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Quantification and distribution of α_1-adrenoceptor subtype mRNAs in human proximal urethra

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1 We performed RNase protection assays and *in situ* hybridization to investigate the ratio of the three α_1-adrenoceptor subtype mRNAs, α1a, α1b and α1d, in human proximal urethra, and their localization in urethral cross-sections. As revealed by the RNase protection assays, α1a was the predominant subtype mRNA in both male and female urethral samples. α1d mRNA was detected only in the female sample, and α1b mRNA was not detected in any of the samples tested. The ratio of the abundance of the subtype mRNAs, α1a : α1b : α1d, was 100 : 0 : 0 in the male urethra and 90 : 0 : 10 in the female urethra.

2 *In situ* hybridization studies showed no significant differences in the cross-sectional distribution of α_1-adrenoceptor subtype mRNAs between male and female urethras. Intense α1a staining was observed in the smooth muscle of the urethra, but α1b and α1d staining was much less intense.

3 Of the three cloned α1 subtypes, α1a is the most likely to be responsible for the contraction of the human urethra. Owing to the side effects of nonselective α_1 drugs, α_1-selective drugs may be clinically superior to nonselective drugs for the treatment of urethral disorders.

Keywords: α_1-Adrenoceptor subtypes; urethra; RNase protection assay; *in situ* hybridization

Introduction

α_1-Adrenoceptors have been classified according to their pharmacological properties into α_{1A} and α_{1B} subtypes (Morrow & Creese, 1986; Han *et al*., 1987; Minneman *et al*., 1988). However, molecular biological techniques have demonstrated the existence of distinct genes coding for three different α_1-adrenoceptor subtypes, α1a, α1b and α1d, in various animals (Cotecchia *et al*., 1988; Minneman *et al*., 1988; Voigt *et al*., 1990; Ramarao *et al*., 1992; Bylund *et al*., 1994). The human α1a (Hirasawa *et al*., 1993), α1b (Ramarao *et al*., 1992) and α1d (Bruno *et al*., 1991) genes have now been cloned and sequenced. To resolve the discrepancy between the pharmacological and molecular properties, Ford *et al*. (1994) proposed a unified nomenclature, which is now recommended by the International Union of Pharmacology (Hieble *et al*., 1995). The three α_1 subtypes are designated α_{1A}, α_{1B} and α_{1D} for the native receptors and α1a, α1b, α1d for the cloned receptors.

The expression of α_1-adrenoceptor subtype mRNAs has previously been investigated in various human tissues, and the major subtype mRNA was found to depend on the type of tissue: α1a in heart, liver, lung and prostate, α1b in spleen, aorta and foetal brain, and α1d in aorta and cerebral cortex (Price *et al*., 1993a; Weinberg *et al*., 1994). The predominance of α1a in the human prostate has been demonstrated by RNase protection assay and *in situ* hybridization as well as by pharmacological techniques such as radioligand binding (Moriyama *et al*., 1996; Nasu *et al*., 1996). However, the abundance of the α_1-adrenoceptor subtype mRNAs in the human urethra have not yet been investigated.

The clinical effect of the nonselective α_1-adrenoceptor antagonist phentolamine, administered for bladder outlet obstruction in patients with benign prostatic hypertrophy (BPH), is to reduce the intraurethral pressure in all portions of the urethra; phentolamine also inhibits noradrenaline-induced contraction of the isolated prostate and prostatic urethra (Furuya *et al*., 1982). α_1-Selective adrenoceptor antagonists, such as prazosin and terazosin have been formed to be more effective for BPH patients than nonselective α-adrenoceptor antagonists. Furthermore, α1a subtype-selective antagonists are already in clinical use for the treatment of BPH to avoid the side effects, such as a lowering of blood pressure, commonly caused by nonselective antagonists. α_1-Adrenoceptor agonists, including norephedrine (Ek *et al*., 1978; Obrink & Bunne, 1978) and midodrine (Jonas, 1977; Nito, 1994), are effective against female stress incontinence. They work by increasing the tone of the smooth muscle of the bladder base and urethra.

A knowledge of the predominant α_1 subtype(s) in the human urethra would greatly aid the development of urethra-selective drugs useful for the relief of stress incontinence or difficulty in urination. However, because of the difficulty of obtaining tissue samples, few studies have been performed on the human urethra. In the present study, we describe the quantification and distribution of α_1-adrenoceptor subtype mRNAs in the male and female human proximal urethra as determined by RNase protection assay and *in situ* hybridization.

