Effects of Castration and Testosterone Administration on Angiotensin II Receptor mRNA Expression and Apoptosis-related Proteins in Rat Urinary Bladder

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Abstract. We investigated the effects of castration and androgen administration on angiotensin II receptor mRNA expression and apoptosis-related proteins in the rat bladders. Sprague-Dawley rats were divided into three groups: the control group (sham operation; n = 8), the castration group (castrated, 8 weeks old, n = 8) and the castration plus testosterone group (1% testosterone gel administrated percutaneously into the dorsum daily for 8 weeks starting at 4 weeks after castration, n = 8). Bladder total RNA was extracted, and real-time PCR was performed to quantitatively measure the mRNA expression of angiotensin converting enzyme (ACE), angiotensin II (A II) receptor type 1 (AT1 receptor) and A II receptor type II (AT2 receptor). Western blotting was performed to determine the expression of apoptosis-related proteins. Expression of AT2 receptor mRNA and caspase-3 protein significantly increased in the rat bladder after castration, and these increases were reduced to control levels by testosterone administration. These results suggest that expression of AT2 receptor and caspase-3 in the bladder is androgen-dependent. Expression of Bcl-2 and Bax protein in the rat urinary bladder was not altered by castration. Expression of mitogen-activated protein (MAP) kinase phosphatase-1 protein in the rat urinary bladder was significantly increased by castration, but this increase was smaller with testosterone administration. These results suggest that expression of AT2 receptor mRNA and apoptosis-related proteins in the rat urinary bladder are affected by the change of androgen environment. The present study was the first to clarify the relationship between AT2 receptor and androgen in the urinary bladder.

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As the size of the elderly population increases, many middle-aged and elderly people are developing health problems. In women psychological and physical symptoms are sometimes induced when menopause causes a rapid decrease in estrogen. On the other hand, while age-induced changes in men are gradual and individual differences are substantial, there have been reports of late-onset hypogonadism (LOH) [1].

The basis of LOH is reduced androgen levels. It is characterized by erectile dysfunction (ED), depressive tendencies, irritability, reduced memory and attention, reduced muscle mass, increased percent body fat and decreased bone mineral density, and it affects middle-aged and elderly men [2–4]. Furthermore, the relationship between male menopause and age-related urinary disturbance has been examined. In the past, bladder outlet obstruction (BOO), such as benign prostatic hypertrophy, was considered to be the main form of urinary disturbance in older men, but in recent years, age-induced disuse bladder dysfunction has been reported, and a new concept known as lower urinary tract symptoms (LUTS) has been introduced [5, 6].

Androgen replacement is generally accepted for the treatment of LOH. Its effectiveness has been con-
firmed, and it has contributed to the mental and physical health of middle-aged and elderly men [7]. However, there have been few reports on the effects of androgen on bladder tissue and function [8]. Takeyumi reported that castration reduced the function of \( \alpha_1 \) adrenergic and muscarinic receptors and that testosterone administration improved dysfunction of these receptors, thus suggesting the involvement of androgen in bladder contraction [9]. In addition, several studies have investigated the effects of androgen on cardiovascular tissues, including myocardial cells, and it has been shown that androgen induces atrophy and fibrosis of cardiovascular smooth muscle cells via angiotensin receptors [10].

In this manner, there is a close relationship between androgen and angiotensin II (A II). Although the bladder and cardiovascular tissues are different types of organs, they both contain smooth muscle cells, and the expression of A II receptors has been demonstrated. Furthermore, Tanabe et al. investigated the effects of A II administration on bladder contraction in rats, and reported that administration of A II type 1 (AT1) and A II type 2 (AT2) receptor antagonists weakened bladder contraction [11]. However, to the best of our knowledge, there have been no studies investigating the relationship between androgen and A II in the bladder. We hypothesized that changes in the androgen environment induce changes in the expression and activity of A II receptors in the bladder. Furthermore, of the various activities of A II receptors, we focused on the effects of these changes with regard to downstream apoptosis-related proteins in the bladder.

