



Evidence for the presence of endothelin ET_A receptors in endothelial cells *in situ* on the aortic side of porcine aortic valve

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1 In the present study, we determined whether ET_A receptors are present on endothelial cells *in situ*, by use of front-surface fluorometry of fura-2-loaded porcine aortic valvular strips and reverse transcription polymerase chain reaction (RT-PCR).

2 Although endothelin-1 (ET-1) and endothelin-3 (ET-3) induced maximum elevations of cytosolic Ca²⁺ concentrations ([Ca²⁺]_i) at 10⁻⁷ M, the peak elevations of [Ca²⁺]_i induced by ET-1 were much greater than those induced by ET-3.

3 The application of ET-1 after ET-3 induced an additional increase in [Ca²⁺]_i, while the application of ET-3 after ET-1 had no effect. A selective ET_A receptor antagonist, BQ-123, partially inhibited the ET-1-induced Ca²⁺ transient but had no effect on ET-3-induced Ca²⁺ transients. These experiments indicated the presence of functioning ET_A receptors in addition to ET_B receptors in endothelial cells *in situ*.

4 The sequence of pig lung ET_A receptor complementary DNA (cDNA) was determined by PCR. RT-PCR, using specific primers for pig ET_A receptor sequence and total RNA from endothelial cells on the aortic side of the aortic valve, gave the expected size of band. This PCR product was sequenced and was found to be identical to the sequence of the pig lung ET_A receptor.

5 The partial sequence of the pig lung ET_B receptor was also determined. RT-PCR for the pig ET_B receptor revealed that endothelial cells of the aortic valve express ET_B receptor messenger RNA (mRNA).

6 We confirmed that functioning ET_A receptors and expression of ET_A receptor mRNA exist in the endothelial cells on the aortic side of porcine aortic valves.

Keywords: Intracellular Ca²⁺ concentration; endothelial cells; endothelin; endothelin receptor; polymerase chain reaction; RT-PCR; PCR cloning

Introduction

Endothelin (ET) was originally described as a potent vasoconstrictor present in the conditioned medium of porcine cultured aortic endothelial cells (Yanagisawa *et al.*, 1988). Subsequent studies led to the discovery of three isoforms of ET (ET-1, -2 and -3), which are different not only in structure but also in function (Inoue *et al.*, 1989). Radioligand binding studies (Watanabe *et al.*, 1989), as well as pharmacological studies (Inoue *et al.*, 1989; Warner *et al.*, 1989) suggested the presence of several subtypes of ET receptors. Recently two distinct ET receptor subtypes have been cloned, one of which preferentially binds to ET-1 and to ET-2 (Arai *et al.*, 1990) while the other binds to ET-1, -2 and -3, with equal affinity (Sakurai *et al.*, 1990). It has been recommended that the former be called the ET_A subtype and the latter be called the ET_B subtype (Vane, 1990). As to the implications of ETs for the cardiovascular systems, it has been postulated that ET_A and ET_B receptors are present on smooth muscle cells and are responsible for vasoconstriction, while ET_B receptors are present on endothelial cells and may be responsible for the release of endothelium-derived relaxing factor (EDRF) (Masaki *et al.*, 1991; Ushio-Fukai *et al.*, 1992; Remuzzi & Benigni, 1993) or prostaglandin I₂ (Suzuki *et al.*, 1991). However, subtypes of ET receptors on endothelial cells have required further study. Physiological or pharmacological studies have indicated the presence of ET_A (Vigne *et al.*, 1990; 1991; Amano *et al.*, 1994), ET_B (Takayanagi *et al.*, 1991; Fujitani *et al.*, 1992) and ET_C (ET-3-specific, Emori *et al.*, 1990; Warner *et al.*, 1992) receptor subtypes in endothelial cells. The expression of ET_B receptor mRNA in endothelial cells has been reported (Ogawa *et al.*, 1991), but not the expression of ET_A receptor mRNA in en-

dothelial cells. Here we show the presence of functioning ET_A receptors and the expression of ET_A receptor mRNA in endothelial cells *in situ*.

We recently developed a new method of investigating Ca²⁺ metabolism of endothelial cells *in situ*, using porcine aortic valve leaflets loaded with fura-2 and front-surface fluorometry (Aoki *et al.*, 1991; 1994; Kuroiwa *et al.*, 1993). Using this method, we found that ET-1 caused a much greater elevation of [Ca²⁺]_i than ET-3, at equimolar concentrations (Aoki *et al.*, 1991). However, if endothelial cells possess a single class of ET_B receptor subtype, ET-1 and ET-3 at an equimolar concentration should elicit a similar size of Ca²⁺ transients, since the ET_B receptor subtype has an equally high affinity for ET-1 and ET-3. To explain this greater response of [Ca²⁺]_i induced by ET-1, we entertained the notion that endothelial cells may have an additional ET receptor subtype other than the ET_B receptor subtype.

In the present study, we characterized Ca²⁺ transients induced by ET-1 and ET-3 and the effect of BQ-123, a selective ET_A receptor antagonist (Ihara *et al.*, 1992) on these transients, the objective being to determine ET receptor subtypes in endothelial cells *in situ*. In the case of the investigation of the additivity of ET-1 and ET-3, the sustained phase of these Ca²⁺ transients were mainly studied, because of the difficulties in investigating the rapid transient phase. These pharmacological investigations indicated that ET_A receptors are present in endothelial cells on the aortic side of the porcine aortic valve. We attempted to detect the ET_A receptor mRNA in endothelial cells at the aortic side of porcine aortic valve. Since the sequence of pig ET_A receptor mRNA has not been described, we determined the sequence of pig ET_A receptor cDNA by PCR using the known cDNA sequences of ET_A receptor from other species (Arai *et al.*, 1990; Lin *et al.*, 1991; Hosoda *et al.*, 1991; Adachi *et al.*, 1991) and designed specific primers for pig ET_A

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receptors. Using specific primers for pig ET_A receptors and total RNA from the endothelial cells on the porcine aortic valve, RT-PCR revealed that ET_A receptor mRNA is present in the endothelial cells from the porcine aortic valve. This is the first account of the presence of functioning ET_A receptors and ET_A receptor mRNA in endothelial cells.

