

Modulation of Alpha 1 Adrenergic Receptors on Urinary Bladder in Rat Spinal Cord Injury Model

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Purpose: Whereas many studies have focused on the vesical changes of the $\alpha 1$ adrenergic receptor (AR) subtypes in partial outlet obstruction, few studies have addressed the modulation of the $\alpha 1$ AR subtypes after spinal cord injury (SCI). Therefore, we studied the modulation of the $\alpha 1$ ARs in urinary bladder in a rat SCI model.

Methods: Four weeks after a SCI, the whole vesical bodies from eight female Sprague-Dawley rats and from eight controls were harvested. The total RNA was extracted from the samples and was used to prepare cDNA. We developed standard plasmid constructs of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and three $\alpha 1$ ARs ($\alpha 1a$, $\alpha 1b$, and $\alpha 1d$) to convert the cycle threshold (Ct) values from real-time polymerase chain reaction (RT-PCR) into subtype mRNA concentrations. The detected Ct values of 16 samples from RT-PCR were interpolated into the standard plasmid curves.

Results: All serially diluted standard samples showed very good linearity. The mRNA expression of GAPDH was higher in the SCI group, whereas the mRNA expression of all $\alpha 1$ ARs was lower in the SCI group than in the control animals. The $\alpha 1a$, $\alpha 1b$, and $\alpha 1d$ mRNA expression in the controls was 81.7%, 3.3%, and 15.1%, respectively, whereas the $\alpha 1a$, $\alpha 1b$, and $\alpha 1d$ mRNA expression in the SCI group was 33.5%, 5.2%, and 60.9%, respectively.

Conclusions: SCI moderates the $\alpha 1$ AR mRNA subtypes in the urinary bladder. The relatively increased $\alpha 1d$ or decreased $\alpha 1a$ AR mRNA expression may be a therapeutic candidate for controlling the symptoms of neurogenic bladder after SCI.

Keywords: Spinal cord injuries; Urinary bladder; Alpha adrenergic receptors

INTRODUCTION

The prevalence of spinal cord injury (SCI) in young patients is increasing, thus leading to an increased need for lifelong care of these patients [1]. For this reason, it is increasingly important to understand the complications in the urinary bladder of SCI individuals who complain of voiding symptoms or impaired bladder emptying.

Detrusor-sphincter-dyssynergia (DSD), a condition characterized by simultaneous contraction of the detrusor and urethral sphincter, is one of the causes of inefficient bladder emptying after SCI [2].

Traditionally, $\alpha 1a$ adrenergic receptor (AR) antagonists have

been used to decrease the maximum urethral closing pressure and the symptoms of DSD after SCI [2,3]; however, recent studies have shown that the roles of the $\alpha 1a$ ARs extend beyond the urethra or the urinary bladder neck. Because all $\alpha 1a$ AR subtypes are expressed in the bladder body, it is reasonable to assume that selective $\alpha 1$ AR antagonists may have on specific roles not only in the bladder neck but also in the bladder body [4-7]. However, the molecular characterization of the AR subtypes in the body of the urinary bladder is not well established.

Three $\alpha 1$ ARs ($\alpha 1a$, $\alpha 1b$, and $\alpha 1d$) have been cloned with the use of molecular technologies and have been characterized pharmacologically [8,9]. Because the $\alpha 1a$ AR subtype predominates in the smooth muscle of the prostate and the proximal urethra,

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this subtype has been assumed to be responsible for the dynamic component of obstruction and the related voiding symptoms [8-12]. Interestingly, the relative expression of the $\alpha 1$ AR subtypes is changed with chronic outlet obstructive lesions in the rat urinary bladder, with a remarkable increase in bladder $\alpha 1d$ AR expression but a decrease in bladder $\alpha 1a$ AR expression [7]. These findings imply that the $\alpha 1d$ AR may be a new therapeutic target for controlling irritable bladder symptoms [12].

The recently developed selective $\alpha 1d$ AR antagonist naftopidil and the selective $\alpha 1a$ AR antagonist silodosin are used for the treatment of lower urinary tract symptoms around the world. Interestingly, the selective $\alpha 1d$ AR antagonist naftopidil improves not only voiding symptoms but also storage symptoms in patients with benign prostatic hyperplasia [13]. The improvement of storage symptoms such as urgency and frequency may be assumed to be from the vesical modulation of $\alpha 1$ AR subtypes in chronic urinary obstructive lesions [7,12,13].

