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Transcriptional regulated plasticity of vascular contractile endothelin ET_B receptors after organ culture

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Abstract

The aim of the present study was to investigate the level of regulation of the contractile endothelin ET_B receptor which appears spontaneously after organ culture of vascular segments. Endothelin-1 elicited a strong contraction while the selective endothelin ET_B receptor agonist, sarafotoxin 6c, had a negligible effect on fresh ring segments of rat mesenteric artery. After organ culture in serum-free Dulbecco's modified Eagle's medium at 37°C (for 1 or 2 days) the endothelin-1-induced contraction was unchanged, whereas sarafotoxin 6c induced, after 1 day, a marked contraction which was further increased at day 2. The contraction induced by sarafotoxin 6c was significantly attenuated by the transcriptional inhibitor, actinomycin D, or the translational inhibitor, cyclohexamide, while the endothelin-1-induced contraction was much less affected. mRNA for endothelin ET_A and endothelin ET_B receptors was present in fresh human omental arteries denuded of endothelium. However, after organ culture, endothelin ET_B mRNA was more prominent than endothelin ET_A mRNA. Furthermore, the mRNA for both receptors was decreased after treatment with actinomycin D but not with cyclohexamide. This suggests that the endothelin ET_A receptor is the dominating contractile receptor in fresh arteries while organ culture induces transcription and subsequent translation of contractile endothelin ET_B receptors. © 1997 Elsevier Science B.V.

Keywords: Endothelin ET_A receptor; Endothelin ET_B receptor; Organ culture; Reverse transcription-polymerase chain reaction; Up-regulation

1. Introduction

The potent vasoconstrictor, endothelin-1, produced by the endothelial cells, is supposed to mediate its effect in mammals through two distinct receptor subtypes, the endothelin ET_A and the endothelin ET_B receptor (Arai et al., 1990; Sakurai et al., 1990; Yanagisawa et al., 1988). In the vascular system, the endothelin ET_A receptor is mainly situated on the smooth muscle cells mediating contraction, while the endothelin ET_B receptor is localized on the endothelial cells mediating vasodilatation and in some vascular regions on the smooth muscle cells mediating contraction (Sakurai et al., 1992). For example, rat and rabbit aorta, and most human arteries demonstrate in vitro the endothelin ET_A receptor as the dominating contractile receptor (Maguire and Davenport, 1995; Moreland et al., 1994; Sumner et al., 1992). However, rabbit jugular and saphenous veins in vitro as well as human resistance and capacitance vessels in vivo have endothelin ET_A and endothelin ET_B receptors which mediate almost equal contractions (Haynes et al., 1995; Moreland et al., 1994; Sumner et al., 1992).

The heterogeneous distribution of the contractile vascular endothelin ET_B receptors between species and between different vessels in the same species is further complicated by the recently discovered plasticity of the endothelin ET_B receptor. In vivo studies in dogs have shown that the endothelin ET_B receptor is upregulated after a chronic increase in blood flow (Barber et al., 1996) or after experimental cerebral vasospasm (Roux et al., 1995) and that there is an enhanced endothelin ET_B receptor-induced coronary vasoconstriction in experimental congestive heart failure (Cannan et al., 1996). In addition, studies of humans demonstrate upregulation of endothelin ET_B receptors in atherosclerotic human coronary arteries (Dagassan et al., 1996). The endothelin ET_B receptors appear to contribute to basal vascular tone induced by endogenous endothelin release in patients with coronary heart diseases (Wenzel et al., 1996). Thus, endothelin ET_B receptor

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plasticity may have both a physiological and a pathophysiological role.

In contrast to smooth muscle cells in culture which tend to differentiate from their normal contractile phenotype (Chaley-Campell et al., 1979), organ culture makes it possible to study receptor regulation in smooth muscle cells with functional contractile capacity. We have shown previously that a contractile effect of the endothelin ET_B receptor appears spontaneously in human omental arteries after 1–5 days of organ culture (Adner et al., 1995, 1996). The aim of the present study was to examine the level of regulation of the contractile endothelin ET_B receptor that occurs after organ culture.

2. Materials and methods

2.1. Tissue preparation and organ culture procedure

Rat mesenteric arteries were used in this study for in vitro pharmacology because of less variation between adjacent segments from the same vessel (unpublished observations). Human omental arteries were used for mRNA detection because the problem of variation does not arise in the long segments used. The protocol was approved by the Ethical Committee of the Lund University.

For in vitro pharmacology male Wistar-Kyoto rats (200–250 g; Møllegården, Denmark) were anaesthetized with CO_2 and killed by a cut through the heart. The superior mesenteric arteries with adherent branches were immediately taken out, immersed in cold (4°C) sterile Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA). The first order of the arterial branch was dissected free from adherent tissue under cold sterile conditions and cut into 1 mm long circular segments.

For RNA extraction and further reverse transcription-polymerase chain reaction (RT-PCR), human omental arteries with connecting fat were removed from patients during abdominal surgery and were immersed in cold sterile DMEM. Within 2 h of their removal, vessels with a resting diameter between 0.2 and 0.5 mm were dissected free from adherent tissue under sterile conditions and prepared as open-ring segments, 20–40 mm long. A scalpel was used to remove the endothelium and adventitia. After preparation, the segments were frozen in liquid nitrogen and stored at −70°C.

Segments from each vessel were divided into two groups: one for immediate experimental analysis (fresh) and the other for organ culture. The segments for organ culture were placed in a well plate, one segment in each well containing DMEM and incubated at 37°C in humidified 5% CO_2 in air. Serum-free DMEM (4500 $mg \cdot l^{-1}$ D-glucose) contained sodium pyruvate (110 $mg \cdot l^{-1}$), L-glutamine (584 $mg \cdot l^{-1}$), and was supplemented with penicillin (100 $U \cdot ml^{-1}$) and streptomycin (100 $\mu g \cdot ml^{-1}$). Segments were incubated for 24-h culture (one day) as time control and with either the transcriptional inhibitor, actinomycin D (5 $\mu g \cdot ml^{-1}$), or the translational inhibitor, cyclohexamide (10 $\mu g \cdot ml^{-1}$) (Schramek et al., 1995). The presence or the absence of endothelium was verified by staining with 5% silver nitrate followed by light microscopy (Abrol et al., 1984).