Methods

Human tissue samples

Male and female patients with invasive bladder cancer (mean age ± s.d., 66.6 ± 7.9 years) were selected for this study, with full informed consent. Specimens of proximal urethra were obtained after total cystoprostatectomy or cystourethrectomy. After tissue had been taken for pathological examina-

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tion, samples for the RNase protection assay were quickly frozen in liquid nitrogen and stored at −130°C. Samples for *in situ* hybridization were embedded in O.C.T. compound, quickly frozen in liquid nitrogen, and stored at −80°C. The stored samples were used for experiments after the corresponding specimens has been shown to be tumour-free.

Preparation of RNA

Total RNA was extracted from human urethra according to the method of Chomczymski and Sacchi (1987). Poly $(A)^+$ RNA was purified on an oligo (dT) resin (Oligotex-dT30 Super; Takara, Kyoto, Japan). Each RNA sample was quantified by its absorbance at 260 nm and stored at −80°C until it was used for the RNase protection assay.

Preparation of RNA probes

Each of the three α_1-adrenoceptor subtype cDNA clones was isolated from a human prostate cDNA library as previously described (Hirasawa *et al*., 1993). Fragments corresponding to the C-terminal region of each α_1-adrenoceptor subtype were selected as probes for the RNase protection assay and *in situ* hybridization assay, and inserted into a pBluescript transcription vector (Stratagene, La Jolla, CA, U.S.A.). We adjusted the α1a fragment to 275 bp (nucleotides 1443–1717), the α1b fragment to 230 bp (nucleotides 2086–2315) and the α1d fragment to 260 bp (nucleotides 1321–1580) in length. Antisense RNA probes for the RNase protection assay were transcribed with T7 RNA polymerase, and sense RNA probes with T3 RNA polymerase, from linearized DNA templates in the presence of [α-^{32}P]-UTP. Each RNA probe was purified by electrophoresis on a 5% polyacrylamide gel containing 8 M urea. Antisense RNA probes for *in situ* hybridization were transcribed with T7 RNA polymerase from linearized DNA templates in the presence of digoxigenin-UTP.

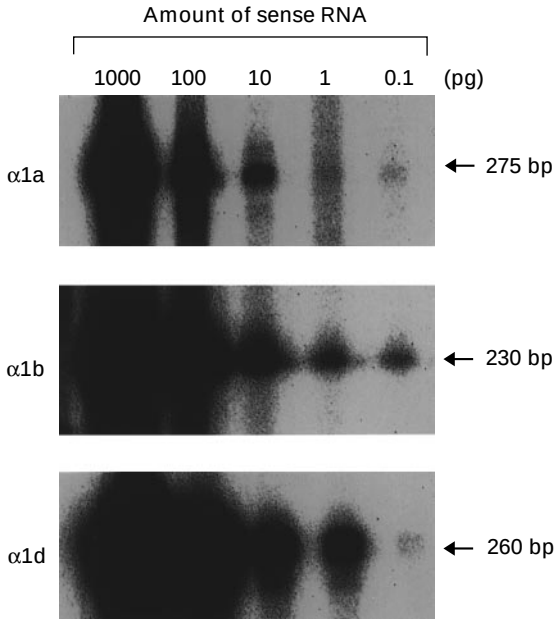

Figure 1 RNase protection assay with each of the *in vitro* transcribed sense RNAs. α1a, α1b and α1d sense RNAs (1000, 100, 10, 1, 0.1 pg) were subjected to the RNase protection assay. After RNase digestion, protected fragments were separated on a 5% polyacrylamide gel containing 8 M urea.

RNase protection assay

RNase protection assays were performed as described by Nasu *et al*. (1996). Poly $(A)^+$ RNA (5 μg) from human urethra was hybridized with each of the radiolabelled RNA probes (1×10^6 d.p.m.) described above. After RNase treatment, protected RNA fragments were electrophoresed on a 5% polyacrylamide gel containing 8 M urea, and an imaging plate was exposed to the dried gel and scanned with an imaging analyser (BAS2000; Fuji Film Co., Ltd., Tokyo, Japan). To quantify the amount of mRNA expressed, sense RNAs corresponding to each antisense RNA probe were transcribed from DNA templates in the absence of [α-^{32}P]-UTP and purified as described above, and standard solutions of the sense RNAs were simultaneously subjected to the RNAse protection assay (Figure 1). The resulting gels were scanned and used to generate standard curves for each probe and the amount of mRNA corresponding to each of the three α1 subtypes expressed in urethral tissues was estimated from these curves.

