspring at the age of postnatal week 24 were euthanized. The results revealed that the adult offspring kidneys in the PCE group exhibited glomerulosclerosis as well as interstitial fibrosis, accompanied by elevated levels of serum creatinine and urine protein. Renal angiotensin II receptor type 2 (AT_2R) gene expression in adult offspring was reduced by PCE, whereas the renal angiotensin II receptor type 1a ($AT_{1a}R$)/AT_2R expression ratio was increased. The fetal kidneys in the PCE group displayed an enlarged Bowman's space and a shrunken glomerular tuft, accompanied by a reduced cortex width and an increase in the nephrogenic zone/cortical zone ratio. Observation by electronic microscope revealed structural damage of podocytes; the reduced expression level of podocyte marker genes, nephrin and podocin, was also detected by q-PCR. Moreover, AT_2R gene and protein expressions in fetal kidneys were inhibited by PCE, associated with the repression of the gene expression of glial-cell-line-derived neurotrophic factor (GDNF)/tyrosine kinase receptor (c-Ret) signaling pathway. These results demonstrated that PCE could induce dysplasia of fetal kidneys as well as glomerulosclerosis of adult offspring, and the low functional programming of renal AT_2R might mediate the developmental origin of adult glomerulosclerosis.

Introduction

Caffeine is a xanthine alkaloid widely present in coffee, tea, soft beverages, food and some analgesic drugs. Because of the effects of relieving fatigue and enhancing mental alertness (van Dam, 2006; Carrieri et al., 2010; Panchal et al., 2012; Sugiura et al., 2012), caffeine containing food and beverages are favored by large number of people, including pregnant women. According to a clinical investigation, about 75% of the women consume caffeine during pregnancy in the USA (Signorello and McLaughlin, 2004). Similarly, the consumption of caffeinated beverages by pregnant women in China has become more and more popular. Recent research indicated that about 48.72% of the pregnant women still consume caffeine after the diagnoses of pregnancy in Chengdu City (Cao and Liu, 2014). However, epidemiological data and animal research have demonstrated that caffeine ingestion during pregnancy gives rise to reproductive and developmental toxicities, which not only increase the risk of premature delivery and spontaneous abortion, but also result in intrauterine growth retardation (IUGR) (Fortier et al., 1993; Kuczkowski, 2009). Our previous study also indicated that caffeine exposure during pregnancy could not only elevate the incidence of IUGR, but also increase the susceptibility of adult offspring to non-alcoholic fatty liver disease induced by high-fat diet (Huang et al., 2012; Xu et al., 2012a; Shen et al., 2014; Wang et al., 2014).

Glomerulosclerosis is a morphological lesion of glomerular injury, defined by the presence of sclerosis in parts of the glomeruli, expansion of the mesangial areas and dilatation of the intraglomerular capillaries (D'Agati et al., 2011). It is the leading cause of steroid-resistant nephrotic syndrome and the most common cause of chronic kidney disease among glomerular diseases (Reidy and Kaskel, 2007; D'Agati et al., 2011). The recent epidemiological data, suggesting that IUGR is a risk factor for glomerulosclerosis, was supported by the finding of higher

* Corresponding author at: Department of Pharmacology, School of Basic Medical Science of Wuhan University, 185 Donghu Road, Wuhan, Hubei, 430071, China. Fax: +86 27 68759222.
E-mail address: wanghui19@whu.edu.cn (H. Wang).

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incidence of glomerulosclerosis among the patients who had low birth weight (Hodgin et al., 2009; Ikezumi et al., 2013). Animal studies also demonstrated that some suboptimal intrauterine environments, such as 50% intrauterine food restriction (Regina et al., 2001), could lead to glomerulosclerosis of adult IUGR offspring. However, it is still unknown whether prenatal caffeine exposure (PCE) could give rise to glomerulosclerosis in adulthood.

In 1988, Brenner, Anderson, and Garcia suggested that renal dysplasia, acquired *in utero*, might be a common denominator in populations with high susceptibility to renal diseases (Brenner et al., 1988). This hypothesis was supported by many subsequent animal experiments (Zimanyi et al., 2006; Boubred et al., 2009; Chen et al., 2010; Plank et al., 2010) and epidemiological data (Lackland et al., 2000; Hodgin et al., 2009; Rajan et al., 2011; Orskov et al., 2012); however, the molecular mechanisms remain to be determined. It is well known that the renin–angiotensin system (RAS) plays a crucial role in kidney development. All the components of the classic RAS were expressed in the fetal kidney 2–3 days after the initiation of metanephric development, which stimulates the expression of glial-cell-line-derived neurotrophic factor (GDNF)/tyrosine kinase receptor (c-Ret) signaling pathway *via* angiotensin II receptors (ATRs), and hence promotes nephrogenesis (Yosypiv, 2008; Song et al., 2010b). However, the effects and the underlying mechanisms of PCE to kidney development are still unclear.

The present study was designed to test the hypothesis that PCE could cause fetal renal dysplasia and induce glomerulosclerosis in adult offspring *via* renal ATRs programming mechanisms. This work will be beneficial in elucidating the kidney developmental toxicity of caffeine and exploring the fetal origin of the susceptibility to glomerulosclerosis in the adult offspring with IUGR.

