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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

The present study aims to systemically characterize the factors that are associated with urinary bladder organ enlargement in the spontaneously hypertensive rats (SHR).

Material and 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)-1 α , IL-6 and TNF α were significantly higher in SHR than those in WKY. 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 those in control rats. However, the smooth muscle content stayed the same in SHR and WKY rats. This was shown as that the ratio of α -smooth muscle actin (SMA) to the nuclear protein histone H3 showed 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. Further study showed that the phosphorylation level of Akt in the urinary bladder was not changed in SHR when compared to WKY. In contrast, the phosphorylation level of ERK1/2 was significantly higher in SHR bladder when compared to WKY.

Significance—These results suggest that inflammation and fibrosis are primary factors that may lead to urinary bladder hypertrophy in SHR.

Keywords

Spontaneously hypertensive rat; urinary bladder; hypertrophy; inflammation

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Conflict of Interest: None

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INTRODUCTION

Since the development of the spontaneously hypertensive rat (SHR) strain in the 1960s [1], SHR has been extensively used in the study of cardiovascular disease and is 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 shows an increase in voiding frequency and a decrease 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 α_1 -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 [10–12]. 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 with an enlarged bladder and thicker bladder wall. Bladder “thick in its coats” (John Hunter, 1788) is accompanied with many diseases and disorders in human and animals suffering from bladder inflammation [13, 14], neurological impairment [15, 16], bladder outlet obstruction with a variety of origin [17–21], 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 contribute to bladder wall thickening have been identified which can be due to excessive production of profibrotic extracellular matrix, increases in the muscular growth, edema and infiltration of immune cells and inflammatory substances [23–25]. 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 have 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 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 contribute to these changes in SHR bladder, the present study examined 1) the mRNA levels of pro-inflammatory factors interleukin

(IL)-1 α , 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 hour 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 Leandro, CA). The expression level of the target protein in control animal 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 15s 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-1 α Forward: CCGCAGCTTTCCAGAGCTGT, Reverse: TCATGGAGGGCAGTCCCCGT; IL-6 Forward: TGTGACAGCCACTGCCTTCCC, Reverse: ACTGGTCTGTTGTGGGTGGTATCCT; TNF α Forward: AGCCCGTAGCCACGTCGTA, 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 C_t method ($C_t = C_t \text{ target gene} - C_t \text{ internal control in the same sample}$). The expression level of target mRNA in 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 ($2^{-C_t \text{ fold}}$) ($C_t = C_t \text{ experimental group} - C_t \text{ control group}$).

Histological analysis

Hematoxylin and Eosin (H&E) Stain to assess the morphology and inflammation of the urinary bladder—Transverse sections of the urinary bladders (10 μm thickness) from all animals were stained with an H&E Stain kit according to the protocol provided by the manufacture (Richard-Allan-Scientific, Kalamazoo, MI). The sections were examined with a Zeiss brightfield microscope. The urinary bladder tissue architecture was characterized by examining the width and protrusion of the urothelium folds, the appearance of smooth muscle bundles, and inflammatory infiltration in the suburothelium and serosal area.

Toluidine Blue Stain to examine the number of mast cells—Transverse sections of the urinary bladders from all animals (10 μm thickness) were stained in a Toluidine Blue solution (0.5% Toluidine Blue O in 7% alcohol and 1% Sodium chloride, pH 2.3) for 2 to 3 min, and washed with water. Sections were dehydrated quickly through 95% and 2 changes of 100% alcohol followed with xylene. The mast cells appeared as violet/red purple with blue background of the tissue sections. The number of mast cells was counted in random non-overlapping microscopic views of the urothelium/suburothelium space and expressed as number of cells per view.

Statistical analysis

Eight SHR and five WKY rats were used. All data were presented as mean $\pm$ SEM. Comparison between SHR and WKY groups was made by using unpaired, two-tailed student's *t*-test. Differences between means at a level of $p < 0.05$ were considered to be significant.

RESULTS

SHR showed bladder organ enlargement and bladder wall thickening

It was reported that SHR had a significantly lower bladder capacity and micturition volume compared to WKY rats when examined with continuous cystometry [7]. In the present study, we examined the voiding frequency of the SHR and WKY animals in metabolic cage under natural condition. The SHR demonstrated a significant increase in voiding frequency (Fig. 1A) suggesting bladder hyperactivity.

The ratio of bladder weight-to-body weight (RBB) was obtained after the bladder was freshly dissected out. The average RBB of WKY animals was similar to those of Sprague Dawley rats that are routinely used in our lab (data not shown). The average RBB of the SHR rats was significantly higher than those of WKY (Fig. 1B) suggesting bladder organ hypertrophy in the SHR.

H&E examination showed inflammatory infiltration in SHR bladder (compare Fig. 1D to C). The urothelium folds (marked as U) were markedly expanded with significantly larger suburothelium spaces and thicker bladder wall. There was no obvious difference in the smooth muscle layer (marked as M) in terms of the size, orientation and tightness of the smooth muscle bundles between SHR and WKY bladders.

