

Basic Science Reports

Both ET_A and ET_B Receptors Mediate Contraction to Endothelin-1 in Human Blood Vessels

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Background Endothelin (ET)-1 has potent vascular effects. Two endothelin receptors have been cloned, namely, the ET_A receptor, which preferentially binds ET-1, and the ET_B receptor, which equally binds ET-1 and ET-3 and preferentially sarafotoxin S6c. We characterized endothelin receptor subtypes on vascular smooth muscle and endothelium of isolated human internal mammary artery (IMA) and vein (IMV) and porcine coronary artery (PCA) using the ET_A antagonists FR139317 and BQ-123, the ET_B ligand sarafotoxin S6c, and the ET_A/ET_B antagonist Ro 47-0203 (bosentan).

Methods and Results In endothelium-denuded IMA and PCA and less so in IMV, FR139317 and BQ-123 (in PCA only) shifted the concentration-contraction curves to ET-1 parallel to the right. However, even at 10⁻⁵ mol/L, FR139317 did not inhibit a high-sensitivity portion of the concentration-contraction curve. Moreover, the ET_B receptor agonist sarafotoxin S6c induced contraction in vessels preincubated with FR139317. IMV was significantly more sensitive to the contractile effect of ET-1 and sarafotoxin S6c than was IMA (*P* < .05). Prolonged

incubation with sarafotoxin S6c (to downregulate ET_B receptors) and FR139317 eliminated the contraction resistant to FR139317. The ET_A/ET_B receptor antagonist bosentan caused a parallel shift of the concentration-contraction curve to the right at all concentrations of endothelin. ET_B receptor mRNA was detected by Northern blot analysis in IMA and aortic smooth muscle cells. In precontracted IMA and PCA with endothelium, sarafotoxin S6c did not cause endothelium-dependent relaxations, whereas transient responses occurred in IMV.

Conclusions Vascular smooth muscle cells of human IMA, IMV, and PCA contain both ET_A and ET_B receptors, whereas the endothelium of IMA and PCA does not express functional ET_B receptors linked to nitric oxide and/or prostacyclin production. Hence, inhibition of endothelin-induced contraction in patients requires the use of combined ET_A/ET_B antagonists. (*Circulation*. 1994;89:1203-1208.)

Key Words • arteries • veins • endothelin

Endothelin (ET)-1 is a 21-amino acid peptide that has strong vasoconstrictor¹⁻³ as well as mitogenic activity.⁴ In addition, under certain conditions it can cause vasodilation⁵: Its plasma levels are very low in normal subjects but are augmented in many disease states such as in certain patients with hypertension,^{6,7} atherosclerosis,⁸ acute myocardial infarction,⁹ and renal failure,¹⁰ suggesting that it may play a pathophysiological role in these situations.

Three isoforms of endothelin (ET-1, ET-2, ET-3) are known,¹¹ but only ET-1 is produced by the vascular endothelium. Two endothelin receptors (ie, ET_A and ET_B) that mediate the vascular effects of ET-1 have been characterized and their cDNAs cloned.^{12,13} The ET_A receptor has a much higher affinity for ET-1 than ET-3, whereas the ET_B receptor has nearly the same affinity for all endothelin isoforms.¹³ ET_A receptors appear to be present mainly on vascular smooth muscle cells, mediating the vasoconstrictor effects of ET-1,

whereas ET_B receptors on the vascular endothelium mediate the transient vasodilator response to ET-1 and ET-3 through release of nitric oxide and/or prostacyclin.^{5,14} Under certain conditions, however, ET_B receptors may also mediate vasoconstrictor response to ET-1.^{15,16} For example, BQ-123, one of the specific antagonists for ET_A receptors, is unable to fully antagonize the vasoconstriction to ET-1 in rats.¹⁶ Endothelin receptor antagonists are likely to be used more frequently both for elucidating the (patho)physiological role of endothelin in various cardiovascular diseases and possibly also for the treatment of these conditions. In this regard, it seems necessary to characterize receptor subtype(s) in various blood vessels and in particular those obtained from humans.