2.2. In vitro pharmacology

Vasomotor reactivity was analysed in temperature-controlled (37°C) tissue baths containing a buffer solution composed of 119 mM NaCl, 15 mM $NaHCO_3$, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.5 mM $CaCl_2$ and 5.5 mM glucose. The solution was continuously equilibrated with 5% CO_2 in O_2 resulting in a pH of 7.4. The vessel segments were mounted on two L-shaped metal prongs. One prong was connected to a force-displacement transducer (FTO3C, Grass Instruments, Quincy, MA, USA) attached to a MacLab unit (ADInstruments, Hastings, UK) for continuous recording of isometric tension by means of the Chart software (ADInstruments). The other prong was connected to a displacement device, allowing adjustment of the distance between the two parallel prongs. A passive tension of 2 mN was applied to the segments. The specimens were subsequently allowed to stabilize at this level of tension for 60 min. The contractile capacity of each tissue segment was tested by exposure to a potassium (K^+)-rich buffer solution (60 mM) which had the same composition as the standard solution except that NaCl was exchanged for an equimolar concentration of KCl. When two reproducible contractions had been obtained (variation less than 10%) the vessels were used for further studies.

Concentration–response curves for the endothelin agonists were obtained by cumulative application of the peptides. Contractile responses of each segment were expressed as percentages of the contraction induced by 60 mM K^+, and the maximum contractile effects of the agonist are given as E_{max} values. The potency of the agonists was expressed as pEC_{50} values (negative logarithm of the molar concentration of agonist inducing half-maximum response). To further investigate the contractile capacity of the vessel, a high concentration of noradrenaline (100 μM) was added to the control vessel segments: for the segments affected by endothelin-1 at the time of maximum effect, and for the segments affected by sarafotoxin 6c after baseline was reached.

2.3. Molecular biology

2.3.1. RNA preparation

The total cellular RNA from human omental arteries was isolated using the TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) following the kit protocol. Briefly the frozen tissue samples were homogenized in TRIzol. RNA was precipitated with isopropanol, washed with 75% ethanol and finally dissolved in 20 μl diethylpyrocarbon-

ate-treated water. Amount and purity of the RNA were evaluated using a DU-64 spectrophotometer (Beckman, Fullerton, CA, USA) at absorption wavelengths of 260 and 280 nm. Preparations with a ratio better than 1.6 were considered pure and were used for the experiments.

2.3.2. Reverse transcription-polymerase chain reaction (RT-PCR)

The reverse transcription of total RNA to cDNA and subsequent PCR were carried with the GeneAmp RNA PCR kit (Perkin Elmer, Foster City, CA, USA) in a Perkin Elmer DNA Thermal Cycler. First-strand cDNA was synthesized from 1 μg total RNA in 20-μl reaction volume using random hexamers as primers. The reaction mixture was incubated at 42°C for 15 min, heated to 99°C for 5 min, and chilled to 5°C for 5 min. A 2-μl portion of the resultant cDNA was amplified by PCR in a final volume of 50 μl, following a standard PCR protocol. Ampli*Taq* DNA polymerase (Perkin Elmer) was used as the thermostable enzyme. The PCR was carried out with the following profile: 2 min at 95°C for 1 cycle; 1 min at 95°C and 1 min at 60°C for 30 or 40 cycles; 7 min at 72°C for 1 cycle; incubation at 4°C for 5 min. A blank (water) was included in all experiments. Control experiments showed that 30 cycles were within the exponential phase of the PCR (not shown). 40 cycles of PCR were only performed in the case of actinomycin-D-treated vessels in order to obtain visible bands.

2.3.3. Oligonucleotide design

RT-PCR assays for endothelin ET_A and endothelin ET_B receptor mRNA were performed using the following primers (Scandinavian Gene Synthesis, Köping, Sweden): endothelin ET_A receptor forward, 5′-TGGCCTTTTGATCACAATGACTTT-3′ (bases 436–459); endothelin ET_A receptor reverse, 5′-TTTGATGTGGCATTGAGCATACAGGTT-3′ (bases 737–711); endothelin ET_B receptor forward, 5′-ACTGGCCATTTGGAGCTGAGATGT-3′ (bases 497–521); endothelin ET_B receptor reverse, 5′-CTGCATGCCACTTTTCTTTCTCAA-3′ (bases 924–901). The primers were designed to span one or more introns so that PCR amplification of the cDNA could be interpreted to reflect tissue mRNA expression rather than contamination by genomic DNA (Arai et al., 1993; Hosoda et al., 1992; O'Reilly et al., 1992).

2.3.4. Verification of PCR products

The identity of the PCR products was verified by restriction analysis and sequencing. Briefly, approximately 80 ng of gel-purified PCR products was directly sequenced with both forward and reverse primers, using the Amplitaq FS cycle sequencing kit (Perkin-Elmer) on a DNA thermocycler (Perkin-Elmer) according to the manufacturer's protocol. After the reaction the samples were purified and run on an ABI Prism 310 DNA sequencer (Perkin-Elmer). The resulting sequences were finally checked for homology to the published sequences in GenBank using the BLAST algorithm of Altschul et al. (1990). In addition restriction analysis of the fragments was performed as described elsewhere (Adner et al., 1996). Briefly, endothelin ET_A receptor fragments were digested with *Bam*HI (Fermentas, Vilnius, Lithuania) generating two fragments, i.e., 108 and 194 basepairs. Endothelin ET_B receptor fragments were digested with *Eco*RV (Boehringer-Mannheim, Mannheim, Germany) generating two fragments, i.e., 100 and 328 basepairs.