Methods

Tissue preparation for fura-2 fluorometry

Aortic roots with an intact aortic valve from adult pigs of either sex were obtained from a local slaughterhouse immediately after the animals had been killed. These roots were placed in ice-cold normal physiological salt solution (PSS) and brought to our laboratory. Usually, 1–2 h elapsed between the animal being killed and the loading of fura-2. The millimolar composition of the normal PSS was: NaCl 123, KCl 4.7, NaHCO₃ 15.5, KH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 1.25 and D-glucose 11.5. The leaflets of the aortic valve were cut in parallel with the midline to obtain approximately 2 mm (width) × 5 mm (length) valvular strips. The centre of each leaflet, *corpus arantii*, was not used. The thickness of the strips was about 0.18 mm (Aoki *et al.*, 1991). We have already described the microscopic verification of the endothelial cells on the aortic side of the aortic valve, shown by the specific uptake of acetylated-low density lipoprotein labelled with 1,1'-diiodotetradecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) and fura-2 in cells with a cobble-stone lining on the surface of the valvular strips. (Aoki *et al.*, 1994).

Fura-2 fluorometry

The strips were incubated for 90 min (37°C) in Dulbecco's modified Eagle's medium, gassed with 5% CO₂ and 95% O₂, containing 5% foetal bovine serum, 1 mM probenecid (Di Virgilio *et al.*, 1989) and 50 µM acetoxymethyl ester of fura-2 (fura-2/AM). After loading with fura-2, the strips were washed at least 5 times with normal PSS and equilibrated for 1 h at

room temperature. Each strip was mounted in a strip holder in a quartz organ bath filled with normal PSS gassed with 5% CO₂ and 95% O₂. The experimental procedures were carried out at 25°C to prevent leakage of the fluorescence dye. Microscopic observation revealed that fura-2 was loaded only in endothelial cells of the sample.

Changes in [Ca²⁺]_i were monitored with a front-surface fluorometer (CAM-OF-1; designed in collaboration with the Japanese Spectroscopic Co., Tokyo, Japan), as described elsewhere (Hirano *et al.*, 1990; Aoki *et al.*, 1991). In brief, the aortic surface of the strip was illuminated by guiding the alternating (400 Hz), 340 nm and 380 nm, excitation light from a xenon light source through quartz optic fibres arranged in a concentric inner circle (diameter: 3 mm). Surface fluorescence of the strip was collected by glass optic fibres arranged in an outer circle (diameter: 7 mm) and introduced through a 500 nm band-pass filter into a photon-counting photomultiplier. Fluorescence intensities (500 nm) at 340 nm (F340) and 380 nm (F380) excitation and its ratio ($R = F340/F380$) were continuously recorded. [Ca²⁺]_i was calculated according to the method described by Grynkiewicz *et al.* (1985). The apparent dissociation constant (K_d) of the fura-2:Ca²⁺ complex at 25°C was 162.0 ± 7.5 nM, determined spectroscopically (Fluorescence Spectrophotometer 650-40; HITACHI, Japan).

Normalization of [Ca²⁺]_i responses

In this study, all of the responses of [Ca²⁺]_i levels were normalized by the response to 10⁻⁵ M ATP, for the following reasons: (1) The response to 10⁻⁵ M ATP was highly reproducible during repetitive application of ATP, at 15 min intervals. (2) The response to ATP was concentration-dependent (10⁻⁶–10⁻³ M). The response to higher concentrations of ATP was a larger elevation of [Ca²⁺]_i than with 10⁻⁵ M ATP, but the full response was not attained until 30 min. (3) The response to ATP was not observed when the surface of the valvular leaflet was rubbed off with a cotton swab to remove the endothelial cells. Thus, the response to ATP derived exclusively from the endothelial cells. Accordingly, 15 min prior to each measurement, the response to ATP (10⁻⁵ M) was registered,

Table 1 Oligonucleotides used for PCR and RT-PCR

EcoRI cassette

5'-HO GTACATATTGTCGTTAGAACGCGTAATACGACTCACTATAGGGAGAG-3'
3'-CATGTATAACAGCAATCTTGCGCATTATGCTGAGTGATATCCCTCTCTTAA OH-5'

Cassette primers

C1: 5'-GTACATATTGTCGTTAGAACGCG-3'
C2: 5'-TAATACGACTCACTATAGGGAGA-3'

Primers for cloning

Sense

RS1: 5'-ATATCCTGTACCATTTTCATCGT-3' (Rat Sense 256–278)
RS2: 5'-CCTTATCTACGTGGTCATTGATCT-3' (Rat Sense 378–401)
PS1: 5'-ATCACCGTCCTCAATCTCTG-3' (Pig Sense 611–630)

Antisense

RA1: 5'-AAGCCACTGCTCTGTACCTG-3' (Rat Antisense 546–565)
RA2: 5'-GGGTACCATGACGAAGCCGA-3' (Rat Antisense 665–684)
PA1: 5'-CAAGGGCAAGGCTGGCTATCA-3' (Pig Antisense 353–373)

Primers for RT-PCR (ET_A)

Sense

PS2: 5'-ATGACTTTGGCGTATTTCTC-3' (Pig Sense 452–471)

Antisense

PA2: 5'-GCTCTTCGCCCTTGATTCA-3' (Pig Antisense 687–706)
PA3: 5'-AACAGCCACAGTCTTTTAC-3' (Pig Antisense 760–779)

Primers for RT-PCR (ET_B)

Sense

RSB: 5'-CAAAGACTGGTGGCTGTTCA-3' (Rat Sense 813–832)

PSB: 5'-TGTTGGCTTCCCTTCATCT-3' (Pig Sense)

Antisense

RAB: 5'-TGGAGCGGAAGTTGTCGTAT-3' (Rat Antisense 1284–1303)

and the resting level and the peak level of the fluorescence ratio were designated 0% and 100%, respectively. $[Ca^{2+}]_i$ levels at 0% and 100% were determined separately and were 65.3 ± 7.5 nM and 185.9 ± 20.8 nM, respectively ($n = 10$) (Aoki *et al.*, 1991).

Cloning of the pig lung ET_A receptor

A part of the lung from an adult pig was obtained from a local slaughterhouse immediately after the animal had been killed. Total RNA was prepared by homogenization with Polytron in guanidinium thiocyanate, extraction with phenol/chloroform and precipitation from isopropanol, according to the method described by Chomczynski & Sacchi (1987). Polyadenylated RNA was enriched with Oligotex-dT30. Double stranded cDNA ligated with Eco RI adaptor was constructed with a TimeSaver cDNA Synthesis Kit. The double stranded cDNA with Eco RI adaptor was further ligated with an Eco RI cassette (Table 1). This double stranded cDNA with Eco RI cassette was amplified using indicated primers. Each PCR product was purified by electrophoresis through a 3% NuSieve agarose gel, cleaned by Gene Clean II, and subcloned into pCR II plasmid vector, using a TA Cloning Kit. cDNA inserts were then sequenced using a T7 Sequencing Kit, as described

by the manufacturer. When determining the cDNA sequence, at least 5 clones were checked to avoid misreading due to possible misincorporation by Taq polymerase.