We pharmacologically characterized endothelin receptor subtypes on the endothelium and vascular smooth muscle of human internal mammary artery (IMA) and vein (IMV) as well as porcine coronary artery (PCA), using the ET_A-selective antagonists FR139317¹⁷ and BQ-123,¹⁸ the ET_B ligand sarafotoxin S6c,¹⁹ and the combined ET_A/ET_B receptor antagonist Ro 47-0203 (bosentan).²⁰

Methods

Blood Vessels and Experimental Setup

Internal mammary arteries and veins were harvested intraoperatively from patients undergoing bypass surgery.^{2,21} Vascular segments from 34 patients (31 men and 3 women; mean

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age, 62 years; range, 31 to 77) were investigated. Twenty-six percent had hypertension (n=9), 15% had diabetes mellitus (n=5), 24% were smokers (n=8), and 38% had hypercholesterolemia defined as >5.2 mmol/L (n=13). The medication of the patients included calcium antagonists (53%, n=18), antiplatelet agents (47%, n=16), nitrates (35%, n=12), β -blockers (38%, n=13), and miscellaneous drugs. Porcine coronary arteries were obtained at a slaughterhouse within 30 minutes of death. Blood vessels were kept in cold modified Krebs-Ringer bicarbonate solution of the following composition (in mmol/L): 118 NaCl, 4.7 KCl, 2.5 CaCl_2 , 1.2 MgSO_4 , 1.2 KH_2PO_4 , 25.0 NaHCO_3 , 0.026 edetate calcium disodium, and 11.1 glucose (control solution). The IMA, IMV, and left anterior descending branch of PCA were dissected free and cleaned of adherent tissue under a dissection microscope. The preparations were cut into rings (4 mm for IMA and IMV, 5 mm for PCA). The endothelium of IMA and PCA was removed mechanically except in protocols 4 and 5 (see below). The rings were suspended between two stirrups in an organ chamber filled with 12.5 mL of control solution (37°C) aerated with 95% O_2 -5% CO_2 , and isometric tension was measured. After equilibrium for 45 minutes under 4 g (IMA, References 2 and 21) or 8 g (PCA, Reference 22) of tension, the absence of endothelium was confirmed by the absence of a relaxation to acetylcholine (10^{-6} mol/L, IMA) or bradykinin (10^{-6} mol/L, PCA).²² Rings that relaxed less than 10% of contraction to norepinephrine (3×10^{-7} mol/L, IMA) or acetylcholine (3×10^{-7} mol/L, PCA) were considered without endothelium. Initial tension for IMV was 1.5 g.²

Protocols

After contraction of each vascular ring with 100 mmol/L KCl (control response), the following protocols were performed.

Effect of FR139317 on Contraction to ET-1 (Protocol 1)

To test the effect of the ET_A receptor antagonist FR139317, IMA and PCA rings without endothelium were preincubated for 30 minutes with FR139317 (10^{-7} , 10^{-6} , 10^{-5} mol/L), and cumulative concentration-contraction curves to ET-1 were constructed. Vehicle (ethanol) did not cause significant effects (data not shown). BQ-123 (10^{-7} , 10^{-6} , 10^{-5} mol/L) was tested in PCA in the same fashion.

Effect of Sarafotoxin S6c on FR139317-Resistant Portion of the Concentration-Contraction Curves to ET-1 (Protocol 2)

Concentration-contraction curves to ET-1 revealed a portion resistant to FR139317 in IMA, IMV, and PCA (see below). To examine whether this was due to ET_B receptor activation, some vessels were preincubated with sarafotoxin S6c (3×10^{-7} mol/L) for 1 hour (to downregulate ET_B receptors) and then with FR139317 (10^{-5} mol/L) for additional 30 minutes. Concentration-contraction curves to ET-1 were constructed for control vessels, vessels preincubated with FR139317, and those preincubated with sarafotoxin S6c and FR139317.