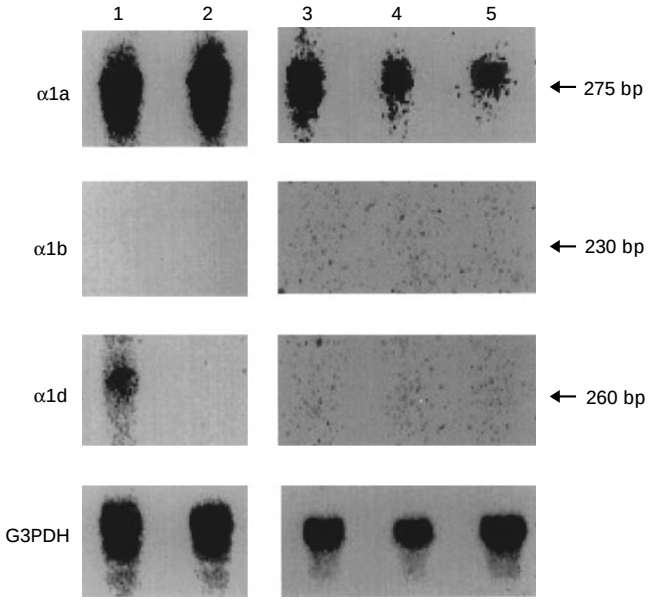

Figure 2 RNase protection assay of α_1-adrenoceptor subtype mRNAs in human proximal urethra. Poly $(A)^+$ RNA (5 μg) from human urethral tissues was hybridized with radiolabelled antisense RNA probes specific for α1a, α1b, α1d or glyceraldehyde-3-phosphate dehydrogenase (G3PDH). After RNase digestion, protected fragments were separated on a 5% polyacrylamide gel containing 8 M urea. Lane 1, female; lane 2, 3, 4 and 5, male.

Table 1 Distribution of α_1-adrenoceptor subtype mRNAs in human urethra

Sex	*Total α1 mRNA*[1] (pg)	*Subtype mRNA*[1] (pg (%)) *α1a*	*α1b*	*α1d*
Female	4.1	3.7 (90)	ND	0.4 (10)
Male	4.9	4.9 (100)	ND	ND
	1.9	1.9 (100)	ND	ND
	1.3	1.3 (100)	ND	ND
	1.2	1.2 (100)	ND	ND

Poly (A)+ RNA (5 μg) from human urethra was used for the RNase protection assay.
[1] The values given for the mRNA represent the weight (pg) of the single stranded protected fragment only.
ND, not detected.

In situ *hybridization*

In situ hybridization assays were performed as described by Moriyama *et al*. (1995). Frozen urethral tissues were cut into slices 10 μm thick, mounted and fixed on slides with 4% paraformaldehyde, and rinsed with 2 × SSC (15 mM sodium citrate, pH 7.0, containing 150 mM NaCl). The specimens were treated with 50 μg ml^{-1} proteinase K, acetylated with 0.25% (v/v) acetic anhydride solution in 0.1 M triethanolamine containing 150 mM NaCl, then preincubated at 45°C for 2 h in hybridization buffer (10 mM Tris-HCl, pH 7.4, containing 0.6 M NaCl, 1 mM EDTA, 16% (v/v) formamide, Denhardt's solution (Denhardt *et al*., 1966), 0.125 mg ml^{-1} salmon sperm DNA, 0.25 mg ml^{-1} yeast tRNA and 10% (w/v) dextran thionate). The specimens were then treated with digoxigenin-labelled probes for 16 h at 45°C in hybridization buffer. The slides were rinsed with 2 × SSC to remove excess probe, they were then treated with 20 μg ml^{-1} RNase A. They were incubated with alkaline phosphatase conjugated anti-digoxigenin antibody and treated with the

