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Analysis of two pharmacologically predicted endothelin B receptor subtypes by using the endothelin B receptor gene knockout mouse

Tadahiko Mizuguchi, [1,]*Mariko Nishiyama, *Kayoko Moroi, †Hirokazu Tanaka, Toshihiro Saito, Yoshiaki Masuda, ‡Tomoh Masaki, #Damiane de Wit, #Masashi Yanagisawa & *Sadao Kimura

The Third Department of Internal Medicine, *Division of Cardiovascular Biology, Center for Biomedical Science and †Department of Obstetrics and Gynecology, Chiba University School of Medicine, Chiba 260, Japan; ‡Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan and #Howard Hughes Medical Institute and Department of Molecular Genetics, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235-9050, U.S.A.

1 This study was performed to clarify whether the endothelin (ET) receptor subtypes mediating two pharmacologically heterogeneous response to ET_B receptor agonists in normal mice are the product(s) of a single ET_B receptor gene.

2 Vasodilator responses to sarafotoxin S6c (S6c) in the thoracic aorta and contractile responses to ET-1 and IRL1620 in the stomach were examined in tissues from normal and ET_B receptor gene knockout mice, in the absence and presence of an ET_A receptor antagonist, BQ-123, or an ET_A/ET_B receptor antagonist, PD142893.

3 In the normal mouse aorta precontracted with phenylephrine, S6c (0.1–100 nM) caused concentration-dependent relaxations ($pD_2 = 8.4$). BQ-123 had no effect on these responses. However, PD142893 almost abolished the relaxations induced by 0.1–300 nM S6c.

4 In aortae taken from ET_B receptor gene knockout mice, S6c up to 1 μM failed to cause relaxations, confirming that ET_B receptors are involved in mediating this response.

5 In normal mouse gastric fundus, 0.1 nM–1 μM ET-1, S6c or IRL1620 caused dose-dependent, BQ-123-insensitive contractions, which were much more resistant to PD142893 than S6c-induced relaxations of the aorta. The pD_2 values for S6c in the absence and presence of PD142893 (10 μM) were 8.12 ± 0.11 and 7.70 ± 0.11, respectively.

6 In the gastric fundus of the ET_B receptor gene knockout mouse, S6c and IRL1620 caused no contractions. ET-1 (0.1 nM–1 μM) caused contractions sensitive to both BQ-123 and PD142893, indicating that only ET_A receptors mediate ET-1-induced contractions of the knockout mouse gastric fundus.

7 Since both the PD142893-sensitive vasodilator response of the aorta and the PD142893-resistant contractile response of the gastric fundus to S6c were completely absent in the ET_B receptor gene knockout mouse, we conclude that the two pharmacologically heterogeneous responses to S6c are mediated by receptors derived from the same ET_B receptor gene.

Keywords: Endothelin; ET_B receptor; aorta; gastric fundus; sarafotoxin S6c; IRL1620; BQ-123; PD142893; knockout mouse

Introduction

Two endothelin (ET) receptors, ET_A and ET_B for three endogenous endothelin isopeptides, ET-1, ET-2 and ET-3, are currently known. The ET_A receptor has a higher affinity for ET-1 and ET-2 than for ET-3, and the ET_B receptor has an equal affinity for all three endothelin isopeptides (Masaki *et al*., 1994). Endothelium-dependent vasodilatation is mediated by ET_B receptors on vascular endothelial cells and some smooth muscle contractions are mediated by ET_B, as well as ET_A receptors (Bax & Saxena, 1994; Masaki *et al*., 1994). However, Warner *et al*. (1993) have shown that vasodilatations induced by ET-1 and a selective ET_B receptor agonist, sarafotoxin S6c (S6c) in the rat mesentery are markedly antagonized by an ET_A/ET_B receptor antagonist, PD142893, but that ET-1- and S6c-induced contractions in the rabbit pulmonary artery and the rat gastric fundus are minimally antagonized by PD142893 or the selective ET_A receptor antagonist, BQ-123. We also found that S6c-induced contractions in rabbit and human saphenous veins are not sensitive to antagonism by PD142893, although contractions induced by another selective ET_B receptor agonist, IRL1620 are very sensitive (Nishiyama *et al*., 1995a,b). Sudjarwo *et al*. (1993, 1994) and Karaki *et al*. (1994) have obtained similar results with the selective ET_B receptor antagonists, IRL1038 and RES-701-1. One possible way to explain these and other similar pharmacologically heterogeneous responses is to assume the presence of two subtypes of ET_B receptor (Sudjarwo *et al*., 1993; 1994; Karaki *et al*., 1994; Nishiyama *et al*.,1995a,b) or a third (ET_C) receptor (Douglas *et al*., 1995).