It is well known that ineffective bladder emptying in SCI patients causes the accumulation of residual urine and bladder hypertrophy similar to like the plasticity of the urinary bladder in a chronic urinary obstructive model [1,2]. Even though the phenotypes in the urinary bladder are similar between these two types of dysfunction, it is clear that the SCI model and the chronic urinary obstructive model have different causal mechanisms and different pathophysiologic or molecular responses. Furthermore, whereas the genetic modulations of the $\alpha 1$ AR subtypes in the urinary bladder are relatively well documented in the chronic obstructive model, there are few reports on the changes of subtypes of $\alpha 1$ AR subtypes in detrusor muscle after SCI. Furthermore, which of these selective antagonists will be more clinically useful in the treatment of voiding symptoms after SCI is not established. For these reasons, we studied the subtype changes of the $\alpha 1$ ARs in the urinary bladder in a rat SCI model. Understanding the pharmacologic modulation of the $\alpha 1$ AR subtypes in the rat model of SCI and the mechanisms underlying this modulation may have implications for the management of neurogenic bladder after SCI.

MATERIALS AND METHODS

Animals

All experimental procedures were conducted in accordance with the National Institutes of Health (NIH) guidelines for investigators and those for animal users developed by the NIH animal research advisory committee. A total of 16 Sprague-Dawley fe-

male rats, with body weights of 250 g, were used. For SCI, the eighth thoracic cord segment of eight rats was exposed under halothane anesthesia and injury was produced by dropping a 2.0 mm diameter rod with a weight of 10 g from a height of 25 mm onto the exposed spinal cord by use of the New York University Impactor (New York, NY, USA) [14]. Eight controls were subjected to the same operation as the SCI rats but without the contusion injury. Animal care after SCI was performed. The post-injury bladder was manually voided twice daily until voluntary voiding occurred. We assessed the changes in locomotor function after SCI and then maintained the animals for 4 weeks. All animals were terminated at four weeks after SCI. The whole bladders were removed and weighed. The harvested bladder was then stored at -80°C . The control rats were sacrificed at the same time as the SCI group.

mRNA Quantification of $\alpha 1$ AR Subtypes: Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted from the whole bladder specimens by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and chloroform. We designed PCR primers for $\alpha 1a$ AR, $\alpha 1b$ AR, $\alpha 1d$ AR, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and all primers spanned at least one intron of the corresponding gene (Table 1). We used the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) to prepare cDNA according to the manufacturer's instructions. To estimate the mRNA expression, calibration curves were made from the measured fluorescence of dilution series of the control plasmids. Then, the concentrations of the unknowns were calculated from standard values. To obtain constructs for plasmid controls ($p\alpha 1a$ AR, $p\alpha 1b$ AR, $p\alpha 1d$ AR, and $pGAPDH$) for quantifying

Table 1. Primers for reverse transcription polymerase chain reaction

Primers	Sequences (5'-3')	Reference
GAPDH	GTCTTCACCCACCACGGAGAAGGC ATGCCAGTGAGCTTCCCGTTCAGC	XR086293.2
Alpha 1a	GTAGCCAAGAGAGAAAAGCCG CAACCCACCACGATGCCAG	NM017191.2
Alpha 1b	GCTCCTTCTACATCCCCTCG AGGGGAGCCAACATAAGATGA	NM016991.2
Alpha 1d	CGTGTGCTCCTTCTACCTACC GCACAGGACGAAGACACCCAC	NM024483.1

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

the mRNA of the AR subtypes and GAPDH, partial cDNAs were amplified from a rat urinary bladder sample by using each AR subtype and GAPDH primers (Table 1). These cDNAs were then cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions. Finally, the DNA sequences of the cloned plasmids were checked with the restriction enzyme *EcoRI* (Fig. 1).