Contractions to Sarafotoxin S6c (Protocol 3)

To test the presence of ET_B receptors, rings were preincubated with FR139317 (10^{-5} mol/L) for 30 minutes (to block ET_A receptors), and cumulative concentration-contraction curves to sarafotoxin S6c were constructed.

Effect of a Combined ET_A/ET_B Receptor Antagonist Bosentan on Contractions to ET-1 (Protocol 4)

In IMA rings preincubated with bosentan (10^{-7} mol/L, 10^{-6} mol/L, 10^{-5} mol/L, 30 minutes), concentration-contraction curves to ET-1 were constructed. Endothelium was not removed in this protocol because protocol 5 (below) showed that

endothelium of IMA did not cause endothelium-dependent relaxation (see "Results").

Effect of Sarafotoxin S6c in Rings With Endothelium (Protocol 5)

To examine whether ET_B receptor stimulation causes endothelium-dependent relaxation, rings were precontracted with norepinephrine (3×10^{-7} mol/L in IMA, 10^{-7} mol/L in IMV) or acetylcholine (3×10^{-7} mol/L in PCA) and exposed to cumulative concentrations of acetylcholine (IMA) or bradykinin (IMV and PCA), respectively. Rings then were washed, preincubated with FR139317 (10^{-5} mol/L, 30 minutes), and cumulative concentration-response curves to sarafotoxin S6c were constructed under the same conditions as above.

Since sarafotoxin S6c did not relax in IMA or PCA (see "Results"), the same protocol was performed after preincubation of these vessels with indomethacin (10^{-5} mol/L, to inhibit possible release of cyclooxygenase-derived endothelium-derived contracting factor⁶) and FR139317 for 30 minutes.

Detection of mRNA for ET_B Receptor

Preparation of RNA

Total RNA was extracted from IMA or aorta by homogenizing the frozen tissue in 4 mol/L guanidine thiocyanate buffer, layering over a 5.6 mol/L CsCl -25 mmol/L sodium acetate cushion, and centrifuging at 175 000g for 24 to 36 hours²³ or by phenol-chloroform extraction according to the single-step method described by Chomczynski and Sacchi.²⁴ Poly(A)+ RNA was purified by two cycles of selection on an oligo(dT) cellulose column (Pharmacia).

Northern Blot Analysis

RNAs were prepared from aorta of organ donor patients or IMA of patients undergoing coronary bypass surgery. Approximately 5 μg of poly(A)+ RNA was subjected to electrophoresis on 1% formaldehyde-agarose gels and transferred to Hybond-N filters (Amersham). The filters were hybridized in 0.5 mol/L Na_2PO_4 (pH 7.2), 1% bovine serum albumin, 7% SDS, and 10 mmol/L EDTA at 65°C with the ^{32}P -labeled cDNA probe prepared by random prime labeling and washed in $2 \times \text{SSC}$, 0.1% SDS- $0.1 \times \text{SSC}$, 0.1% SDS at 65°C. Filters were hybridized with a 0.4-kb ET_A receptor cDNA probe or a 1.4-kb ET_B receptor cDNA (both generous gifts of Dr T. Sakurai, University of Tsukuba, Japan) and as control with a 1.9-kb *EcoRI* fragment of human β -actin cDNA probe. Membranes were exposed to Kodak XRP x-ray film using an intensifying screen at -70°C for 24 hours.

