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Conversion of big-endothelin-1 elicits an endothelin ET_A receptor-mediated response in endothelial cells

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Abstract

Functional conversion of big-endothelin-1 to endothelin-1 and characterization of endothelin receptor subtype were investigated in cultured rat aortic endothelial cells. Exogenous endothelin-1 and big-endothelin-1 both increased arachidonic acid release and inositol phosphate production dose dependently. Endothelin-1 was more potent than big-endothelin-1 as indicated by EC_{50} values: 0.5 ± 0.1 nM and 10.0 ± 2.0 nM for endothelin-1-induced arachidonic acid release and inositol phosphate formation, respectively, versus 1.0 ± 0.4 nM and 35.0 ± 6.0 nM for big-endothelin-1-induced responses. Big-endothelin-1, but not endothelin-1 actions were inhibited by phosphoramidon. Comparative studies of endothelin receptor agonists and antagonists showed that endothelin-3 but not sarafotoxin S6c stimulated arachidonic acid release and inositol phosphate formation. The responses to big-endothelin-1 and endothelin-1 were specifically inhibited by the selective endothelin ET_A receptor antagonist, [cyclo-D-Trp-D-Asp-Pro-D-Val-Leu] (BQ-123) but not by the selective endothelin ET_B receptor antagonist [*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methyl-Leu-D-Trp-(COMe)-D-NLeu-ONa] (BQ-788). [^{125}I]Endothelin-1 binding was inhibited by endothelin-1, endothelin-3 and BQ-123 but not by BQ-788. These results indicate that the pharmacological responses to big-endothelin-1 in aortic endothelial cells are due to the extracellular phosphoramidon-sensitive conversion to endothelin-1. Endothelin effects are mediated through endothelin ET_A receptors in these cells.

Keywords: Endothelin-1; Endothelin-converting enzyme; Phospholipase C; Phospholipase A_2; Phosphoramidon

1. Introduction

The story of the endothelin system has been documented since the discovery of endothelin-1 (Yanagisawa et al., 1988). Nonetheless, much remains to be learned regarding the respective interaction of the endothelin system, including big-endothelin, with endothelium versus smooth muscle cells in health and disease. Two components of this system have been investigated: one is the processing pathway of endothelin-1, and the other is the nature of endothelin receptors. The relevant data indicating how endothelin-1 is produced from its precursor big-endothelin-1 are: first, phosphoramidon depresses the amount of endothelin-1 in the culture medium of endothelial cells, while increasing the content of unprocessed big-endothelin-1 (Ikegawa et al., 1991; Onhaka et al., 1991; Sawamura et al., 1991). Second, the phosphoramidon-sensitive endothelin converting enzyme activity detected in endothelial cells is located in the membrane compartment (Shields et al., 1991; Corder et al., 1993; Ohnaka et al., 1993). Third, the recent cloning of endothelin converting enzyme from endothelial cells (Shimada et al., 1994; Xu et al., 1994) may provide a major step forwards understanding endothelin-1 processing. And fourth, systemically administered phosphoramidon blocks the pressor effects of big-endothelin-1 in vivo (Pons et al., 1992; Pollock et al., 1993; Corder and Vane, 1994). Nevertheless the ability of endothelium to convert the circulating extracellular big-endothelin-1 and therefore to generate autocrine intracellular signals in response to big-endothelin-1 remain to be demonstrated.

Regarding endothelin receptors, current knowledge assesses the existence of two main classes: endothelin ET_A receptors, which display a higher affinity and selectivity for endothelin-1 than endothelin-3 and endothelin ET_B receptors, characterized as non-isopeptide-selective (endothelin-1 = endothelin-2 = endothelin-3) and which selec-

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tively bind sarafotoxin S6c. They have both been successfully cloned (Arai et al., 1990; Sakurai et al., 1990). The intracellular signalling pathways of endothelin receptors have been extensively investigated. Endothelin-1 binding to its receptors stimulates phosphatidyl inositol turnover via phospholipase C activation, Ca^{2+} mobilization, arachidonic acid release via phospholipase A_2 activation and the production of relaxing factors like nitric oxide and prostaglandins by endothelial cells (Resink et al., 1990; Filep et al., 1991). Classically, endothelin ET_A receptors are abundant in the heart and arterial smooth muscle, while endothelin ET_B receptors are mainly found in the vascular endothelium. It was initially thought that endothelin ET_B receptors mediated vasodilator effects and endothelin ET_A receptors vasoconstrictor effects. This view has recently been challenged with the discovery of endothelin ET_B receptor-mediated vasoconstriction (Ihara et al., 1992; Moreland et al., 1992; Sudjarwo et al., 1993; Teerlink et al., 1994). The physiological responses drawn by endothelin receptors as well as their tissular distribution do not seem to be sharply demarcated, and further receptor subtypes may well be revealed, as suggested by atypical agonist and antagonist profiles (Moreland et al., 1994; Clark and Pierre, 1995).

The current study reports the pharmacological interaction between extracellular big-endothelin-1 and an endothelial cellular model designed in our laboratory, primary rat aortic endothelial cells. Conversion and intracellular signal generation were monitored by measuring the release of arachidonic acid and inositol phosphates. Moreover, the endothelin receptor subtype mediating these responses was pharmacologically characterized.