Two microliters of the standard plasmid was added to 25 μ L of reaction mixture containing each forward primer (300 nM), reverse primer (300 nM), 0.5 unit of uracil-N-glycosylase (Sigma Chemicals, Balcatta, WA, USA), and 2X QuantiTech SYBR Green PCR Master Mix (Qiagen GmbH, Hilden, Germany). Real-time PCR was performed in a Rotor-Gene (Rotor Gene 6.0, Corbett, Sydney, Australia) according to the manufacturer's instructions. Amplification consisted of 2 minutes at 50°C for carryover prevention, 15 minutes at 95°C, 10 seconds denaturation at 95°C, and annealing at 52°C for 15 seconds and 72°C for 20 seconds for 40 cycles. Melting curve analysis was performed and concentration values were measured. Negative controls were also included. The detected cycle threshold (Ct) values were interpolated into the standard curves of the plasmid constructs, and the mRNA expression in the samples was calculated [15].

Statistical Analyses

The changed mRNA concentration in each $\alpha 1$ AR subtype of

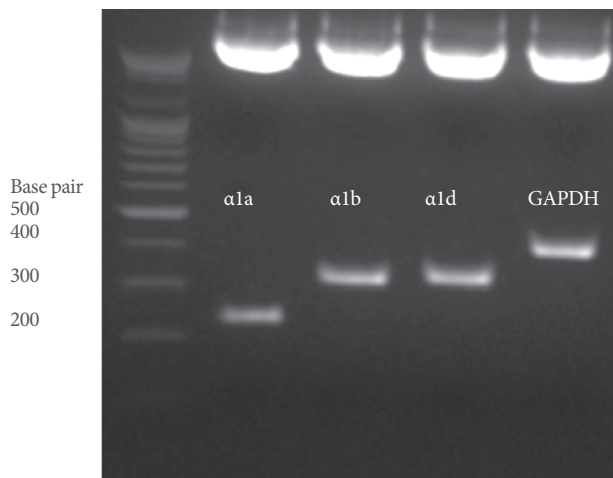


Fig. 1. The cloned control plasmids, p $\alpha 1a$ AR, p $\alpha 1b$ AR, p $\alpha 1d$ AR, and pGAPDH were cut with restriction enzyme (*EcoRI*). We can see the fragmented or restricted segments, measuring about 200- to 400 bp. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

SCI rats was compared with the controls by using the Mann-Whitney test. The statistical difference in the adjusted mRNA expression of the $\alpha 1$ AR subtypes in the controls and the SCI group was determined by Kruskal-Wallis analysis with *post-hoc* Mann-Whitney test. P-values < 0.05 were considered statistically significant.

RESULTS

All rats showed a typical posture of SCI, the hind limbs were drooped and paralyzed at day 1 after the contusion injury. Significant increase in bladder wet weight was noted in the SCI group (Fig. 2).

Standardization of Real-Time PCR

The linear relationships of all cloned plasmids from 10^0 to 10^5 starting copies/reaction revealed high correlations between the copy numbers and the Ct values. pGAPDH also generated a good log-linear regression plot (Fig. 3).

Changes in mRNA Expression of $\alpha 1$ AR Subtypes

The SCI group showed statistically increased mRNA expression of GAPDH at 4 weeks after injury compared with the control group (Table 2, Fig. 4A) ($P=0.041$). The expression of the $\alpha 1$ AR subtypes was adjusted by the expression of GAPDH in each sample. The adjusted $\alpha 1$ AR subtype expression in the control group was 371.50 ± 118.19 for $\alpha 1a$, 15.11 ± 7.97 for $\alpha 1b$, and 68.62 ± 40.99 for $\alpha 1d$ AR. There were significant differences in AR subtype expression in the urinary bladder in controls ($P=0.001$) (Fig. 4B).

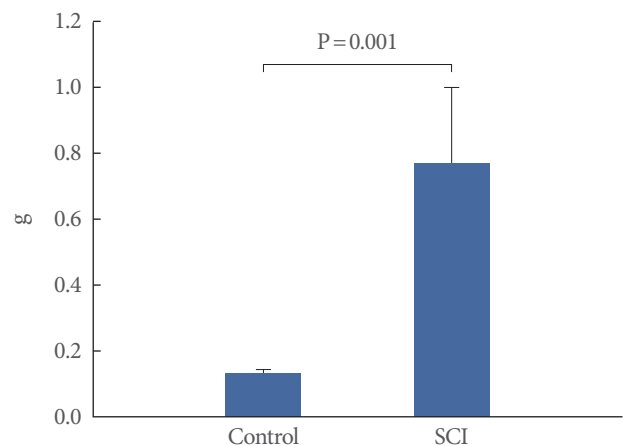


Fig. 2. Weight of urinary bladder in the control and the spinal cord injury (SCI) group. Data are reported as the mean \pm SD.

