

## Expression of $\alpha 1$ -Adrenergic Receptor Subtypes and Angiotensin II Type 1 Receptor in the Prostate of Spontaneously Hypertensive Rats

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**Background:** To clarify the mechanisms of lower urinary tract symptoms (LUTS) associated with benign prostate hyperplasia (BPH), we investigated the expression of  $\alpha 1$ -adrenergic receptor (AR) subtypes and the angiotensin II type 1 receptor (AT1) within the prostate of spontaneously hypertensive rats (SHRs).

**Methods:** Twelve male 25-week-old SHRs and Wistar Kyoto (WKY) rats were randomly separated into two groups (n = 6 each). One group was given 20 ml 0.9% sodium chloride solution (saline) orally per kg-body weight daily for one week. The other group received no treatment. After 7 days of saline loading, systolic blood pressure (SBP) and prostate weight were measured. The prostates were immunohistochemically analyzed for  $\alpha 1$ -AR subtypes and AT1.

**Results:** After 7 days, the SBP and prostate weight of saline-loaded SHRs tended to increase, but was not significantly different compared to the untreated rats. The expression of  $\alpha 1$ -AR subtypes and AT1 within the prostates of saline-loaded SHRs was higher than in the untreated ones. In contrast, the expression in the saline-loaded WKY rat prostates did not increase compared to the untreated ones.

**Conclusion:** Increased numbers of  $\alpha 1$ -AR subtypes and AT1 in saline-loaded SHR prostates might play important roles in the development of LUTS associated with BPH. *Shinshu Med J 58 : 103–114, 2010*

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**Key words:** spontaneously hypertensive rats, benign prostate hyperplasia,  
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### I Introduction

Persistent hypertension is a risk factor for stroke, heart attack, heart failure, and arterial aneurysm and is a leading cause of chronic renal failure. It increases not only the risk of developing cardiovascular disease and diabetes, but also that of lower urinary tract symptoms (LUTS). In clinical cases, many patients complaining of LUTS due to benign prostate hypertrophy (BPH) or overactive bladder have persistent hypertension<sup>1)</sup>. However, the

relationship(s) between persistent hypertension and BPH are not clear.

Spontaneously hypertensive rats (SHRs), which are used as a model of cardiovascular disease<sup>2)3)</sup>, also develop erectile dysfunction<sup>4)–6)</sup> and prostate hypertrophy<sup>7)</sup>. The rat prostate consists of five lobes: the ventral, lateral type 1, lateral type 2, and dorsal lobes, as well as the coagulating gland<sup>8)</sup>. We focused on the expression of  $\alpha 1$ -adrenergic receptor (AR) subtypes and angiotensin II type 1 receptor (AT1) in the prostatic ventral and lateral-dorsal lobes of SHRs. The  $\alpha 1$ -AR subtypes regulate cell growth as well as mediate prostate smooth muscle contraction in the human prostate<sup>9)</sup>. The angiotensin II peptide increases blood pressure<sup>10)</sup>

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and binds to at least two receptor subtypes, AT1 and angiotensin II type 2 receptor (AT2). It also promotes cell growth and smooth muscle tone within the prostate<sup>11)12)</sup>. Previously, we showed that oral loading with 0.9 % sodium chloride solution (saline) increases the expression of tachykinins, transient receptor potential vanilloid 1 (TRPV1), and P2X3 purine receptors within the urinary bladder of SHR<sup>13)</sup>. These rats then develop detrusor overactivity<sup>13)</sup>. In this study we investigated whether the expression of the  $\alpha$ 1-AR subtypes and AT1 within the prostate of SHR was also increased by saline loading.

## II Materials and Methods

### A Animals

Male SHR (SHR/Izm, n = 12, 350–380 g, SLC Inc., Shizuoka, Japan) and Wistar Kyoto rats (WKY, n = 12, 390–430 g, SLC Inc., Shizuoka, Japan) at 25 weeks of age were used for these experiments. All were maintained under a 12-hour alternating light-dark cycle with food and water ad libitum. The animals were treated in accordance with The National Institutes of Health Animal Care Guidelines and the guidelines approved by the Animal Ethics Committee of Shinshu University School of Medicine.

### B Administration of Saline Solution

The SHR were randomly separated into two groups (n = 6 each). One, the saline-loaded group, was administered with 20 ml of 0.9 % sodium chloride solution (saline) per kg-body weight daily for 7 days. The saline was delivered with a syringe via a stomach feeding tube inserted through the mouth. The other group did not receive any treatment (untreatment controls). After 7 days of saline loading, body weight was measured and systolic blood pressure (SBP) was indirectly measured with a tail-cuff apparatus (BP-98A-L, Softron, Tokyo, Japan) while the rats were quietly restrained without anesthesia in a plastic chamber. The WKY rats were also treated as above.

### C Tissue processing

Following SBP measurement, the experimental

rats were anesthetized with intraperitoneal ketamine hydrochloride (75 mg/kg body weight, Sankyo Eeru Medicine. Co., Tokyo, Japan) and xylazine (15 mg/kg body weight, Bayer, Leverkusen, Germany). The prostate was removed along with the urinary bladder, urethra, and seminal gland. The animals were then euthanized by inhalation of diethyl ether as approved by the Animal Ethics Committee. The urinary bladder and seminal gland were resected from the prostate, which was then weighed.