2. Materials and methods

2.1. Isolation of rat aortic endothelial cells

Rat aortic endothelial cells were isolated and cultured as previously described (Battle et al., 1994). Briefly, thoracic aortae were collected into Ca^{2+} and Mg^{2+}-free Hank's buffered salt solution. After the removal of the adventitia, the remaining media plus intima was cut into little rings and then digested with collagenase (1242 U/ml; Eurobio) for 40 min at 37°C. The endothelial cells were harvested from the rings by flushing. They were then separated from the rings by sterile filtration, collected by centrifugation ($2500 \times g$, 5 min) and resuspended in Dulbecco's minimum essential medium (Boehringer, Meylan, France) supplemented with: 15% (v/v) horse serum (Boehringer), 4% (v/v) foetal calf serum (Dutscher, Brumath, France), 75 μg/ml endothelial cell growth supplement (Sigma), 50 μg/ml heparin (Sigma), 5% (v/v) L-glutamine, 1% (v/v) antibiotic and 20 mM HEPES. After plating on rat fibronectin, cells were incubated at 37°C in a 5% CO_2 atmosphere with 95% humidity, for 40 min. This short period allowed only endothelial cells to adhere. Cells were washed gently to remove non-adherent cells and fresh medium was added.

Endothelial cells were characterized by morphological and immunohistological criteriae (Fig. 1). Cells stained positively to von Willebrand's factor and to a specific vascular endothelial cell antibody (Duijvestijn et al., 1992), and negatively for smooth muscle α-actin (Gabbiani et al., 1981). Cell cultures from passages 1–6 were used.

2.2. [^{3}H]Arachidonic acid release assay

Confluent cultures in 24-well plates were labelled to equilibrium with [^{3}H]arachidonic acid (150 Ci mmol^{-1}; Amersham, France) for 20 h in serum-free and HEPES-free medium under a 5% CO_2 atmosphere at 37°C. Cells were then rinsed in order to eliminate unincorporated radioactivity: 3 rinses with phosphate-buffered saline, 1 rinse with HEPES-buffered medium without bicarbonate, and 2 rinses of 20 min with HEPES-buffered medium with 0.1% fatty acid-free bovine albumin serum. In a first set of experiments, incubations of endothelial cells with different concentrations of endothelin-1 or big-endothelin-1 or with vehicle were performed for different times (10, 20, 30 or 40 min). The maximal responses were reached at 20 min, independently of the peptide concentration (data not shown). Thus, further experiments were performed after a 20 min incubation with drugs or vehicle. The assay was stopped by removing the incubation medium which was centrifuged ($1000 \times g$; 5 min) in order to collect 350 μl of the medium containing the released arachidonic acid for scintillation counting.

The supernatant radioactivity was not separated chromatographically. The term '[^{3}H]arachidonic acid release' therefore refers to the egression of [^{3}H]arachidonic acid plus related ^{3}H-labelled metabolites which globally reflected the phospholipase A_2 activity.

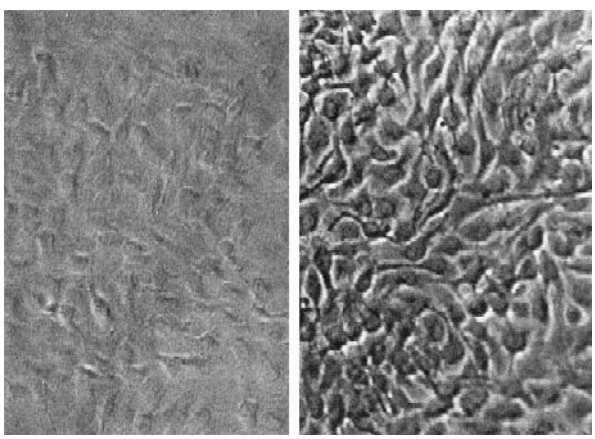

Fig. 1. Immunostaining of rat endothelial cells using a specific rat endothelial cell antibody (RECA): control (left), positive staining (right).

2.3. [^{3}H]Inositol phosphate formation assay

Confluent cultures in 24-well plates were labelled to equilibrium with [^{3}H]D-*myo*-inositol (15 Ci mmol^{-1}; Amersham) for 20 h in serum-free and HEPES-free medium under a 5% CO_2 atmosphere at 37°C. Cells were then rinsed twice with HEPES-buffered medium without bicarbonate. As for arachidonic acid release, the time required to obtain the maximal inositol phosphate production in response to endothelin-1 or big-endothelin-1 was previously determined and correspond to 20 min (data not shown). After incubation of cells with drugs or vehicles in the presence of 20 mM LiCl, inositol phosphate formation was stopped by removing the medium and adding ice-cold 10 mM formic acid (750 μl/well). After 30 minutes at 4°C, the extracts were frozen at −20°C. The thawed samples were applied onto columns containing 600 μl anion exchange resin (AG1-X8, 200–400 mesh; Bio-Rad, France) that were previously equilibrated with 4 ml water and 4 ml 2.5 mM NH_3; 3 ml 5 mM NH_3 solution (yielding a final pH of 8–9) were simultaneously added with each acidic sample so that neutralization occurred on the columns. Free [^{3}H]inositol and [^{3}H]glycerophosphoinositol were eluted with 4 ml 40 mM ammonium formate buffer and total [^{3}H]inositolphosphates with 4 ml 2 M ammonium formate buffer. The total labelled inositol phosphates quantified by liquid scintillation counting reflected the phospholipase C activity.