Since cDNA sequences for ET_A receptor for bovine (Arai *et al.*, 1990), rat (Lin *et al.*, 1991) and man (Hosoda *et al.*, 1991; Adachi *et al.*, 1991) have been published, 4 primers, RS1, RS2, RA1 and RA2 (Table 1), based on the rat sequence, were designed from conserved regions among these species. First PCR was performed to determine the sequence of the core region of the pig lung ET_A receptor cDNA. An aliquot of cDNA synthesized from pig lung mRNA was amplified by PCR using primers RS1 and RA2, at reduced stringency and under thermal cyclor conditions of 94°C for 30 s, 40°C for 1 min and 72°C for 2 min for a total of 40 cycles. This PCR product was purified by electrophoresis, subcloned into pCR II plasmid vector and then sequenced (core sequence). Based on this core sequence of pig ET_A receptor cDNA, primers PS1 and PA1 were designed. A second PCR was performed to determine the sequence of the 5' region of the pig lung ET_A receptor cDNA. An aliquot of cDNA ligated with cassette was amplified by PCR using primers C1 and RA1, under thermal cyclor conditions of 94°C for 30 s, 40°C for 1 min and 72°C for 2 min, for a total of 30 cycles. Then, an aliquot of this second PCR was further amplified with primers C2 and PA1, under thermal cyclor conditions of 94°C

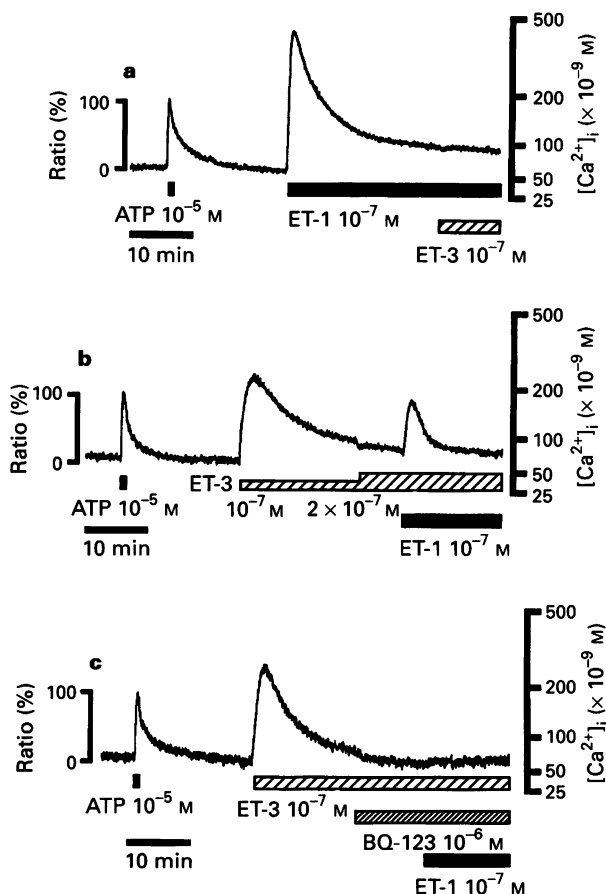


Figure 1 Representative traces showing the additivity of $[Ca^{2+}]_i$ transients induced by endothelin-1 (ET-1) and ET-3, using fura-2-loaded endothelial cells in aortic side of aortic valvular strips. In each panel (a, b, c), the response to 10^{-5} M ATP was recorded as a control. (a) ET-3 (10^{-7} M) was added during the sustained phase of the $[Ca^{2+}]_i$ transient induced by 10^{-7} M ET-1; (b) First, ET-3 (10^{-7} M) was added during the sustained phase of the $[Ca^{2+}]_i$ transient induced by 10^{-7} M ET-3. Subsequently, ET-1 (10^{-7} M) was added during the sustained phase of the $[Ca^{2+}]_i$ transient induced by 10^{-7} M ET-3. (c) ET-1 (10^{-7} M) was added during the sustained phase of the $[Ca^{2+}]_i$ transient induced by 10^{-7} M ET-3 in the presence of 10^{-6} M BQ-123.

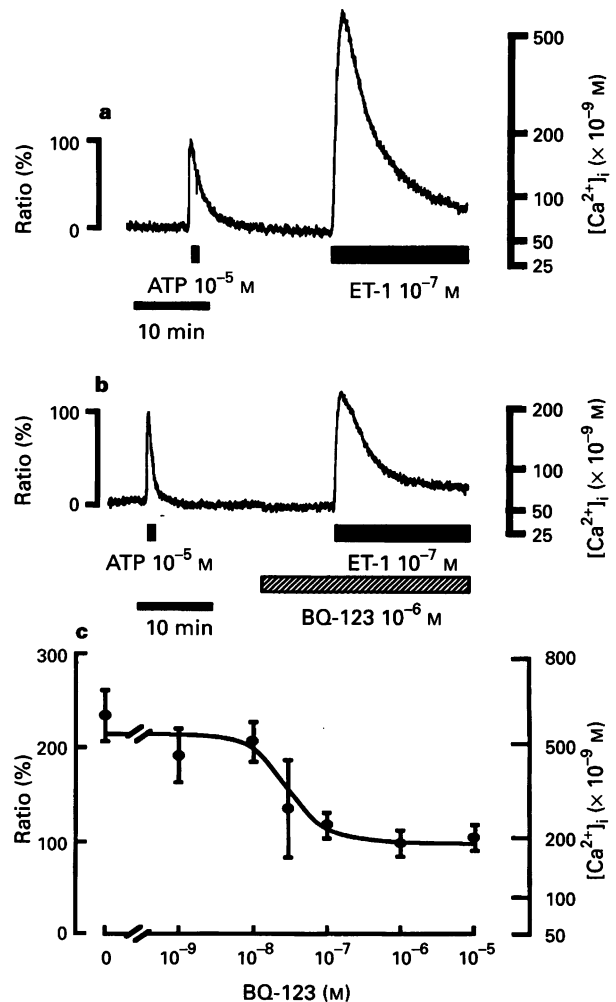


Figure 2 Representative time course of changes in endothelial $[Ca^{2+}]_i$ induced by 10^{-7} M endothelin-1 (ET-1) in the absence (a) and presence (b) of 10^{-6} M BQ-123, and concentration-response curve of the effect of BQ-123 (c). ET-1 caused rapid and transient elevations of $[Ca^{2+}]_i$. BQ-123 at maximum effect, only partially inhibited the elevation of $[Ca^{2+}]_i$ in a concentration-dependent manner. The resting level and peak response to 10^{-5} M ATP were designated 0% and 100%, respectively. Values are mean \pm s.e.mean ($n = 4$).

