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Cytokines induce increased endothelin ET_B receptor-mediated contraction

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Abstract

The effect of cytokines on the induction of contractile endothelin ET_B receptors during organ culture was examined. Ring segments of rat superior mesenteric artery were used fresh or incubated for 24 h in Dulbecco's modified Eagle's medium alone, or with either interleukin-1β, tumor necrosis factor-α (TNF-α) or interleukin-2. In fresh arterial segments there was no endothelin ET_B receptor-induced contraction. After incubation, the selective endothelin ET_B receptor agonist sarafotoxin 6c evoked a contraction of $22 \pm 6\%$ relative to that induced by 60 mM K^+. The endothelin ET_B receptor-induced contraction was further increased to $125 \pm 25\%$ and $157 \pm 29\%$ by interleukin-1β and TNF-α, respectively, while interleukin-2 did not alter the endothelin ET_B receptor-induced contraction. The identity of the contractile receptor was confirmed as the endothelin ET_B receptor by the use of an additional specific endothelin ET_B receptor agonist, IRL 1620, and by antagonist experiments with FR 139317 and IRL 2500. The endothelin-1-induced contraction was not altered by either of the cytokines. Reverse transcriptase–polymerase chain reaction revealed increased levels of endothelin ET_B mRNA, relative to endothelin ET_A mRNA following organ culture, suggesting that contractile endothelin ET_B receptors appear via de novo transcription. None of the cytokines changed the ratio of endothelin ET_A and endothelin ET_B receptor mRNA, indicating that the further increased sarafotoxin 6c-induced contraction is mediated through an enhancement of intracellular signalling mechanisms.

Keywords: Interleukin-1β; TNF-α (tumor necrosis factor-α); Interleukin-2; Endothelin-1; Sarafotoxin 6c; IRL 1620; Endothelin ET_B receptor

1. Introduction

Endothelin-1 is the most potent vasoconstrictor described to date (Yanagisawa et al., 1998). Its action in mammals is mediated through two distinct G-protein coupled receptors: endothelin ET_A and ET_B receptors (Arai et al., 1990; Sakurai et al., 1990). The endothelin ET_A receptor is present in the smooth muscle cell layer, mediates vasoconstriction and may contribute to the maintenance of vascular tone (Haynes and Webb, 1994). The endothelin ET_B receptor was initially thought to be present exclusively in the endothelium where it induces a transient reduction in vascular tone via release of nitric oxide or prostacyclin (De Nucci et al., 1988; Lodge et al., 1995). Lately, endothelin ET_B receptors have been shown to mediate a contractile smooth muscle response in some vascular regions, mainly in low pressure systems such as veins (Bigaud and Pelton, 1992; Gardiner et al., 1994; Lodge et al., 1995). Plasticity of smooth muscle cells expressing endothelin ET_B receptors have been shown in several pathological conditions: in healthy individuals, the subcutaneous and coronary arteries express contractile endothelin ET_A receptors but not contractile endothelin ET_B receptors (Wenzel et al., 1994; Dagassan et al., 1996). However, in the subcutaneous arteries of patients with coronary artery disease as well as in coronary arteries of patients with atherosclerosis, contractile endothelin ET_B receptors have been found (Dagassan et al., 1996; Wenzel et al., 1996). In addition, upregulation of endothelin ET_B receptors appear after experimental subarachnoid hemorrhage (Roux et al., 1995).

The phenomenon of inducible receptors as a response to pathological or altered physiological conditions has been described to be modulated by inflammatory mediators (Hakonarson et al., 1996; Donaldsson et al., 1997). Inter-

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leukin-1β has been implicated in the altered response of the bradykinin B_1 receptor and of β-adrenoceptors. Interleukin-1β is thought to participate in the activation of acute phase responses (Dinarello et al., 1996). Since both endothelin ET_A and ET_B receptors have binding sites for acute phase reaction regulatory elements in their promotor regions, the molecular prerequisites for receptor regulation by cytokines are present (Arai et al., 1993).