Drugs

The following drugs were used (all from Sigma Chemical Co, St Louis, Mo, unless otherwise stated): ET-1 and FR139317 (Fujisawa Pharmaceutical Co, Ltd, Osaka, Japan); sarafotoxin S6c (Bachem, Bubendorf, Switzerland); acetylcholine, bradykinin, norepinephrine, indomethacin, and bosentan (F. Hoffmann-La Roche, Ltd, Basel, Switzerland). ET-1 and sarafotoxin S6c were dissolved in 0.1% bovine serum albumin. FR139317 was dissolved in 50% ethanol (stock solution, 10^{-5} mol/L) and diluted with distilled water. The final concentration of ethanol in the organ chamber was less than 0.3%. Indomethacin was dissolved in distilled water containing 5×10^{-3} mol/L carbonate. All other drugs were dissolved in distilled water.

Data Analysis and Statistics

Contractions are expressed as a percentage of the control response to 100 mmol/L KCl. In protocol 1, the concentration of ET-1 (expressed as negative log molar) exhibiting 50% of the response to 100 mmol/L KCl (pD_2) was calculated separately for each ring. The area under the concentration-contraction curves (in arbitrary units) also was calculated in

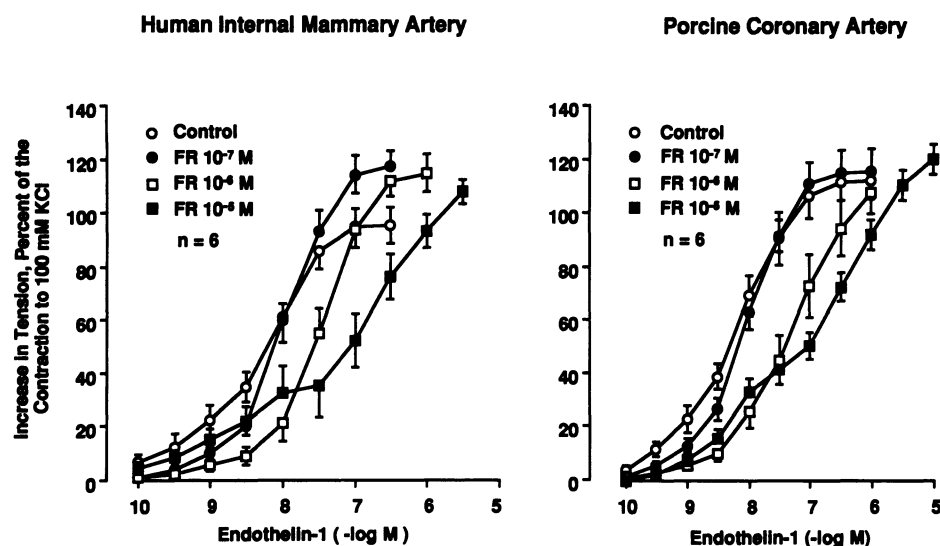


Fig 1. Line plots show contractions to endothelin (ET)-1 in the human internal mammary artery (left) and porcine coronary artery (right). The effect of the ET_A antagonist FR139317 (FR) is shown. Values are mean $\pm$ SEM.

protocol 3. ANOVA followed by Scheffé's test was used to compare the pD_2 values at each concentration of FR139317 (protocol 1) and bosentan (protocol 4), the area under the curves, and the maximal contractions in response to sarafotoxin S6c (protocol 3). The Student's unpaired t test was used to compare the pD_2 values for ET-1-induced contractions in control vessels of IMA and IMV. All values are expressed as mean $\pm$ SEM. Under all circumstances, n refers to the number of patients studied (one ring per patient per series of experiments). A two-tailed value of $P < .05$ was considered statistically significant.

Results

Effect of FR139317 on ET-1-Induced Contraction

ET-1 caused concentration-dependent contractions in all blood vessels, but IMV was more sensitive than

IMA (log shift, 2512-fold; Figs 1 and 2; $P < .05$). Incubation with FR139317 had no vascular effects on its own, but in IMA and PCA, it shifted the concentration-contraction curves to higher concentrations of ET-1 parallel to the right, whereas maximal contraction was not different (Fig 1). In IMA, the pD_2 values were 8.2 ± 0.1 in the absence and 8.1 ± 0.1 (10^{-7} mol/L), 7.6 ± 0.1 (10^{-6} mol/L), and 7.2 ± 0.3 (10^{-5} mol/L, $P < .05$) in the presence of FR139317. In PCA, similar results were obtained using the ET_A antagonists FR139317 or BQ-123 ($n = 5$, data not shown).