### D Immunohistochemistry

For immunohistochemistry, the prostates were rinsed with phosphate buffered saline (PBS) and fixed in 4 % paraformaldehyde with 4 % sucrose in 0.1 M phosphate buffer for 12 hours at 4°C, and then embedded in paraffin. Each sample was cut into 5  $\mu$ m thick serial sections. The sections were deparaffinized, rehydrated, and rinsed three times with PBS for 5 minutes at 4°C. Antigen retrieval was achieved by immersion of the sections in 10 mM sodium citrate and microwaving at 100°C for 5 minutes. The specimens were coated with 1.5 % normal donkey serum (Chemicon International Inc., Temecula, CA, USA) and 1.5 % non-fat milk in PBS for 1 hour at 4°C. Some sections were then incubated with proliferative cell nuclear antigen (PCNA, 1 : 250, mouse monoclonal, Thermo Fisher Scientific Anatomical Pathology, Cheshire, UK), a marker for proliferating cells. Other sections were incubated with anti-angiotensin II type 1 receptor antibody (Angiotensin II receptor, 1 : 250, mouse monoclonal, Novus Biologicals, Inc., Littleton, UK) for 12 hours at 4°C. The sections were rinsed with PBS at 4°C, and then incubated with donkey anti-mouse IgG secondary antibody conjugated with Alexa fluor 594 (1 : 250, Molecular Probes, Eugene, OR, USA) for 1 hour at 4°C. Following rinsing, double staining of each section was achieved by incubation with antibodies for the  $\alpha$ 1A adrenergic receptor ( $\alpha$ 1A-AR, 1 : 250, rabbit polyclonal, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), the  $\alpha$ 1D adrenergic receptor ( $\alpha$ 1D-AR, 1 : 250, rabbit polyclonal, Santa Cruz Biotechnology Inc.), or the  $\alpha$ 1B adrenergic

receptor ( $\alpha$ 1B-AR, 1 : 250, goat polyclonal, Santa Cruz Biotechnology Inc.) antibody for 12 hours at 4° C. After rinsing with PBS, they were incubated with donkey anti-rabbit or anti-goat IgG secondary antibody conjugated with Alexa fluor 488 (1 : 250, Molecular Probes) for 1 hour at 4°C. Finally, cell nuclei were counterstained with 5  $\mu$ g/ml 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes). The slides were coated with Fluorescent Mounting Medium (Dako Cytomation, Carpinteria, CA, USA). Other sections from each sample were stained with hematoxylin and eosin (H&E) or the ApopTag® ISOL fluorescence apoptosis detection (DNase Types I & II, Chemicon International Inc.) technique, which identifies both caspase-dependent and -independent apoptosis. All samples were observed with a Leica DAS Microscope (Leica Microsystems GmbH, Wetzlar, Germany). Also, we confirmed that negative controls without  $\alpha$ 1-ARs and AT1 antibody were not visualized.

Observers, who were not aware of the treatment status, made six observations of each sample, and then semi-quantitatively evaluated the presence of fluorescence markers in each observed area. The numbers of PCNA-positive cells and fluorescently tagged spots were counted, and the observed areas were estimated using Image-Pro® Plus (Media Cybernetics, Inc., Bethesda, MD, USA). The density of PCNA-positive cells and fluorescently tagged spots was then calculated and expressed as cells/ $\mu$ m<sup>2</sup> or spots/ $\mu$ m<sup>2</sup>.

### E Statistical Analysis

Results were expressed as means  $\pm$  standard error of the means. The non-repeated measures Student's t-test was used to compare untreated controls with saline-loaded SHR. Repeated measures Student's t-test was used to compare staining of the ventral and laterodorsal prostate in each group. Differences with  $P < 0.05$  were considered significant.

## III Results

### A Blood pressure and prostate weight

Prior to saline loading, there was no difference between the systolic blood pressures (SBP) of the untreated control and saline-loaded groups (189.5  $\pm$  4.2 mmHg, 197.1  $\pm$  4.3 mmHg, respectively). After 7 days of saline loading, the SBP of the saline-loaded SHR, 205.5  $\pm$  5.4 mmHg, tended to increase, but was not significantly different from the controls, 192.2  $\pm$  4.6 mmHg. At that time, the prostate weight in saline-loaded animals, 1.24  $\pm$  0.03 g, was not significantly different from that of the controls, 1.18  $\pm$  0.017 g. Also, the SBP (146.83  $\pm$  3.7 mmHg) and the prostate weight (1.23  $\pm$  0.05 g) of the saline-loaded WKY rats were not significantly different from these of the controls (110.17  $\pm$  8.5 mmHg, 1.36  $\pm$  0.08 g, respectively).

### B Immunohistochemistry of the ventral lobes

At 7 days after saline loading, there were no obvious differences between the prostate ventral lobes of the untreated (Fig. 1A) and saline-loaded SHR (Fig. 1B) when viewed by standard bright field microscopy. Numerous proliferating epithelial cells in both groups were positive for PCNA antibody (Fig. 1C,D), but there was no significant difference between them (Table 1). There were one or two caspase-dependent and -independent apoptotic cells present in both groups (Fig. 1E,F).

