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Pharmacological characterization of TA-0201, an endothelin receptor antagonist, with recombinant and human prostate endothelin receptors

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Abstract

The pharmacological profile of *N*-(6-(2-(5-bromopyrimidine-2-yloxy)ethoxy)-5-(4-methylphenyl)pyrimidin-4-yl)-4-(2-hydroxy-1,1-dimethylethyl) benzensulfonamide sodium salt sesquihydrate (TA-0201), a new antagonist of endothelin receptors, was examined, using human recombinant and prostate endothelin receptors. In binding experiments with [^{125}I]endothelin-1, TA-0201 showed extremely high affinity for recombinant endothelin ET_A receptors ($pK_i = 10.7$), as compared with that for recombinant endothelin ET_B receptors ($pK_i = 7.8$). Endothelin ET_A and ET_B receptors coexisted in human prostate with different proportions (endothelin ET_A receptor: approximately 70%), which were distinguished by TA-0201 in the same manner as with recombinant receptors. Human prostate strips contracted in response to endothelin-1 and sorafotoxin S6c, but the maximum contraction induced by endothelin-1 was approximately three times greater than that induced by sarafotoxin S6c. The response to endothelin-1, but not to sarafotoxin S6c, was inhibited by TA-0201 and cyclo(D-Asp-Pro-D-Val-Leu-D-Trp) (BQ123) (endothelin ET_A receptor antagonist) but not by BQ788 (endothelin ET_B receptor antagonist). These results suggest that TA-0201 is a highly selective endothelin ET_A receptor antagonist and will be useful for understanding the physiological and pathological roles of the endothelin ET_A receptor in human prostate and other tissues.

Keywords: Endothelin receptor antagonist; TA-0201; Endothelin receptor; Prostate, human

1. Introduction

Endothelin-1 was first isolated from the culture medium of porcine endothelial cells as a potent vasoconstricting peptide (Yanagisawa et al., 1988). Thereafter, two other isopeptides were identified and named endothelin-2 and endothelin-3 (Inoue et al., 1989). The endothelins have a variety of important physiological functions associated with the cardiovascular, renal, pulmonary and nervous systems (Masaki and Yanagisawa, 1992; Rubanyi and Polokoff, 1994). These activities are exerted through at least two distinct endothelin receptors (ET_A and ET_B). Endothelin ET_A receptors show higher affinity for endothelin-1 and endothelin-2 than for endothelin-3, whereas endothelin ET_B receptors have nearly equal affinity for endothelin-1, endothelin-2 and endothelin-3 (Arai et al., 1990; Sakurai et al., 1990).

In 1993, Langenstroer et al. first demonstrated the predominant occurrence of endothelin-1 and its contractile function in human prostate. Endothelin receptors have been also demonstrated in human prostate (Kobayashi et al., 1994; Kondo et al., 1995; Imajo et al., 1997; Prayer-Galetti et al., 1997). These results suggest that endothelin-1 may be involved in prostate functions, such as contraction, especially in benign prostatic hypertrophy (Kondo et al., 1995; Brun et al., 1996; Hiraoka et al., 2000).

TA-0201 is a newly developed, non-peptide antagonist selective to endothelin ET_A receptors (Hoshino et al., 1998). Prolonged treatment with TA-0201 ameliorates cardiac dysfunction and extends the life span of hamsters with hereditary heart failure (Yamauchi-Kohno et al., 1999). TA-0201 also improves pulmonary hypertension (Ohnishi et al., 1998; Yamauchi-Kohno et al., 1997) and cerebral vasospasm (Kikkawa et al., 1999), suggesting pathophysiological roles for endothelin ET_A receptors in some cardiovascular disorders. However, the effects of TA-0201 on human prostate remain to be examined. In the present study,

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we characterized the pharmacological profile of TA-0201, using recombinant and human prostate endothelin receptors.

2. Materials and methods

Human prostate specimens were obtained from patients with symptomatic benign prostatic hypertrophy who underwent open prostatectomy. All patients gave informed consent and the project was approved by the Ethics Committee of Fukui Medical University.