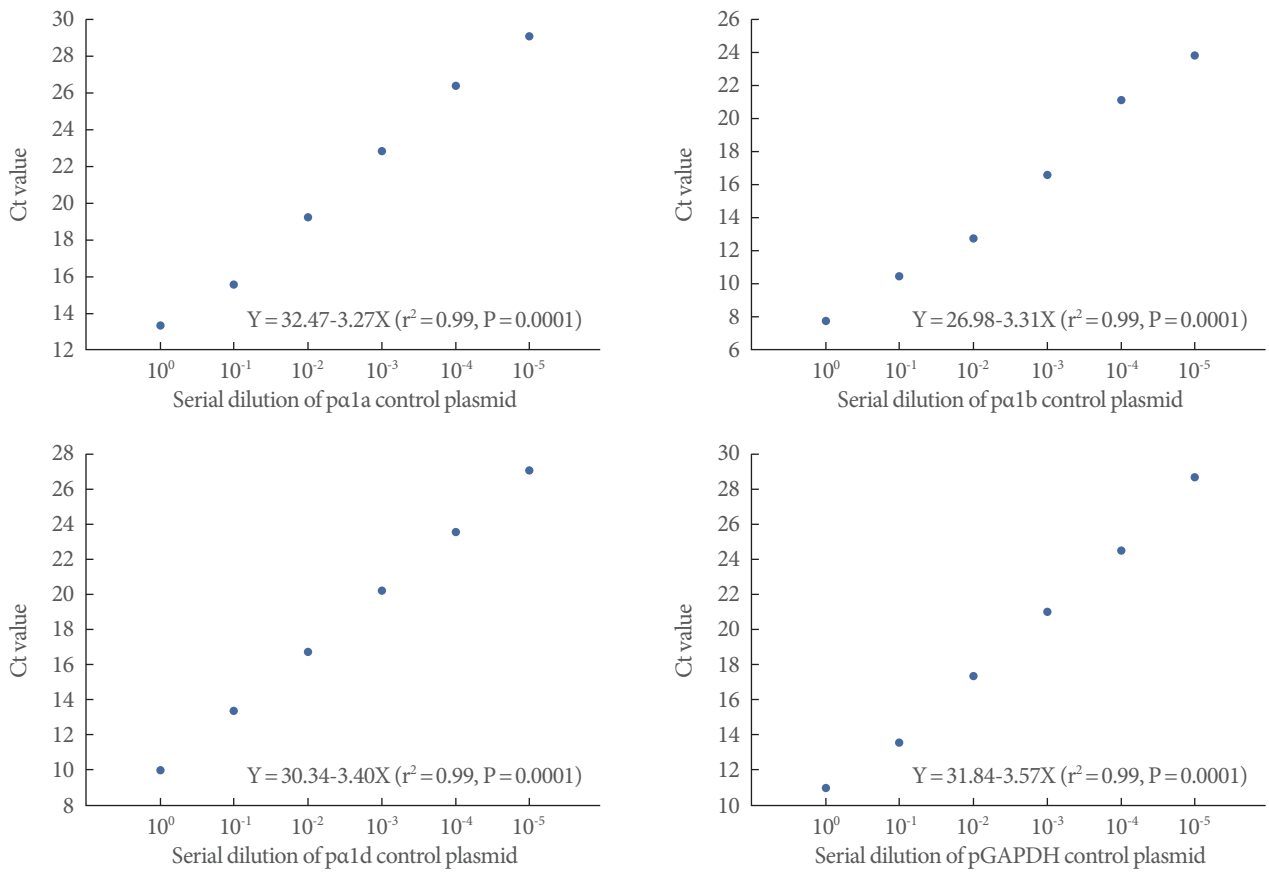


Fig. 3. A linear relationship between the cycle number reaching above threshold of fluorescence and the corresponding log-transformed cDNA values of control plasmids.