**Materials and Methods**

*Animals and Tissues*

Male Sprague-Dawley rats (8-week-old; 280–330 g) were used in this experiment. Animals were housed under controlled conditions (22 ± 2°C, 55 ± 5% humidity, 12 h light/dark cycle) and had ad libitum access to water and laboratory chow (CE-2, Nippon CLEA, Tokyo, Japan). For examination, rats were divided into 3 groups: the control group (sham operation; \( n = 8 \)), the castration group (\( n = 8 \)) and the castration plus testosterone (T) group (castration + T group; \( n = 8 \)), to which 1% T gel (1% AndroGel\textsuperscript{TM}; 50 \( \mu \)g/rat/day) was applied daily to the surface of the dorsum for 8 weeks beginning at 4 weeks after castration.

All rats were sacrificed by decapitation at 20-week-old, 12 weeks after surgery, after which whole blood was collected, and serum was separated by centrifugation (1500 \( \times \) g, 15 min) and was stored at –20°C until use. Bladders were also collected, and were snap frozen in liquid nitrogen and stored at –80°C until use. The protocol for the present site investigation was approved by Animal Research Committee, St. Marianna University Graduate School of Medicine where the present study was conducted.

**Measurement of testosterone**

Testosterone concentration in serum was determined using a commercial testosterone RIA kit (DiaSorin Inc., Stillwater MN, USA).

**Expression analysis of A II receptors and ACE mRNA**

Total RNA was extracted from frozen bladder tissues using RNeasy Fibrous Tissue Mini Kit (Qiagen, Tokyo, Japan). Total RNA was treated with DNase inhibitor (PNase-free DNase set, QIAGEN) to remove contaminating genomic DNA.

First-standard cDNA was produced using 2 \( \mu \)g of total RNA in the presence of transcriptase and random decamers with a commercial reverse transcription kit (Retoro-script, Ambion, Austin, TX, USA). The reverse-transcribed mixture (1 \( \mu \)l) was used as template for subsequent real-time PCR. Real-time PCR was performed according to the SYBR method using an ABI prism 7000 Sequence Detector (Applied Biosystems, Foster City, CA, USA). PCR primers were chosen with the assistance of Primer Express software version 2.0 (Applied Biosystems), as shown in Table 1. The PCR mixture (50 \( \mu \)l) for single measurements contained 25 \( \mu \)l of 2 \( \times \) SYBR Green PCR Master Mix, each primer at 900 nM, and 1 \( \mu \)l of sample cDNA. Amplification and detection were performed with the ABI prism 7000 under the following thermal cycling conditions: 2 min at 50°C, 15 min at 95°C, and 40 cycles at 95°C for 1 min, each of the primers’ annealing temperatures for 1 min, and 72°C for 1 min. Each measurement was carried once.

Analysis of the results was based on the comparative Ct (threshold cycle) method according to the manufacturer’s instructions (Applied Biosystems), where Ct represents the cycle number at which a fluorescent
signal, which is associated with an exponential increase in PCR products, crosses a given threshold. External standard curves were generated by amplification of 9-fold dilutions of GAPDH and the product of interest. Results were calculated as relative differences in target Ct values normalized against GAPDH.

Detection of apoptosis-related proteins

Individual bladder samples were divided in half along the long axis and were stored at –80°C. Half of these tissues were pooled together and homogenated in 800 µl of ice-cold lysis buffer (pH 7.4) containing 300 mM NaCl, 50 mM NaPi (pH 7.8), 2 mM dithiothreitol, 1% polyoxyethylene (9) nonylphenyl ether plus 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged at 4°C for 20 min at 4000 × g. Aliquots containing 50 µg of protein were electrophoresed on 12.5% SDS-polyacrylamide denaturing gels (ATTO, Tokyo, Japan), and each gel was blotted onto a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK), according to the manufacturer’s instructions. Membranes were placed in blocking buffer (5% skim milk in wash buffer; Tris-buffered saline with 0.1% Tween 20) overnight at room temperature and blots were then incubated with primary antibody (anti-rabbit caspase-3 polyclonal antibody; Chemicon International, Temecula, CA, USA) for 2 h at room temperature. Blots were then incubated with a secondary horseradish peroxidase conjugated anti-rabbit antibody (Bio-Rad, Hercules, CA, USA) for 1 h. All other primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA): anti-mouse Bcl-2 monoclonal antibody; anti-rabbit Bax polyclonal antibody; and anti-rabbit mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) polyclonal antibody. After washing of the secondary antibody, signals were visualized using ECL plus Western blotting detection reagents (Amersham Biosciences) according to the manufacturer’s instructions. Protein band intensities were measured using a densitometer (AE-6920-MF densitograph Ver. 2.5, ATTO).