Although these pharmacological data suggest strongly the presence of multiple functional endothelin receptor subtypes, confirmation by molecular cloning is essential to establish their existence. However, at present, only two endothelin receptors, ET_A and ET_B have been cloned. In the present study, therefore, we examined PD142893-sensitive vasodilator responses of the aorta and PD142893-resistant contractile responses of the gastric fundus in tissues from normal mice and from ET_B receptor gene knockout mice, in order to clarify whether the endothelin receptor subtypes mediating these two types of response are the product(s) of a single ET_B receptor gene.

[1] Author for correspondence at: Division of Cardiovascular Biology, Center for Biomedical Science, Chiba University School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260, Japan.

Methods

Animals

The targeted disruption of the mouse ET_B receptor gene has been described previously (Hosoda *et al*., 1994). We used the homozygous ET_B receptor null (−/−) mice, which could be distinguished by their white-spotted coat colour; because these homozygous null mice often died within 3–4 weeks of birth, mice of either sex aged 14–19 days were used in this study. The genotype of each littermate was determined, with DNA extracted from the tail tip, by polymerase chain reaction (PCR) analyses with the primers for the exon 3 or those for the neomycin resistance cassette introduced in place of the exon 3 (Hosoda *et al*., 1994). C57BL/6J mice of either sex aged 14–22 days were purchased from CLEA JAPAN, Inc. Both C57BL/6J mice and homozygous wild type (+/+) littermates of knockout animals were used as normal controls.

Relaxation of the thoracic aorta

Mice were killed by decapitation under ether anaesthesia. Ring preparations of the thoracic aorta with intact endothelium were prepared. In some cases, the endothelium was removed by gently rubbing the intimal surface with a thin stainless steel rod. Each ring was mounted horizontally in a 5 ml organ chamber filled with Krebs solution of the following composition (mM): NaCl 118.4, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.9, KCl 4.7, $NaHCO_3$ 25.0 and D-glucose 10.1, aerated with 95% O_2 and 5% CO_2, and maintained at 37°C. Mechanical responses were recorded isometrically under a resting load of 10 mN. After an equilibration period of 90 min, the viability of the preparation was confirmed by application of 60 mM KCl (1.64 ± 0.04 mN in normal mice ($n=235$) and 1.65 ± 0.06 mN in knockout mice ($n=79$)). After washing and a further 60 min equilibration, 1 μM acetylcholine was applied to the rings precontracted by 1 μM phenylephrine, to verify the presence of an intact endothelium. The relaxations induced by acetylcholine were similar in preparations isolated from normal mice and from ET_B receptor gene knockout mice, and only the preparations which showed relaxations of more than 80% were used in this study. Then, 150 min later, a single dose of S6c was applied to each ring precontracted by 1 μM phenylephrine. Resultant relaxant responses were expressed as percentages of the contractions induced by 1 μM phenylephrine (2.21 ± 0.06 mN in normal mice ($n=179$) and 2.41 ± 0.09 mN in knockout mice ($n=72$)).

Contractions of the gastric fundus

Longitudinal muscle strips prepared from the mouse gastric fundus were suspended in 5 ml organ chambers containing Krebs solution described above. Contractile responses were measured isometrically under a resting load of 2 mN. After an equilibration period of 60 min, the tissues were contracted twice with 10 μM acetylcholine. Then, ET-1, S6c or IRL1620 was applied cumulatively. Contractions to these agonists were expressed as percentages of the response to 10 μM acetylcholine (2.9 ± 0.2 mN in normal mice ($n=37$) and 3.1 ± 0.3 mN in knockout mice ($n=24$)).

Statistics

Concentration-response curves were analysed by a curve-fitting computer programme (SigmaPlot). Maximal responses (E_{max}) and pD_2 values are expressed as the means ± s.e.mean. The data were evaluated statistically by Student's t test and $P<0.05$ was taken as significant.