2.1. Membrane preparations

Chinese hamster ovary (CHO) cells expressing recombinant human endothelin ET_A or ET_B receptors were kindly provided by Professor S. Miwa (Hokkaido University) and membranes were prepared as described previously (Takagi et al., 1995). Briefly, the harvested cells were suspended in ice-cold homogenate buffer (20 mM Tris–HCl, 5 mM EDTA, 0.25 M sucrose, 100 μM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, 0.5 mg/ml pepstatin, pH 7.5), sonicated and centrifuged at 3000 × *g* for 10 min. The supernatant was then centrifuged at 80,000 × *g* for 30 min, and the resulting pellet was resuspended in assay buffer (50 mM Tris–HCl, 20 mM $MgCl_2$, pH 7.5) and used for binding experiments.

The transition zone of prostate isolated from benign prostatic hypertropy patients was minced and homogenized in 20 volumes of ice-cold homogenate buffer with a Polytron homogenizer (setting 8, 15 s × 3). The homogenate was centrifuged at 3000 × *g* for 15 min. The supernatant was decanted through four layers of gauze and centrifuged at 80,000 × *g* for 30 min. The resulting pellet was resuspended in the same volume of assay buffer for the binding experiments. All procedures were performed at 4 °C.

2.2. Radioligand binding experiments

The membranes were incubated at 30 °C for 120 min with 0.1 nM [^{125}I]endothelin-1 in assay buffer containing 0.05% bovine serum albumin. The incubation volume was 0.25 ml in all experiments. Reactions were terminated by rapid filtration through a Brandel cell harvester (Biomedical Research and Developmental Laboratories, Gaithersburg, MD, USA) onto Whatman GF/C filters (Whatman International, Maidstone, Kent, UK) presoaked in 0.1% bovine serum albumin for 60 min. The filters were washed three times with 4 ml ice-cold 50 mM Tris–HCl (pH 7.4) and then the filter-bound radioactivity was determined with a gamma counter (Aloka, Model ARC-300). Non-specific binding was defined as radioactivity in the presence of 1 μM nonlabeled endothelin-1. Assays were conducted in duplicate. The affinity constants for the antagonists were calculated using the LIGAND program. Protein concentrations were quantified by the method of Bradford using bovine serum albumin as standard (Bradford, 1976).

2.3. Functional experiments

The transition zone of human prostate was cut into strips (about 7 × 3 mm). Each strip was mounted vertically in an organ bath containing 5 ml of modified Krebs–Henseleit solution of the following composition in mM: NaCl 112, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.0, $NaHCO_3$ 25.0, NaH_2PO_4 1.2, and glucose 11.5. The medium was bubbled with a gas mixture consisting of 95% O_2 and 5% CO_2, and allowed to equilibrate under a resting tension (1 g) for at least 1 h at 37 °C before experiments started. The change of tension of muscle strips in response to various pharmacological agents was recorded by means of an isometric force-displacement transducer (T7-30, Orientec, Japan) and an oscillograph (FJG-4128, Nihon Koden, Tokyo, Japan).

After equilibration, responses to 100 μM noradrenaline were obtained twice with a 60-min interval, during which the bath medium was exchanged repeatedly at 20- to 30-min intervals. Ninety minutes after the second response to noradrenaline, endothelin-1 or sarafotoxin S6c was cumulatively applied once to each strip and the response to endothelin-1 or sarafotoxin S6c was normalized against the second standard response to noradrenaline obtained in the same strip. Because the responses to endothelin or sarafotoxin S6c were not reproducible in the same strip, the experiments with antagonists were done in parallel with antagonist-free experiments and the responses were also normalized against the standard responses to noradrenaline in the same strips. The antagonists were applied for 60 min before and during application of endothelin-1 or sarafotoxin S6c.

2.4. Data analysis

Experimental values are given as means ± S.E.M. Analysis of radioligand binding data was performed with PRISM (Graphpad Software, CA, USA), nonlinear curve-fitting program. Saturation and competition binding data were first fitted to a one- and then to a two-site model, and the two-site model was accepted only if it resulted in a significant

Table 1
Binding affinity of TA-0201 and other drugs at recombinant human endothelin receptors

Drug	pK_i	
	ET_A	ET_B
TA-0201	10.7 ± 0.5	7.8 ± 0.1
Endothelin-1	9.1 ± 0.1	10.2 ± 0.2
Sarafotoxin S6c	6.1 ± 0.1	10.3 ± 0.1
BQ123	6.6 ± 0.1	< 5
BQ788	5.0 ± 0.3	7.8 ± 0.1

Membranes from CHO cells expressing human endothelin ET_A and ET_B receptors were incubated with 100 pM [^{125}I]endothelin-1 in the presence of unlabeled drugs. Binding affinity (pK_i) of each drug is shown as mean ± S.E.M. of three experiments.