2.4. Binding studies

Confluent endothelial cells in 24-well plates were washed with an Earle's salt buffer (in mM: 140 NaCl, 5 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 5 glucose, 25 HEPES, 1 mg/ml bovine serum albumin, pH 7.4). For saturation binding assays, cells were incubated for 1 h at room temperature with 1–60 pM [^{125}I]endothelin-1 in absence (total binding) or in presence (non-specific binding) of 400 nM unlabelled endothelin-1. Non-specific binding was 10–20% of total binding. For competitive binding assays, cells were incubated with 30 pM [^{125}I]endothelin-1 and increasing concentrations of various competing ligands (endothelin-1, endothelin-3, BQ-123 and BQ-788). After the incubation period, cells were rinsed with Earle's buffer (3 times), solubilized in 1 M NaOH and the cell-bound radioactivity was counted. The data were analyzed with a non-linear least squares curve fitting procedure (Ebda-Ligand, Elservier Biosoft Cambridge, UK) (Munson and Rodbard, 1980).

2.5. Drugs

Endothelin-1 (21 amino acids) was purchased from France Biochem (France) and [^{125}I]endothelin-1 from NEN (France). Phosphoramidon was purchased from Sigma Chemicals (France). Big-endothelin-1 (39 amino acids), endothelin-3 (21 amino acids), sarafotoxin S6c (Williams et al., 1991), bosentan (i.e., Ro 47-0203; 4-*tert*-butyl-*N*-[6-(2-hydroxy)-ethoxy-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]benzenesulphonamide)), a non-specific endothelin receptor antagonist (Clozel et al., 1994), BQ-123 [cyclo (D-Trp-D-Asp-Pro-D-Val-Leu], a specific endothelin ET_A receptor antagonist (Ihara et al., 1992), and BQ-788 (*N*-*cis*-2,6-dimethylpiperidinocarbonyl-L-γ MeLeu-D-Trp(COMe)-D-NLe-ONa), a specific endothelin ET_B receptor antagonist (Ishikawa et al., 1994), were synthesized

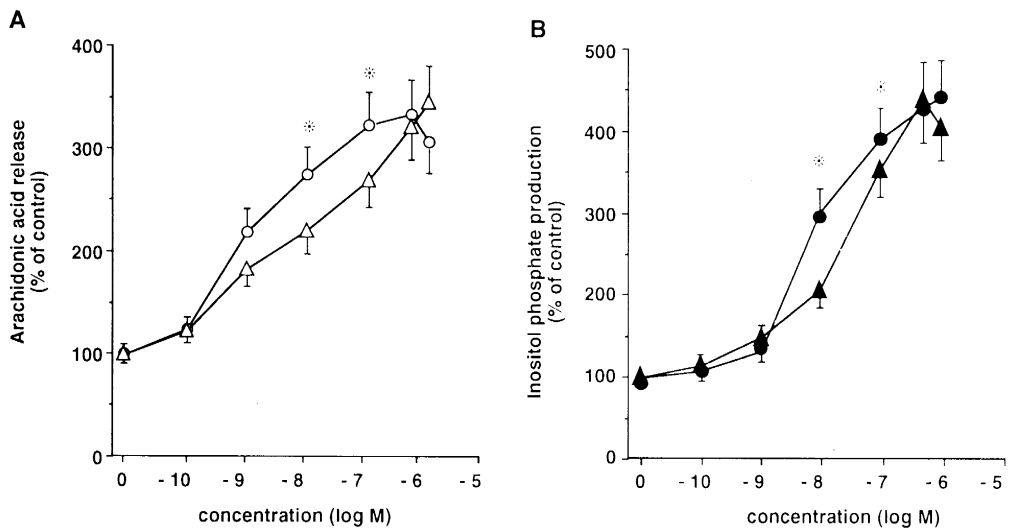

Fig. 2. Dose-response for endothelin-1 and big-endothelin-1 in endothelial cells. Arachidonic acid release and inositol phosphate production were measured as described in materials and methods. (A) Concentration-dependent arachidonic acid release in response to endothelin-1 (○) and big-endothelin-1 (△). (B) Concentration-dependent inositol phosphate production in response to endothelin-1 (●) and big-endothelin-1 (▲). Results are means ± S.E.M. of 3 independent experiments performed in triplicate. * $P < 0.05$, big-endothelin-1 versus endothelin-1.

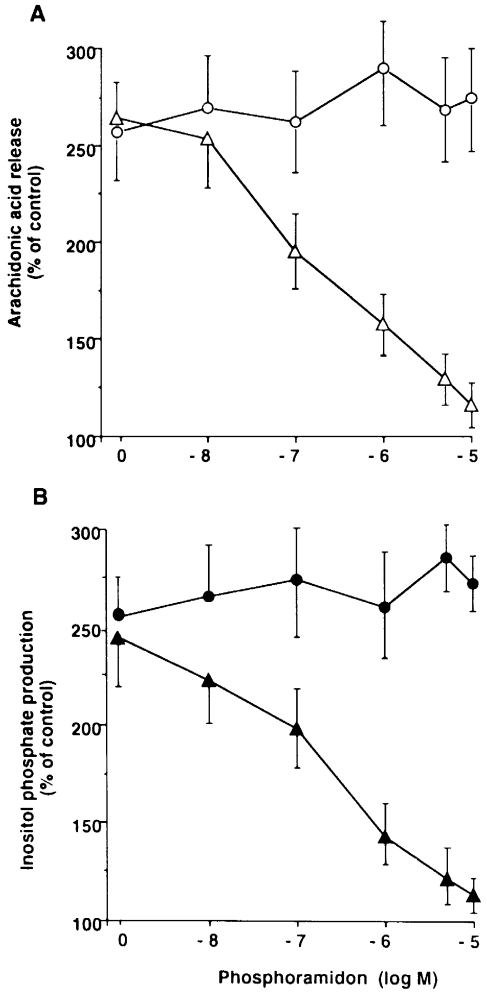

Fig. 3. Effect of phosphoramidon on the responses induced by 10^{-8} M endothelin-1 or big-endothelin-1 by endothelial cells. (A) Effect of phosphoramidon on arachidonic acid release induced by endothelin-1 (○) or big-endothelin-1 (△). (B) Effect of phosphoramidon on inositol phosphate production induced by endothelin-1 (●) or big-endothelin-1 (▲). Results, expressed as percentages of control, are means ± S.E.M. of 4 independent experiments performed in triplicate.