Drugs and chemicals

ET-1 and S6c were purchased from Peptide Institute Inc. BQ-123 (cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-)) (sodium salt) and PD142893 (Ac-D-Dip-Leu-Asp-Ile-Ile-Trp) (sodium salt) were supplied by Nippon Chemiphar Co., Ltd and Warner-Lambert Co., respectively, and were dissolved in phosphate-buffered saline (pH 7.2) containing 0.05% bovine serum albumin. IRL1620 (Suc-[Glu^9,$Ala^{11,15}$]-ET-1(8-21)) was a gift from CIBA-GEIGY Japan and dissolved in 0.01 M NaOH. The antagonists were applied 15–20 min before the addition of the agonists.

Results

Relaxation responses of the mouse aorta

As shown in Figure 1, S6c (0.1–100 nM) caused concentration-dependent relaxations of endothelium-intact aortic rings prepared from normal C57BL/6J mice ($pD_2=8.4$, $E_{max}=22.9 \pm 2.8\%$). S6c produced no response in aortae without endothelium (data not shown). Although 3 μM BQ-123 antagonized very effectively ET-1-induced contraction of normal mouse aortae without endothelium (data not shown), S6c-induced relaxations of the endothelium-intact preparations were not influenced by the same concentration of BQ-123, ($pD_2=8.5$, $E_{max}=20.8 \pm 3.2\%$). However, in the presence of 10 μM PD142893, S6c in concentrations as high as 300 nM failed to elicit relaxations, and 1 μM S6c caused only modest relaxations. In contrast, in the aorta of the ET_B receptor gene knockout mouse, S6c up to 1 μM had no effect. The wild type littermates showed responses similar to those observed in the C57BL/6J mice (data from 8 wild type littemates, not shown).

Contractile responses of the normal mouse gastric fundus

ET-1, S6c and IRL1620 produced concentration-dependent contractions of the longitudinal muscle of the normal C57BL/6J mouse gastric fundus (Figure 2). The apparent pD_2 values and the maximal responses for ET-1 and S6c are summarized in Table 1. Neither 5 μM atropine nor 1 μM tetrodotoxin influenced these contractions (data not shown). BQ-123 (3 μM) did not significantly affect the concentration-response curves to any of these three agonists. In contrast to its marked antagonistic effect in the normal mouse aorta, PD142893 (10 μM) had a weak effect in the stomach preparations. The maximal response and the apparent pD_2 values for ET-1 were not changed in the presence of this antagonist (Figure 2a, Table 1). Contractions induced by 0.3–3.0 nM S6c were suppressed by 10 μM PD142893, while those induced by concentrations

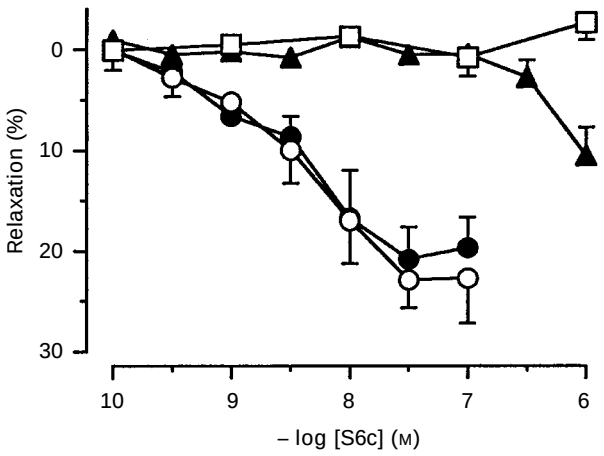

Figure 1 Sarafotoxin S6c (S6c)-induced relaxations of the aorta. Concentration-response curves for S6c in the absence of antagonists (○) and in the presence of 3 μM BQ-123 (●) or 10 μM PD142893 (▲) in aortae from normal C57BL/6J mice and aortae from knockout mice (in the absence of antagonists) (□). Vertical lines represent s.e.mean ($n=6-19$).