2.3.5. Densitometric analysis of PCR products

After RT-PCR, a 10 μl aliquot from each PCR product was separated on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide, in 1 × TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0; Sambrook et al., 1989). The gels were photographed on an UV box using Polaroid 667 film. The pictures were scanned on a Umax-S6e flatbed scanner and analyzed densitometrically with the NIH-Image software (v1.60) (Rasband and Bright, 1995). Before calculation of the ratio between endothelin ET_A receptor and endothelin ET_B receptor band densities, the endothelin ET_A receptor values were corrected for the decreased ethidium bromide incorporation of the shorter endothelin ET_A receptor fragment, using the equation of Menzo et al. (1992):

$$\text{corrected density} = (\text{measured density}) \times (428/302).$$

2.4. Drugs

Endothelin-1 and sarafotoxin 6c (Auspep, Parkville, Australia) were dissolved in 0.1% (w/v) bovine serum albumin in double-distilled water and further diluted in buffer solution. Actinomycin D, cyclohexamide, noradrenaline and all other reagents were purchased from Sigma, if not otherwise stated.

2.5. Statistics

The data are expressed as mean values ± S.E.M. and *n* refers to the number of rats or patients from whom the vessel segments were obtained. The Wilcoxon signed-rank test was used for paired analysis, with Friedman multiple analysis. The Mann-Whitney *U*-test and Kruskal-Wallis test for multiple groups, together with Bonferroni correction were used for unpaired analyses. Differences were considered significant at P values < 0.05.

3. Results

3.1. Vasomotor responses

3.1.1. Potassium-induced contraction

The K^+-induced contraction, used as reference for the contractile capacity of the rat mesenteric artery, was not

different in any of segments exposed to endothelin-1 and sarafotoxin 6c. Neither was there any difference in contractile capacity between fresh segments (6.49 ± 0.79 mN, $n = 7$) and segments cultured for 1 day (5.45 ± 0.90 mN, $n = 8$) or 2 days (5.10 ± 1.05 mN, $n = 6$). Further, there was no difference in the K^+-induced contraction in vessels cultured with actinomycin D at day 1 (6.78 ± 0.74 mN, $n = 8$) or at day 2 (4.32 ± 0.39 mN, $n = 6$) and also not for cyclohexamide at day 1 (5.25 ± 0.82 mN, $n = 8$) or at day 2 (6.01 ± 1.26 mN, $n = 6$), compared to the contraction of control arteries.

3.1.2. Contractile effects of endothelin-1 and sarafotoxin 6c in fresh and cultured segments

The endothelin-1-induced maximal contractions in fresh segments, in segments cultured for 1 day and in segments cultured for 2 days did not differ significantly as to absolute values (8.99 ± 1.03 mN, 9.78 ± 1.01 and 5.36 ± 1.12, respectively), whereas the E_{max} values measured as percent of K^+-induced contraction differed significantly between all the three groups (Table 1). The potency of endothelin-1 showed a trend to increase during culturing but the values were not significantly different from each other. Sarafotoxin 6c did not cause any statistically significant contraction in the fresh segments (Fig. 1), but elicited a clear-cut contraction in segments cultured for 1 and for 2 days (Table 1). The E_{max} for sarafotoxin 6c was 70% of that for endothelin-1, day 1, and 98% of that for endothelin-1, day 2. The potency of sarafotoxin 6c was higher after 2 days' culture compared to that with arteries cultured for 1 day. It was interesting that, after reaching its maximum, the contraction with endothelin-1 was stable and did not reach baseline even after 2 h with several washes, whereas the sarafotoxin 6c-induced contraction after reaching its maximum, rapidly decreased back to its baseline within 30 min (not shown).

3.1.3. Noradrenaline-induced contraction

At the maximum effect of endothelin-1, a single dose of noradrenaline (100 μM) did not cause a further contraction

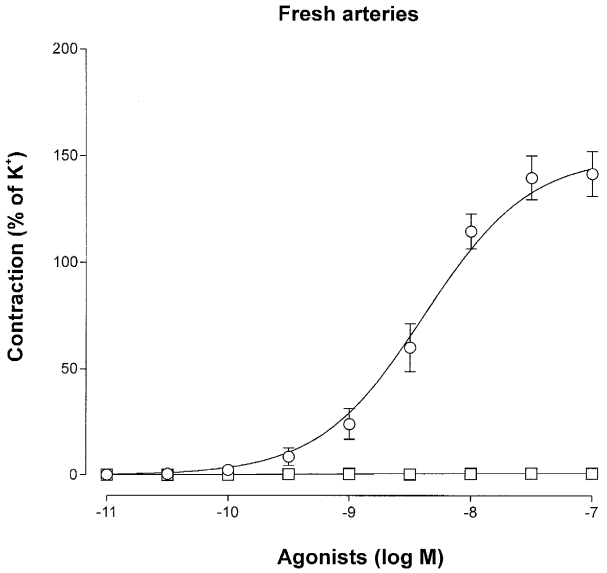

Fig. 1. Contraction of fresh rat mesenteric artery induced by cumulative concentrations of endothelin-1 (○) and sarafotoxin 6c (□). The contraction of each segment tested was calculated as a percentage of the potassium (K^+)-induced contraction in the same segment, and each point represents the mean for all segments tested, with error bars representing S.E.M. $n = 7$.

stronger than the one endothelin-1 had elicited, in either fresh or cultured arteries ($n = 6$–8), which indicated that endothelin-1 alone has the capacity to induce a contraction close to the maximum of the vessel with only the endothelin ET_A receptor and with both endothelin ET_A and endothelin ET_B receptors. When the segments had reached baseline after the concentration–response curve for sarafotoxin 6c, noradrenaline elicited, as compared to the K^+-induced contraction (not shown), a contraction of $123 \pm 3\%$ (6.54 ± 0.62 mN, $n = 6$) in fresh arteries, $141 \pm 5\%$ (7.03 ± 0.72 mN, $n = 6$) in arteries cultured for 1 day and $99 \pm 7\%$ (5.60 ± 0.97 mN, $n = 6$) in arteries cultured for 2 days.