The presence of  $\alpha$ 1A-,  $\alpha$ 1D-,  $\alpha$ 1B-ARs, and AT1 on the epithelial cells in the ventral lobes were visualized by immunohistochemistry (Fig. 2). The expression of  $\alpha$ 1A-,  $\alpha$ 1D-, and  $\alpha$ 1B-ARs in the saline-loaded SHR was significantly higher than in the controls (Table 1). There was also significantly more epithelial AT1 in the ventral lobe of the saline-loaded SHR compared to untreated controls (Fig. 2, Table 1). In contrast, the presence of  $\alpha$ 1A-,  $\alpha$ 1D-,  $\alpha$ 1B-ARs, and AT1 in the ventral lobes of the saline-loaded WKY rats did not increase compared to the untreated controls (Table 2). None of the  $\alpha$ 1-AR subtypes appeared to co-localize with the AT1.

### C Immunohistochemistry of lateral-dorsal lobes

At 7 days after saline loading, there were no obvious differences between the prostate lateral-dorsal lobes of the untreated (Fig. 3A) and saline-loaded SHR (Fig. 3B) when viewed by standard

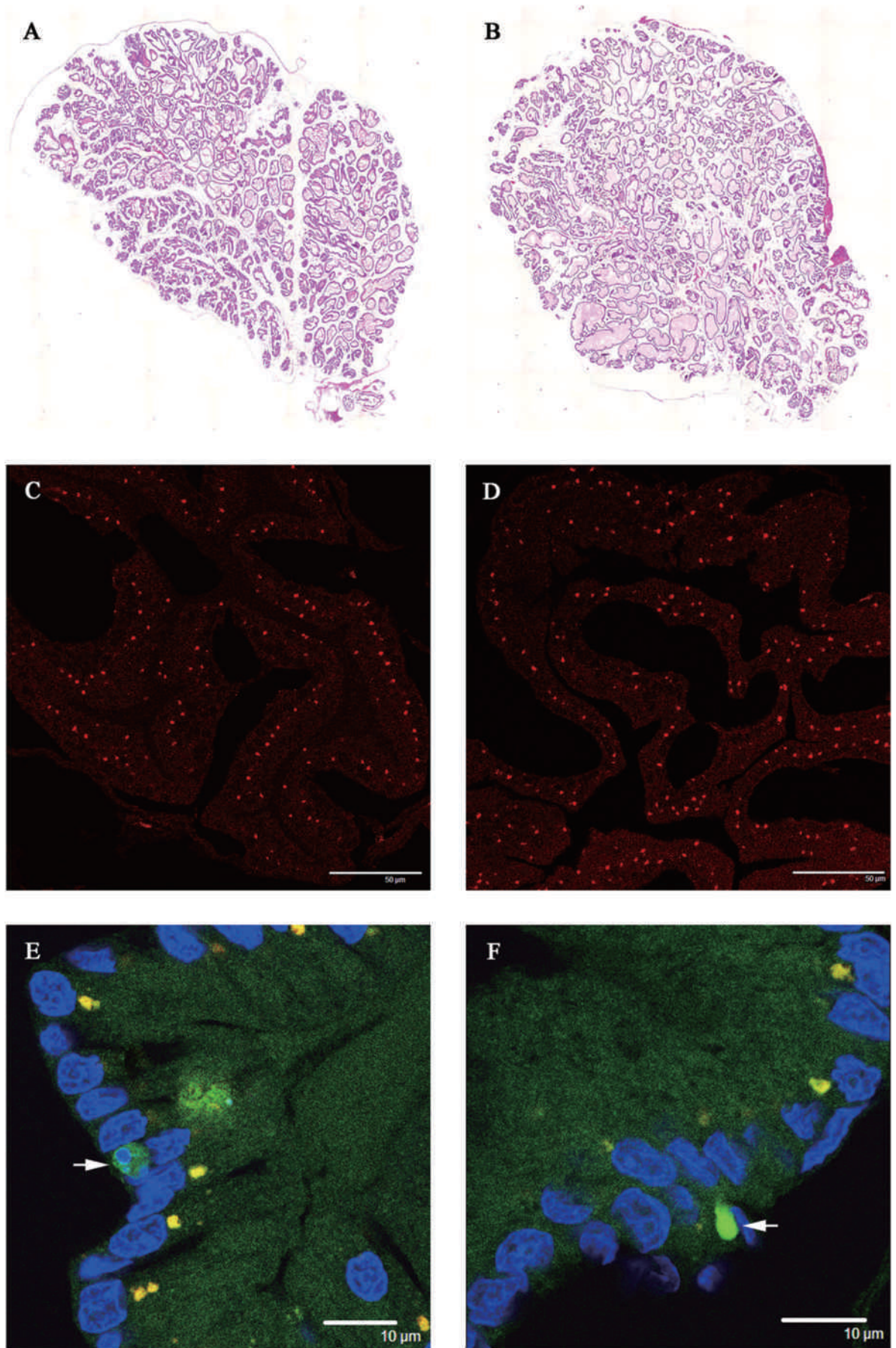


Fig.1

Table 1 PCNA-positive cells, epithelial  $\alpha$ 1-AR subtypes, and Angiotensin II type 1 receptor in the ventral and lateral-dorsal lobes of SHR