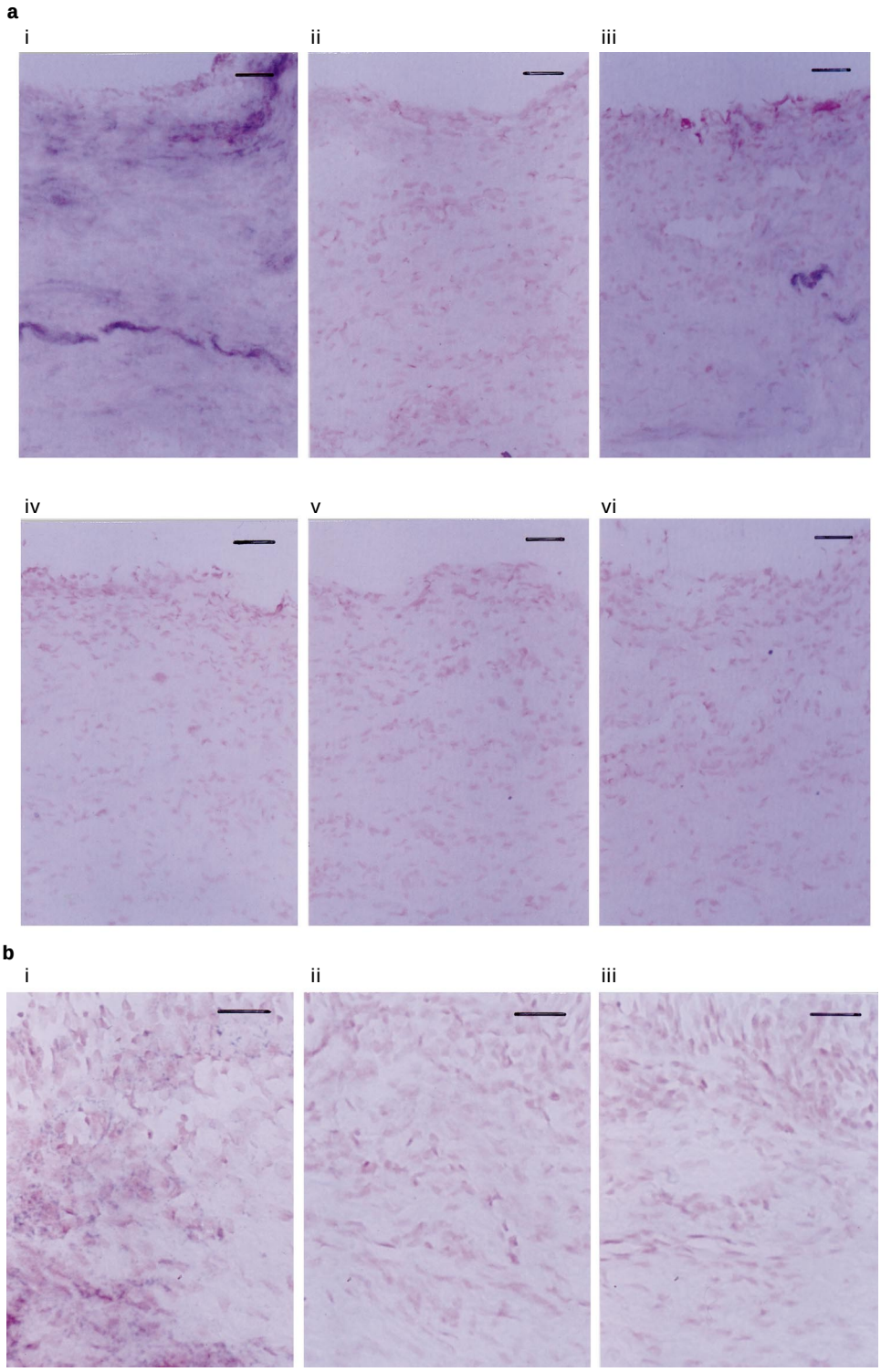

Figure 3 Microscopic views of *in situ* hybridization signals of α_1-adrenoceptor subtype mRNAs in human proximal urethra. (a) Female urethra; (b) male urethra: (i) α1a antisense probe, (ii) α1b antisense probe, (iii) α1d antisense probe, (iv) α1a sense probe, (v) α1b sense probe, (vi) α1d sense probe. Bar = 25 μm.

substrate solution (100 mM Tris-HCl, pH 9.5, containing 50 mM $MgCl_2$, 100 mM NaCl, 20 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate, and 37.5 mg ml^{-1} nitroblue tetrazolium) for 4 h to visualize the bound probes. The stained slides were examined under a light microscope and photographed.

Results

Quantification of α_1-adrenoceptor subtype mRNAs in human urethra

The subtype selectivity of the α1a, α1b and α1d RNA probes was verified by Northern blot hybridization with Chinese hamster ovary (CHO) cell lines, each of which expresses one of the human α_1-adrenoceptor subtypes (α1a, α1b or α1d), as described previously (Nasu *et al*., 1996). Sense RNAs corresponding to the α_1-adrenoceptor subtype mRNAs were synthesized and purified as described in the Methods section. The electrophoresis patterns of the RNase protection assay with various concentrations of the sense RNAs (Figure 1) indicated that all three antisense probes were reactive and that the intensity of the bands increased in a dose-dependent manner. The band position in the gel corresponds to the predicted size of the antisense RNA probe.