However, FR139317 did not antagonize the effect of ET-1 completely; rather, a small contraction occurring at 10^{-10} to 3×10^{-8} mol/L of ET-1 was unmasked, particularly at 10^{-5} mol/L FR139317 in IMA and PCA (Fig 1). This

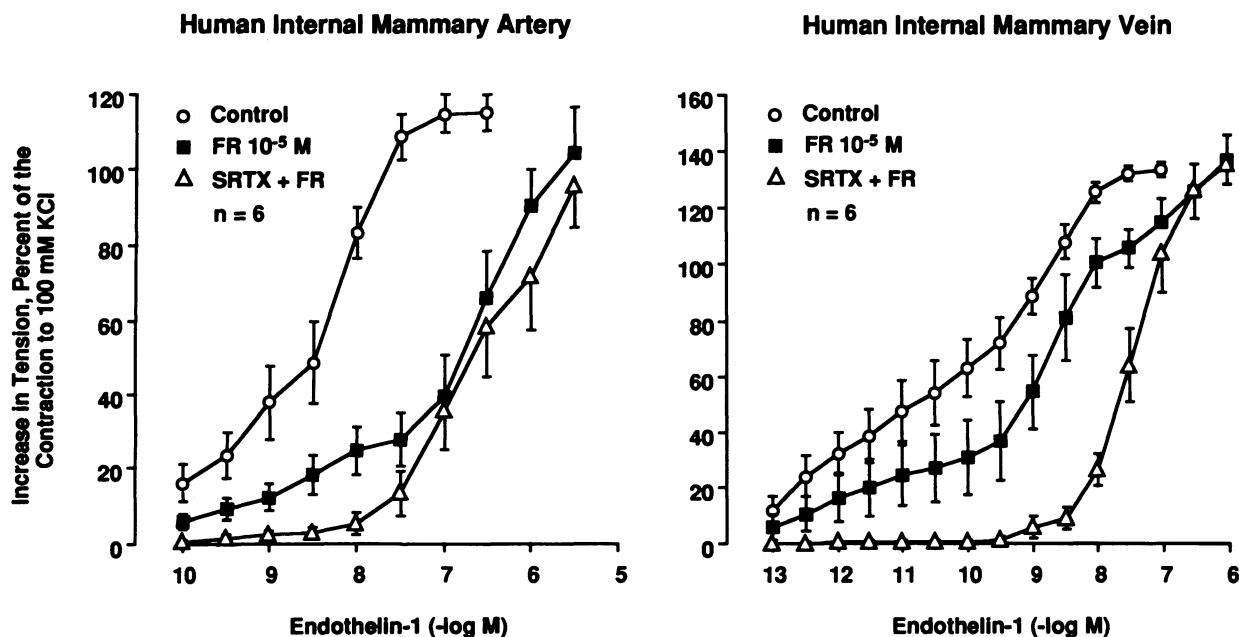


Fig 2. Line plots show contractions to endothelin (ET)-1 in the human internal mammary artery (left panel) and internal mammary vein (right panel). The role of ET_A and ET_B receptors was investigated using the ET_A antagonist FR139317 (FR) (10^{-5} mol/L) and sarafotoxin S6c (SRTX) preincubation (3×10^{-7} mol/L for 60 minutes to downregulate ET_B receptors). Values are mean $\pm$ SEM.