	Ventral lobe		Lateral-dorsal lobe	
	Untreated	Saline-loaded	Untreated	Saline-loaded
PCNA positive cells (cells/ $\mu\text{m}^2$ )	0.30 $\pm$ 0.03	0.31 $\pm$ 0.03	0.01 $\pm$ 0.002 ††	0.03 $\pm$ 0.01**§§
$\alpha$ 1A adrenergic receptor (spots/ $\mu\text{m}^2$ )	2.05 $\pm$ 0.51	5.27 $\pm$ 1.15*	0.58 $\pm$ 0.11 †	1.53 $\pm$ 0.24**§
$\alpha$ 1D adrenergic receptor (spots/ $\mu\text{m}^2$ )	1.49 $\pm$ 0.25	7.80 $\pm$ 1.39**	1.47 $\pm$ 0.21	6.03 $\pm$ 1.95*
$\alpha$ 1B adrenergic receptor (spots/ $\mu\text{m}^2$ )	1.31 $\pm$ 0.19	2.73 $\pm$ 0.27**	1.81 $\pm$ 0.18	6.08 $\pm$ 1.24**§
Angiotensin II type 1 receptor (spots/ $\mu\text{m}^2$ )	0.35 $\pm$ 0.03	0.58 $\pm$ 0.11*	1.09 $\pm$ 0.23 †	6.20 $\pm$ 2.54*§

\*P<0.05, \*\*P<0.01, compared with untreated control SHRs

† P<0.05, †† P<0.01, compared with ventral prostate of untreated control SHRs

§ P<0.05, §§ P<0.01, compared with ventral prostate of saline-loaded SHRs

bright field microscopy. The lateral-dorsal lobe of the untreated controls had few PCNA-positive cells (Fig. 3C). In contrast, the same region of saline-loaded SHRs had significantly more PCNA-positive cells than controls (Fig. 3D, Table 1). One or two caspase-dependent and -independent apoptotic cells were present in each group (Fig. 3E,F).

There were significantly more  $\alpha$ 1A-,  $\alpha$ 1D-, and  $\alpha$ 1B-ARs within the lateral-dorsal lobe epithelium of the saline-loaded SHRs than in the untreated controls (Fig. 4, Table 1). Also, there was significantly more epithelial AT1 in the saline-loaded SHRs compared to the controls (Fig. 4, Table 1). In contrast, the presence of  $\alpha$ 1A-ARs,  $\alpha$ 1D-ARs,  $\alpha$ 1B-ARs and AT1 in the lateral-dorsal lobes of the saline-loaded WKY rats did not increase compared to the untreated controls (Table 2). As in the ventral lobe, none of the  $\alpha$ 1-AR subtypes appeared to co-localize with the AT1.

#### D Comparison between ventral and lateral-dorsal lobes

In the untreated control SHRs, there were

significantly more PCNA-positive cells in the ventral lobes than in the lateral-dorsal lobes (Table 1). The expression of the  $\alpha$ 1A-ARs within the ventral lobe epithelial cells was also higher than that in the lateral-dorsal lobes. In contrast, the expression of neither the  $\alpha$ 1D- nor the  $\alpha$ 1B-ARs within the ventral lobes was significantly different from that in the lateral-dorsal lobes. However, expression of epithelial AT1 within the ventral lobes was significantly lower than in the lateral-dorsal lobes. In the untreated WKY rats, there were no differences between ventral and lateral-dorsal lobes (Table 2).

After 7 days of saline loading, the number of PCNA-positive proliferating cells in the ventral lobes was significantly greater than that in the lateral-dorsal lobes (Table 1). The expression of the  $\alpha$ 1A-ARs within the ventral lobe epithelium was also significantly greater than that in the lateral-dorsal lobes. In saline-loaded SHRs, there was no difference in the expression of the  $\alpha$ 1D-AR between the ventral and lateral-dorsal lobes. However, the expression of both the  $\alpha$ 1B-AR and the AT1 was

Fig. 1 Ventral lobes in untreated control and saline-loaded SHRs. At 7 days after saline loading, there were no obvious differences between the prostate ventral lobes of the untreated (A) and saline-loaded SHRs (B) when viewed and analyzed by image processing. H&E stain, x40. Both the control (C) and saline-loaded (D) SHRs had numerous proliferating epithelial cells that were positive for PCNA antibody (red). Bar = 50  $\mu\text{m}$ . Apoptotic cells induced by caspase-dependent or -independent pathways (green, arrow) were rarely present in the (E) control or (F) saline-loaded SHRs. Bar = 10  $\mu\text{m}$  Blue : nuclei.