for 30 s, 55°C for 1 min and 72°C for 2 min, for a total of 30 cycles. A third PCR was performed to determine the sequence of the 3' region of the pig lung ET_A receptor cDNA. An aliquot of cDNA was amplified with primers C1 and RS2, under thermal cycler conditions of 94°C for 30 s, 40°C for 1 min and 72°C for 2 min, for a total of 30 cycles. Then, an aliquot of this third PCR was further amplified using primers C2 and PS1, under thermal cycler conditions of 94°C for 30 s, 55°C for 1 min and 72°C for 2 min, for a total of 30 cycles.

Detection of pig ET_A receptor mRNA in endothelial cells on the aortic side of porcine aortic valves by RT-PCR

Porcine aortic valves from 4–5 pigs were isolated and the surface of the aortic side of the valves was scraped with a rubber policeman. Total RNA was isolated from the scrapings, according to the methods by Chomczynski & Sacchi (1987). For RT-PCR, 3 primers, PS2, PA2 and PA3 (Table 1), were designed according to the cDNA sequence for pig ET_A receptor (Figure 4). RT-PCR was performed as described elsewhere (Nishimura *et al.*, 1992). In brief, first-strand cDNA was synthesized from total RNA obtained from porcine aortic valve endothelial cells, using M-MLV reverse transcriptase and antisense primer PA3. An aliquot of RT product was amplified by PCR using primers PS2 and PA2, under thermal cycler conditions of 94°C for 30 s, 55°C for 1 min and 72°C for 1 min, for a total of 45 cycles. For control, RT-PCR procedures were carried out in the same manner, except that M-MLV reverse transcriptase was omitted during reverse transcription. The expected size of the PCR product was 255 bp. A portion of the PCR mixture was electrophoresed in a 3% NuSieve agarose gel. The gel was stained with ethidium bromide and photographed. The 255 bp bands were cut from the gel, cleaned by Gene Clean II, subcloned into pCR II plasmid vector and then sequenced.

Detection of pig ET_B receptor mRNA in endothelial cells on the aortic side of porcine aortic valves by RT-PCR

Since the sequence of the pig ET_B receptor mRNA has not been described, we chose the primers, based on the rat sequence, from the conserved regions among the bovine (Saito *et al.*, 1991), rat (Sakurai *et al.*, 1990) and human (Nakamuta *et al.*, 1991) ET_B sequences. Using these primers (RSB and RAB; Table 1) and the total RNA prepared from the pig lung, RT-PCR gave an expected size of band (491 bp). This PCR product was partially sequenced (data not shown). According to this sequence, we made one more primer (PSB; Table 1) and performed RT-PCR using the primers (PSB and RAB) and the total RNA from the endothelial cells on the aortic valve. The expected size of this PCR product was 304 bp.

Chemicals

Synthetic ET-1 and ET-3 were obtained from Peptide Institute Co. Ltd. (Osaka, Japan), sodium salt of ATP was purchased from Boehringer Mannheim (Germany), fura-2/AM and EGTA were purchased from Dojindo (Kumamoto, Japan). BQ-123 (cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-)) was kindly donated by Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan). M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase was purchased from BRL (Gaithersburg, U.S.A.). NuSieve 3:1 agarose, Oligotex-dT30, Eco RI, Eco RI cassette and cassette primers, C1 and C2, were from TaKaRa (Kyoto, Japan). RNase inhibitor and ϕ X174/Hinc II digest were purchased from TOYOBO (Osaka, Japan). Taq DNA polymerase was from Wako (Osaka, Japan). Gene Clean II was from Funakoshi (Osaka, Japan). TimeSaver cDNA Synthesis Kit and T7 Sequencing Kit were from Pharmacia LKB Biotechnology. TA Cloning Kit was Invitrogen (San Diego, U.S.A.). Oligonucleotides for primers were synthesized by Sawady Technology Inc. (Tokyo, Japan). All other chemicals were of the highest grade commercially available.

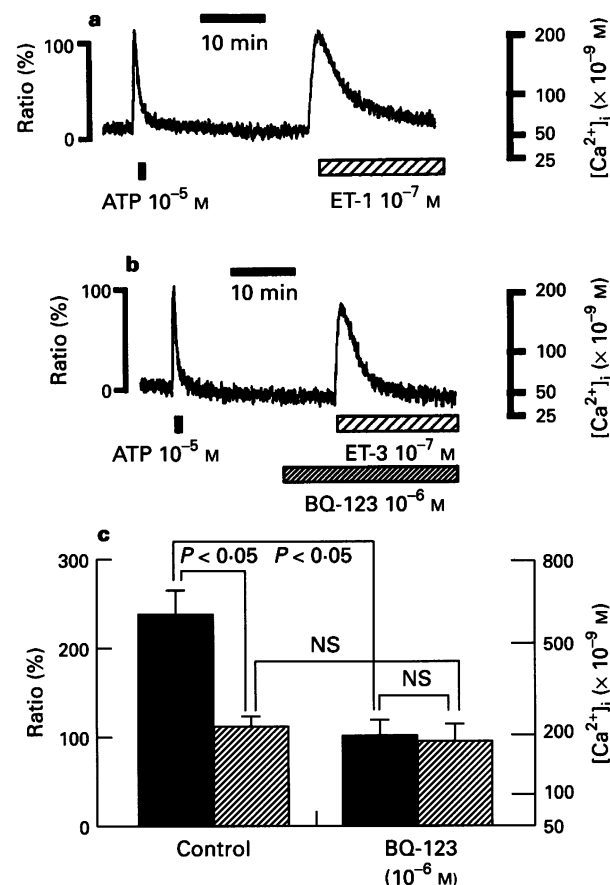


Figure 3 Representative time courses of changes in endothelial $[Ca^{2+}]_i$ induced by 10^{-7} M endothelin-3 (ET-3) in the absence (a) and presence (b) of 10^{-6} M BQ-123, and peak responses of $[Ca^{2+}]_i$ induced by 10^{-7} M ET-1 (stippled columns) or ET-3 (hatched columns) in the absence (Control) and presence of 10^{-6} M BQ-123 (c). In (c) the resting level and peak response to 10^{-5} M ATP were designated 0% and 100%, respectively. Values are mean \pm s.e.mean (NS, not significant; $n = 4$).