Detection of apoptosis

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) was performed for detection of apoptotic cell. Four percent paraformaldehyde-fixed bladder from 20-week-old SD rats was embedded in paraffin and cut at 4 µm. Deparaffinized sections were incubated with 10 µg/mL proteinase K at 37°C for 15 min and next with 0.3% H₂O₂/methanol. TdT reaction was performed by incubating the tissue with reaction buffer (200 mM/L potassium cacodylate, 25 mM/L Tris-HCl, 0.25 mg/mL BSA, 1 mM/L CoCl₂, 0.01 mM/L Biotin-dUTP (Roche Diagnostics, Mannheim, Germany), 560 U/mL TdT (Takara bio, Otsu, Japan) at 37°C for 60 min, and the section washed with 0.02 M phosphate buffered saline PBS, pH 7.4. Next the section was incubated with Vectstain Elite ABC Reagent based on biotin-avidin-peroxidase complex technique (Vectstain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) after washing PBS and color reaction developed using diaminobenzidine (DAB) as the chromagen. The sections were counterstained by Myer’s hematoxylin for nuclear staining. We used Apop Taq Positive Control Slide (Chemicon International, Temecula, CA, USA) for positive control.
Statistical analysis

Results are presented as means ± SE. Statistical differences between means in the sham operation, castration and replacement groups were compared by ANOVA, followed by Bonferroni test. P values of <0.05 were considered statistically significant. Arbitrary densitometric units for protein bands from each animal were used to assess differences among the various groups.

**Results**

Total body weight, prostate and bladder weights, and bladder-body ratios are shown in Table 2. The initial body weight of the three groups was similar, but at the end of the 12-week study period, the castration and castration + T groups weighed less than the control group (P<0.05). Bladder-body ratios did not differ among the three groups.

In the castration group, serum T levels were below...
the detection threshold. In the castration + T group, serum T levels increased to about 8-fold those in the control group; however, these levels varied between individuals.

Fig. 1A shows the quantitative expression of angiotensin converting enzyme mRNA, as detected by real-time PCR in the three groups. The castration and castration + T groups showed slight increases in the expression of this mRNA when compared with the control group (*p<0.05, **p<0.05).

Fig. 1B shows the quantitative expression of AT1 mRNA detected by real-time PCR in the three groups. There were no significant differences in AT1 mRNA expression among the three groups (not significant; NS).

Fig. 1C shows the quantitative detection of AT2 mRNA in the urinary bladders of the three groups by real-time PCR. AT2 mRNA expression in the urinary bladder was significantly increased by castration (p<0.001), but this increase was less marked in the castration + T group (p<0.014). However, AT2 mRNA expression in castration + T group did not recover to the levels seen in the control group (p<0.001).

Western blotting analysis of caspase-3 protein expression in rat urinary bladder is shown in Fig. 2. Panel A shows the polyclonal antibody against caspase-3 bound to the band at 32 kDa. In the castration group, expression of caspase-3 protein in the urinary bladder was significantly higher than in the control group. The histogram (panel B) shows the means ± SE of the arbitrary densitometric readings for caspase-3 in the control group, castration group, and castration + T group. Castration induced a significant increase of casepase-3 protein in the urinary bladder. Furthermore, in the castration + T group, expression of caspase-3 protein decreased to the same levels as in the control group (p<0.001).

We also examined the expression of apoptosis-related proteins (MKP-1, Bax and Bcl-2) in the urinary bladder of each group. As shown in Figs. 3 and 4, expression of Bax and Bcl-2 protein did not significantly vary among the three groups. As shown in Fig. 5, MKP-1 protein expression in the urinary bladder was significantly elevated by castration (p<0.001). The increase in MKP-1 protein expression in the urinary bladder in the castration group was less marked with T administration, but this change was not statistically significant (Fig. 5).

