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The urinary bladder of spontaneously hypertensive rat demonstrates bladder hypertrophy, inflammation, and fibrosis but not hyperplasia

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ABSTRACT

Aims: The present study aims to systemically characterize the factors that are associated with urinary bladder organ enlargement in spontaneously hypertensive rats (SHR).
Main methods: We compared the SHR to age-matched normotensive Wistar-Kyoto (WKY) control rats in the levels of bladder pro-inflammatory factors, collagen expression (type I), and detrusor smooth muscle growth.
Key findings: Our results showed that enhanced inflammatory responses and fibrosis were key factors that were closely associated with bladder wall thickening in SHR. Specifically the mRNA levels of inflammatory factors interleukin (IL)-1α, IL-6 and TNFα were significantly higher in SHR than those in WKY rats. The SHR also had a higher number of mast cells in the suburothelium space. Type I collagen production was also significantly higher in SHR when compared to that in control rats. However, the smooth muscle content stayed the same in SHR and WKY rats. This was shown by the results that the ratio of α-smooth muscle actin (SMA) to the nuclear protein histone H3 had no difference between these two rat strains. The mRNA and protein levels of proliferating cell nuclear antigen (PCNA) also showed no change in the urinary bladder of SHR and WKY rats. Further study showed that the phosphorylation level of Akt in the urinary bladder was not changed in SHR when compared to WKY rats. In contrast, the phosphorylation level of ERK1/2 was significantly higher in SHR bladder when compared to that of WKY rats.
Significance: These results suggest that inflammation and fibrosis are primary factors that may lead to urinary bladder hypertrophy in SHR.

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Introduction

Since the development of the spontaneously hypertensive rats (SHR) strain in the 1960s [1], SHR have been extensively used in the study of cardiovascular disease and are considered to be a good animal model of human primary hypertension [2]. Subsequent studies show that this strain of rats also has significant urological complications demonstrated as development of microscopic kidney stones and bladder calculi [3], hyperactive voiding [4,5], a shorter filling phase and greater frequency of intravesical pressure rises [6]. SHR often show increases in voiding frequency and decreases in bladder capacity and micturition volume [4,7]. The urinary bladder dysfunction can be attributable to two levels of changes that include abnormal function of the neural pathway and alterations in the urinary bladder per se. In response to intrathecal doxazosin, an α1-adrenoceptor antagonist, nonvoiding contractions in SHR is markedly reduced [7]. Efferent and afferent neuronal hypertrophy is also seen within the micturition pathways of SHR [8]. In the SHR bladder, smooth muscles demonstrate a reduced expression level of smooth muscle myosin heavy chain B (MHCb) [9]. MHCb is preferentially expressed in cardiac vessels and the urinary bladder [9], and its mutation can cause organ hypertrophy [10,11,12]. So far there is no report on SHR bladder hypertrophy and the contributing factors have not yet been fully characterized.

Bladder hypertrophy is a medical condition where there is an enlarged bladder and a thicker bladder wall. Bladder "thick in its coats" (John Hunter, 1788) is accompanied by many diseases and disorders in humans and animals suffering from bladder inflammation [13,14], neurological impairment [15,16], bladder outlet obstruction with a variety of origin [17,18,19,20,21], social stress [22], or as a natural effect of aging [18]. The urinary bladder is made of four layers. The innermost urothelium layer, which is made of epithelium cells, acts as a permeability barrier protecting underlying tissues against noxious urine components; the next suburothelium space contains nerves, blood vessels and connective tissues; the muscular layer is called the detrusor muscle which controls the distension and contraction of the urinary bladder, and is surrounded by the outer layer, a serous membrane. Factors that

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contribute to bladder wall thickening have been identified which include excessive production of profibrotic extracellular matrix, increases in the muscular growth, edema and infiltration of immune cells and inflammatory substances [23,24,25]. Growth factors such as nerve growth factor (NGF) [13,26], transforming growth factor-beta (TGF)β [24], and basic fibroblast growth factor (BFGF) [27] have substantial roles in promoting collagen production and smooth muscle hypertrophy. Systemic release of pro-inflammatory cytokines and chemokines from distinct tissue compartments [28,29] also has a modulatory role in the organizational and functional alterations in micturition reflex pathways [23,30,31].

In SHR, artery hypertrophy has been noticed [32,33]. Artery wall hypertrophy and stiffness also appear in essential hypertensive patients [34]. It is not known whether the urinary bladder in SHR also demonstrates hypertrophic characteristics. We have found that the ratio of the bladder weight-to-body weight in SHR was significantly higher than those in age-matched WKY rats, which was accompanied by bladder wall thickening examined by H&E stain. To characterize the factors that were associated with these changes in SHR bladder, the present study examined 1) the mRNA levels of pro-inflammatory factors interleukin (IL)-1α, IL-6, tumor necrosis factor (TNF) α as well as mast cell expression; 2) the protein levels of type I collagen; 3) the mRNA and protein levels of proliferating cell nuclear antigen (PCNA); and 4) the relative level of α-smooth muscle actin to nuclear protein histone H3. These results will suggest whether inflammation, fibrosis and smooth muscle growth have roles in SHR bladder hypertrophy.