by Roussel-Uclaf (Romainville, France). Big-endothelin-1, endothelin-1, endothelin-3 and sarafotoxin S6c were dissolved in 0.1 M acetic acid, whilst phosphoramidon and BQ-123 were dissolved in DMSO/ethanol (1:9), and BQ-788 in ethanol. All drugs were stored at −20°C. They were diluted to working concentrations in medium with 1% bovine serum albumin.

2.6. Statistical analysis

The data are expressed as means ± S.E.M. They were subjected to a two-way factorial analysis of variance in order to examine the effect of different parameters. When the F value indicated overall significance, specific comparisons were performed using the Scheffé F-test. Statistical significance was assessed for $P < 0.05$.

3. Results

3.1. Effect of exogenous big-endothelin-1 and endothelin-1 on arachidonic acid release and inositol phosphate formation by endothelial cells

Addition of endothelin-1 to cultured endothelial cells prelabelled on one hand with [^{3}H]arachidonic acid, on the other hand with [^{3}H]*myo*-inositol triggered dose-dependent increases in arachidonic acid release (Fig. 2A) and inositol phosphate production (Fig. 2B). The maximal effect (3.32 ± 0.50- and 4.4 ± 0.43-fold increases in arachidonic acid release and inositol phosphate accumulation respectively) occurred after a 20-min stimulation. The concentration of endothelin-1 required to produce a half-maximal response (EC_{50}) was 0.5 ± 0.1 nM for arachidonic acid release and 10.0 ± 2.0 nM for inositol phosphate formation. Similarly,

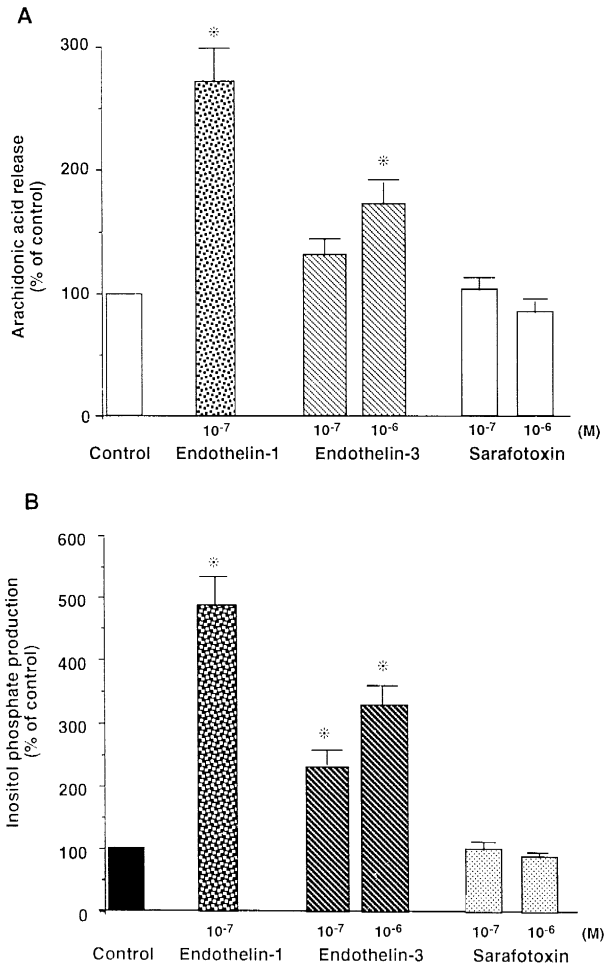

Fig. 4. Agonist-induced responses in endothelial cells. (A) Stimulation of arachidonic acid release in response to endothelin-1, endothelin-3 or sarafotoxin S6c. (B) Stimulation of inositol phosphate production in response to endothelin-1, endothelin-3 or sarafotoxin S6c. Results, expressed as percentages of control, are means ± S.E.M. of 3 independent experiments performed in triplicate. Statistically significant differences are indicated as follows: * $P < 00.5$, compared to control.