Table 1
Contractile effects of ET-1 and sarafotoxin 6c (S6c) in rat mesenteric arterial segments

		Control			Actinomycin D (5 μg/ml)			Cyclohexamide (10 μg/ml)		
		n	E_{max}	pEC_{50}	n	E_{max}	pEC_{50}	n	E_{max}	pEC_{50}
Day 0	ET-1	7	142 ± 11	8.45 ± 0.08						
	S6c	7	1 ± 1	N.D.						
Day 1	ET-1	8	205 ± 26 [b]	8.89 ± 0.37	8	132 ± 10 [a]	8.75 ± 0.21	8	177 ± 14 [a]	8.65 ± 0.13
	S6c	8	138 ± 12 [b]	9.04 ± 0.09	8	2 ± 1 [a]	9.23 ± 0.35	8	34 ± 11 [a]	8.96 ± 0.12
Day 2	ET-1	6	106 ± 4 [b,c]	9.45 ± 0.39	6	99 ± 6	9.35 ± 0.22	6	104 ± 6	8.90 ± 0.32
	S6c	6	104 ± 5 [b,c]	9.69 ± 0.26 [c]	6	62 ± 8 [a]	9.52 ± 0.15	6	42 ± 15 [a]	9.35 ± 0.15

Segments were used fresh (day 0), cultured for 1 day (day 1); without (control) and with actinomycin D or cyclohexamide, or cultured for 2 days (day 2); the first day without treatment and the second day without (control) and with actinomycin D or cyclohexamide. Responses were characterized by E_{max} values (maximum contractile effect expressed as a percent of 60 mM K^+-induced contraction) and pEC_{50} values (negative logarithm of the molar concentration that produced half-maximum contraction) and expressed as mean with S.E.M. n represents the number of animals.

[a] $P < 0.05$ compared to control.

[b] $P < 0.05$ compared to day 0.

[c] $P < 0.05$ compared to day 1.

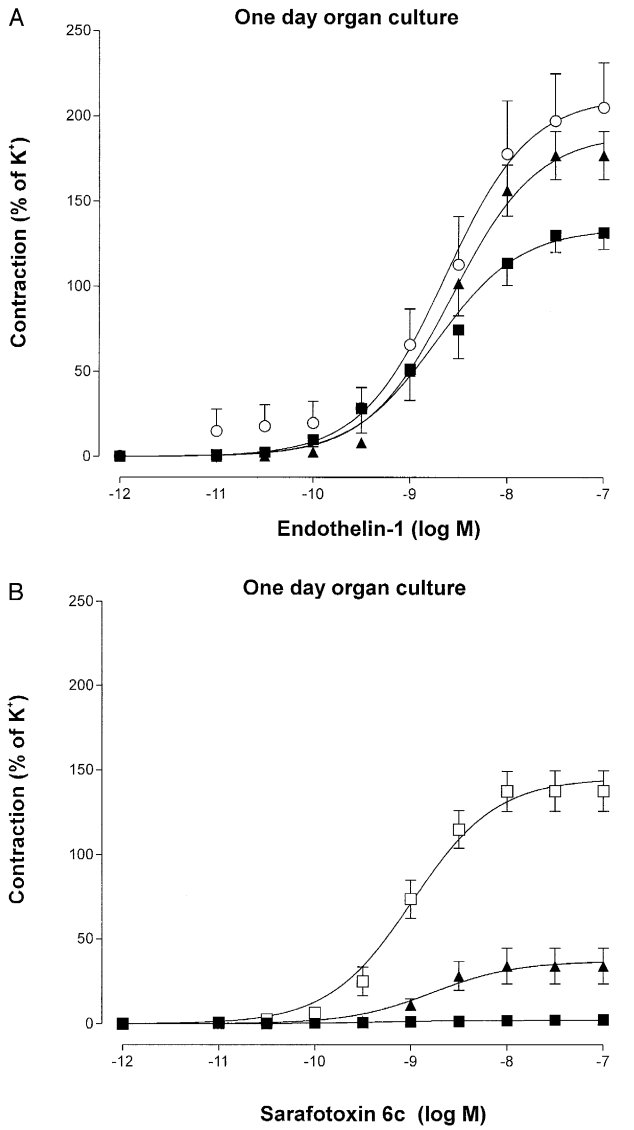

Fig. 2. Contraction of rat mesenteric artery induced by cumulative concentrations of endothelin-1 (○) (A) and sarafotoxin 6c (□) (B). The segments had been in organ culture for 1 day without or together with the transcriptional inhibitor, actinomycin D (5 μg/ml) (■), or the translational inhibitor, cyclohexamide (10 μg/ml) (▲). The contraction of each segment tested was calculated as a percentage of the potassium (K^+)-induced contraction in the same segment, and each point represents the mean for all segments tested, with error bars representing S.E.M. $n = 8$.

3.1.4. Contractile effects after treatment with actinomycin D and cyclohexamide

In arteries cultured with the transcriptional inhibitor actinomycin D (5 μg/ml) for 1 day, the E_{max} for endothelin-1 was attenuated and endothelin-1 elicited a contraction of 68% compared to endothelin-1 applied the same day (Fig. 2A). Sarafotoxin 6c induced a contraction that was only 1% of that seen with endothelin-1 and that was not significantly different from the negligible effect in fresh arteries (Fig. 2B).

Segments cultured together with the translational inhibitor, cyclohexamide (10 μg/ml), had an E_{max} for endothelin-1 that was 86% of that seen in cultured control arteries (Fig. 2A). The sarafotoxin 6c contraction was attenuated by the cyclohexamide treatment and yielded an E_{max} that was 16% of that with endothelin-1 the same day (Fig. 2B).