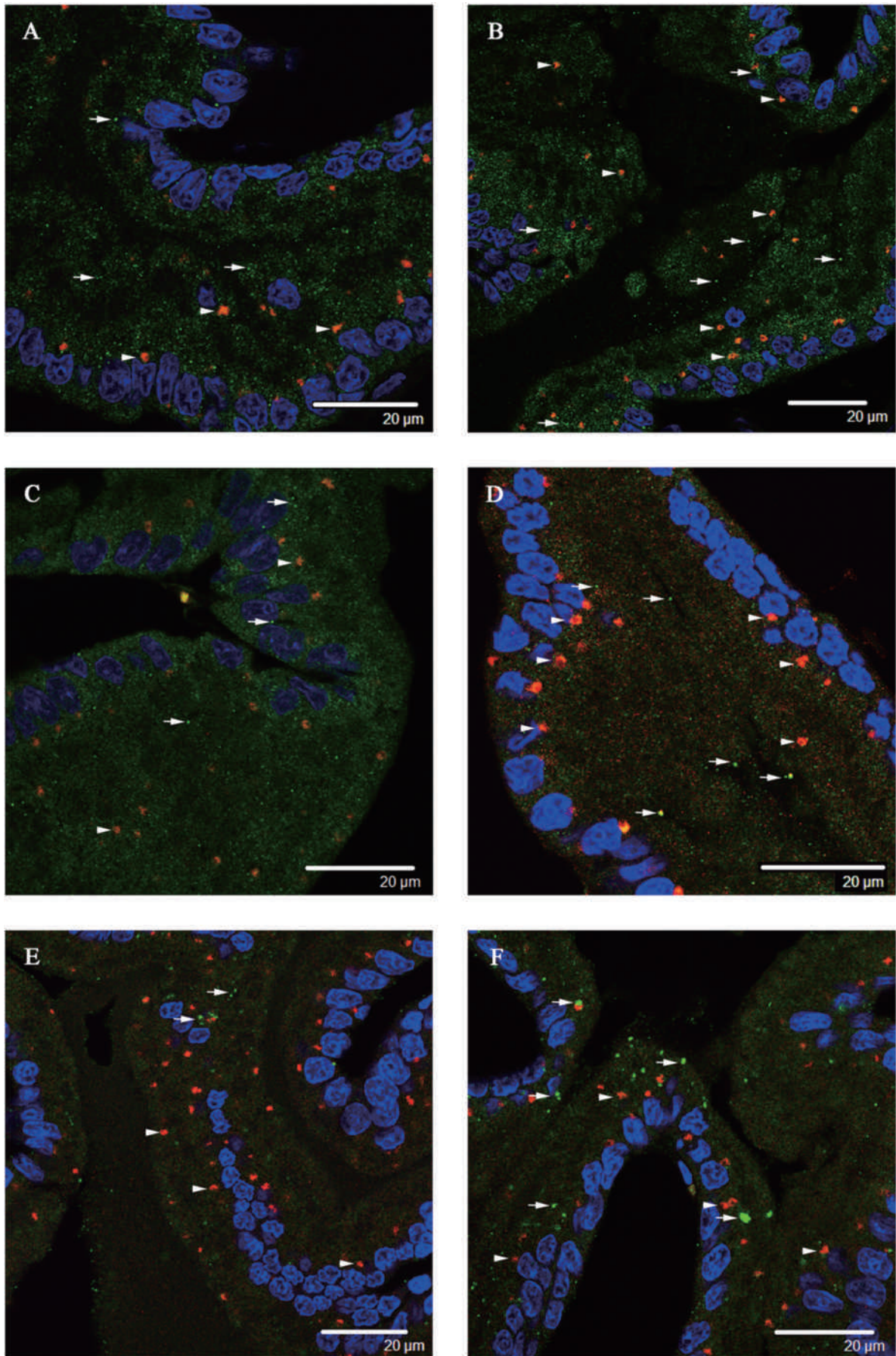


Fig. 2

Table 2 Epithelial  $\alpha$ 1-AR subtypes and AngiotensinII type 1 receptor in the ventral and lateral-dorsal lobes of WKY rat

	Ventral lobe		Lateral-dorsal lobe	
	Untreated	Saline-loaded	Untreated	Saline-loaded
$\alpha$ 1A adrenergic receptor (spots/ $\mu\text{m}^2$ )	0.005 $\pm$ 0.0012	0.004 $\pm$ 0.0007	0.003 $\pm$ 0.0008	0.003 $\pm$ 0.0010
$\alpha$ 1D adrenergic receptor (spots/ $\mu\text{m}^2$ )	0.004 $\pm$ 0.0007	0.004 $\pm$ 0.0004	0.003 $\pm$ 0.0002	0.003 $\pm$ 0.0005
$\alpha$ 1B adrenergic receptor (spots/ $\mu\text{m}^2$ )	0.005 $\pm$ 0.0004	0.004 $\pm$ 0.0003	0.005 $\pm$ 0.0005	0.005 $\pm$ 0.0009
Angiotensin II type 1 receptor (spots/ $\mu\text{m}^2$ )	0.004 $\pm$ 0.0004	0.003 $\pm$ 0.0006	0.004 $\pm$ 0.0010	0.004 $\pm$ 0.0008

significantly lower in the ventral lobes compared to the lateral-dorsal lobes. In the saline-loaded WKY rats, there were no differences between ventral and lateral-dorsal lobes (Table 2).