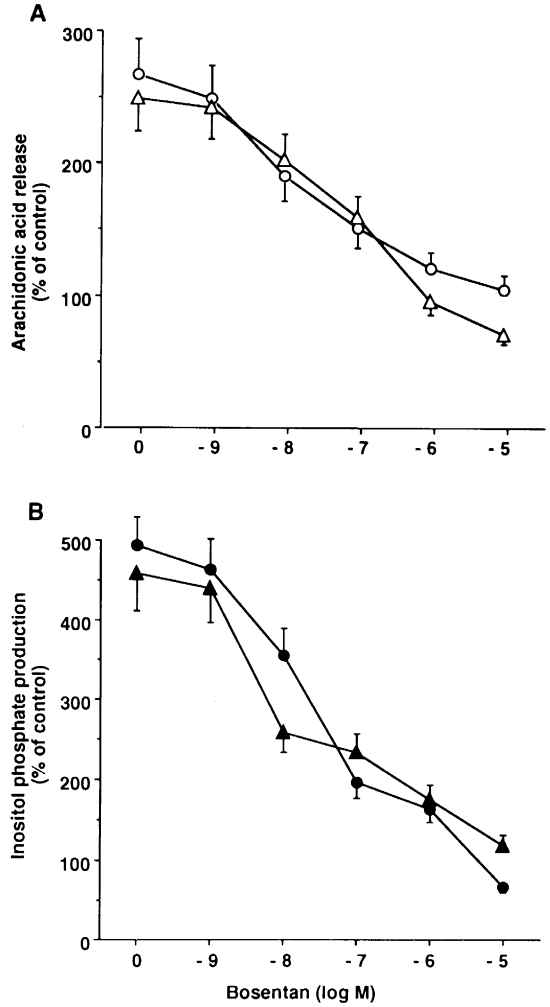

Fig. 5. Effect of bosentan on the responses of endothelial cells to 10^{-8} M endothelin-1 or big-endothelin-1. (A) Effect of bosentan on arachidonic acid release induced by endothelin-1 (○) or big-endothelin-1 (△). (B) Effect of bosentan on inositol phosphate production induced by endothelin-1 (●) or big-endothelin-1 (▲). Results are means ± S.E.M. of 6 independent experiments performed in triplicate.

exogenous big-endothelin-1 raised arachidonic acid release and inositol phosphate formation as shown in Fig. 1A,B. The maximal effect was almost the same than that of endothelin-1 (3.21 ± 0.17- and 4.42 ± 0.24-fold increases for arachidonic acid and inositol phosphates, respectively). However, responses were delayed so that maximal effects were observed after only a 30-min stimulation period and EC_{50} values were higher (1.0 ± 0.4 nM for arachidonic acid and 35.0 ± 6.0 nM for inositol phosphates) than those observed for endothelin-1.

3.2. Effect of phosphoramidon on arachidonic acid release and inositol phosphate formation induced by exogenous big-endothelin-1 and endothelin-1

Both big-endothelin-1-stimulated arachidonic acid liberation (Fig. 3A) and inositol phosphate production (Fig. 3B) were significantly and dose dependently reduced by phosphoramidon (10^{-8}–10^{-5} M). The arachidonic acid release and inositol phosphate production induced by 10^{-8} M big-endothelin-1 were inhibited by 89.3 ± 7.0% and 90.6 ± 1.8%, respectively, with 10^{-5} M phosphoramidon. The decrease in arachidonic acid release had an IC_{50} (the concentration of inhibitor that suppresses half of the response) of 0.10 ± 0.02 μM, while the decrease in inositol phosphate production had an IC_{50} of 0.35 ± 0.05 μM. It has been verified that the inhibitor alone elicited no responses (data not shown). As expected, the responses to endothelin-1 were unaffected by phosphoramidon (Fig. 3A,B).

3.3. Effect of endothelin receptor agonists on arachidonic acid release and inositol phosphate production by endothelial cells

The experiments depicted in Fig. 4 were conducted with sarafotoxin S6c, a specific agonist for endothelin ET_B

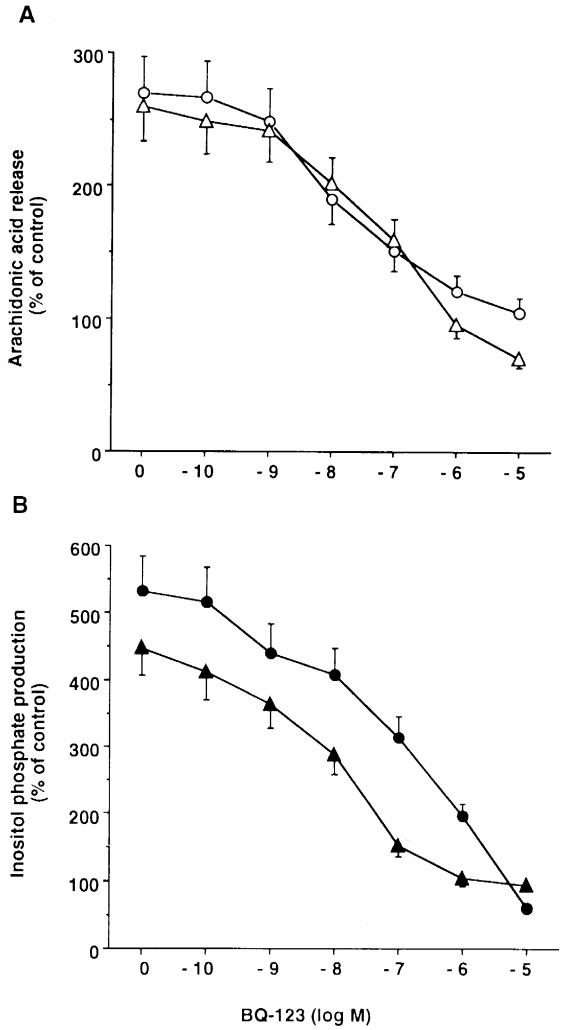

Fig. 6. Effect of BQ-123 on the responses of endothelial cells to 10^{-8} M endothelin-1 or big-endothelin-1. (A) Effect of BQ-123 on arachidonic acid release induced by endothelin-1 (○) or big-endothelin-1 (△). (B) Effect of BQ-123 on inositol phosphate production induced by endothelin-1 (●) or big-endothelin-1 (▲) ($n = 4$).

receptor subtype and endothelin-3 which binds to endothelin ET_A receptor less efficiently than endothelin-1 to elucidate the receptor subtype that may mediate the stimulation of arachidonic acid release (Fig. 4A) and inositol phosphate formation (Fig. 4B) from endothelial cells. Sarafotoxin elicited no responses at the concentrations tested (10^{-7} and 10^{-6} M). On the contrary, endothelin-3 raised arachidonic acid and inositol phosphate production. However, endothelin-3-induced effects were weaker than those induced by 10^{-7} M endothelin-1 ($P < 0.05$). The difference in the potencies of endothelin-1 and endothelin-3 was more marked for inositol phosphate production: the increase in inositol phosphate formation induced by 10^{-7} M endothelin-1 was twice that of 10^{-7} M endothelin-3. Taken together, these results suggest the presence of endothelin ET_A receptors on endothelial cells and make it possible that they mediate the effects of endothelin-1 and big-endothelin-1.