Since actinomycin D and cyclohexamide strongly diminished the endothelin ET_B receptor-induced contraction after 1 day of incubation, we studied further the effect of the same agents in segments which, after 1 day, had developed the response of contractile endothelin ET_B receptors. However, the E_{max} for endothelin-1 after 1 day of

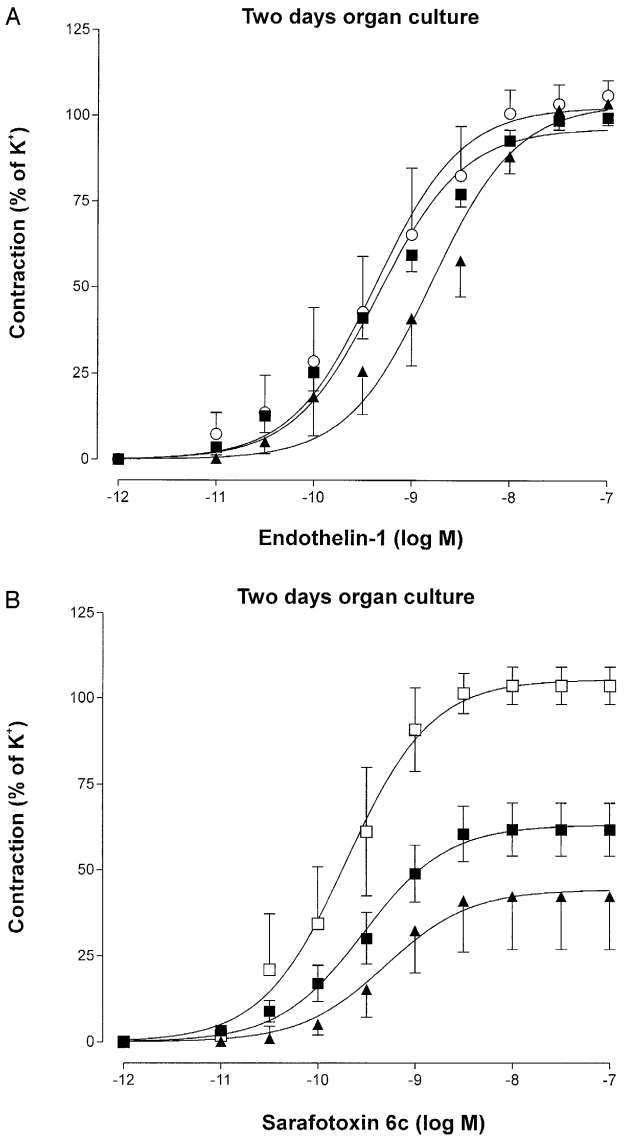

Fig. 3. Contraction of rat mesenteric artery induced by cumulative concentrations of endothelin-1 (○) (A) and sarafotoxin 6c (□) (B). All segments had been in organ culture for 1 day without any treatment and thereafter were further cultured 1 day without or together with the transcriptional inhibitor, actinomycin D (5 μg/ml) (■), or the translational inhibitor, cyclohexamide (10 μg/ml) (▲). The contraction of each segment tested was calculated as a percentage of the potassium (K^+)-induced contraction in the same segment, and each point represents the mean for all segments, tested with error bars representing S.E.M. $n = 6$.

culture (with no treatment) and a further day of subsequent culture with actinomycin D or cyclohexamide was not affected (95% and 99% of the endothelin-1 effect same day, respectively)(Fig. 3A). However, the E_{max} induced by sarafotoxin 6c was decreased to 58% and 40% of that with endothelin-1, respectively (Fig. 3B).

3.2. mRNA for the endothelin ET_A and ET_B receptor

RT-PCR analysis was performed on RNA extracted from endothelium-denuded human omental arteries that were treated in the same way as the vessels for in vitro pharmacology. After 30 cycles of amplification, products of the expected sizes (302 basepairs for endothelin ET_A and 428 basepairs for endothelin ET_B receptors) were detected in RNA preparations from fresh arteries and arteries cultured without actinomycin or cyclohexamide (time control) (Fig. 4). The identity of the products was verified by DNA sequencing, which showed no dissimilarities from the published sequences (Arai et al., 1993; Hosoda et al., 1992) and by restriction analysis, that yielded products of the expected size after cleavage (see Section 2). In fresh arteries, the endothelin ET_A/ET_B receptor ratio of intensity for the bands was 1.78 ± 0.18 ($n = 4$) which indicated that the amount of mRNA for the endothelin ET_A receptor exceeded the amount of mRNA for the endothelin ET_B receptor (Fig. 4B). However, after 1 day of organ culture there was a greatly increased expression of mRNA for the endothelin ET_B receptor. This was verified by densitometric analysis that yielded an endothelin ET_A/ET_B receptor ratio of 0.61 ± 0.09 ($n = 4$) which was significantly lower than that seen for the fresh arteries ($P = 0.017$). No visible bands were detected from arteries incubated with actinomycin D after 30 cycles of PCR, whereas 40 cycles of PCR generated visible products corresponding to the endothelin ET_A and the endothelin ET_B receptors (Fig. 4A). This demonstrates that actinomycin D attenuated the transcription of mRNA for both receptors. The ratio of endothelin ET_A and endothelin ET_B receptor mRNA tended to resemble the pattern from fresh segments (1.83 ± 0.68, $n = 3$) (Fig. 4B). In contrast, arteries treated with cyclohexamide, after 30 cycles of amplification, showed visible bands with a pattern similar to that of the time control with an endothelin ET_A/ET_B receptor ratio of 0.58 ± 0.12 ($n = 3$) (Fig. 4B) and was significantly different from fresh artetries ($P = 0.036$). This indicates that cyclohexamide did not affect the expression of mRNAs for either endothelin ET_A or endothelin ET_B receptors.