Western blot analyses of Bcl-2 in the urinary bladder. Sham operation (sham, n = 8); Castration (n = 8); Castration + testosterone (T) (n = 8). Histogram showing the means ± SE the arbitrary densitometric readings of the Bcl-2. (NS, not significant.)
Fig. 4. Western blot analyses of Bax in the urinary bladder. Sham operation (sham, n = 8); Castration (n = 8); Castration + testosterone (T) (n = 8). Histogram showing the means ± SE the arbitrary densitometric readings of Bax. (NS, not significant.)

Fig. 5. Western blot analyses of MKP-1 in the urinary bladder. Sham operation (sham, n = 8); Castration (n = 8); Castration + testosterone (T) (n = 8). Histogram showing the means ± SE the arbitrary densitometric readings of the MKP-1. (*, p<0.001; NS, not significant.)

Fig. 6. Urinary bladder sections were assayed for apoptosis with TUNEL staining. TUNEL positive cell was not detected in the sham operation control rat (A). There was no change on the expression of TUNEL positive cell in the castrated-rat (B). Apop Taq Positive Control Slide was simultaneously stained with TUNEL staining for positive control (C).
Discussion

In the present study, we examined the expression of A II receptor mRNA in the urinary bladder of castrated rats and castrated rats receiving testosterone. It is known that androgen increases A II levels and plays a role in the subsequent increase in contractile force of vascular smooth muscles and in the elevation of blood pressure [12]. Furthermore, there are gender differences in myocardial atrophy and fibrosis, and reports have shown that androgen is involved, and that atrophy and fibrosis of cardiovascular smooth muscle cells are mediated by the AT1A receptor [13–15]. The role of AT2 receptor remains unclear, but it has been generally accepted that the balance between AT1 and AT2 receptors is important for various disorders.

The AT1 receptor has been shown to correlate with vasoconstriction, cellular proliferation, reactive oxygen species (ROS) production and endothelin release, while the AT2 receptor has been shown to correlate with inhibition of cell proliferation, vasodilatation, apoptosis, nitric oxide (NO) production and collagen synthesis [16, 18]. In this manner, androgen is closely related to A II and its receptors, and thus we investigated the relationship between androgen and expression of AT2 receptor mRNA in the urinary bladder.

In the present study, we clarified that expression of AT2 receptor mRNA in the bladder changes in an androgen-dependent manner. Furthermore, we investigated the effects of the castration and androgen administration on the expression of apoptosis-related proteins in the urinary bladder because the AT2 receptor appears to be involved in apoptosis.

Several studies have investigated the relationship between A II receptors and apoptosis, and have found a particularly close relationship between the AT2 receptor and apoptosis. AT2 receptor expression in PC12W (rat pheochromocytoma cell line) and R3T3 (mouse fibroblast cell line) cells is high, and in these cells, apoptosis is induced via the AT2 receptor [19]. Diep et al. conducted a thorough study using spontaneously hypertensive rats and reported that when severe cardiomyopathy was induced using A II, marked myocardial apoptosis was seen and this apoptosis was suppressed by an AT2 receptor antagonist (PD123319) [20]. These findings suggest that A II induces apoptosis via AT2 receptor activation. In this manner, AT2 receptor-mediated apoptosis has been documented in vessels and cardiac smooth muscle cells.

Because our results suggested that the AT2 receptor, which is involved in apoptosis, was expressed in the bladder and that its expression was regulated by androgen, we investigated the expression of caspase-3 protein, the final factor for apoptosis which is a key effector in the apoptotic pathway. When compared with the control group, the expression of caspase-3 in the bladder was significantly elevated in the castration group, but there were no significant differences between the control and castration + T groups. In the urinary bladder of the castration group, as with the expression of AT2 receptor, the level of caspase-3 protein probably increased to induce apoptosis. On the other hand, there were no significant differences in the level of caspase-3 protein between the control and castration + T groups, thus suggesting that caspase-3 protein levels are androgen dependent. These findings suggest that changes in the androgen environment affect apoptosis induction via A II receptors.