Materials and methods

Experimental animals and reagents

Age-matched SHR and WKY male rats were purchased (in collaboration with Dr. Gea-Ny Tseng) from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Animal use and care were in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and National Institutes of Health guidelines, and were approved by the Institutional Animal Care and Use Committee (IACUC). SHR and WKY rats were housed in a clean, quiet and well-ventilated animal room under a diurnal 12 hour light cycle. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Real-time PCR reagents were purchased from Applied Biosystems (Foster City, CA). Specialized kits were used according to manufacturer's instructions.

Protein extraction and western blot

The urinary bladder was homogenized in solubilization buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 100 mM NaF supplemented with protease inhibitor cocktail (P8340, 1:100, Sigma-Aldrich) and phosphatase inhibitor cocktail 1 (P2850, 1:100, Sigma-Aldrich). The homogenate was centrifuged at 20,200 *g* for 10 min at 4 °C, and the supernatant was removed to a fresh tube for further analysis. The protein concentration was determined using Bio-Rad DC protein assay kit.

Protein extracts were further separated on a 7.5–15% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk in Tris-buffered saline for 1 h and then incubated with primary antibodies against PCNA (1:1000, Cell Signaling Technology), type I collagen (Cell Signaling Technology), α-smooth muscle actin (1:3000, EMD Millipore Corporation), phospho-Akt (1:1000, Cell Signaling Technology), and phospho-ERK1/2 (1:1000, Cell Signaling Technology). Total Akt (1:2000, Cell Signaling Technology), total ERK1/2 (1:1000, Cell Signaling Technology), histone H3 (1:1000, Abcam plc.) and β-actin (1:5000, Sigma) were used as internal control for phospho- and non-phospho-proteins. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody. The bands were identified by ECL-exposed films that were then digitized and performed for densitometric quantification using the software FluorChem 8800 (Alpha Innotech, San Leabdro, CA). The expression level of the target protein in control animals from each independent experiment was considered as 1, and the relative expression level of the target protein in experimental animals was adjusted as a ratio to control animals.

RNA extraction and real-time PCR

Total RNA was extracted using a RNA extraction kit RNAqueous (Ambion, TX). RNA concentration was determined spectrophotometrically. cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ABI). Following reverse transcription, quantitative real-time PCR was performed on StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, ABI) under a condition of 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For inflammatory factors, real-time PCR was performed using SYBR Green as indicator. The sequences of primers were IL-1α forward: CCGCAGCTTTCCCAGAGCTGT, reverse: TCATGGAGGGCAGTCCCCGT; IL-6 forward: TGTTGACAGCCACTGCCTTCCC, reverse: ACTGGTCTGTTGTGGGTGGTATCCT; TNFα forward: AGCCCGTAGCCCACGTCGTA, reverse: ATGCCATTGGCCAGGAGGGC. Taqman probes were used for PCNA. The level of target mRNA was normalized against the expression of the internal control 18S or β-actin and was calculated with the ΔCt method (ΔCt = Ct target gene − Ct internal control in the same sample). The expression level of target mRNA in the control group from each independent experiment was considered as 1, and the relative expression level of target mRNA in experimental groups was adjusted as a ratio to its control in each independent experiment and expressed as fold changes ($2^{-\Delta\Delta Ct}$ fold) (ΔΔCt = ΔCt experimental group − ΔCt control group).

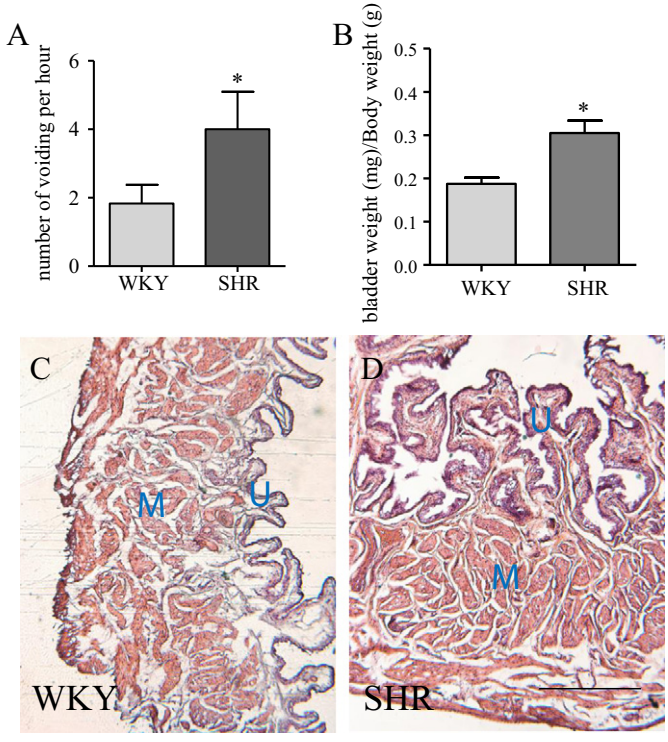

Fig. 1. SHR demonstrated bladder hyperactivity, hypertrophy and wall thickening. The SHR showed higher voiding frequency than WKY rats when examined by a noninvasive method (A). The ratio of bladder weight-to-body weight was higher in SHR than in WKY rats (B) SHR also demonstrated urinary bladder wall thickening and enhanced infiltration examined by H&E stain (compare D to C). *, $p < 0.05$. U: urothelium; M: muscle layer. Bar = 200 μm.

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