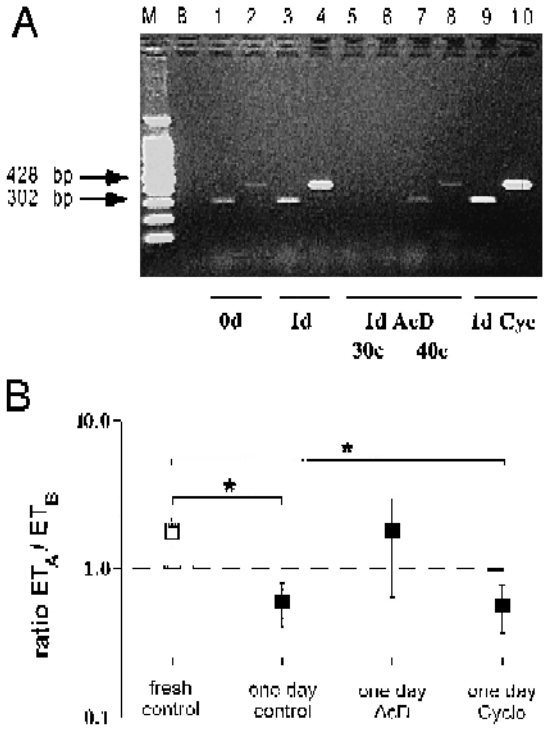

Fig. 4. RT-PCR analysis from RNA which was isolated from endothelium-denuded human omental arteries. (A) Representative electrophoresis from one patient; a 10-μl aliquot from each PCR product was separated on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. The sizes of the fragments were 302 basepairs for the endothelin ET_A receptor product and 428 basepairs for the endothelin ET_B receptor product. Lane M: 100 basepairs ladder; lane B: blank (H_2O); lanes 1 and 2: fresh artery, 30 cycles; lanes 3 and 4: artery cultured for 1 day, 30 cycles; lanes 5 and 6: artery cultured for 1 day with actinomycin D, 30 cycles; lanes 7 and 8: artery cultured for 1 day with actinomycin D, 40 cycles; lanes 9 and 10: artery cultured for 1 day with cyclohexamide, 30 cycles. (B) Compiled ratios of mRNA for endothelin ET_A and endothelin ET_B receptors from fresh arteries ($n = 5$) and arteries cultured for 1 day; control ($n = 4$), treated with actinomycin D (AcD) ($n = 3$) or cyclohexamide (Cyclo) ($n = 3$). Ratios are calculated as the densitometrically analyzed signal for the endothelin ET_A receptor product divided by the signal for the endothelin ET_B receptor product for each sample, corrected for different lengths (see Section 2), each point representing the mean with error bars (S.E.M.). * $P < 0.05$ compared with fresh arteries.

4. Discussion

In this study we demonstrated that in fresh rat mesenteric artery, contraction is mediated through only endothelin ET_A receptors and that mRNA for both endothelin ET_A and endothelin ET_B receptors was present in endothelium-denuded human omental arteries. In contrast, segments which had been cultured for 1 or 2 days showed a strong and potent endothelin ET_B receptor-mediated contraction. The spontaneous increase in the contractile effect for the endothelin ET_B receptors after organ culture was dependent on de novo transcription and translation of mRNA. Thus, these in vitro data demonstrated that the plasticity in the smooth muscle cell expression of contractile endothelin ET_B receptors is regulated at the transcriptional level.

The contractile effect of endothelin-1 and the lack of effect of sarafotoxin 6c in the fresh rat mesenteric artery suggest that the contraction was mediated through mainly endothelin ET_A receptors. This is in accordance with the results of pharmacological characterization in human

omental arteries (Adner et al., 1996). The potency of the contraction induced by endothelin-1 in our study was of a magnitude similar to that in a study of the third branch from the mesenteric artery in the rat (Deng et al., 1995), while the sarafotoxin 6c-induced contraction was about 14% of the endothelin-1-induced contraction in the latter study. It has been shown that the ET_B receptor is most prominent in the smaller resistance arteries (Ekelund et al., 1994) and the greater effect of sarafotoxin 6c in the study by Deng et al. (1995) may have been due to the fact that the third branch of the mesenteric artery has a smaller diameter than the mesenteric branch used in our study.

The maximum contraction with endothelin-1, in reference to the 60 mM K^+ contractile capacity, was significantly different in all three groups, i.e., the fresh arteries, and the arteries cultured for 1 or for 2 days. However, when one compares the contraction of endothelin-1 measured as absolute values (mN) no statistically significant differences are seen. It has been demonstrated in other studies that organ culture affects the K^+-induced contraction (De Mey et al., 1989; Mauger et al., 1975) and evokes both increased and decreased contractions at different times after organ culture (De Mey et al., 1991). Therefore, we assume that the amount of contractile endothelin ET_A receptors does not change much during culturing. Instead, we believe that the changes in E_{max} values (measured as % of K^+) are due to altered K^+-induced contraction in the segments exposed to organ culture. The minor change in the endothelin-1-induced contractions after treatment with actinomycin D and cyclohexamide further supports this hypothesis. Also, the effect induced by noradrenaline followed a pattern similar to that seen with endothelin-1, having the strongest contraction at day 1 and the weakest at day 2.

We now found a marked increase in the sarafotoxin 6c-induced contraction after organ culture for 1 day. This increased effect of sarafotoxin 6c in the cultured mesenteric artery was similar to that seen in an earlier study with human omental arteries and another selective endothelin ET_B receptor agonist, IRL 1620 (Adner et al., 1995), suggesting that there is an increase in the effect of contractile endothelin ET_B receptors in arteries after organ culture. This is consistent with the pharmacological characterization performed on human omental arteries (Adner et al., 1996), showing that the endothelin ET_B receptor has the capability to produce about 70% of the total endothelin-1-induced contraction in segments cultured for 5 days. Further, several other vessels, such as rat superior mesenteric artery, femoral artery and femoral vein, share this ability to evoke endothelin ET_B receptor-induced contraction after organ culture (unpublished observations). Both this and a recent study (Adner et al., 1996) showed a more intense signal from the RT-PCR products for the endothelin ET_B receptor than for the endothelin ET_A receptor in the cultured arteries. This difference was not seen in fresh segments and since our study was not quantitative, the difference in the ratio of mRNA from cultured arteries may indicate a decrease of endothelin ET_A receptor mRNA, an increase of endothelin ET_B receptor mRNA, or both. In accordance with results of the pharmacological analysis in the earlier study that demonstrated the presence of endothelin ET_A receptor after 5 days' culture (Adner et al., 1996), we now suggest that the relatively higher amount of endothelin ET_B receptor mRNA reflects a true increase due to de novo transcription. Thus, the increase in endothelin ET_B receptor mRNA in the human omental arteries, together with the increased contractile effect induced by the endothelin ET_B receptor in the rat mesenteric branch, suggest that there is an upregulation of smooth muscle contractile endothelin ET_B receptors in cultured arteries. However, this remains to be confirmed by binding studies.