Table 2
Binding affinity of TA-0201 and other drugs in human prostate membranes

Drug	pK_i high (%)	pK_i low (%)
TA-0201	9.7 ± 0.3 (70)	7.2 ± 0.3 (30)
Endothelin-1	10.0 ± 0.2	–
Sarafotoxin S6c	10.6 ± 0.6	–
BQ123	7.1 ± 0.2	–
BQ788	8.8 ± 1.0 (26)	6.3 ± 0.1 (74)

Membranes were incubated with 100 pM [^{125}I]endothelin-1 in the presence of unlabeled drugs. Mean ± S.E.M. of three to four experiments.
(%): percentage proportion of each affinity site in total specific binding sites.

improvement of the fit, as judged by an *F*-test comparison with a *P* value less than 0.05.

2.5. Drugs

The drugs used were as follows: *N*-(6-(2-(5-bromopyrimidine-2-yloxy)ethoxy)-5-(4-methylphenyl)pyrimidin-4-yl)-4-(2-hydroxy-1,1-dimethylethyl) benzensulfonamide sodium salt sesquihydrate (TA-0201) from Medical Chemistry Research Laboratory, Tanabe Seiyaku (Saitama, Japan); endothelin-1 and sarafotoxin S6c from Peptide Institute (Osaka, Japan); cyclo(D-Asp-Pro-D-Val-Leu-D-Trp) (BQ123) and 2,6-dimethylpiperidinecardpnyl-γ-methyl-Leu-N_{in}-(methoxycarbonyl)-D-Trp-D-Nle (BQ788) from American Peptide (California, USA); 1-noradrenaline bitartate, leupeptin, pepstatin and phenylmethylsulfonyl fluoride from Sigma (St. Louis, MO, USA); and [^{125}I]endothelin-1 from DuPont NEN (Boston, USA).

3. Results

3.1. Radioligand binding study

Binding of [^{125}I]endothelin-1 to human recombinant endothelin receptors (ET_A or ET_B) expressed in CHO cells was inhibited by TA-0201 and other drugs in a concentration-dependent manner. Table 1 shows the binding affinity of the tested drugs. Endothelin-1 showed high affinity and a 10-fold selectivity for the endothelin ET_A receptor. BQ788 and sarafotoxin S6c had higher affinity for the endothelin ET_B receptor than the endothelin ET_A receptor, while BQ123 was selective for the endothelin ET_A receptor. TA-0201 showed approximately 1000 times higher affinity for the endothelin ET_A receptor than for the endothelin ET_B receptor, and the affinity (pK_i = 10.7 ± 0.5) was the higher than that of endothelin-1 (9.1 ± 0.1), sarafotoxin S6c (6.1 ± 0.1), BQ123 (6.6 ± 0.1) and BQ788 (5.0 ± 0.3) at the endothelin ET_A receptor (n = 3 in each drug).

[^{125}I]endothelin-1 also bound to human prostate membranes. Scatchard plots of the saturation binding revealed a linear relationship, suggesting the presence of a single affinity site. The estimates of affinity and B_{max} were 99 ± 14 pM and 1014 ± 16 fmol/mg protein, respectively (n = 3). This binding was also inhibited by TA-0201 and other drugs in a concentration-dependent manner. Endothelin-1 inhibited binding monophasically with a high affinity (pK_i = 10.0 ± 0.2). TA-0201 and BQ788 showed shallow competition curves. The high-affinity site for TA-0201 and the low-affinity site for BQ788 accounted for approximately