Materials and methods

Chemicals and reagents. Caffeine was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Creatinine (Cr), urea nitrogen (UN) and urine protein assay kits were obtained from Jiancheng Bioengineering Institute (Nanjing, China). TRIzol® reagent was purchased from Life technologies (Carlsbad, CA, USA). PrimeScript® RT reagent Kit with gDNA Eraser and SYBR® Premix Ex Taq™ Kit were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). All oligonucleotide primers of rat were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). RIPA buffer was from Applygen Technologies Inc. (Beijing, China). Protein Assay Kit was obtained from Bio-Rad Laboratories, Inc. (Beijing, China). Tween-20 was from Sigma-Aldrich Co. (Shanghai, China). The enhanced chemiluminescence kit (ECL) and goat anti-rabbit IgG were obtained from Thermo Pierce Biotechnology Inc. (Rockford, IL, USA). Polyclonal antibodies of rat angiotensin II receptor type 1(AT_1R) (AB124505) and angiotensin II receptor type 2 (AT_2R) (NBP1-77368) were from Abcam Inc. (Cambridge, MA, USA) and Novus Biotechnology Inc. (Novus, CA, USA) respectively. Polyclonal antibody of rat GDNF (PB0045) was produced by Boster Inc. (Wuhan, China). All chemicals and reagents were of analytical grade.

Animals and treatment. Animal experiments were performed in the Center for Animal Experiments of Wuhan University (Wuhan, China), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Wuhan University School of Medicine (Permit Number: 14016).

The designed experiments were divided into 2 separate batches, including the experiments of the adult offspring fed with normal diet and the experiments of the fetal offspring. Each experiment was independent to the other, including breeding, processing and detection of the experimental indicators.

Wistar rats (with weights of 260–300 g for males and 200–240 g for females) were obtained from the Experimental Center of Hubei Medical Scientific Academy (No. 2009-0004, Hubei, China). Animals were housed under standard conditions (room temperature: 18–22 °C; humidity: 40–60%) and allowed free access to rat chow and distilled water. After one week of acclimation before subjected to experimental conditions, 2 females were mated with 1 male every night. Upon confirmation of mating by the appearance of sperm in a vaginal smear, the day was taken as gestational day (GD) 0. Pregnant females were then transferred to individual cages and randomly divided into 2 groups: control and PCE groups. In the PCE group, the rats were administered 120 mg/kg of caffeine intragastrically once daily from GD9 until term delivery (GD20) (Xu et al., 2012b), the rats in the control group were given the same volume of distilled water. Twelve to fourteen randomly selected pregnant rats in each group were kept until normal delivery (GD21), and on postnatal day 1 (PD1), the number of pups was normalized to 8 pups per litter to assure adequate and standardized nutrition until weaning (postnatal week 4, PW4). After weaning, one male pup per litter was randomly selected and fed with standard lab chow. The 24 h urine samples of rats were collected using metabolic cages at PW23 for urine protein analysis. At PW24, the offspring rats were anesthetized with isoflurane and decapitated. Serum was prepared and stored at −80 ° C for Cr and UN analyses. Kidneys were dissected, randomly selected, partly fixed in 10% formalin solution for histological examination, and the rest were immediately frozen in liquid nitrogen and stored at −80 °C for gene and protein expression analyses.

In the experiment of fetal rats, the pregnant rats were randomly divided into 3 groups: control, PCE 30 and 120 mg/kg groups. The rats were treated with different doses of caffeine with the same route and time duration as described above in the adult offspring experiments. The rats in the control group were given the same volume of distilled water. The rats were anesthetized with isoflurane and euthanized on GD20, thirteen dams with 10–14 fetuses were selected from each group. IUGR was diagnosed when the body weight of an animal was two standard deviations less than the mean body weight of the control group (Engelbregt et al., 2001). Three fetal kidneys from 3 dams (one kidney per litter) in each group were randomly selected and routinely fixed for histological and ultra-structural examination, the rest of the fetal kidneys were immediately frozen in liquid nitrogen and stored at −80 °C for gene and protein expression analyses.

Blood and urine sample analysis. Serum Cr, UN and urine protein levels were detected by assay kits following the manufacturer's protocol.

Histological examination. Kidney tissue was fixed overnight in 10% formalin solution and embedded in paraffin, sectioned into 4 μm thick slices and stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) reagent and Masson's trichrome (Masson). The sections were observed and photographed with an Olympus AH-2 light microscope (Olympus, Tokyo, Japan).

At least 30 glomeruli on each PAS-stained slide were randomly selected for the evaluation of glomerulosclerosis under 400× magnification using a semiquantitative scoring system from 0 to 4: grade 0, normal appearance; grade 1, involvement of up to 25% of the glomerulus; grade 2, involvement of 25–50% of the glomerulus; grade 3, involvement of 50–75% of the glomerulus; and grade 4, involvement of 75–100% of the glomerulus. A glomerulosclerotic index (GSI) was then established using the formula: $GSI\ (\%) = (1 \times n_1 / n + 2 \times n_2 / n + 3 \times n_3 / n + 4 \times n_4 / n) \times 100$, where n_x is the glomeruli number in the rat with a given score (x), n is the total glomeruli number (Hohenstein et al., 2007). Similarly, tubulointerstitial injury, defined as tubular atrophy, dilatation, thickening of the basement membrane and protein cast, was assessed by semiquantitative analysis. Thirty cortical fields from each animal were examined at 200× magnification and graded according to a scale of 0–4: 0, no tubulointerstitial injury; 1, 25% of the tubulointerstitium injured; 2, 25%–50% of the tubulointerstitium injured; 3, 51%–75% of the tubulointerstitium injured; and 4, 76%–100% of the tubulointerstitium injured.