3.4. Effect of endothelin receptor antagonists on the responses induced by big-endothelin-1 and endothelin-1 from endothelial cells

In order to ascertain the above conclusion, the effects of the endothelin ET_A-ET_B receptor antagonist bosentan, the selective endothelin ET_A receptor antagonist BQ-123 and, the selective endothelin ET_B receptor antagonist BQ-788 were tested on the responses to big-endothelin-1 and endothelin-1. Bosentan (Fig. 5) and BQ-123 (Fig. 6) reduced both responses to big-endothelin-1 and endothelin-1 dose dependently with a complete inhibition occurring at 10^{-5} M. On the contrary, BQ-788 altered neither big-endothelin-1 nor endothelin-1-stimulated responses (Fig. 7). At a concentration as high as 10^{-5} M BQ-788, no decreases

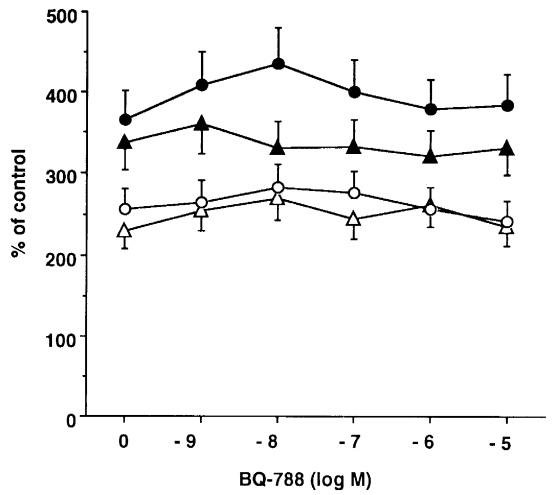

Fig. 7. Effect of BQ-788 on the responses of endothelial cells to 10^{-8} M endothelin-1 or big-endothelin-1. (A) Effect of BQ-788 on arachidonic acid release induced by endothelin-1 (○) or big-endothelin-1 (△). (B) Effect of BQ-788 on inositol phosphate production induced by endothelin-1 (●) or big-endothelin-1 (▲) ($n = 3$).

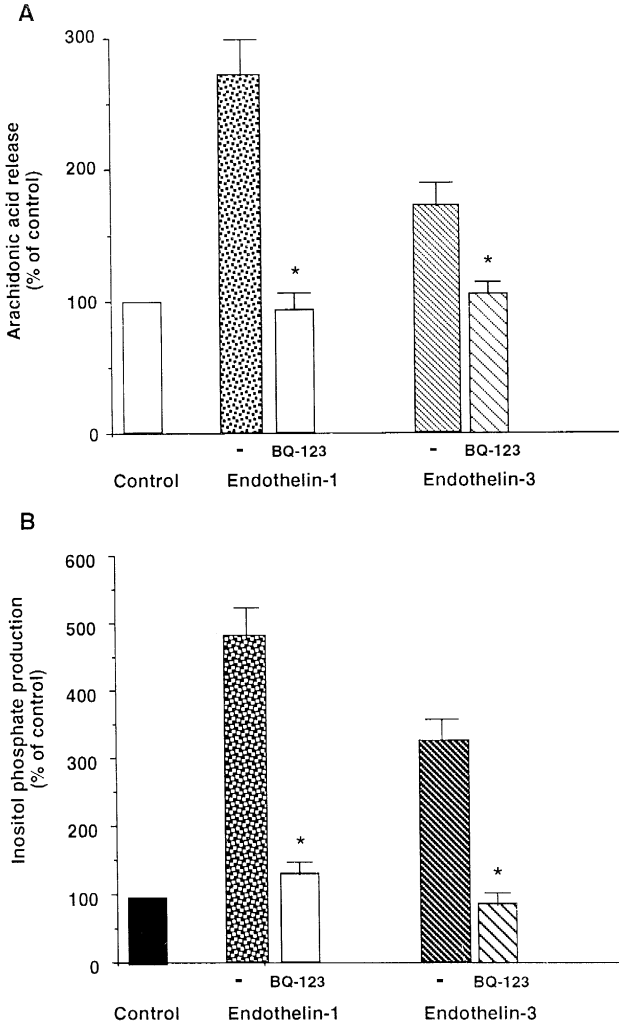

Fig. 8. Effect of BQ-123 on the responses of endothelial cells to endothelin-1 or endothelin-3. (A) Effect of BQ-123 on arachidonic acid release induced by 10^{-7} M endothelin-1 or 10^{-6} M endothelin-3. (B) Effect of BQ-123 on inositol phosphate production induced by 10^{-7} M endothelin-1 or 10^{-6} M endothelin-3. Results are means ± S.E.M. of at least 3 independent experiments performed in triplicate. * $P < 0.05$, significantly different from endothelin-1 or endothelin-3 alone.

in inositol phosphate production and arachidonic acid release was observed. It is noticeable that BQ-123 also inhibited the increases in arachidonic acid release (Fig. 8A) and inositol phosphate formation (Fig. 8B) induced by endothelin-3; a complete blockage was achieved with 10^{-6} M BQ-123 plus 10^{-6} M endothelin-3. These observations are consistent with the previous conclusion arguing that the effects of endothelin-1, big-endothelin-1 and endothelin-3 are triggered through the activation of endothelin ET_A receptors.