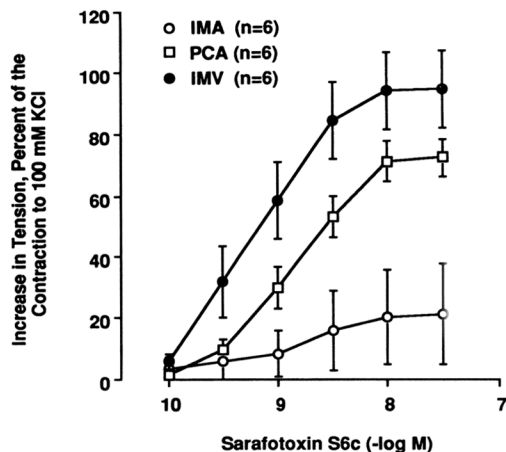


Fig 3. Line plot shows endothelin (ET_B) receptor activation by sarafotoxin S6c in the human internal mammary artery (IMA), internal mammary vein (IMV), and porcine coronary artery (PCA). Experiments were performed in the presence of the ET_A antagonist FR139317 (10^{-5} mol/L). Values are mean $\pm$ SEM; IMA vs IMV for maximal contraction and area under the curves, $P < .05$.

response was even more pronounced in the IMV at concentrations of 10^{-13} to 10^{-8} mol/L ET-1 (Fig 2).

Effect of Sarafotoxin S6c in Quiescent Vessels

Certain quiescent vessels were preincubated with sarafotoxin S6c (in the presence of 10^{-5} mol/L FR139317). Under these conditions, sarafotoxin S6c (3×10^{-7} mol/L) induced immediate contractions in all vessels ($27 \pm 12\%$ of KCl 100 mmol/L in IMA, $106 \pm 4\%$ in IMV, $61 \pm 19\%$ in PCA), which decreased spontaneously, and only a small contraction ($2 \pm 1\%$ in IMA, $4 \pm 2\%$ in IMV, $21 \pm 5\%$ in PCA) was left after 1 hour. Readdition of sarafotoxin S6c did not cause contraction after this incubation. If concentration-contraction curves to ET-1 were performed after preincubation of the vessels with sarafotoxin S6c, the portion of the endothelin-induced contraction resistant to FR139317 was suppressed in IMA and IMV (Fig 2). Similar results were obtained in PCA ($n=6$, data not shown).

In the presence of FR139317 (10^{-5} mol/L), sarafotoxin S6c evoked concentration-dependent contractions in IMA, IMV, and PCA (Fig 3). The maximal contraction was greater and the area under the curve larger in IMV than IMA ($P < .05$; pD_2 in IMV, 9.0 ± 0.3 ; IMA was not calculable). Maximal contraction of PCA to sarafotoxin S6c also was more pronounced than that of IMA ($P < .05$; Fig 3; pD_2 , 8.6 ± 0.1).

Effects of Bosentan on Contraction to ET-1

In IMA, bosentan shifted the concentration-contraction curves to the right in a completely parallel fashion, contrary to the case of FR139317 (Fig 4). The pD_2 values of ET-1 were 8.3 ± 0.1 under control conditions and 8.3 ± 0.1 (10^{-7} mol/L), 7.9 ± 0.1 (10^{-6} mol/L, $P < .05$), and 7.2 ± 0.1 (10^{-5} mol/L, $P < .05$) in the presence of bosentan.

Northern Blot Analysis

ET_B receptor mRNA was detected in IMA as well as in aortic smooth muscle cells (Fig 5).

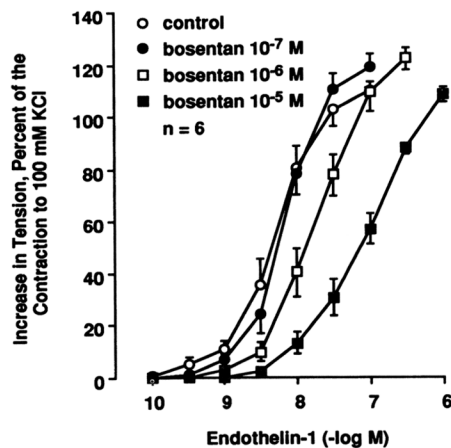


Fig 4. Line plot shows contractions to endothelin (ET)-1 in the human internal mammary artery. The effect of the combined ET_A/ET_B antagonist bosentan is shown. Values are mean $\pm$ SEM.