If fresh arteries are cultured in the presence of the transcriptional inhibitor, actinomycin D, the contraction induced by sarafotoxin 6c is almost completely abolished after 1 day of organ culture. This suggests that the sarafotoxin 6c-induced contraction after 1 day of culture was mediated by newly transcribed and synthesized endothelin ET_B receptors, in accordance with the hypothesis of receptor upregulation. Even though the contractile capacity for the vessel (as measured by the K^+-induced contraction) seemed to be unaffected by the treatment with actinomycin D, it is possible that the attenuated effect of sarafotoxin 6c was due to a decrease of special mediators in the intracellular signal transduction system for the endothelin ET_B receptor. However, even though the mRNA for the receptors in human omental arteries was not quantitated, the absence of detectable signals from the PCR after 30 cycles indicates that the most likely reason for the negligible effect of sarafotoxin 6c on actinomycin D-treated rat mesenteric arterial segments is blunted transcription of the receptor. The visible band for both receptors after 40 cycles of PCR in arteries treated with actinomycin D may represent a remainder of mRNA which had not yet been degraded. In segments cultured for the same time period in the presence of the translational inhibitor, cyclohexamide, there was a similar, but smaller, reduction of the contractile effect induced by sarafotoxin 6c, leaving the K^+-induced contraction unaffected. Also, this effect could have resulted from a decrease in the endothelin ET_B receptor-associated second messenger system. However, since the decrease in the sarafotoxin 6c-mediated contraction after treatment with actinomycin D seems to have been caused by decreased transcription of the endothelin ET_B receptor, it is more likely that the reduced effect in segments treated with cyclohexamide was due to inhibition of subsequent translation of the receptor.

In contrast to that in the arteries cultured for 1 day, the effect of cyclohexamide was more pronounced than that of actinomycin D in arteries cultured for 2 days. This may have been the result of the increased amount of the mRNA for the endothelin ET_B receptor after 1-day culture without treatment not having disappeared immediately after expo-

sure to actinomycin D and still having been translated into new receptors during a certain period. Thus, cyclohexamide, which was less effective than actinomycin D, can possibly decrease the ongoing translational process immediately and may therefore be more effective in this situation.

The smaller decrease in the endothelin-1 receptor-mediated contraction after treatment with actinomycin D or cyclohexamide can be explained by the fact that endothelin ET_A receptors are normally more abundant in the smooth muscle membrane (Davenport et al., 1995) and that the decrease results from a normal turnover rate and abolished production of newly synthesized receptor. Even if the effect of endothelin ET_A receptors is close to the maximum contractile effect of the vessel, as shown by the addition of noradrenaline on top of the endothelin-1 contraction in fresh arteries, it is possible that the endothelin-1-induced contraction on day 1 is due to a summed effect of both endothelin ET_A and endothelin ET_B receptors. Therefore the decreased contraction in treated segments could be due to absence of endothelin ET_B receptors. In contrast, application of actinomycin D or cyclohexamide during the second day of organ culture did not affect the endothelin-1-induced contraction. This may be because endothelin-1, with equal binding affinity to endothelin ET_A and endothelin ET_B receptors (Masaki et al., 1994) activates both receptors, each possessing a contractile effect near the maximum of the vessel at day 2. Unfortunately, how actinomycin D or cyclohexamide affects the endothelin ET_A receptor cannot be understood from the results of our organ culture assay, since no selective endothelin ET_A receptor agonist yet exists.

The RT-PCR results in several studies, even though not quantitative, have yielded similar strong signals for both the endothelin ET_A and endothelin ET_B receptor of different human vascular regions (Adner et al., 1996; Davenport et al., 1995; Nilsson et al., 1997; Opgaard et al., 1996). This reflects neither autoradiography results, showing 85% binding of endothelin ET_A receptors and 15% of endothelin ET_B receptor in human vascular smooth muscle cells (Davenport et al., 1995), nor results of functional studies, showing negligible endothelin ET_B receptor-mediated contraction (Adner et al., 1996; Maguire and Davenport, 1995; Nilsson et al., 1997; Opgaard et al., 1996). However, our data further suggest that the amount of mRNA of the endothelin ET_A receptor exceeds the amount for the endothelin ET_B receptors in ordinary fresh arteries, whereas the mRNA for the endothelin ET_B receptor is more prominent in cultured arteries. The estimated high level of mRNA for the endothelin ET_B receptor after 1 day may indicate rapid and effective transcription and, thus, may be increased in conditions of environmental stress. This could be reflected by the upregulation of endothelin ET_B receptors in arteries exposed to increased flow (Barber et al., 1996) and by the different pathological states which have been shown to be associated with upregulation of endothelin ET_B receptors (Roux et al., 1995; Cannan et al., 1996; Dagassan et al., 1996; Wenzel et al., 1996). The exact nature of the initiation of the increased transcription of the endothelin ET_B receptor is not known. Even if the promoter regions of the two receptors are different there are no major differences which could explain this phenomenon (Arai et al., 1993). Thus, future investigations have to be performed in order to understand the process of regulation of endothelin receptors.

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References

Abrol, R.P., Hughes, W.M., Krueger, G.A., Cook, D.A., 1984. Detection of endothelium in cerebral blood vessels. J. Pharmacol. Methods 12, 213–219.

Adner, M., Erlinge, D., Nilsson, L., Edvinsson, L., 1995. Upregulation of a non-ET_A receptor in human arteries in vitro. J. Cardiovasc. Pharmacol. 26 (Suppl. 3), S314–S316.

Adner, M., Cantera, L., Ehlert, F., Nilsson, L., Edvinsson, L., 1996. Plasticity of contractile endothelin-B receptors in human arteries after organ culture. Br. J. Pharmacol. 119, 1159–1166.

Altschul, F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Arai, H., Hori, S., Aramori, I., Ohkubo, H., Nakanishi, S., 1990. Cloning and expression of a cDNA encoding an endothelin receptor. Nature 348, 730–732.

Arai, H., Nakao, K., Takaya, K., Hosoda, K., Ogawa, Y., Nakanishi, S., Imura, H., 1993. The human endothelin-B receptor gene. J. Biol. Chem. 268, 3363–3470.