In the cardiovascular system, AT2 receptor-mediated apoptosis does not mainly involve p53 in the apoptotic pathway mediated via Bcl-2 and Bax, but rather is mostly mediated by MAP kinase activation [17, 20]. Of the various MAP kinases, MKP-1 has been shown to be activated by the AT2 receptor and subsequently induces apoptosis [17, 21]. We obtained the same result as shown in Figs. 3 and 4, which showed there were no significant intergroup differences in the expression of Bcl-2 and Bax (mitochondrial apoptotic pathway) in the urinary bladder. However, as shown in Fig. 5, MKP-1 expression was significantly higher in the castration group. These results suggest that the increase in the caspase-3 expression was induced by an increase in MKP-1 expression, which was mediated by the AT2 receptor. We conjectured that the castration-induced elevation of AT2 expression increases caspase-3 expression through MKP-1, but we could not demonstrate the involvement of AT2. It is necessary to elucidate the roles of the androgen and angiotensin systems in the urinary bladder. We will add a castrated and AT2 inhibitor-treated group in the experiment to clarify the relationship between the androgen and angiotensin systems.

Morphologically, we could not detect apoptotic cell in the control and castrated rat by TUNEL method. The result suggested that the change of the apoptosis-related proteins in our results could not histologically cause apoptotic cell death in the bladder in the castrated rat. Our results suggest that changes in the androgen
environment alter the expression of AT2 receptor mRNA and then influence apoptosis-related proteins. Although caspase-3 expression in the urinary bladder was significantly increased by castration, histologically, apoptosis was not increased. The caspase-3 expression level in the control group was about 70% of that in the castrated group, but no morphological apoptosis was noted. Based on these findings, the caspase-3 expression level in the control group was not sufficient to induce morphological apoptosis, and the caspase-3 level which increased by about 1.4 fold following castration may have still been insufficient to induce apoptosis. These findings suggest that it is difficult to induce apoptosis in urinary bladder tissue. In this study, we investigated the relationships between castration and the influence of androgen administration and between the increased AT2 expression and apoptosis. Caspase-3 expression was altered, but no histological change in apoptosis was noted, suggesting that the increased AT2 by castration was more closely associated with signals other than apoptosis. We are planning another experiment to investigate actions associated with MKP-1 other than apoptosis to clarify the action associated with AT2. We believe that this is one of the age related changes in the bladder caused by age related reductions in androgen level. The findings after castration suggested that testosterone deficiency (deficiency of androgen action) increased ACE and AT2 expression. However, testosterone administration did not enable to recover to ACE and AT2 resume the control levels. Although its cause was unclear, a markedly high testosterone level (marked androgen action) after castration may have inversely inhibited ACE and AT2 expression. The action of inhibin, which is a testis-derived FSH inhibitor, is independent from testosterone action [22, 23]. To elucidate the crosstalk mechanism between the angiotensin and androgen systems in the urinary bladder, investigations of COX-1 expression are necessary. Functions of AT2 receptor are not well understood and recent reports have demonstrated that AT2 receptor may be implicated in inhibition of cell proliferation, vasodilatation, NO production and collagen synthesis in addition to apoptosis. Further examinations are necessary to clarify the relationship between apoptosis as well as the functions of AT2 receptor and androgen environment in the bladder.

References

4. Wu CY, Yu TJ, Chen MJ (2000) Age related testosterone administration in the castrated group may have been at least partially related to testicular factors, such as inhibin. Percutaneous administration of testosterone gel has been shown to lead to the maintenance of a constant blood testosterone level for a longer time in humans compared to injection, and is considered to be closer to physiological state. Percutaneous administration of testosterone gel that recovers the blood testosterone level to the normal level in castrated rats may provide an androgen-supplemented model in a state close to normal. We are planning to establish testosterone-supplemented animals in a state close to normal by improving the dose and administration method of testosterone gel, and investigate androgen dependence of AT2 expression in the urinary bladder using this model.