Statistical analysis

All data are expressed as mean \pm standard error with number of observations ($=n$). Student's *t* test was used to determine statistical difference between two mean values. Analysis of variance was used to analyze the concentration-response of a drug. *P* values < 0.05 were considered to be statistically significant. The four-parameter logistic model (De Lean *et al.*, 1978) was used to fit the sigmoidal curve to the concentration response of a drug.

Results

$[Ca^{2+}]_i$ transients induced by ET-1 and ET-3

ET-1 caused a rapid elevation of $[Ca^{2+}]_i$ which reached a peak level within 1 min, then gradually declined to reach a sustained state of elevation, as shown in Figure 1a and Figure 2a. ET-3 also caused a similar biphasic elevation of $[Ca^{2+}]_i$ (Figure 1b, 1c and Figure 3a). Previously, we have shown the full concentration-response curves of porcine aortic valvular endothelial cells for ET-1 and ET-3 both in the presence and absence of extracellular Ca^{2+} , and reported that peak levels are concentration-dependent, and at 10^{-7} M, both ET-1 and ET-3 induced maximum elevations of $[Ca^{2+}]_i$ (Aoki *et al.*, 1991). The initial rapid phase was thought to be due to the intracellular Ca^{2+} release and the sustained phase was due to the Ca^{2+} influx from the extracellular space (Aoki *et al.*, 1991; 1994). As is obvious from these traces, at 10^{-7} M, the initial peak elevation of $[Ca^{2+}]_i$

induced by ET-1 was much greater than that induced by ET-3. Statistical analysis revealed that this difference was significant ($P < 0.05$, Figure 3c). Figures 1a and 1b also show the additivity of $[Ca^{2+}]_i$ transients induced by ET-1 and ET-3 examined. The addition of 10^{-7} M ET-3 during the sustained phase induced by 10^{-7} M ET-1 led to no additional change in $[Ca^{2+}]_i$ (Figure 1a). However, the addition of 10^{-7} M ET-1 during the sustained phase induced by 2×10^{-7} M ET-3 did induce a further elevation in $[Ca^{2+}]_i$ (Figure 1b). This additional elevation of $[Ca^{2+}]_i$ induced by ET-1 may not be due to a concentration of ET-3 inadequate to bind its receptors, since the additional application of 10^{-7} M ET-1 after 10^{-7} M ET-3 induced no further elevation in $[Ca^{2+}]_i$ (Figure 1b). These observations indicated the presence of receptors other than ET_B, probably ET_A receptors in endothelial cells *in situ* in porcine aortic valves. Thus, we examined the effect of BQ-123, an ET_A receptor antagonist (Ihara *et al.*, 1992), on ET-induced Ca^{2+} transients in the following experiments.

Effects of BQ-123 on ET-1- and ET-3-induced $[Ca^{2+}]_i$ transients

As shown in Figure 1c, the additional increase in $[Ca^{2+}]_i$ induced by 10^{-7} M ET-1 after the application of 10^{-7} M ET-3 was abolished in the presence of 10^{-6} M BQ-123. Figure 2a and 2b shows the effect of 10^{-7} M ET-1 on $[Ca^{2+}]_i$ in endothelial cells *in situ*, in the absence and presence of 10^{-6} M BQ-123, respectively. BQ-123 (10^{-6} M) markedly inhibited the elevation of $[Ca^{2+}]_i$ induced by 10^{-7} M ET-1 (from $234 \pm 27\%$ to $98.6 \pm 14.4\%$ at peak level, $n = 4$). Figure 2c shows the effects of various concentrations of BQ-123 on the peak elevations of $[Ca^{2+}]_i$ induced by 10^{-7} M ET-1. BQ-123 inhibited the peak level of $[Ca^{2+}]_i$ induced by ET-1 in a concentration-dependent manner ($P < 0.05$ by analysis of variance, $n = 4$ for each concentration). The IC_{50} of BQ-123 was 3×10^{-8} M and, at a concentration of 10^{-7} M or higher, BQ-123 revealed its maximal inhibitory effect; however, it was only a partial inhibition.

Since ET-1 stimulates both ET_A and ET_B receptors, while ET-3 preferentially stimulates ET_B receptors (Vane, 1990), it

can be postulated that $[Ca^{2+}]_i$ transients induced by ET-3 may be mediated mainly by ET_B receptors. Figure 3a and 3b shows the responses of $[Ca^{2+}]_i$ in endothelial cells to ET-3 (10^{-7} M) in the absence and presence of 10^{-6} M BQ-123. Both in the absence and presence of BQ-123, ET-3 caused a biphasic elevation of $[Ca^{2+}]_i$. BQ-123 had no effect on the peak level nor on the time courses of $[Ca^{2+}]_i$. The peak elevations of $[Ca^{2+}]_i$ induced by ET-3 were $110.6 \pm 11.7\%$ ($n = 4$) and $94.5 \pm 16.5\%$ ($n = 5$) in the absence and presence of 10^{-6} M BQ-123, respectively. Figure 3c summarizes the effects of BQ-123 (10^{-6} M) on the peak elevations of $[Ca^{2+}]_i$ induced by 10^{-7} M ET-1 and 10^{-7} M ET-3. BQ-123 (10^{-6} M) inhibited Ca^{2+} transients induced by 10^{-7} M ET-1 significantly ($P < 0.05$ by Student's *t* test, $n = 4$), but had no effect on the elevation of $[Ca^{2+}]_i$ induced by 10^{-7} M ET-3 ($P > 0.05$ by Student's *t* test, $n = 4$ in the absence and $n = 5$ in the presence of BQ-123). In addition, in the presence of 10^{-6} M BQ-123, ET-1 (10^{-7} M) elevated $[Ca^{2+}]_i$ to a level comparable to that induced by ET-3 (10^{-7} M) in the absence and presence of BQ-123.