Barber, D.A., Michener, S.R., Ziesmer, S.C., Miller, V.M., 1996. Chronic increases in blood flow upregulate endothelin-B receptors in arterial smooth muscle. Am. J. Physiol. 39, H65–H71.

Cannan, C.R., Burnett, J.C., Lerman, A., 1996. Enhanced coronary vasoconstriction to endothelin-B-receptor activation in experimental congestive heart failure. Circulation 93, 646–651.

Chaley-Campell, J., Campell, G.R., Ross, R., 1979. The smooth muscle cell in culture. Physiol. Rev. 59, 2–61.

Dagassan, P.H., Breu, V., Clozel, M., Kunzli, A., Vogt, P., Turina, M., Kiowski, W., Clozel, J.P., 1996. Up-regulation of endothelin-B receptors in atherosclerotic human coronary arteries. J. Cardiovasc. Pharmacol. 27, 147–153.

Davenport, A.P., O'Reilly, G., Kuc, R.E., 1995. Endothelin ET_A and ET_B mRNA and receptors expressed by smooth muscle in the human vasculature: majority of the ET_A sub-type. Br. J. Pharmacol. 114, 1110–1116.

De Mey, J.G.R., Uitendaal, M.P., Boonen, H.C.M., Vrijdag, M.J.J.F., Daemen, M.J.A.P., Struyker-Boudier, H.A.J., 1989. Acute and long-term effects of tissue culture on contractile reactivity in renal arteries of the rat. Circ. Res. 65, 1125–1135.

De Mey, J.G.R., Uitendaal, M.P., Boonen, H.C.M., Schiffers, P.M.H., Fazzi, G.E., 1991. Growth responses in elastic, muscular and resistance arterial segments of the rat. Blood Vessels 28, 372–385.

Deng, L.-Y., Li, J.-S., Schiffrin, E.L., 1995. Endothelin receptor subtypes in resistance arteries from humans and rats. Cardiovasc. Res. 29, 532–535.

Ekelund, U., Adner, M., Edvinsson, L., Mellander, S., 1994. Effects of selective ET_B-receptor stimulation on arterial, venous and capillary functions in cat skeletal muscle. Br. J. Pharmacol. 112, 887–894.

Haynes, W.G., Strachan, F.E., Webb, D.J., 1995. Endothelin ET(A) and ET(B) and receptors cause vasoconstriction of human resistance and capacitance vessels in vivo. Circulation 92, 357–363.

Hosoda, K., Nakao, K., Tamura, N., Arai, H., Ogawa, Y., Suga, S., Nakanishi, S., Imura, H., 1992. Organization, structure, chromosomal assignment, and expression of the gene encoding the human endothelin-A receptor. J. Biol. Chem. 267, 18797–18804.

Maguire, J.J., Davenport, A.P., 1995. ET(A) receptor-mediated constrictor responses to endothelin peptides in human blood vessels in vitro. Br. J. Pharmacol. 115, 191–197.

Masaki, T., Vane, J.R., Vanhoutte, P.M., 1994. V. International Union of Pharmacology nomenclature of endothelin receptors. Pharmacol. Rev. 46, 137–142.

Mauger, J.P., Worcel, M., Tassin, J., Courtois, Y., 1975. Contractility of smooth muscle cells of rabbit aorta in tissue culture. Nature 255, 337–338.

Menzo, S., Bagnarelli, P., Giacca, M., Manzin, A., Varaldo, P.E., Clementi, M., 1992. Absolute quantitation of viremia in human immunodeficiency virus infection by competitive reverse transcription and polymerase chain reaction. J. Clin. Microbiol. 30, 1752–1757.

Moreland, S., McMullen, D., Abboa-Offei, B., Seymour, A., 1994. Evidence for a differential location of vasoconstrictor endothelin receptors in the vasculature. Br. J. Pharmacol. 112, 704–708.

Nilsson, T., Cantera, L., Adner, M., Edvinsson, L., 1997. Presence of contractile endothelin-A and dilatory endothelin-B receptors in human cerebral arteries. Neurosurgery 40, 346–353.

Opgaard, O.S., Cantera, L., Adner, M., Edvinsson, L., 1996. Endothelin-A and -B receptors in human coronary arteries and veins. Regul. Pept. 63, 149–156.

O'Reilly, G., Charnock-Jones, D.S., Davenport, A.P., Cameron, I.T., Smith, S.K., 1992. Presence of messenger ribonucleic acid for endothelin-1, endothelin-2, and endothelin-3 in human endometrium and a change in the ratio of ET_A and ET_B receptor subtype across the menstrual cycle. J. Clin. Endocrinol. Metab. 75, 1545–1549.

Rasband, W.S., Bright, D.S., 1995. NIH-Image: a public domain image processing program for the Macintosh. Microbeam Anal. Soc. J. 4, 137–149.

Roux, S., Löffler, B.M., Gray, G.A., Sprecher, U., Clozel, M., Clozel, J.P., 1995. The role of endothelin in experimental cerebral vasospasm. Neurosurgery 37, 78–85.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., Masaki, T., 1990. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 348, 732–735.

Sakurai, T., Yanagisawa, M., Masaki, T., 1992. Molecular characterization of endothelin receptors. Trends Pharmacol. Sci. 13, 103–108.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schramek, H., Sorokin, A., Watson, R.D., Dunn, M.J., 1995. Endothelin-1 and PDGF BB induce MEK mRNA and protein expression in mesangial cells. J. Cardiovasc. Pharmacol. 26, S95–S99.

Sumner, M.J., Cannon, T.R., Mundin, J.W., White, D.G., Watts, I.S., 1992. Endothelin ET_A and ET_B receptors mediate vascular smooth muscle contraction. Br. J. Pharmacol. 107, 858–860.

Wenzel, R.R., Duthiers, N., Noll, G., Bucher, J., Kaufmann, U., Lüscher, T.F., 1996. Endothelin and calcium antagonists in the skin microcirculation of patients with coronary artery disease. Circulation 94, 316–322.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T., 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411–415.