The AR subtype expression of the SCI group differed from that of the controls. The adjusted $\alpha 1$ AR expression in the SCI group was 15.90 ± 12.13 for $\alpha 1a$, 2.48 ± 2.01 for $\alpha 1b$, and 28.94 ± 17.61 for $\alpha 1d$ AR (Fig. 4C). Furthermore, there were significant differences in AR subtype expression in the urinary bladder in the SCI group ($P=0.001$). The $\alpha 1d$ AR was marginally more expressed than the $\alpha 1a$ AR in the SCI group ($P=0.05$) (Fig. 4C). The SCI group had statistically less mRNA expression of the $\alpha 1$ ARs than did the control group ($\alpha 1a$, $P=0.000$; $\alpha 1b$, $P=0.001$; and $\alpha 1d$, $P=0.016$) (Fig. 4D).

DISCUSSION

The communication between the outlet and the bladder body is under the control of the central nervous system as well as the sympathetic, parasympathetic, and somatic nerves that innervate the bladder body muscle and urinary bladder neck [16,17]. The interplay between the urinary bladder and the upper con-

trol organs can be disrupted as a result of trauma or disease [1-3,16,17]. In the case of spinal cord transection, voluntary control of voiding is eliminated and is accompanied by an uncoordinated bladder and external urethral sphincter activity, termed DSD [1-3,16,17]. In addition, a high intravesical pressure can cause damage to the upper urinary tract, leading to vesical muscular hypertrophy [2,18]. Even though many studies have focused on pathophysiological alterations to the bladder neck or posterior urethra after SCI, few studies have been done regarding changes in the enlarged vesical body or hypertrophied detrusor muscle that might accompany SCI [3,4].

Our results showed that the SCI group had statistically increased mRNA expression of GAPDH at 4 weeks after injury compared with the control group (Fig. 4A). GAPDH is one of the most commonly used housekeeping genes for comparing gene expression data [19,20]. The housekeeping genes are standards for normalizing the expression levels of a target gene. Because very few reports have evaluated the GAPDH changes in