Cloning of the pig lung ET_A receptor

These pharmacological experiments indicated that ET_A receptors are present on endothelial cells of the porcine aortic valve. Therefore, we searched for the expression of ET_A receptor mRNA in endothelial cells *in situ*. Since the sequence of pig ET_A receptor mRNA has not been reported, we determined this sequence to design specific primers for pig ET_A receptors. Figure 4 shows the sequence of pig lung ET_A receptor cDNA. Similarities of the pig cDNA sequence compared with bovine (Arai *et al.*, 1990), rat (Lin *et al.*, 1991) and human (Hosoda *et al.*, 1991; Adachi *et al.*, 1991) cDNA sequences were 92.29%, 83.49%, and 91.04% in the coding regions, respectively. The deduced amino acid sequence of pig ET_A receptor was 96.96%, 92.52% and 97.20% identical to those of the bovine, rat and human ET_A receptor, respectively. These findings strongly indicated that the pig ET receptor cloned in the present study belongs to the ET_A subtype.

AAAAAAAAAGAGGGGTGTAACGTGACAGAATAAGAGATTTCCTCAGATTGTCATCAAG

ATGGAACCTTCTGCTTCAGGGTGTCTTTGGGTGGCCCTGTGGGATCGTAACTAGTATACCTCGAGAGCCAGCAGCAAACTGAGCACTACGCTGGAGATTTCACACATTT
MetGluThrPheCysPheArgValSerPheTrpValAlaLeuLeuGlyCysValIleSerAspAsnProGluSerHisSerThrAsnLeuSerThrHisValAspPheThrThrPhe

CGTGGACAGAACTCAGTTTGGTGGTTACCACTCATGACCCAGCAATCTGGCCCTCCAGCAATGGCTCCATGCACAACTACTGCCACAGCAGACCAAGATTACTTCAGCTTTCAAA
ArgGlyThrGluPheSerLeuValValThrThrHisArgProThrAsnLeuAlaLeuProSerAsnGlySerMetHisAsnTyrCysProGlnGlnThrLysIleThrSerAlaPheLys

TACATTAACTGTGATATCGTGTACTATTTCATCGTGGGAATGGTGGGAATGCAACCTGCTCAGGATCATTTACAGAACAGTGATGAGGAACGGCCCAACGGCGTGATAGCC
TyrIleAsnThrValIleSerCysThrIlePheIleValGlyMetValGlyAsnAlaThrLeuLeuArgIleIleTyrGlnAsnLysCysMetArgAsnGlyProAsnAlaIleAla

AGCCTTGGCCCTGGAGAACCTCATCTATGTGGTCATTGATCTCCTATCAATGTATTAAAGCTGTGGCTGGGCGCTGGCCCTTTGAGAACCATGACTTTGGCGTATTCTCTGCAAGTTG
SerLeuAlaLeuGlyAspLeuIleTyrValValIleAspLeuProIleAsnValPheLysLeuLeuAlaGlyArgTrpProPheGluAsnHisAspPheGlyValPheLeuCysLysLeu

TTCCCTTTTTCAGAACCTCAGTGGGGATCACTGCTCAATCTCTGGCTCTCAGTGTGACAGGTACAGAGCAGTTGCTCTGGAGTGTGTTGAGGAATCGGGATTCCATTG
PheProPheLeuGlnLysSerSerValGlyIleThrValLeuAsnLeuCysAlaLeuSerValAspArgTyrArgAlaValAlaSerTrpSerArgValGlnGlyIleGlyIleProLeu

GTCACAGCCATTGAGATTGTCTCCATCTGGATCTTCTTTTATCTGGCCATCCCTGAAGCAATCGGCTTTGTCTGCTGGTCCCTTTGAATACAGGGGGAAGAGCAGCAAAACCTGTATG
ValThrAlaIleGluIleValSerIleTrpIleLeuSerPheIleLeuAlaIleProGluAlaIleGlyPheValMetValProPheGluTyrLysGlyGluGluHisLysThrCysMet

CTCAATGOCAGCTCAAAATTCATGGAGTTCTATCAAGACGTAAAGACTGGTGGCTGTGGGTCTACTTCTGCATGCCCTGGTGTGTTACTGCCATCTCTACACTCTCATGACTTGT
LeuAsnAlaThrSerLysPheMetGluPheTyrGlnAspValLysAspTrpTrpLeuPheGlyPheTyrPheCysMetProLeuValCysThrAlaIlePheTyrThrLeuMetThrCys

GAGATGTTAAACAGAGAAATGGAAGCTGAGAAATGCGCTCAGTGAACCTGAAACAGCTGAGAGGTGGCAAAACAGTGTCTGCTGGTGTAAATTTGCTCTGTGCTGGTTC
GluMetLeuAsnArgArgAsnGlySerLeuArgIleAlaLeuSerGluHisLeuLysGlnArgArgGluValAlaLysThrValPheCysLeuValValIlePheAlaLeuCysTrpPhe

CCTCTTCAATTAAGCGTATTTTGAAGAAACCGTGTATGATGAGATGACAGAACCGATGTGAATTACTTAGTTTCTGCTCTCATGGACTATATTGGTATTAACCTGGCAACCATG
ProLeuHisLeuSerArgIleLeuLysLysThrValTyrAspGluMetAspLysAsnArgCysGluLeuLeuSerPheLeuLeuLeuMetAspTyrIleGlyIleAsnAlaThrMet

AACTCATGTATAAACCACTAGCTCTATATTTGTGAGCAAGAAATTTAAATTTGTTCCAGTCTTGGCTCTGCTGCTGTACAGTCCAAAGTCTGATGAACCTCAGTCCCATG
AsnSerCysIleAsnProIleAlaLeuTyrPheValSerLysLysPheLysAsnCysPheGlnSerCysLeuCysCysCysTyrGlnSerLysSerLeuMetThrSerValProMet

AATGGACAGCATCCAGTGGAGAACCATGAACAAACAACCATAACTGAGAGGAGCAGCCACAGGACAGTATAAATCTCTAAAGAACATTTCATCCATGTACAT
AsnGlyThrSerIleGlnTrpLysAsnHisGluGlnAsnAsnHisAsnThrGluArgSerSerHisLysAspSerIleAsn***

Figure 4 Nucleotide and deduced amino acid sequences for pig ET_A receptor. Nucleotides and amino acids are numbered on the right. The seven putative hydrophobic transmembrane domains are underlined.