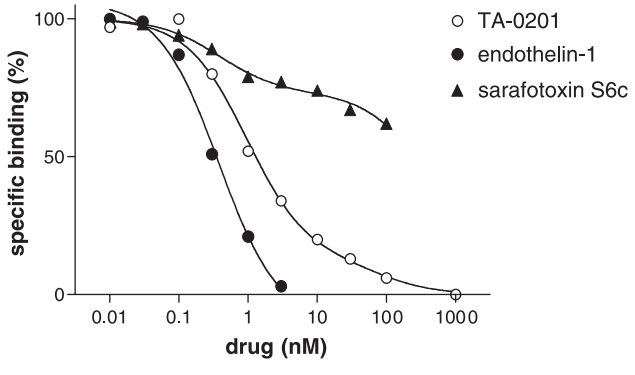

Fig. 1. Competition curves for TA-0201 (open circle), endothelin-1 (close circle) and sarafotoxin S6c (close triangle) to displace [^{125}I]endothelin-1 binding to human prostate membranes. This figure shows a representative example of three to four experiments. Each point represents the mean of duplicate determinations.

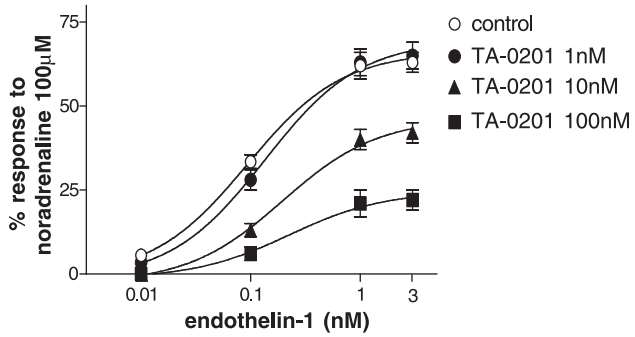

Fig. 2. Effects of TA-0201 on the contractile response induced by endothelin-1 in human prostate. Contraction induced by endothelin-1 is expressed as a percentage of the maximal response elicited by 100 μM noradrenaline in each sample. Each point represents mean ± S.E.M. of three to five preparations.

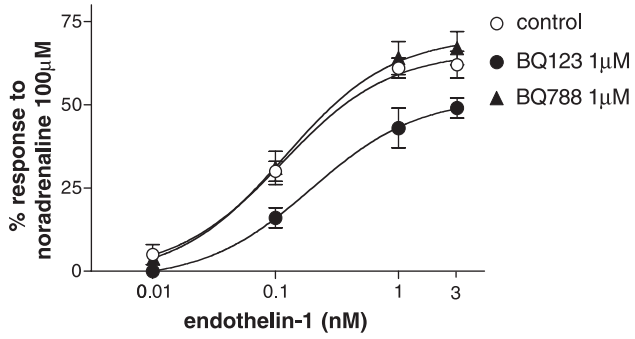

Fig. 3. Effects of BQ123 and BQ788 on the contractile response induced by endothelin-1 in human prostate. Each point represents mean ± S.E.M. of five preparations. Other experimental conditions are the same as Fig. 2.

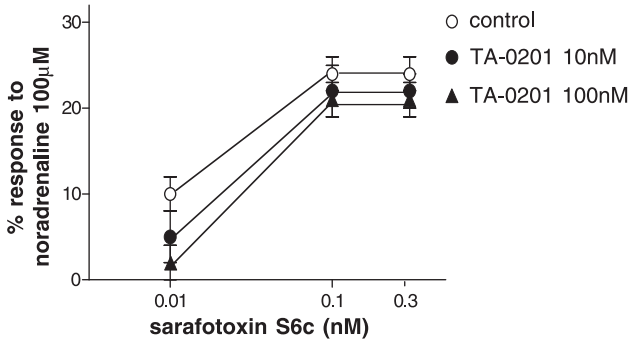

Fig. 4. Effects of TA-0201 on the contractile response induced by sarafotoxin S6c in human prostate. Each point represents mean ± S.E.M. of five preparations. Other experimental conditions are the same as Fig. 2.

70% of total specific binding sites (Table 2). BQ123 (0.1 nM–10 μM) and sarafotoxin S6c (0.01 nM–0.1 μM) did not completely inhibit [^{125}I]endothelin-1 binding: approximately 30% and 65% of the binding sites remained uninhibited, respectively. Fig. 1 shows the representative data for endothelin-1, TA-0201 and sarafotoxin S6c obtained for human prostate membranes.

