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Aminoguanidine attenuates hypertension, whereas 7-nitroindazole exacerbates kidney damage in spontaneously hypertensive rats: The role of nitric oxide

Chien-Fu Huang [a], Chien-Ning Hsu [b,c], Shao-Ju Chien [a], Ying-Jui Lin [a], Li-Tung Huang [a,d], You-Lin Tain [a,e,*]

[a] Department of Pediatrics, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University, College of Medicine, Kaohsiung, Taiwan
[b] Department of Pharmacy, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan
[c] Graduate Institute of Clinical Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan
[d] Department of Traditional Chinese Medicine, Chang Gung University, Linkow, Taiwan
[e] Center for Translational Research in Biomedical Sciences, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University, College of Medicine, Kaohsiung, Taiwan

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ABSTRACT

Nitric oxide (NO) deficiency contributes to hypertension and end-organ damage. Three nitric oxide synthase (NOS) isoforms have been identified: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Whether selective nNOS or iNOS inhibition exacerbates kidney damage in spontaneously hypertensive rats (SHRs) remains unclear. Seven-week-old SHRs were randomly assigned to 4 groups ($n=8$ for each group): group 1, SHRs receiving no treatment; group 2 (SHR+7-NI), SHRs given7-nitroindazole (7-NI, nNOS inhibitor) in their drinking water (10 mg/kg/day); group 3 (SHR+salt), SHRs given 1% NaCl; and group 4 (SHR+AG), SHRs given0.1% aminoguanidine (AG; iNOS inhibitor) in drinking water. The mean arterial pressure of SHRs treated with salt was significantly elevated compared with untreated controls. While AG caused a decrease of mean arterial pressure at 8 and 12 weeks of age in SHRs, both 7-NI and salt exacerbated kidney injury. In addition, AG significantly increased L-arginine levels and the L-arginine-to-asymmetric dimethylarginine (ADMA) ratio in the kidney. Salt treatment decreased renal nNOS-α protein levels and reduced dimethylarginine dimethylaminohydrolase (DDAH) activity. Salt and AG treatment increased nNOS-β and L-citrulline levels in SHR kidneys. AG attenuates hypertension development by upregulation of L-citrulline-to- L-arginine conversion and an increase in the L-arginine-to-ADMA ratio in SHR kidneys. 7-NI impairs renal function but has no effect on blood pressure, suggesting reno-protective role for the nNOS. Salt exacerbates kidney damage mainly through decreasing renal nNOS-α protein levels and DDAH activity. Our findings highlight the protective role of the nNOS/NO pathway in the development of kidney damage in SHRs.

1. Introduction

Nitric oxide (NO), an endogenous vasodilator, regulates systemic and local blood flow. Evidence supports the contribution of NO deficiency to hypertension and end-organ damage (Thomas et al., 2001; Baylis, 2008). In the spontaneously hypertensive rat (SHR), doubt remains as to whether NO deficiency is involved in different phases of hypertension since both increased (Vaziri et al., 1999; Wu and Yen, 1999) and decreased (Hayakawa and Raij, 1998; Koeners et al., 2007) NO has been reported in this model. There are 3 nitric oxide synthase (NOS) isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Both iNOS-induced oxidative stress and NO deficiency due to a decrease in constitutive NOS-activity (eNOS and nNOS) are linked to hypertension (Wilcox, 2005).

SHRs develop hypertension at 4–6 weeks of age, along with features of hypertensive end-organ damage (Pinto et al., 1998). Although renal damage has been found in older SHRs (Zhou et al., 2002), adults are less vulnerable to kidney damage (Griffin et al., 2001). Specifically, renal damage was accelerated in SHRs when treated with N^{w}-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor (Qiu et al., 1999). Renal damage in SHRs can also be exacerbated by high salt intake, through a blood pressure (BP)-independent mechanism (Frohlich and Varagic, 2005); however, NO production is also enhanced, leading to improved excretion of sodium and stabilization of BP in normotensive rats (Shultz and Tolins, 1993). Furthermore, L-NAME—induced kidney

* Corresponding author at: Department of Pediatrics, Kaohsiung Chang Gung Memorial Hospital, 123 Dabi Road, Niausung, Kaohsiung 833, Taiwan.
Tel.: +886 975056995; fax: +886 7 7338009.
E-mail address: tainyl@hotmail.com (Y.-L. Tain).

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damage is exacerbated by high salt intake in SHRs (Vaskonen et al., 1997). Thus, the upregulation of constitutive NOS is proposed as an adaptive mechanism to prevent end-organ damage in the SHR kidney (Hayakawa and Raij, 1998).

In salt-sensitive hypertension, NO derived from nNOS in the macula densa regulates afferent arteriolar diameter and tubuloglomerular feedback (TGF) (Tojo et al., 2004). However, TGF is unresponsive to the selective nNOS inhibitor, 7-nitroindazole (7-NI) in SHRs (Welch et al., 2000). These findings suggest that nNOS/NO may be involved in a protective mechanism in SHR kidneys. In addition, aminoguanidine, an iNOS inhibitor has been shown to attenuate hypertension development in SHRs (Hong et al., 2000; Wang et al., 2007). Although it is established that the non-selective NOS inhibitor, L-NAME, worsens renal damage, it remains unclear whether selective nNOS or iNOS inhibition can exacerbate kidney damage in SHRs.

In addition to decline in NOS, possible mechanisms for NO deficiency include L-arginine (the substrate for NOS) deficiency, inactivation by oxidative stress, or an increase of asymmetric dimethylarginine (ADMA) (Baylis, 2008). Similar to L-NAME, ADMA is a NOS inhibitor which competes with L-arginine for NOS binding to reduce NO production. We found that renal ADMA levels and the L-arginine-to-ADMA ratios were restored in 24-week-old SHRs; hence, we propose that these mechanisms might protect SHRs against kidney damage.