SHR bladder showed an enhanced inflammatory response characterized by the elevated levels of pro-inflammatory factors and mast cell numbers

To confirm the histological observation on the enhanced inflammatory responses in the SHR urinary bladder, we performed real-time PCR analysis of pro-inflammatory factors IL-1 α , IL-6 and TNF α and examined mast cell profiles in the urinary bladder. Results showed that the levels of interleukins IL-1 α and IL-6, and TNF α were significantly higher than those in WKY rats (Fig. 2). Mast cell numbers were also significantly higher in the SHR than WKY bladder (Fig. 3, blue cells). The average number of mast cells in WKY bladder was 2.7 ± 0.9 per microscopic field (100x); the average number of mast cells in SHR bladder was 10.2 ± 1.8 per microscopic field (100x) (Fig. 3C). The mast cells were mainly located in the urothelium crypt and suburothelium space (Fig. 3, arrows).

The type I collagen protein levels were increased in SHR bladder

Increased production and deposition of extracellular matrix often exist in hypertrophied visceral organs. Type I collagen forms the main constituent of the extracellular matrix in visceral organs. Western blot results showed that protein levels of type I collagen in SHR were significantly higher (2.4 folds, $p < 0.05$) than those in WKY bladder (Fig. 4) suggesting an increase in the degree of fibrosis in the SHR bladder.

There was no smooth muscle hypertrophy or hyperplasia in the SHR bladder

Smooth muscle hypertrophy and hyperplasia can increase the muscular content of the urinary bladder. Both real-time PCR and western blot showed that there were no significant differences in the expression levels of PCNA between SHR and WKY rats (Fig. 5) suggesting that there was no hyperplasia in the SHR bladder. The smooth muscle

hypertrophy was examined by testing the ratio of smooth muscle specific cytoplasmic protein α -smooth muscle actin to the nucleus protein histone H3. Western blot showed that the relative levels of smooth muscle actin stayed the same in SHR bladder and WKY bladder (Fig. 6).

The level of phospho-ERK1/2 but not phospho-Akt was increased in SHR bladder compared to WKY

It was reported that the level of NGF was increased in the SHR bladder when compared to WKY [35]. The Akt and ERK1/2 pathways are two major pathways downstream of NGF and are regulated by endogenous NGF in the urinary bladder during cystitis [13]. Examination of the activity (phosphorylation) of Akt and ERK1/2 by western blot (Fig. 7A) showed that the Akt activity was not changed in SHR bladder when compared to WKY (Fig. 7B); however the ERK1/2 activity in the urinary bladder was significantly increased in SHR (Fig. 7C).

DISCUSSION

The present study demonstrates that elevated inflammatory process and collagen production are primary factors that contribute to urinary bladder wall thickening in SHR. Although bladder smooth muscle hyperplasia and muscle cell hypertrophy are important factors resulting in bladder hypertrophy, smooth muscle growth is not seen in SHR bladder. In SHR, the urinary bladder shows an increase in the bladder weight-to-body weight ratio and an increase in the thickness of bladder wall when compared to WKY rats. Real-time PCR and western blot show that the mRNA levels of pro-inflammatory factors IL-1 α , IL-6 and TNF α and the protein level of type I collagen are significantly higher in SHR bladder than in WKY. This suggests an enhanced inflammatory response and fibrosis in the urinary bladder of SHR. Examination of the level of α -smooth muscle actin and PCNA shows no change between SHR and WKY, suggesting unaltered smooth muscle content in SHR-induced bladder hypertrophy. Enhanced inflammatory responses in the urinary bladder of SHR are also shown as having a higher number of mast cells than those in WKY. It has been reported that the level of NGF is higher in bladders from adult SHR when compared to WKY rats [4]. Characterization of the NGF downstream intracellular signaling molecules showed that the ERK1/2 pathway but not the Akt pathway is activated in the SHR bladder. These results suggest that SHR-associated bladder hypertrophy is associated with specific physiological events involving an increased inflammatory response and collagen upregulation in the urinary bladder.