#### IV Discussion

The SBP of the SHR reaches 180 to 200 mmHg around 5–6 weeks of age<sup>14)–16)</sup> and is promoted by the administration of salt<sup>17)18)</sup>. A high concentration of salt increases the risk of death due to cerebrovascular accidents. Thus, we adopted a saline loading protocol. However, the protocol did not significantly increase the SBP further above the existing hypertensive state. It also did not significantly increase prostate weight.

The ventral lobes within both SHR groups had numerous PCNA - positive proliferating cells compared to apoptotic cells. Yamashita et al. reported that the ventral lobe in SHR is hyperplastic and predominantly composed of glandular epithelium<sup>19)</sup>. Our results also support these data even though our saline-loading conditions did not induce more ventral lobe hyperplasia than in untreated SHR. In contrast, the number of proliferating cells within the lateral-dorsal lobes of the saline-loaded SHR was significantly higher than that of the untreated controls. Thus, our saline-loading conditions in-

duced a small degree of hyperplasia in the lateral-dorsal lobes, while not increasing the SBP and prostate weight.

Expression of the three  $\alpha$ 1-AR subtypes within both lobes of the saline-loaded SHR was significantly higher than in the untreated controls. The  $\alpha$ 1A-AR plays a predominant role in mediating smooth muscle contraction activated by catecholamines in the prostate<sup>20)21)</sup>. The  $\alpha$ 1B-AR mediates the blood pressure response to adrenergic stimulation<sup>22)23)</sup>, and the  $\alpha$ 1D-AR regulates cell proliferation in the human prostate<sup>9)24)</sup>. Therefore, our results suggest that the increased  $\alpha$ 1-AR subtypes, especially  $\alpha$ 1D-AR, might mediate the hyperplastic response induced by saline loading.

The expression of AT1 within both of the lobes of the saline-loaded SHR was also significantly higher than in the untreated controls. When angiotensin II peptide binds to AT1 in the adrenal cortex, aldosterone is released and increases blood pressure<sup>10)</sup>. In the prostate, angiotensin II peptide also binds to AT1 and promotes cell growth and smooth muscle tone<sup>11)12)</sup>. In human BPH, angiotensin II peptide is increased, whereas AT1 is decreased<sup>11)</sup>. Similarly, an increased level of angiotensin II peptide is associated with a decrease of AT1 in the prostates of SHR<sup>25)</sup>. In our model, the

Fig. 2  $\alpha$ 1-AR subtypes and AT1 within the ventral lobes. There were fewer  $\alpha$ 1A-,  $\alpha$ 1D-, and  $\alpha$ 1B-ARs (A, C, and E, green, arrows) within the epithelial cells of the ventral lobes in the untreated control SHR than in the saline-loaded ones (B, D, and F, green, arrows). Similarly, the expression of AT1 (A, C, and E, red, arrowheads) within the epithelial cells of the ventral lobes of the control SHR was also lower than in the saline-loaded ones (B, D, and F, red, arrowheads). Bars = 10  $\mu\text{m}$ , Blue : nuclei.