Poly $(A)^+$ RNA from human proximal urethral tissues was analysed by the RNase protection assay to determine the amount of each α_1-adrenoceptor subtype mRNA. Radiolabelled sense RNA probes corresponding to each subtype did not reveal any mRNA expressed in the urethral tissues (data not shown). Each antisense RNA probe revealed a specific protected fragment of the predicted size (Figure 2), and the amounts of α_1-adrenoceptor mRNA detected in the human urethral samples were calculated from standard curves as described in Methods (Table 1). α1a mRNA was the predominant receptor subtype mRNA in human urethral tissues, accounting for 90–100% of the total α_1-adrenoceptor mRNA. The expression levels of mRNA for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) did not vary between the samples (Figure 2). α1d mRNA was detected only in the female sample (10% of the total α1 mRNA), and α1b mRNA was not detected in any of the samples tested, male or female.

In situ *hybridization*

In situ hybridization was performed to investigate the localization of α_1-adrenoceptor subtype mRNAs within cross-sections of male and female proximal urethras. There was no detectable staining upon treatment with sense RNA probes (Figure 3a(iv–vi)). The localization of α_1-adrenoceptor subtype mRNAs was very similar in the male and the female samples, and α1a was clearly detected in the urethral smooth muscle (Figure 3a(i–iii) and b). In contrast, α1b and α1d were only faintly detected in the samples.

Discussion

The expression of the three α_1-adrenoceptor subtype mRNAs has been investigated in various human tissues (Price *et al*., 1993a; Weinberg *et al*., 1994). Functional studies of human prostate samples have shown that the predominant α_1 subtype in this tissue has the pharmacological characteristics of the native α_{1A}, which appears to be identical to the cloned α1a (Lepor *et al*., 1993; Chapple *et al*., 1994). RNase protection assays have confirmed that α1a is the predominant subtype mRNA in the human prostate (Price *et al*., 1993b; Nasu *et al*., 1996).

An investigation of functional α_1-adrenoceptors in the urethra of rat, rabbit, dog and human by radioligand binding assays revealed that the α_{1A} subtype is predominant (Testa *et al*., 1993). By use of WB4101 and 5-methylurapidil, α_{1A}-selective antagonists, and chlorethylclonidine (CEC), an α_{1B} selective antagonist, Hatano *et al*. (1994) found distinct α_1-adrenoceptor subtype populations in the human urethra and a branch of the iliac artery, and concluded that artery was primarily α_{1B} and urethra probably α_{1A}. Taniguchi *et al*. (1996) also found that NS-49 ((**R**)-3′-(2-amino-1-hydroxyethyl)-4′-fluoromethanesulphonanilide hydrochloride) (Obika *et al*., 1995), an α1a-selective agonist, selectively increases intraurethral pressure in dogs and causes selective contraction of the dog isolated urethra, with little or no effect on the arteries. However, the ratio of α_1-adrenoceptor subtype mRNAs and their distribution within the human urethra have not yet been determined. Furthermore, the distribution of the receptor proteins has not been studied because of the lack of subtype-specific antibodies. The aim of the present study was to identify α_1-adrenoceptor subtype that might be responsible for mediating the contraction of the human urethra and to determine the distribution of the three subtypes within the urethra. Thus, our study may shed light on the variation in efficacy between the nonselective α_1-adrenoceptor antagonists or agonists used in the treatment of BPH or stress incontinence.

Accordingly, we have quantified each α_1-adrenoceptor subtype mRNA expressed in the human proximal urethra by using an RNase protection assay. The predominant subtype mRNA was α1a in both male and female tissue samples, where it accounted for 90–100% of the total α_1-adrenoceptor mRNA. Although α1d mRNA was detected in the female sample and not in any of the male samples, we can draw no firm conclusion from this difference on the basis of the single female sample analysed. Because α1b mRNA was not detected in male or female samples, this receptor subtype is probably not relevant to the contraction of the human urethra. These findings are supported by our *in situ* hybridization results. Through *in situ* hybridization, we found that the α1a mRNA was localized primarily in the smooth muscle, and that this was the main subtype mRNA along the length of the urethra (data not shown). At present, no specific antibodies against α_1-adrenoceptor subtypes are available, so *in situ* hybridization of the corresponding mRNAs may be the best guide yet available to the tissue localization of α_1-adrenoceptor subtypes.

Our finding that α1a is the predominant subtype mRNA in the human posterior urethra, both male and female, is in agreement with the results of the binding and functional studies described above. Of the three cloned α_1 subtypes, α1a is the most likely to be responsible for the contraction of the urethra, which leads directly to obstruction of the urinary tract in BPH patients. In view of the cardiovascular side effects associated with nonselective α_1 antagonists, our data suggest that α_{1A}-selective antagonists may be superior to nonselective antagonists for the treatment of BPH patients.

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