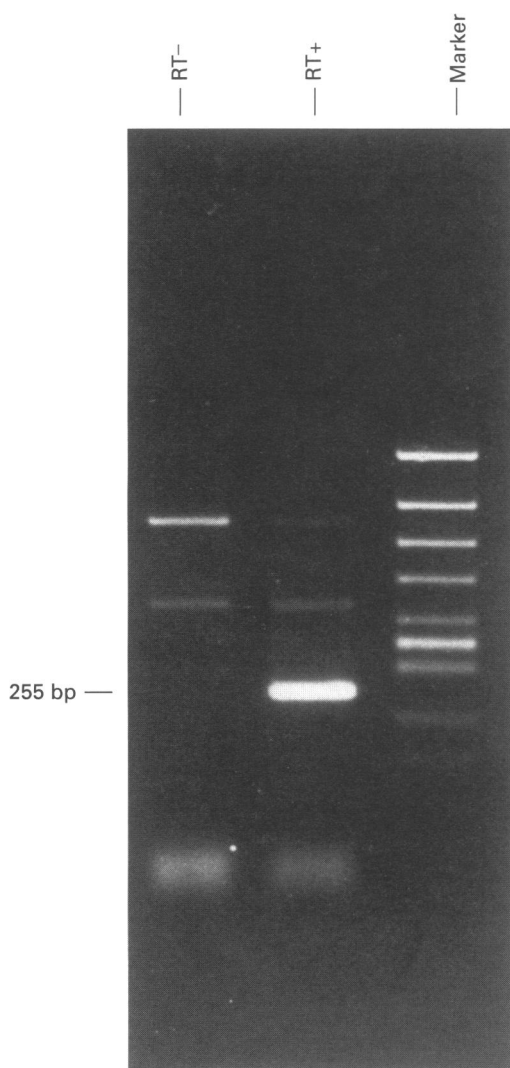


Figure 5 Detection of ET_A receptor mRNA in endothelial cells of porcine aortic valves by reverse transcription polymerase chain reaction (RT-PCR). In RT (+) experiment, 255 bp band was detected. In RT (-) experiment, reverse transcriptase was omitted during RT reaction and 255 bp band was not detected. ϕ X174/Hinc II digest was used as a molecular size marker (Marker). The size of each band was, from top to bottom, 1057, 770, 612, 495, 392, 345 + 341 + 335, 297 + 291, 210 and 162 bp.

Detection of pig ET_A receptor mRNA in endothelial cells on the aortic side of porcine aortic valves by RT-PCR

Having acquired the sequence of pig ET_A receptor cDNA, we asked whether endothelial cells in pig aortic valves express mRNA of the ET_A receptor. As shown in Figure 5, using total RNA prepared from the endothelial cells on pig aortic valves and the specific primers for pig ET_A receptor, RT-PCR revealed the band of expected size (255 bp), in agarose gel electrophoresis. The sequence of this RT-PCR product was found to be identical to the sequence of pig lung ET_A receptor. Possible amplification of the genomic ET_A receptor sequence was excluded since the band of the expected size was detected only when reverse transcriptase was added (Figure 5). Thus, it seems clear that the ET_A receptor mRNA is present in endothelial cells on the aortic side of porcine aortic valves.

Detection of pig ET_B receptor mRNA in endothelial cells on the aortic side of porcine aortic valves by RT-PCR

In the next step, we also searched for the expression of ET_B receptor mRNA in endothelial cells *in situ*. The PCR product

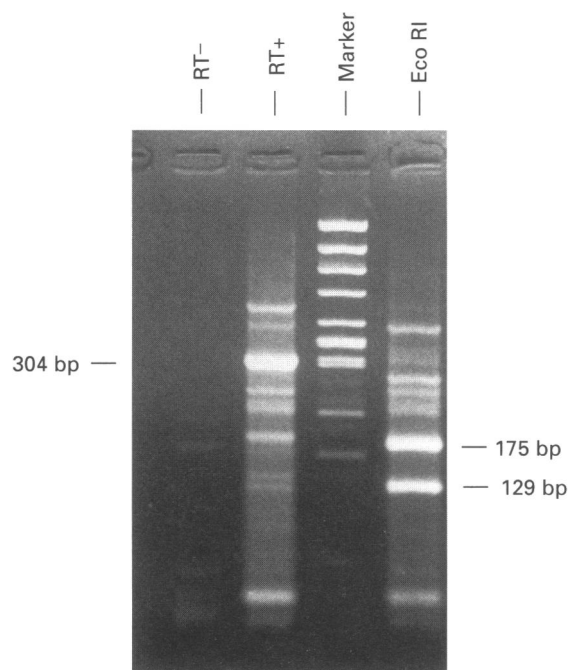


Figure 6 Detection of ET_B receptor mRNA in endothelial cells of porcine aortic valves by reverse transcription polymerase chain reaction (RT-PCR). The RT reaction carried out with 50 ng of total RNA from the endothelial cells of porcine aortic valves and RAB primer, in a total volume of 20 μ l. An aliquot (1 μ l) of RT product was amplified by PCR using primers PSB and RAB, under thermal cycling conditions of 94°C for 30 s, 55°C for 1 min and 72°C for 0.5 min, for a total of 35 cycles. In RT (+) experiment, 304 bp band was detected. In RT (-) experiment, reverse transcriptase was omitted during RT reaction and 304 bp band was not detected. This 304 bp band was digested into 129 and 175 bp bands by Eco RI (Eco RI). ϕ X174/Hinc II digest was used as a molecular size marker (Marker). The size of each band was, from top to bottom, 1057, 770, 612, 495, 392, 345 + 341 + 335, 297 + 291, 210 and 162 bp.

obtained by the RT-PCR using the primers RSB and RAB was partially sequenced (275 bases were determined, data not shown). Similarities of this sequence compared with the corresponding region of the bovine (Saito *et al.*, 1991), rat (Sakurai *et al.*, 1990) and human (Nakamuta *et al.*, 1991) ET_B receptor cDNA sequences were 93.45%, 85.45% and 93.09%, respectively. These analyses strongly indicated that the PCR product obtained by these primers derived from the pig ET_B receptor cDNA. These sequencing data also indicated that the pig ET_B receptor cDNA preserves Eco RI site at the corresponding region of the bovine (1128-1133), rat (1128-1133) and human (1131-1136) ET_B receptor cDNA. As shown in Figure 6, RT-PCR, using the primers PSB and RAB (Table 1) and the total RNA from the endothelial cells from the aortic valve, gave an expected size of band (304 bp), only when reverse transcriptase was added. This PCR product was digested by Eco RI into two expected size of bands (129 bp and 175 bp). These results indicated that ET_B receptor mRNA is present in endothelial cells of the porcine aortic valves.