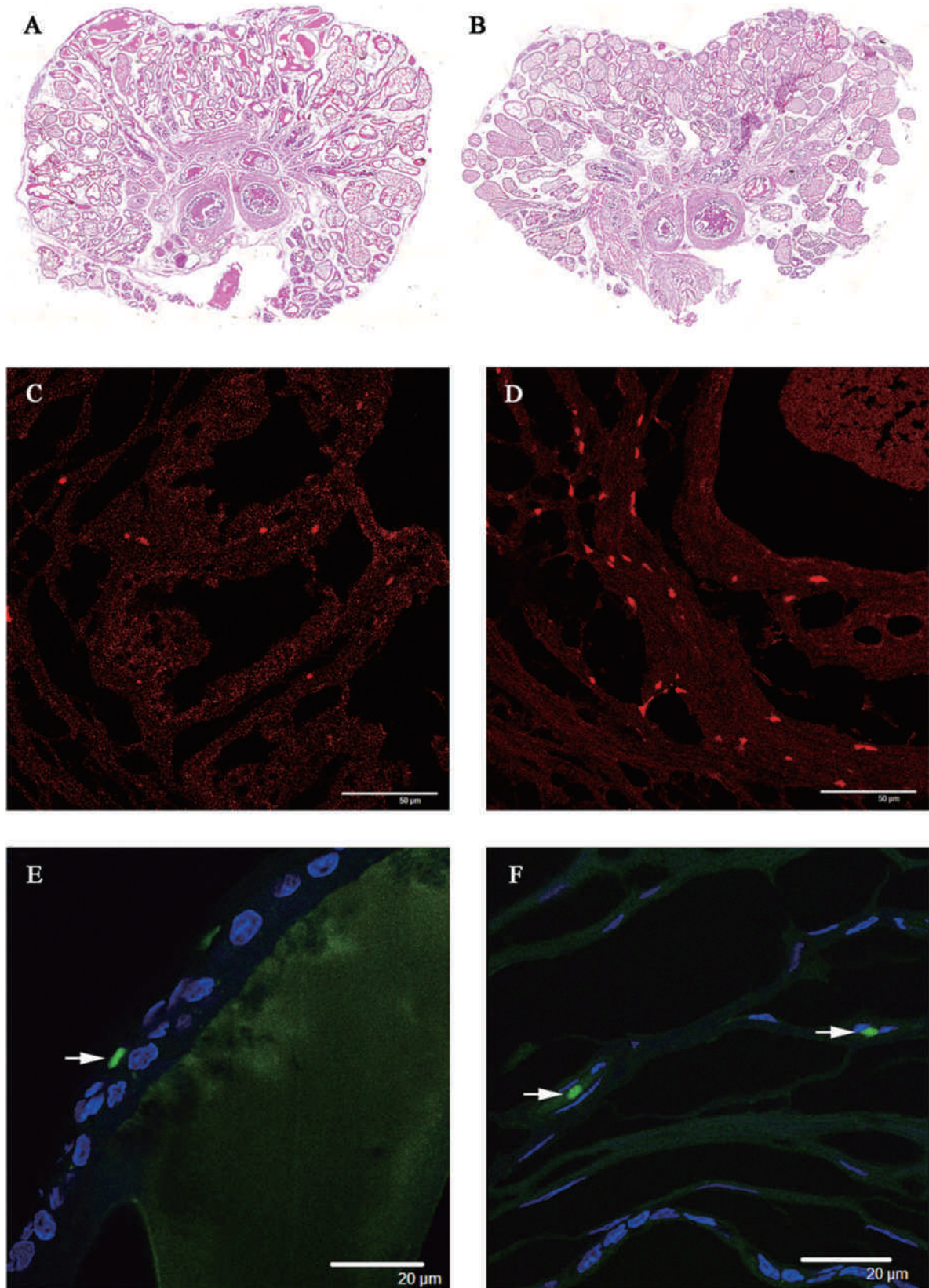


Fig. 3



increase in AT1 suggested that the angiotensin II peptide of saline-loaded SHR might be decreased in the prostate, though this must be determined in future studies. Thus, the absence of elevated SBP might be due to a decrease in angiotensin II peptide.

In each SHR group, the number of proliferating cells and the expression of  $\alpha$ 1-AR subtypes and AT1 were different between the ventral and lateral-dorsal lobes. In the untreated control SHR, the number of proliferating cells and the expression of epithelial  $\alpha$ 1A-ARs in the ventral lobes were significantly greater than those in the lateral-dorsal lobes. In contrast, the expression of epithelial AT1 in the control ventral lobes was significantly less than in the lateral-dorsal lobes. The expression of  $\alpha$ 1D- and  $\alpha$ 1B-ARs was the same in the two lobes.

In the saline-loaded SHR, the number of proliferating cells and the expression of epithelial  $\alpha$ 1A-ARs in the ventral lobes were also greater than in the lateral-dorsal lobes. In contrast, the expression of epithelial  $\alpha$ 1B-ARs and AT1 in the ventral lobes was significantly less than in the lateral-dorsal lobes while the  $\alpha$ 1D-ARs in saline-loaded SHR was the same in both lobes. The increased presence of lateral-dorsal  $\alpha$ 1B-ARs due to saline loading might have induced ischemia due to enhanced vascular smooth muscle contraction in the SHR prostates.

In this study, we showed that saline loading increased the expression of  $\alpha$ 1-AR subtypes and AT1 within the prostates of SHR. Similarly, we previously showed that the loading of saline could increase the expression of tachykinins, TRPV1, and P2X3 receptors within the urinary bladders of SHR<sup>13</sup>. While the mechanism(s) of increased receptor expression are not yet established, it is clear

that our saline-loading conditions for the SHR increase the levels of receptors and peptides associated with LUTS. The combination of persistent hypertension and hyperglycemia or hyperlipidemia is a well-known metabolic syndrome. It increases the risk of developing not only cardiovascular disease and diabetes, but also LUTS. In addition, the saline loading did not have any effects on the expressions of  $\alpha$ 1-AR subtypes and AT1 within the prostates of WKY rats. Therefore, our SHR model will be useful in the investigation of LUTS induced by this metabolic syndrome.

The present study suggested that an AT1 blocker might be useful for the treatment of LUTS in BPH to suppress angiotensin II activity of the prostate, just as an  $\alpha$ 1-AR blocker is the first choice drug for LUTS in BPH to suppress  $\alpha$ 1-AR activity of the prostate. Furthermore, control of hypertension and sodium intake would help to prevent BPH progression.

## V Conclusion

Saline loading did not significantly increase the SBP and the prostate weight of experimental rats. However, it did increase the number of proliferating cells within the lateral-dorsal lobes of SHR. Furthermore, saline loading increased the expression of prostate  $\alpha$ 1-AR subtypes and AT1 in both the ventral and lateral-dorsal lobes of SHR. Changes of 1-AR and AT1 in saline-loaded SHR might be one of the mechanisms of LUTS associated with BPH.