3.5. [^{125}I]endothelin-1 binding and displacement assays on endothelial cells

These experiments were undertaken to support further the above conclusion and to examine whether rat aortic

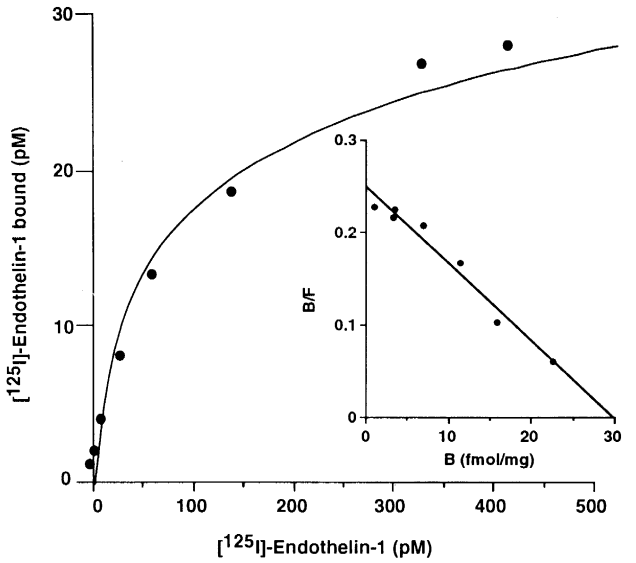

Fig. 9. Saturation curve and Scatchard analysis of [^{125}I]-endothelin-1 binding to endothelial cells. Specific binding was defined in the presence of 4×10^{-7} M endothelin-1. Data are from one representative experiment performed in duplicate.

endothelial cells also express endothelin ET_B receptors. The data of Fig. 9 show that [^{125}I]endothelin-1 binding to endothelial cells was specific, saturable and of high affinity. The apparent dissociation constant (K_d) and the maximum binding (B_{max}) were 122 ± 9 pM and 22 ± 4 fmol/mg. Analysis of the data revealed a straight line ($r = 0.97$), which is consistent with a single class of binding sites. The results of the competitive binding of unlabelled endothelin-1, endothelin-3, BQ-123 or BQ-788 with [^{125}I]endothelin-1 are gathered in Table 1. It is apparent that endothelin-1 was the most potent competitor ($K_i = 0.14 \pm 0.02$ nM), whereas endothelin-3 was 300-fold weaker. Moreover, BQ-123 displayed a K_i value of 2.3 ± 0.6 nM while BQ-788 was without effect up to 10^{-6} M. Binding studies therefore confirmed the presence of endothelin ET_A receptors on rat aortic endothelial cells and indicated that these cells do not express endothelin ET_B receptors.

Table 1
Effects of various agonists and antagonists on [^{125}I]-endothelin-1 binding to endothelial cells

	K_i (nM)
Agonist	
Endothelin-1	0.14 ± 0.02
Endothelin-3	44.4 ± 11.3
Antagonist	
BQ-123	2.3 ± 0.6
BQ-788	> 1000

Binding studies were performed as explained in Section 2. Each experiment was performed in duplicate and repeated 2–5 times. The data presented are means ± S.E.M. values.

4. Discussion

Evidence now argues for the specific conversion of big-endothelin-1 to endothelin-1 by an endothelin converting enzyme in vivo (Matsumara et al., 1990; McMahon et al., 1991) and in vitro (Ikegawa et al., 1991; Fujita et al., 1994; Hisaka et al., 1994). However, the cellular site of big-endothelin-1 conversion (cytoplasmic or membranous) is still uncertain and the physiological relevance of in vitro endothelin converting enzyme activity is not well established. In this report, the functional role of endothelin converting enzyme was examined in primary cultured endothelial cells using the arachidonic acid and inositol phosphate release assays to monitor the formation of biologically active endothelin-1 from big-endothelin-1.

In such a system, we have observed that big-endothelin-1 stimulates arachidonic acid release and inositol phosphate formation via a phosphoramidon-sensitive mechanism. These results argue against a direct interaction of big-endothelin-1 on endothelin receptors (Clozel et al., 1993) and suggest that big-endothelin can be extracellularly converted. It has been demonstrated that phosphoramidon is 100-fold more efficient to inhibit exogenous than endogenous conversion of big-endothelin-1 in transfected cells (Xu et al., 1994). Recently, the cell surface location of endothelin converting enzyme-1 has been confirmed in EA.hy926, a human endothelial cell line (Barnes et al., 1995). However, the presence of a cytosolic endothelin converting enzyme activity in endothelial cells cannot be ruled out (Ikegawa et al., 1991; Sawamura et al., 1991; Shields et al., 1991; Barnes et al., 1995).

The inhibition profile of this apparent cell surface conversion of exogenous big-endothelin-1 exhibited IC_{50} values, of the same order of magnitude as that of the purified enzyme (Ohnaka et al., 1993). Much higher concentrations of phosphoramidon were required to inhibit endogenous big-endothelin-1 conversion (Corder et al., 1993; Xu et al., 1994). Nevertheless, these results provide evidence that primary cultured aortic endothelial cells contain an endothelin converting enzyme which can extracellularly process big-endothelin-1 to mature endothelin-1 efficiently.