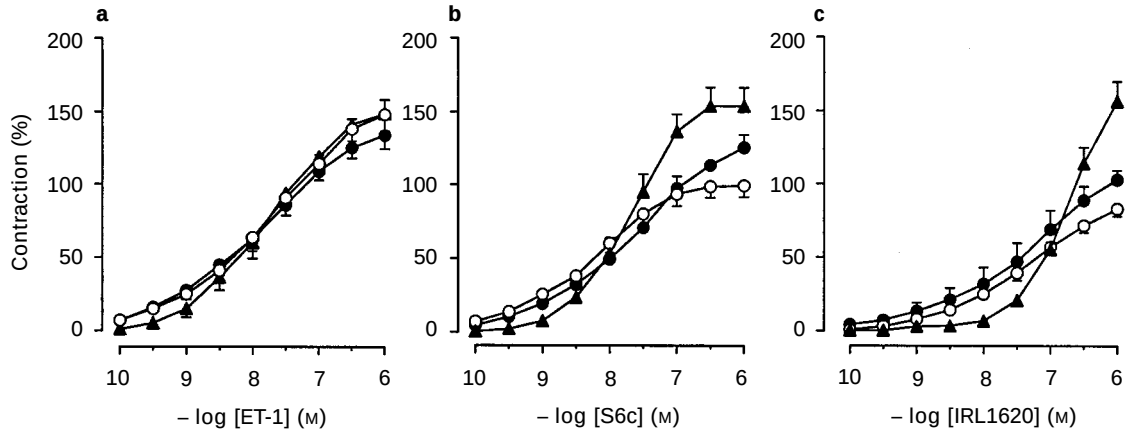

Figure 2 Concentration-response curves for endothelin-1 (ET-1) (a), sarafotoxin S6c (S6c) (b) and IRL1620 (c) in the gastric fundus of normal C57BL/6J mice, in the absence of antagonists (○) and in the presence of 3 μM BQ-123 (●) or 10 μM PD142893 (▲). Vertical lines represent s.e.mean ($n=6-9$).

Table 1 Contractile effects of endothelin-1 (ET-1) and sarafotoxin S6c (S6c) on the gastric fundus of the normal mouse

Agonists/Antagonists	n	pD_2	E_{max} (%)
ET-1	8	7.68±0.15	158.2±12.9
ET-1 + BQ-123 (3 μM)	6	7.83±0.21	145.5±15.9
ET-1 + PD142893 (10 μM)	6	7.79±0.24	161.0±13.1
S6c	9	8.12±0.11	102.3±7.9
S6c + BQ-123 (3 μM)	5	7.81±0.22	128.7±12.7
S6c + PD142893 (10 μM)	7	7.70±0.11*	165.3±13.2*

Values are expressed as the means±s.e.mean of n experiments. E_{max}: maximal responses, expressed as percentages of the maximal tension induced by 10 μM acetylcholine. pD_2 values were calculated by taking the values at 1 μM ET-1 or S6c as the E_{max}. *$P<0.05$, compared with the value in the absence of BQ-123 or PD142893.

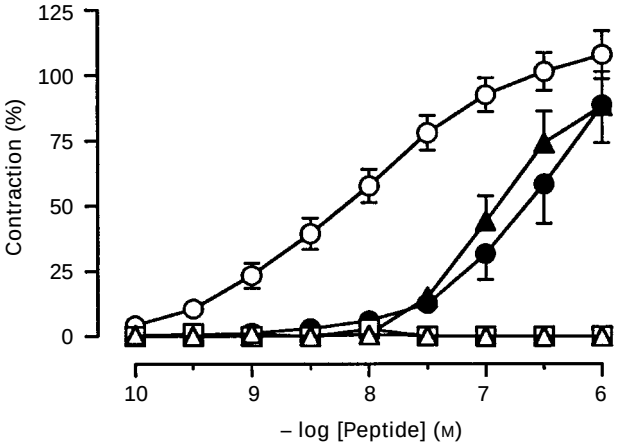

Figure 3 Contractile effects of endothelin-1 (ET-1), sarafotoxin S6c (S6c) and IRL1620 in the gastric fundus of ET_B receptor gene knockout mice. Concentration-response curves for ET-1 in the absence (○) and in the presence of 3 μM BQ-123 (●) or 10 μM PD142893 (▲), and those for S6c (□) and IRL1620 (△) in the absence of antagonists are shown. Vertical lines represent s.e.mean ($n=6-12$).

higher than 300 nM were increased (Figure 2b). The pD_2 values for S6c in the absence and presence of PD142893 were 8.12±0.11 and 7.70±0.11, respectively, indicating only a 2.6 fold rightward shift of the concentration-response curve. Similar results were obtained in the case of IRL1620 (Figure 2c). The wild type littermates of the knockout mouse showed responses closely similar to those observed in C57BL/6J mice (data from 8 wild type littermates, not shown).