Effect of Sarafotoxin S6c in Precontracted Vessels With Endothelium

In IMA, IMV, and PCA with endothelium contracted with norepinephrine or acetylcholine, respectively, acetylcholine (IMA) or bradykinin (IMV and PCA) exhibited potent relaxation, demonstrating the presence of endothelium ($n=5$; IC_{50} in IMA, 7.3 ± 0.1 ; IMV, 8.4 ± 0.2 ; PCA, 8.6 ± 0.1 ; maximal relaxation in IMA, $92 \pm 7\%$; IMV, $86 \pm 9\%$; PCA, $99 \pm 9\%$). However, under the same experimental conditions, IMA and PCA did not relax to sarafotoxin S6c but contracted to higher concentrations ($n=5$, data not shown). Relaxation did not occur even in the presence of indomethacin ($n=5$, data not shown). In contrast, in four of the five IMV rings, higher concentrations of sarafotoxin S6c evoked

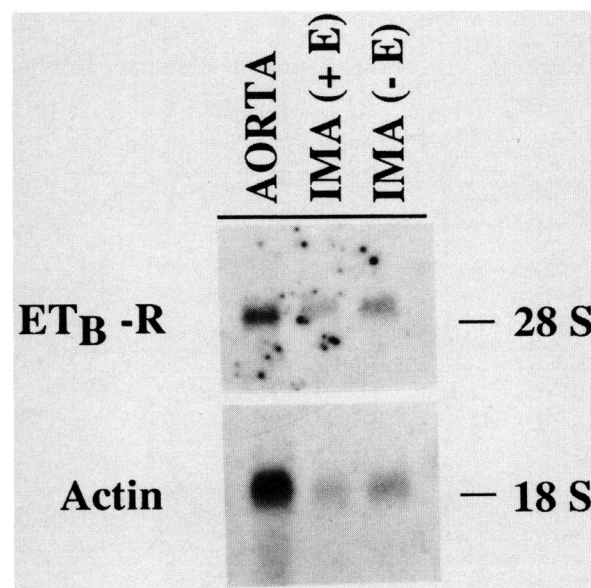


Fig 5. Northern blots show expression of endothelin (ET_B) receptor (ET_B -R) mRNA in human blood vessels: mRNA of smooth muscle cells of aorta [left] and internal mammary artery with [IMA (+E), middle] or without [IMA (-E), right] endothelium is shown. Actin served as control.

modest (6% to 33% of norepinephrine-induced contraction) and transient relaxations followed by sustained contractions.

Discussion

The major findings are that both ET_A and ET_B receptors contribute to ET-1-induced contraction in smooth muscle of human mammary artery and vein and PCA and that the endothelium of the arteries does not express functional ET_B receptors linked to release nitric oxide or prostacyclin, whereas modest transient responses occur in the vein.

Endothelins and sarafotoxins have a high degree of sequence homology. Sarafotoxins are toxins purified from the venom of the Middle Eastern burrowing asp, *Atractaspis engaddensis*.^{25,26} Endothelins and sarafotoxins have potent biological effects, eg, vasoconstriction, vasodilation, and potentiation of the effect of other vasoconstrictors and mitogenesis.^{1-4,6,27,28} Endothelin also has been implicated in atherosclerosis^{8,29} and in the extension of myocardial infarction.³⁰