A cell surface mechanism raises the question of the efficiency of endothelin converting enzyme. It has been reported that endothelin-1 never exceeds 2% of the exogenously supplied big-endothelin-1 concentration in vivo (Emoto and Yanagisawa, 1995) or in vitro (Ikegawa et al., 1991). This low conversion rate could be explained because big-endothelin-1 is converted to endothelin-1 at local membrane sites. As endothelin-1 has an extremely high affinity for its receptors (apparent dissociation constant in the subnanomolar range) it can be supposed that, in proportion as big-endothelin-1 conversion occurs, endothelin-1 is trapped by its receptor so that the measurable concentration of endothelin-1 is lower than the total endothelin-1 production. Frelin and Guédin (1994) have proposed such a predominant clearance mechanism involving binding to

functional receptors rather than an equilibrium between free and bound endothelin.

We initially analyzed endothelin receptors on primary cultured endothelial cells using various endothelin receptor agonists. The results indicated that both endothelin-1 and endothelin-3 raised arachidonic acid release and inositol phosphate production, although the responses to endothelin-1 were twice higher than those to endothelin-3. In contrast, Sarafotoxin S6c, an endothelin ET_B receptor selective agonist, triggered neither arachidonic acid release nor inositol phosphate accumulation. This profile of agonist action is consistent with that of the endothelin ET_A receptors found in tissues such as rabbit carotid artery and rat aorta smooth muscle (Moreland et al., 1994). It therefore suggests that primary cultured rat aortic endothelial cells contain endothelin ET_A receptors coupled to phospholipase C and phospholipase A_2.

Characterization of receptors based on the rank order of agonist potencies can give misleading information. Consequently, further experiments with the mixed antagonist, bosentan, the endothelin ET_A receptor selective antagonist, BQ-123, and the endothelin ET_B receptor selective antagonist, BQ-788 were undertaken. BQ-788 did not inhibit the endothelin-1-induced increases in arachidonic acid and inositol phosphate. In contrast, bosentan and BQ-123 completely abolished arachidonic acid liberation and inositol phosphate formation induced by endothelin-1 or endothelin-3. These results demonstrate that these cells bear endothelin ET_A receptors which are functionally coupled to phospholipase C and phospholipase A_2. It has been widely recognized that endothelins increase arachidonic acid release and inositol phosphate production by activation of endothelin ET_B receptors in vascular endothelial cells, whilst activation of endothelin ET_A receptors occurs in vascular smooth muscle cells (Sakurai et al., 1992). But the results of recent studies suggest that this scheme requires revision. Indeed, the coexistence of endothelin ET_A and ET_B receptors has been found to mediate vascular wall contraction (Sumner et al., 1992; Fukuroda et al., 1994), and the presence of these two types of receptors has been reported in porcine aortic smooth muscle cells and in the endothelium of porcine aortic valve (Aoki et al., 1993). Hence, the receptor pattern may be more complex than previously described.

The present report describes endothelin ET_A receptor-mediated responses to endothelin-1 in primo-cultured rat endothelial cells from large artery origin. Although the pharmacological study indicates that the intracellular signal transduction via phospholipase C and phospholipase A_2 is endothelin ET_A receptor-dependent, the presence of endothelin ET_B receptors cannot be ruled out if they were uncoupled to phospholipase C and phospholipase A_2. Binding assays confirm the absence of endothelin ET_B receptors on rat aortic endothelial cells. It is therefore more than likely that these cells express exclusively endothelin ET_A receptors which have an affinity ($K_d = 100$ pM) similar to that reported for vascular smooth muscle endothelin ET_A receptors (Arai et al., 1990; Ihara et al., 1992; Ishikawa et al., 1994). This finding contrasts with the current data supporting a vascular endothelium localization of endothelin ET_B receptors (Sakurai et al., 1990; Hirata et al., 1993). The data of the literature suggest that the ratio of the endothelin receptors varies from one vascular bed to another in the same species, and from one species to another in the same vascular bed.

This paper demonstrates that endothelin-1 activates phospholipase A_2 and phospholipase C via endothelin ET_A receptors in endothelial cells. Phospholipase A_2 stimulation results in the release of arachidonic acid from membrane phospholipids. It can be supposed that the consequent endothelial production of prostaglandins – the most important being prostacyclin – exerts vasodilator effects on the underlying vascular smooth muscles. It has been shown that phospholipase C activation via endothelin ET_B receptors leads to the production of inositol phosphates, the mobilization of Ca^{2+} and the release of the relaxing factor, nitric oxide (Hirata et al., 1993). However, very little is known about the signal transduction of ET_A receptors in vascular endothelial cells, as most of signalling pathway studies focus on smooth muscle endothelin ET_A receptors. Amano et al. (1994) provided evidence that the binding of endothelin-1 to endothelin ET_A receptor was responsible for the increase in intracellular Ca^{2+} in the endothelium of rabbit aortic valve. Further studies will be needed to clearly establish the physiological relevance of endothelial ET_A receptors.

In summary, the present study demonstrates that big-endothelin-1-induced signals in endothelial cells are due to the extracellular conversion of big-endothelin-1 to mature endothelin-1, and that primary cultured rat aortic endothelial cells exclusively express endothelin ET_A receptors which are coupled to phospholipase A_2 and phospholipase C. The understanding of the physiological and pathophysiological roles of the endothelin system requires a detailed knowledge of two key features: first, endothelin-1 processing via endothelin converting enzyme and the cascade of events following its binding to endothelin receptors; second, the bio-availability of this peptidic-peptidasic system within the plasma-endothelium compartment versus the interstitium-smooth muscle cell compartment within the arterial wall.

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