Contractile responses of the gastric fundus of the ET_B receptor gene knockout mouse

In gastric fundus strips isolated from ET_B receptor gene knockout mice, there were no responses to S6c or IRL1620 (Figure 3). On the other hand, ET-1 elicited concentration-dependent contractions similar to those observed in the normal mouse stomach ($pD_2=8.03\pm0.15$, $E_{max}=113.7\pm9.4\%$). In the knockout mouse, the concentration-response curve for ET-1 was clearly shifted to the right by either 3 μM BQ-123 or 10 μM PD142893 (Figure 3).

Discussion

Vasodilator responses induced by endothelins are mediated by ET_B receptors on endothelial cells. In the present study, a selective ET_B receptor agonist, S6c caused endothelium-dependent relaxation responses in the normal mouse aorta. These responses were not affected by the ET_A receptor antagonist, BQ-123, but were very effectively antagonized by the ET_A/ET_B receptor antagonist, PD142893. Moreover, the relaxation response was completely absent in the ET_B receptor gene knockout mouse. These results clearly indicate the involvement of the ET_B receptor, on which PD142893 is an effective antagonist.

However, in the normal mouse stomach, the same concentration of PD142893 was not an effective inhibitor of S6c- or IRL1620-induced contractions (Figure 2). Since the contractions induced by these selective ET_B receptor agonists were not affected by BQ-123, these responses are mediated by ET_B receptor(s). However, compared to its ability to antagonize S6c-induced vasodilator responses in the aorta, PD142893 was much less effective in antagonizing S6c-induced contractions in the stomach. In our preliminary experiments, a selective ET_B receptor antagonist, BQ-788 was also ineffective as an antagonist of S6c-induced contractions in this tissue. These observations might be explained by the presence of additional endothelin receptors, and there have been several studies suggesting the presence of multiple subtypes of ET_B receptor (Warner *et al*., 1993; Sudjarwo *et al*., 1993; 1994; Karaki *et al*., 1994; Douglas *et al*., 1995; Nishiyama *et al*., 1995a,b). However, in the present study, both the PD142893-resistant con-

tractile response to S6c in the stomach and the PD142893-sensitive vasodilator response to S6c in the aorta, were absent in the ET_B receptor gene knockout mouse. Therefore, it must be concluded that these two pharmacologically heterogeneous responses to S6c are mediated by receptors derived from the same ET_B receptor gene.

In the stomach, S6c- and IRL1620-induced maximal contractions were increased by PD142893 (Figure 2). Since acetylcholine-induced contractions in this tissue were not influenced by PD142893 (data not shown), contractility of the smooth muscle does not seem to be affected, and endothelin receptors may be responsible for these potentiation phenomena. However, the involvement of prostanoids or nitric oxide released by stimulation of endothelin receptors is unlikely, because similar results could also be obtained in the presence of indomethacin, a cyclo-oxygenase inhibitor, or N^G-nitro-L-arginine methyl ester, a nitric oxide synthase inhibitor, in this tissue (data not shown). It has been shown that ET-1 activates small conductance Ca^{2+}-activated K^+ channels in rat gastric smooth muscle, and that ET-1-induced contractions in this tissue can be partly counteracted by activation of the K^+ channels (Gray *et al*., 1995). If it is presumed that S6c has such dual effects in the mouse stomach, and that PD142893 can reduce K^+ channel activation more effectively than contractions induced by S6c, it is possible that the maximal contraction to S6c could be augmented by this antagonist.

ET-1-induced contractions in the normal mouse stomach were insensitive to BQ-123 and PD142893. However, those in the stomach of the ET_B receptor gene knockout mouse were sensitive to both these antagonists, indicating that the contractile responses to ET-1 are mediated by the ET_A receptor. Futhermore, in our preliminary experiments, [^{125}I]-ET-1 binding sites in the normal mouse stomach were found to consist of 75% ET_A receptors and 25% ET_B receptors. Therefore, in the normal mouse stomach, both ET_A and ET_B receptors may be involved in mediating ET-1-induced contractions.