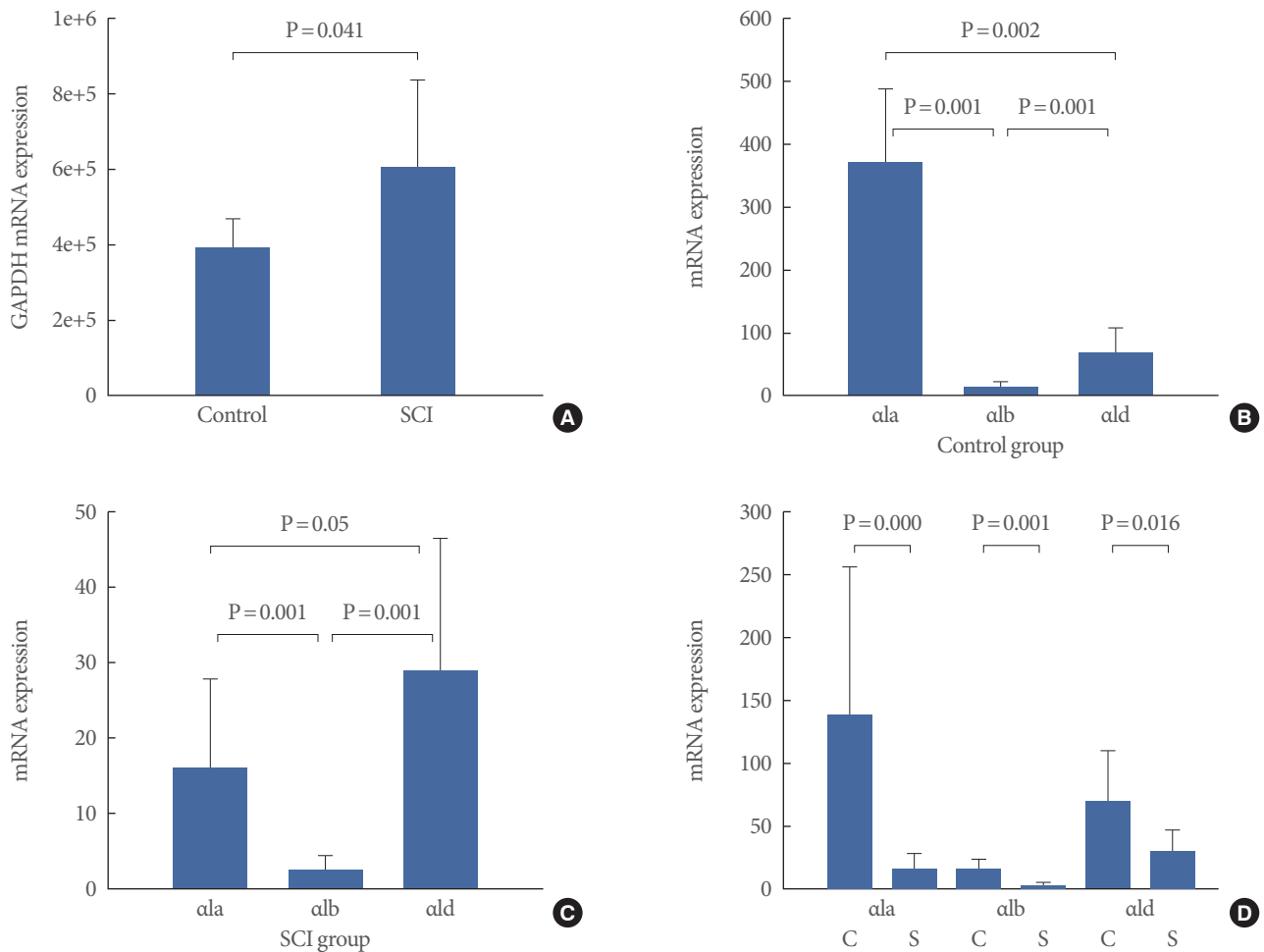


Fig. 4. The changes in mRNA expression of $\alpha 1$ adrenergic receptor (AR) subtypes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (A) GAPDH mRNA expression in the control and the spinal cord injury (SCI) group. (B) Alpha1 AR subtype mRNA expression in the control group. (C) Alpha1 AR subtype mRNA expression in the SCI group. (D) Expression of each $\alpha 1$ AR subtype between the control and the SCI group. Data are reported as the mean \pm SD. C, control group; S, SCI group.

Table 2. Change in mRNA expression of $\alpha 1$ adrenergic receptor (AR) subtypes in urinary bladder after spinal cord injury

	mRNA expressions (mean \pm SD)		
	Control	Spinal cord injury	P-value
GAPDH	385,398 \pm 85,128	607,590 \pm 229,095	0.041
Alpha 1a AR _{adj} GADPH	371.50 \pm 118.19	15.90 \pm 12.13	0.000
Alpha 1b AR _{adj} GADPH	15.11 \pm 7.97	2.48 \pm 2.01	0.001
Alpha 1d AR _{adj} GADPH	68.62 \pm 40.99	28.94 \pm 17.61	0.016

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

urinary bladder after SCI, we can extrapolate the significance of the GAPDH change in the urinary bladder after SCI from the findings of the partial bladder neck obstruction model. It is well

known that the DNA content and ^3H -thymidine incorporation in the urinary bladder are increased in partially obstructed urinary bladders [21,22]. Our data showed that the expression of GAPDH in the SCI group was 2 times that in the controls (Table 2). For these reasons, we adjusted mRNA expression of the $\alpha 1$ AR subtypes to reflect the housekeeping effect of GAPDH.

The $\alpha 1$ AR is a member of the large family of G protein coupled receptors that mediate the actions of the endogenous catecholamines norepinephrine and epinephrine, resulting in smooth muscle contractions in body organs [4-10]. Three subtypes, $\alpha 1a$, $\alpha 1b$, and $\alpha 1d$, have been identified in various mammalian organs and have been characterized by either molecular or pharmacokinetic techniques [4-11]. It is well known that the three $\alpha 1$ AR subtypes expressed in the prostate play a major role in

prostatic smooth muscle contraction [11]. The switch on the AR mediates lower urinary tract symptoms and pathophysiological mechanisms for benign prostatic hyperplasia [23,24]. Quantification of $\alpha 1$ AR mRNA expression within human prostatic tissue has revealed that the $\alpha 1a$ AR predominates, followed by the $\alpha 1d$ AR and $\alpha 1b$ AR [23,24]. Furthermore, the mRNA expression of the $\alpha 1$ AR subtypes in the prostate may be a predictor of the efficacy of subtype selective AR antagonists in patients with benign prostatic hyperplasia [7,23,24].