3.2. Isometric tension studies

Endothelin-1 and sarafotoxin S6c produced concentration-dependent contractions in human prostate strips. However, the responses were not reproducible in the same preparation and the second application of the peptide produced a lower amplitude of contraction as compared with that for the first concentration response (data not shown). Therefore, 100 μM noradrenaline was at first applied twice with a 60-min interval, to check the responsiveness of the strip, and then endothelin-1 or sarafotoxin S6c was added once to each preparation. That is, the responses to endothelin-1 or sarafotoxin S6c were compared with the second (standard) response to 100 μM noradrenaline, which had been obtained beforehand in the same preparation. The maximum contraction induced by endothelin-1 and sarafotoxin S6c was approximately 70% and 25% of the standard response to noradrenaline, respectively (Figs. 2–4). TA-0201 (10 and 100 nM) and BQ123 (1 μM) shifted the concentration-response curve for endothelin-1 to the right and reduced the maximum response, showing insurmountable inhibition (Figs. 2 and 3). The pD_2' value, which is the antagonist concentration to inhibit the maximum contraction by 50% (Van Rossum, 1963), was 7.8 for TA-0201 or 5.4 for BQ123, respectively. BQ788 at 1 μM did not inhibit the contractile response to endothelin-1 (Fig. 3). TA-0201 had little effect on the concentration-response curve for sarafotoxin S6c (Fig. 4).

4. Discussion

Many peptide and nonpeptide antagonists for endothelin receptors have been developed (Ohlstein et al., 1996). TA-0201 is a newly synthesized, nonpeptide antagonist for endothelin receptors. It has been reported that this compound shows high selectivity for native endothelin ET_A receptors compared with native endothelin ET_B receptors (Hoshino et al., 1998). In the present study with recombinant endothelin receptors, we confirmed the conclusion and further suggest that TA-0201 has the highest selectivity (approximately 1000 times selective for endothelin ET_A receptors) among the drugs developed so far (Ohlstein et al., 1996). Such high selectivity was also detected in the binding study with human prostate membranes.

TA-0201 biphasically inhibited [^{125}I]endothelin-1 binding to human prostate membranes. The proportion of high-affinity site was approximately 70% of total specific binding, which was consistent with the sites detected with BQ123 (endothelin ET_A receptor antagonist) or the low-affinity sites for BQ788 (endothelin ET_B receptor antagonist). In accordance with this, the contractile response to endothelin-1 was inhibited by TA-0201 and BQ123 but not by BQ788. The amplitude of the contraction induced by endothelin-1 was approximately three times greater than that of the contraction induced by sarafotoxin S6c (endothelin ET_B receptor agonist). These results, together with previous reports (Langenstroer et al., 1993; Brun et al., 1996; Hiraoka et al., 2000), suggest that the predominant functional endothelin receptor in human prostate is the endothelin ET_A receptor subtype.

Because endothelin-1 occurs and its receptors are expressed in the human prostate (Langenstroer et al., 1993; Kobayashi et al., 1994; Kondo et al., 1995; Prayer-Galetti et al., 1997), the endothelin-1/endothelin receptor system may be involved in several physiological functions of the prostate. Prostate contraction disturbs micturition, especially in patients with benign prostatic hypertrophy. Therefore, this symptom is a target of pharmacotherapy. Alpha-1 adrenoceptor antagonists have been widely used in patients with benign prostatic hypertrophy, to overcome the urinary disturbance (Lepor, 1993; Monda and Olsterling, 1993; De Mey, 1998). In the present study, endothelin-1 produced a significant contraction of human prostate and the maximum amplitude of the contraction was approximately 70% of that induced by noradrenaline. Furthermore, the contractile response to endothelin-1 persisted for a long time (Langenstroer et al., 1993; unpublished observations). These lines of evidence suggest that the endothelin-1/endothelin receptor system regulates the basal tonus of the prostate and might be related to urinary disturbance in patients with benign prostatic hypertrophy. In addition, it is likely that other physiological functions, such as cell proliferation, are predominantly regulated by the endothelin system in the human prostate (Rubanyi and Polokoff, 1994).

In conclusion, the present study with recombinant and native endothelin receptors indicates that TA-0201 is a highly selective antagonist of the endothelin ET_A receptor and will be useful in the regulation of the endothelin system in the human prostate and other tissues.

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