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Fig. 3 Lateral-dorsal lobes in untreated control and saline-loaded SHR. At 7 days after saline loading, there were no obvious differences between the prostate lateral-dorsal lobes of the untreated (A) and saline-loaded SHR (B) when viewed and analyzed by image processing. H&E stain, x40. (C) The control SHR had few PCNA-positive epithelial cells (red). Bar = 50  $\mu$ m. (D) In contrast, the saline-loaded SHR had more PCNA-positive epithelial cells than the controls (red). Bar = 50  $\mu$ m. Apoptotic cells induced by caspase-dependent or -independent pathways (green, arrow) were rarely present in the (E) untreated or (F) saline-loaded SHR. Bar = 20  $\mu$ m, Blue : nuclei.

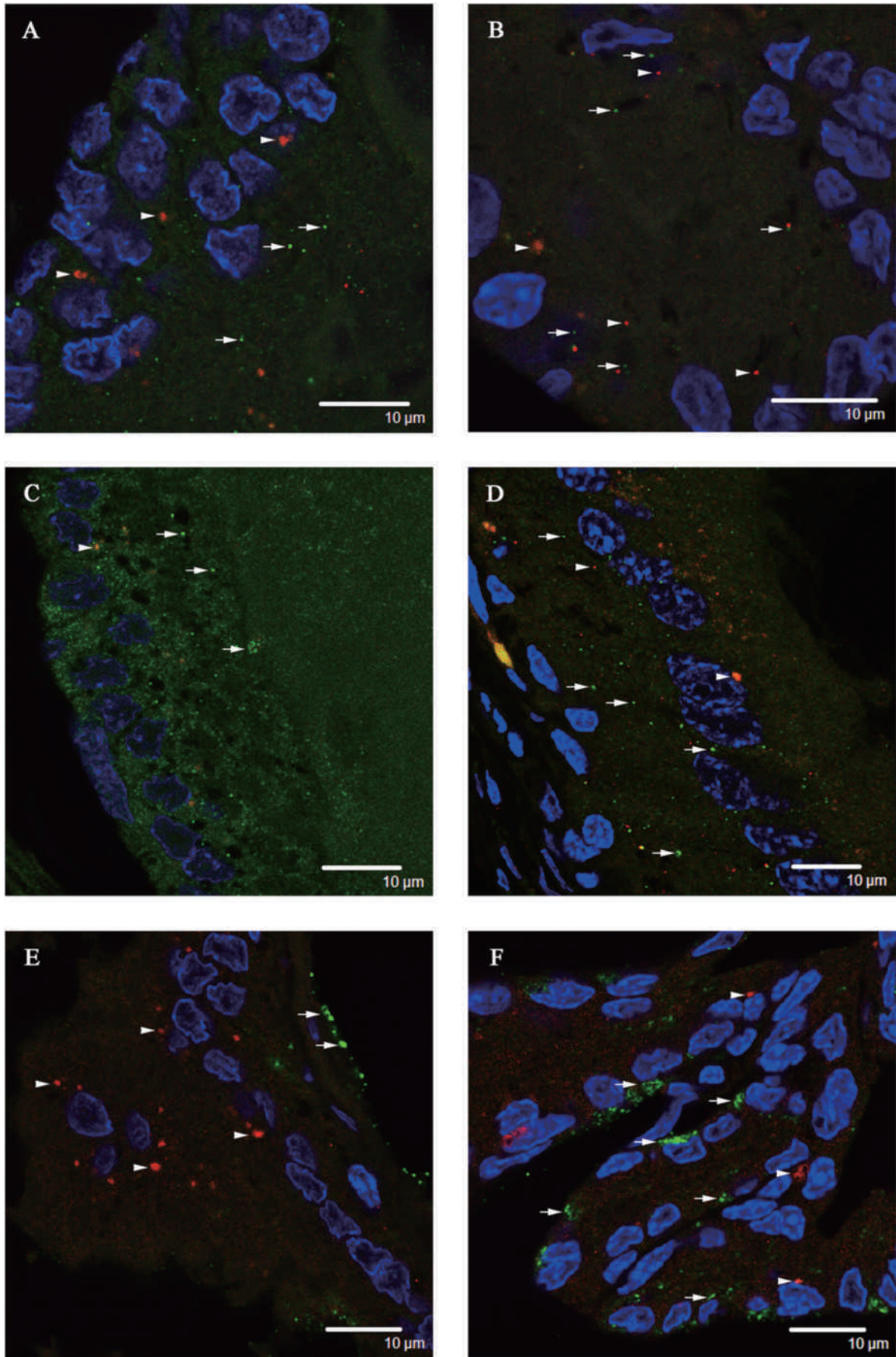


Fig. 4

Fig. 4  $\alpha$ 1-AR subtypes and AT1 within the lateral-dorsal lobes. There were fewer  $\alpha$ 1A<sup>-</sup>,  $\alpha$ 1D<sup>-</sup>, and  $\alpha$ 1B-AR (A, C, and E, green, arrows) within the epithelial cells of the untreated control lateral-dorsal lobes than in the saline-loaded ones (B, D, and F, green, arrows). Similarly, the expression of AT1 within lateral-dorsal lobe epithelium in the control SHR<sup>s</sup> (A, C, and E, red, arrowheads) was also lower than in the saline-loaded ones (B, D, and F, red, arrowheads). Bars = 10  $\mu$ m, Blue : nuclei.

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