Two receptors, ET_A and ET_B,^{12,13} mediate the action of endothelins. It is assumed that ET_A receptors are expressed in smooth muscle to mediate vasoconstriction and ET_B receptors on the endothelium. In our study, however, ET_B receptor activation by sarafotoxin S6c did not cause endothelium-dependent relaxation in the arteries. A concomitant release of cyclooxygenase-derived, endothelium-derived contracting factors⁶ can be excluded, as vessels preincubated with indomethacin (to block cyclooxygenase) did not show any relaxation. This contrasts with observations in experimental animals^{5,14,31} and suggests that depending on species and vascular bed, functional endothelial endothelin receptors are or are not expressed. An expression of endothelial ET_B receptors with no functional link to nitric oxide or prostacyclin production cannot be excluded, however. In contrast, the mammary vein modestly responded to endothelial ET_B activation with transient relaxation followed by sustained contraction. The fact that low-dose ET-1 increases flow in human forearm circulation³ indicates that in humans, endothelial endothelin receptors linked to nitric oxide and prostacyclin are expressed in small but not large arteries.

Endothelin antagonists are crucial tools to unravel the (patho)physiological role of endothelin.^{18,32} BQ-123 and FR139317 are highly selective competitive antagonists for ET_A receptors.^{17,18,33,34} FR139317 and BQ-123 (in PCA) shifted the concentration-contraction curves to ET-1 to the right, whereas maximal contractions remained similar. Remarkably, however, at low concentrations of ET-1, a contractile response resistant to FR139317 or BQ-123 remained, suggesting the presence of contractile receptor(s) other than ET_A on vascular smooth muscle.

To investigate ET_B receptors in smooth muscle, we used sarafotoxin S6c, which is a highly selective agonist for brain ET_B receptors.¹⁹ In the presence of FR139317, sarafotoxin S6c caused contractions in mammary arteries and particularly in veins and PCA. Sarafotoxin S6c, on the other hand, can be used as an antagonist because endothelin receptors are downregulated after prolonged incubation with an agonist.³⁵⁻³⁷ Preincubation with sarafotoxin S6c induced an immediate contraction, but only a very small amount of tension was left after 1

hour, suggesting downregulation of ET_B receptors. Importantly, the portion of the ET-1-induced contractions resistant to FR139317 was suppressed by preincubation with sarafotoxin S6c. Therefore, the receptor involved is shared by sarafotoxin S6c and ET-1 and hence must be an ET_B receptor.^{15,16,18,31,38,39} The presence of ET_B receptors on vascular smooth muscle also was detected by Northern blot analysis. Furthermore, the use of the combined ET_A/ET_B receptor antagonist bosentan demonstrated a parallel shift of the concentration-response curve to ET-1.

The fact that vasoconstrictor ET_B receptors are present in human mammary arteries and veins as well as in PCA has important implications for drug development and therapeutic use of endothelin antagonists in patients. Indeed, to prevent endothelin-induced responses, a combined ET_A/ET_B antagonist such as bosentan is required, particularly at lower concentrations of endothelin (which are more likely to be achieved in vivo⁶). The results obtained in PCA, which was used as a model for the human coronary artery, suggest that ET_B receptors also contribute to contractile responses in the coronary circulation and that activation of these receptors may be involved in coronary spasm⁴⁰ and extension of myocardial infarction.³⁰ As the contraction to sarafotoxin S6c was much larger in mammary veins than in arteries, ET_B receptors may be especially important in the venous system. The increased sensitivity of veins² is related to a larger contribution of ET_B receptors. Circulating endothelin levels are higher in venous than in arterial blood⁴⁰ and are increased in heart failure,⁴¹ a clinical syndrome with excess venous tone. If ET_B receptors—as suggested by our preliminary results with IMA resistance arteries—also contribute to peripheral vascular resistance, combined endothelin antagonists will give more insights into the role of endothelin in hypertension (see References 6 and 42). A potential disadvantage of combined ET_A/ET_B receptor antagonist may be blockade of nitric oxide or prostacyclin release in certain blood vessels.^{1,3,5} However, the fact that such molecules fully block the vasoconstrictor effect of ET-1 appears to be more important.

Acknowledgments

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