Some studies have shown that IRL1620-induced contractions are sensitive to certain ET_B receptor antagonists, including PD142893 and RES-701-1, while S6c-induced contractions are resistant to the same antagonists in the same tissues, such as human and rabbit saphenous veins (Sudjarwo *et al*., 1994; Nishiyama *et al*., 1995a,b). However, in the present study IRL1620-induced contractions of the mouse stomach were rather insensitive to PD142893, like S6c-induced contractions. It seems possible that this discrepancy is caused by the sequence difference between the mouse and the human ET_B receptors, in particular, in the extracellular loop regions (Sakamoto *et al*., 1991; Yanagisawa, 1995).

In spite of accumulating pharmacologial evidence for the existence of multiple ET_B receptor subtypes, these results indicate that the PD142893-sensitive and -resistant responses to S6c are mediated by ET_B receptors derived from a single gene. If endothelin receptor antagonists are considered as potentially useful drugs for therapeutic purposes, further studies are required to elucidate the underlying mechanism responsible for the pharmacological heterogeneity.

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References

BAX, W.A. & SAXENA, P.R. (1994). The current endothelin receptor classification: time for reconsideration? *Trends Pharmacol. Sci*., **15,** 379–386.

DOUGLAS, S.A., BECK, G.R. JR., ELLIOT, J.D. & OHLSTEIN, E.H. (1995). Pharmacological evidence for the presence of three distinct functional endothelin recptor subtypes in the rabbit lateral saphenous vein. *Br. J. Pharmacol*., **114,** 1529–1540.

GRAY, G.A., BREU, V. & CLOZEL, M. (1995). Endothelin receptors that modulate contraction of the rat fundus. *J. Cardiovasc. Pharmacol*., **26 (Suppl. 3)** S126–S129.

HOSODA, K., HAMMER, R.E., RICHARDSON, J.A., BAYNASH, A.G., CHEUNG, J.C., GIAID, A. & YANAGISAWA, M. (1994). Targeted and natural (Piebald-Lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell*, **79,** 1267–1276.

KARAKI, H., SUDJARWO, S.A., HORI, M., TANAKA, T. & MATSUDA, Y. (1994). Endothelin ET_B receptor antagonist, RES-701-1: effects on isolated blood vessels and small intestine. *Eur. J. Pharmacol*., **262,** 255–259.

MASAKI, T., VANE, J.R. & VANHOUTTE, M. (1994). International union of pharmacology nomenclature of endothelin receptors. *Pharmacol. Rev*., **46,** 137–142.

NISHIYAMA, M., MOROI, K., SHAN, L.-H., YAMAMOTO, M., TAKASAKI, C., MASAKI, T. & KIMURA, S. (1995a). Two different endothelin B receptor subtypes mediate contraction of the rabbit saphenous vein. *Jpn. J. Pharmacol*., **68,** 235–243.

NISHIYAMA, M., TAKAHARA, Y., MASAKI, T., NAKAJIMA, N. & KIMURA, S. (1995b). Pharmacological heterogeneity of both endothelin ET_A- and ET_B-receptors in the human saphenous vein. *Jpn. J. Pharmacol*., **69,** 391–398.

SAKAMOTO, A., YANAGISAWA, M., SAKURAI, T., TAKUWA, Y., YANAGISAWA, H. & MASAKI, T. (1991). Cloning and functional expression of human cDNA for the ET_B endothelin receptor. *Biochem. Biophys. Res. Commun*., **178,** 656–663.

SUDJARWO, S.A., HORI, M., TAKAI, M., URADE, Y., OKADA, T. & KARAKI, H. (1993). A novel subtype of endothelin B receptor mediating contraction in swine pulmonary vein. *Life Sci*., **53,** 431–437.

SUDJARWO, S.A., HORI, M., TANAKA, T.,MATSUDA, Y., OKADA, T. & KARAKI, H. (1994). Subtypes of endothelin ET_A and ET_B receptors mediating venous smooth muscle contraction. *Biochem. Biophys. Res. Commun*., **200,** 627–633.

YANAGISAWA, M. (1995). *GenBank Database*. Accession: U32329.

WARNER, T.D., ALLCOCK, G.H., CORDER, R. & VANE, J.R. (1993). Use of the endothelin antagonists BQ-123 and PD142893 to reveal three endothelin receptors mediating smooth muscle contraction and the release of EDRF. *Br. J. Pharmacol*. **110,** 777–782.

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