Because lower urinary tract symptoms or pathophysiological changes in the urinary bladder resulting from bladder outlet obstruction are common disorders in urology, there are ample studies about $\alpha 1$ AR receptor subtype modulation after partial bladder outlet obstruction. For this reason, we compare the $\alpha 1$ AR mRNA modulation in the SCI model with that in the bladder outlet obstruction model induced by partial urethral ligation. Control rats show predominantly $\alpha 1a$ AR expression (68 to 74%), followed by $\alpha 1d$ AR (21 to 28%) and $\alpha 1b$ AR (4 to 5%) along the whole urinary bladder [7]. However, the obstructive rat urinary bladder shows predominantly $\alpha 1d$ AR expression (77 to 74%), followed by $\alpha 1a$ AR (21 to 24%) and $\alpha 1b$ AR (2 to 3%) along the whole urinary bladder [7]. Barendrecht et al. [5] evaluated the mRNA expression of the $\alpha 1$ AR subtypes by use of the real-time PCR method in rats with bladder outlet obstruction induced by partial urethral ligation. In that study, the mean relative expressions (mRNA expression fold of sham) were $\alpha 1d$ AR > $\alpha 1b$ AR > $\alpha 1a$ AR [5]. Interestingly, the mean relative expression of all $\alpha 1$ AR subtypes was higher in the bladder outlet obstruction group than in the sham group; however, discrepancies in subtype expression in urinary bladder were reported by Hampel et al. [7]. In control animals, 70% of $\alpha 1$ AR mRNA was the $\alpha 1a$ AR subtype, 5% was $\alpha 1b$ AR and 25% was $\alpha 1d$ AR, whereas in obstructed bladder, $\alpha 1$ AR expression changed to 23% $\alpha 1a$ AR, 2% $\alpha 1b$ AR, and 75% $\alpha 1d$ AR [7]. In addition, the mRNA expression of the $\alpha 1a$ AR subtype was dramatically reduced in the partially obstructed group compared with the control rats [7]. Our results were consistent with the results from Hampel et al.'s report [7] except for one thing. The total expression of $\alpha 1d$ AR more increased than in the control animals after 6 weeks of partial outlet obstruction in Hampel et al.'s study [7]. Even though the adjusted $\alpha 1d$ AR mRNA expression was dominant among the $\alpha 1$ AR subtypes, we found that the mRNA expression of the $\alpha 1d$ AR definitively decreased less than in the control animals. Although the reasons for these differences are not yet clear, the differences may be due to different experimen-

tal designs or animal models. Further studies may be needed to elucidate the meaning of the absolute decrease in all $\alpha 1$ AR subtypes in the SCI model.

Because the $\alpha 1d$ AR subtype has a 10- to 100-fold higher affinity for the endogenous neurotransmitters norepinephrine and epinephrine than does the $\alpha 1a$ or $\alpha 1b$ AR subtype, this finding provides a potentially important rationale for targeting the $\alpha 1d$ AR subtype when treating voiding symptoms after SCI [7].

To characterize the function of the $\alpha 1$ ARs in the urinary bladder, quantitative evaluation of mRNA and protein must be performed. However, it is methodologically difficult to quantitatively analyze genetic expression by use of western blotting or immunohistochemical staining. Furthermore, because the three $\alpha 1$ ARs have very similar amino acid sequences, it is very difficult to develop unique and specific antibodies for each subtype. Real-time reverse transcriptase PCR is becoming a widely used method to quantify the gene expression from cells, tissues, or tissue biopsies [5,23,24].

A limitation of this study is that we did not evaluate the physiologic role of the changes in $\alpha 1$ AR modulation. Urodynamic studies in animals or contractile responses of harvested strips to selective $\alpha 1$ AR antagonists such as silodosin or naftopidil may extend our findings in the future. In addition, we evaluated $\alpha 1$ AR mRNA expression in whole bladder specimens. Because the $\alpha 1$ ARs are widely distributed throughout the whole urinary bladder, some expression of $\alpha 1$ ARs in the mucosa or submucosa may have affected the mRNA expression values in our experiment.

In conclusion, SCI modulates the $\alpha 1$ AR mRNA subtypes in rat urinary bladder. The relatively increased the $\alpha 1d$ or decreased the $\alpha 1a$ AR mRNA expression may be a therapeutic candidate for controlling symptoms of neurogenic bladder after SCI. Further studies will be needed to evaluate the action of selective $\alpha 1$ AR subtype antagonists on the urinary bladder after SCI.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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