Bladder hypertrophy is often seen in animal models with bladder injuries and also sometimes in animals with neurological disorders. Studies by us and others show that cystitis causes a significant increase in bladder weight which is associated with bladder inflammation and increases in the expression of growth factors and collagen production [13, 36, 37]. Bladder outlet obstruction also causes bladder hypertrophy that is due to bladder inflammation and increased fibrosis [23]. Bladder smooth muscle hyperplasia and hypertrophy are also seen in bladder dysfunction associated with several diseases [38, 39]. To examine the factors that are associated with the increases in bladder weight in SHR, we

examined the major physiological changes, i.e. collagen production and deposition, inflammation, and smooth muscle hypertrophy and hyperplasia, in the urinary bladder by use of a variety of techniques. Our results demonstrate that in SHR the urinary bladder shows significant inflammation and an increase in the levels of type I collagen; however, the mRNA and protein levels of PCNA, and the relative level of α -smooth muscle actin to histone H3 are not changed when compared to WKY rats. This is consistent to that in SHR there is no hyperplasia determined even though there is smooth muscle hypertrophy in aortas [40]. It is also reported that SHR heart demonstrates a higher level of interstitial fibrosis [41]. The causes of arterial smooth muscle hypertrophy and myocardial fibrosis are unknown. Inhibition of cytokines, p38 MAPK and Akt improves cardiac structure and performance in SHR by reducing collagen deposition and left ventricular hypertrophy [42]. Consistently, our results showed an increased level of cytokine production in the urinary bladder and the up-regulation of ERK1/2 activity. However, the Akt activity is not increased in the urinary bladder in SHR as it is in the heart [43]. The Akt/mTor pathway plays a critical role in the development of cardiac hypertrophy [43]. The unchanged Akt activity in the urinary bladder may explain the unaltered smooth muscle proliferation in the bladder of SHR. Similar phenomenon has also been seen in the urinary bladder of animals with colitis. After 2,4,6-Trinitrobenzenesulfonic acid treatment, the urinary bladder becomes overactive which is accompanied by an increase in the mast cell numbers with no change in the activity of Akt [44–46].

The increases in inflammatory responses in the urinary bladder of SHR may have effects on NGF production in the organ. It is reported that NGF expression can be regulated by pro-inflammatory mediators [47]. The activated mast cells could be a source to release NGF [48]. In SHR it is noted that mast cell numbers are significantly higher in the urinary bladder than that in WKY rats. Increases in NGF production in the bladder, in turn, can either sensitize the sensory nerve terminals leading to sensory hyperactivity or regulate gene expression in the urinary bladder. We have reported that NGF is able to regulate collagen production in cystitis bladder through activation of the Akt and ERK1/2 pathways [13]. In the present study, we found that both collagen and the ERK1/2 pathway are up-regulated in the SHR bladder; this is in line with the findings that excessive production of NGF and hyper-innervation of the target tissues are demonstrated in SHR [35, 49, 50].

CONCLUSION

To conclude this study, we demonstrate that bladder inflammation and fibrosis are primary factors in SHR-associated urinary bladder hypertrophy. Bladder smooth muscle hyperplasia is not involved. SHR-associated bladder hyperactivity is believed to be attributable to changes in the afferent and efferent pathways that supply the urinary bladder [8]. Increases in the mast cells in the SHR bladder and increases in the inflammatory factors produced by these cells can act on the sensory nerve terminals and increase neuronal activity and ultimately lead to bladder hyperactivity.

Acknowledgments

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Figure 1. SHR demonstrates bladder hyperactivity, hypertrophy and wall thickening

The SHR shows higher voiding frequency than WKY rats when examined by a noninvasive method (A). The ratio of bladder weight-to-body weight is higher in SHR than in WKY (B). SHR also demonstrates 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 μ m.

Figure 2. SHR shows elevated levels of inflammatory cytokines in the urinary bladder
The levels of IL-1 α (A), IL-6 (B) and TNF α were higher in SHR bladder than those in WKY rats. *, $p < 0.05$.

Figure 3. SHR shows a higher level of mast cell profile in the urinary bladder

There are a higher number of mast cells in the urinary bladder of SHR than those of WKY (compare B to A). The number of mast cells in each microscopic field (x100) was counted and shown in C. The mast cells are mainly located in the area of suburothelium space (blue cells in A and B, arrows). *, $p < 0.05$. Bar = 50 μm .

Figure 4. SHR shows an increased type I collagen level in the urinary bladder
Western blot (A) shows an increase in the protein level of type I collagen in SHR bladder when compared to WKY (B). *, $p < 0.05$.

Figure 5. No hyperplasia is found in SHR bladder

Western blot (A) and real-time PCR (B) confirms that the expression level of proliferation marker PCNA stays the same in SHR and WKY bladders suggesting that there is no obvious cellular hyper-proliferation in the urinary bladder of SHR when compared to WKY rats.

Figure 6. There is no difference in the detrusor smooth muscle content between SHR and WKY rats

Western blot (A) shows that the ratios of smooth muscle actin to H3 are the same in SHR and WKY rats (B). This suggests that in detrusor smooth muscle cells, the cytoplasmic protein content stays in ratio with the nuclear protein level, indicating an unlikelihood of detrusor smooth muscle cellular hypertrophy.

Figure 7. An enhanced ERK1/2 but not Akt activity is shown in SHR bladder

Western blot (A) shows that the phospho-Akt level stays the same in SHR bladder when compared to WKY (B). The level of phospho-ERK1/2 is significantly higher in SHR bladder than those in WKY (C). *, $p < 0.05$.