We used SHRs, SHRs with specific NOS inhibition, and SHRs with high salt as models of hypertension, which allowed us to examine whether the NOS-ADMA-NO pathway protects SHRs from renal damage. To manipulate NOS, we selectively inhibited nNOS with 7-NI, or iNOS with aminoguanidine.

2. Materials and methods

2.1. Animals and experimental design

This experiment was approved and performed under the Guidelines for Animal Experiments of Chang Gung Memorial Hospital and Chang Gung University. The treatment of animals conformed to the US National Institutes of Health guidelines. Male SHRs at 6 weeks of age were obtained from BioLASCO Taiwan Co., Ltd., Taipei, Taiwan. Rats were housed and maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility with free access to tap water and standard rat chow. At 7 weeks of age, rats were randomly assigned to1 of 4 groups ($n=8$ for each group): group 1, untreated SHRs; group 2 (SHR+7-NI), SHRs receiving 7-nitroindazole (7-NI; 10 mg/kg/day) in their drinking water (Wangensteen et al., 2003; Sedlakova et al., 2009); group 3 (SHR+salt), SHRs receiving NaCl (1%) in their drinking water (Wangensteen et al., 2003; Sedlakova et al., 2009), and group 4 (SHR+AG), SHRs treated by addition of 0.1% aminoguanidine (AG) to their drinking water (Wang et al., 2007). The dose of 7-NI was chosen to inhibit nNOS, but not other forms of NOS in-vivo (Chrysant et al., 1979; Wangensteen et al., 2003). The dose of AG used in this study was previously shown to inhibit iNOS effectively (Nilsson, 1999; Wang et al., 2007). The amount of drinking water was measured daily to ensure all groups consumed equal amounts of the drugs. BP was measured in conscious rats by an indirect tail-cuff method (BP-2000; Visitech Systems, Inc., Apex, NC, USA) after being systematically trained at 6, 8, 10, and 12 weeks of age (Tain et al., 2011). The average of 3 stable consecutive blood pressure measurements was used. All rats were killed at the age of 12 weeks. Urine was collected for 24 h before sacrifice to enable determination of total protein by the Bradford method and measurement of NOx ($NO_2^- + NO_3^-$) levels by the Greiss reaction. Heparinized blood samples were collected and kidneys harvested. One kidney was snap-frozen and the other perfusion-fixed and used for immunohistochemistry.

For the histological analysis, kidneys were formalin fixed andembedded in paraffin, and 4-μm sections were stained with periodic acid-Schiff (PAS). Histopathological evaluation of glomerular injury was performed as previously described (Tain et al., 2010).

2.2. Detection of L-arginine, L-citrulline, and ADMA

Plasma and tissue L-arginine, L-citrulline, and ADMA levels were measured using high-performance liquid chromatography (HP series 1100; Agilent Technologies Inc., Santa Clara, CA, USA) with the derivatization reagent, *o*-phthalaldehyde-3-mercaptopropionic acid (OPA-3MPA), used as previously described (Tain et al., 2010). Standards were 1–100 μM of L-arginine and L-citrulline, and 0.5–5 μM of ADMA. The recovery rate was approximately 90–105%, and levels of these substances in tissue were corrected for protein concentration and presented as μM/mg protein.

2.3. Western blotting

Western blot analysis was performed as previously described (Tain et al., 2011). A mouse monoclonal antibody (Santa Cruz, SantaCruz, CA, USA) was used for nNOS-α detection, and a C-terminal rabbit polyclonal antibody (Affinity BioReagents, Golden, CO, USA) for nNOS-β. A mouse monoclonal antibody (Transduction Laboratories, 1:250 dilution, 1-h incubation), followed by a goatanti-mouse IgG-HRP secondary antibody, was used to detect eNOS. For detection of protein arginine methyltransferase-1 (PRMT-1), we used a rabbit anti-rat PRMT-1 antibody (Millipore, Billerica, MA, USA; 1:2000 dilution). For detection of dimethylarginine dimethylaminohydrolase (DDAH), we used a goat anti-rat DDAH-1 antibody (Santa Cruz; 1:500 dilution, overnight incubation) and a goat anti-rat DDAH-2 antibody (Santa Cruz; 1:100 dilution, overnight incubation) followed by a secondary donkey anti-goat antibody. For detection of argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL), a goat anti-rat ASS antibody (Santa Cruz; 1:200 dilution, overnight incubation) and a rabbit anti-rat ASL antibody (Santa Cruz; 1:200 dilution, overnight incubation) were applied. For the detection of cationic amino acid transporter-1 (CAT-1), we used a rabbit anti-rat antibody (Abcam, Cambridge, MA, USA; 1:250 dilution); for arginase II, we used a rabbit antibody (Santa Cruz; 1:1000 dilution, overnight incubation) followed by a goat anti-rabbit secondary antibody. Bands of interest were visualized using enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA, USA) and quantified by densitometry (Quantity One Analysis software; Bio-Rad, Hercules, CA, USA) as integrated optical density (IOD) after subtraction of the background. The IOD was corrected for Ponceau Red staining (PonS) to account for any variations in total protein loading and protein abundance represented as IOD/PonS.

2.4. DDAH activity

The activity of DDAH was determined by a colorimetric assay measuring the rate of citrulline production, as recently optimized in our laboratory (Tain and Baylis, 2007). Kidney cortex was homogenized in sodium phosphate buffer. Tissue homogenate was preincubated with urease for 15 min, and then 100 μL (2 mg) of homogenate was incubated with 1 mM ADMA for 45 min at 37 °C. After deproteinization, the supernatant was incubated with a color mixture at 60 °C for 110 min. Each sample was analyzed with a