Discussion

[Ca²⁺]_i transients identified in endothelial cells *in situ* in porcine aortic valves suggested the presence of ET_A receptors. We found earlier that, at an equimolar concentration, ET-1 caused a greater response of [Ca²⁺]_i than did ET-3 in endothelial cells on porcine aortic valves (Aoki *et al.*, 1991). We observed a similar phenomenon in the present study (Figure 1a, b, c, Figure 2a, and Figure 3a, c). When endothelial cells possess a

single class of ET_B receptor subtype, ET-1 and ET-3 should cause a similar size of Ca²⁺ transients, since the ET_B receptor subtype has an equally high affinity for ET-1 and ET-3 (Vane, 1990). Thus, the difference in size between ET-1-induced and ET-3-induced [Ca²⁺]_i transients suggested the presence of an ET receptor subtype other than the ET_B receptor subtype. Since [Ca²⁺]_i transients could be induced by the addition of ET-1 even after application of a saturating amount of ET-3 (Figure 1b), and since [Ca²⁺]_i transients could not be induced by the addition of ET-3 after the application of ET-1 (Figure 1a), a population of the ET receptor has a higher affinity for ET-1 than for ET-3, which is compatible with the characteristics of the ET_A receptor subtype. Consistent with this hypothesis, the former [Ca²⁺]_i transient could be blocked by BQ-123, a selective antagonist of the ET_A receptor (Ihara *et al.*, 1992) (Figure 1c). Furthermore, part of the elevation in [Ca²⁺]_i induced by ET-1 was inhibited by a low concentration of BQ-123 (10⁻⁷ M), while the remainder was insensitive to higher concentrations of BQ-123 (up to 10⁻⁵ M). The extent of 10⁻⁷ M ET-1-induced elevation in [Ca²⁺]_i resistant to BQ-123 was comparable to that of 10⁻⁷ M ET-3-induced elevations in [Ca²⁺]_i, which was not affected by the ET_A antagonist. Thus, part of ET-1-induced Ca²⁺ transients may be mediated by the ET_A subtype of receptor, while the Ca²⁺ transient induced by ET-3 and BQ-123-resistant Ca²⁺ transient induced by ET-1 may be mediated by some subtype other than ET_A, presumably ET_B.

The presence of the ET_A receptor subtype in endothelial cells on porcine aortic valves was confirmed by RT-PCR, as shown in Figure 5. In addition, we also confirmed the presence of the ET_B receptor mRNA in endothelial cells on porcine aortic valves (Figure 6). Thus, we conclude that endothelial cells on the aortic side of porcine aortic valves have functioning ET_A receptors and the ET_A receptor mRNA as well as ET_B receptor mRNA.

Subtypes of ET receptors on endothelial cells remain open to question. Takayanagi *et al.* (1991) suggested the presence of ET_B receptors on rat aortic endothelial cells, as deduced from binding studies. Fujitani *et al.* (1992) also reported that ET_B receptors are present in primary culture of endothelial cells of the human umbilical vein also deduced from binding studies. The significant expression of ET_B receptor mRNA has been noted in human cultured umbilical vein endothelial cells (Ogawa *et al.*, 1991). Thus, it appears that the presence of ET_B receptors on endothelial cells has gained general acceptance (Masaki *et al.*, 1991; Remuzzi & Benigni, 1993). On the other hand, only two groups have reported the presence of ET_A receptors on endothelial cells, detected in binding studies (Vigne *et al.*, 1990) and functional studies (Vigne *et al.*, 1991; Amano *et al.*, 1994). The expression of ET_A receptor mRNA has apparently not been reported. Thus, this is a first documentation

of the expression of ET_A receptor mRNA in endothelial cells. It would be interesting to note that some investigators had suggested the presence of ET_C receptors (ET-3-specific subtype) on cultured bovine aortic endothelial cells (Emori *et al.*, 1990) and bovine native aortic endothelial cells (Warner *et al.*, 1992), detected in binding study and functional study, respectively. Recently, cDNA clone for the ET_C receptor subtype has been reported in non-mammalian tissue (Karne *et al.*, 1993). However, the expression of ET_C receptor mRNA in endothelial cells has not been reported.

In the cardiovascular system, endocardial endothelial cells and the aortic valvular endothelial cells, as well as the vascular endothelial cells, play important roles in the antithrombotic, anticoagulatory and vasorelaxant actions (Jaffe, 1985). It has been reported that valvular endothelial cells can produce endothelium-derived relaxing factor (Ku *et al.*, 1990; Aoki *et al.*, 1994). Recently, the cardiac valvular endothelial cells have been used as a model for the *in situ* endocardial and/or vascular endothelial cells in the investigation of the regulation of Ca²⁺ homeostasis (Aoki *et al.*, 1991; 1994; Laskey *et al.*, 1994; Amano *et al.*, 1994; Li & van Breemen, 1995). These valvular preparations have an advantage in measuring the change in [Ca²⁺]_i of the *in situ* endothelial cells, since there is no underlying cardiomyocytes or vascular smooth muscle cells (Aoki *et al.*, 1994), which, if present, would interfere the fura-2 signal. Although the clinical implication of the present study is unknown, it is tempting to speculate that ET-1, once released from endothelial cells as a vasoconstrictor, would induce large [Ca²⁺]_i transients in the valvular endothelial cells mediated by ET_A as well as ET_B receptors to secrete anticoagulatory and vasorelaxant factors as an autocrine or paracrine transmitter.

In conclusion, we provide the first evidence for the presence of mRNA for the ET_A receptor, which contributes to the elevation of [Ca²⁺]_i in endothelial cells *in situ*. Since ET-1 is the only member of the endothelin family proven to be expressed in endothelial cells (Van, 1990), the ET_A receptor, as well as ET_B receptor in endothelial cells, may play an important role in the regulating vascular tonus.

We thank M. Ohara for helpful comments on the manuscript. This study was supported in part by Grants-in-Aid for Developmental Scientific Research (No. 03557043) and for General Scientific Research (No. 04454268, 05837015, 05837016) from the Ministry of Education, Science and Culture, Japan, and Grants from the Uehara Memorial Foundation, from Yokoyama Rinshouyaku, from Japanese Heart Foundation, from Kaibara Morikazu Medical Science Promotion Foundation, from Mochida Memorial Foundation and from The Tokyo Biochemical Research Foundation. We also thank K. Kajishima for secretarial services.

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(Received November 14, 1994

Revised April 24, 1995

Accepted May 12, 1995)