In a previous study, it was observed that de novo expression of the endothelin ET_B receptor can be elicited in human omental arteries following organ culture (Adner et al., 1996). In the present study, we have examined whether the inflammatory mediators interleukin-1β, tumor necrosis factor-α (TNF-α) and interleukin-2 may affect endothelin-1 and the selective endothelin ET_B receptor agonist, sarafotoxin 6c-induced responses in isolated ring segments of the rat superior mesenteric artery and if a changed response is regulated at the transcriptional level as studied by reverse transcriptase–polymerase chain reaction (RT–PCR).

2. Materials and methods

2.1. Tissue preparation and organ culture procedure

The rat superior mesenteric artery was chosen since a previous study has shown that this vessel moderately upregulates endothelin ET_B receptors following 24 h of incubation with Dulbecco's modified Eagle's medium (DMEM) (Adner et al., 1998b). Male Wistar–Kyoto rats (250–300 g, Möllegården, Denmark) were anaesthetized with CO_2 and killed by exsanguination. The animal experiments were approved by the Lund University Animal Ethics Committee. The superior mesenteric artery was removed, dissected free from adherent tissue and cut into 1 mm long circular segments. Each segment was either used immediately or placed in a well containing 1 ml of DMEM, supplemented with penicillin and streptomycin, and incubated for 24 h at 37°C in humidified 5% CO_2 in air. Test segments were incubated with interleukin-1β (10 and 100 ng/ml), TNF-α (10 and 100 ng/ml) or interleukin-2 (100 and 200 ng/ml).

2.1.1. Removal of the endothelium

In separate experiments the endothelium was removed with a 10 s infusion of Triton X-100 0.1% v/v (Sigma, St. Louis, USA). The segments were incubated after removal of the endothelium. The functional integrity of the vessel segments was assessed prior to the experiment by obtaining a concentration–response curve to acetylcholine, 0.01 μM to 1 μM, after pre-contraction of the artery with noradrenaline 10 μM.

2.2. In vitro pharmacology

After incubation the segments were immersed in temperature-controlled (37°C) tissue baths containing a bicarbonate buffer solution. The solution was continuously aerated with 5% CO_2 in O_2 resulting in a pH of 7.4. Each

Table 1
Contractile responses for sarafotoxin 6c, IRL 1620 and endothelin-1 in segments cultured 1 day in interleukin-1β, TNF-α and interleukin-2

Sarafotoxin 6c	*n*	K^+ (mN) ± S.E.M.	E_{max} (mN) ± S.E.M.	E_{max} (% of K^+) ± S.E.M.	pEC_{50} ± S.E.M.
Fresh	6	6.16 ± 1.00[a]	0.12 ± 0.07[a]	2 ± 1[a]	N.D.
Control	8	4.16 ± 0.37	0.86 ± 0.22	22 ± 6	9.19 ± 0.18
Interleukin-1, 10 ng/ml	6	4.43 ± 0.28	3.33 ± 1.30[a]	76 ± 29[a]	8.75 ± 0.09
Interleukin-1, 100 ng/ml	6	3.70 ± 0.59	4.05 ± 0.88[a]	121 ± 25[a]	9.15 ± 0.27
TNF-α, 10 ng/ml	6	2.02 ± 0.22[a]	1.97 ± 0.86	112 ± 55	8.71 ± 0.16
TNF-α, 100 ng/ml	6	2.35 ± 0.44[a]	3.68 ± 0.98[a]	157 ± 29[a]	8.71 ± 0.24
Interleukin-2, 100 ng/ml	7	2.84 ± 0.36[a]	0.65 ± 0.15	25 ± 7	8.94 ± 0.18
Interleukin-2, 200 ng/ml	6	3.11 ± 0.58	0.40 ± 0.15	18 ± 10	9.03 ± 0.12
IRL 1620					
Control	6	2.70 ± 0.21	1.35 ± 0.47	56 ± 21	8.83 ± 0.19
Interleukin-1, 100 ng/ml	6	2.38 ± 0.52	2.82 ± 0.70[a]	133 ± 25[a]	8.41 ± 0.07
TNF-α, 100 ng/ml	6	2.17 ± 0.29	4.24 ± 0.51[a]	204 ± 22[a]	8.36 ± 0.08
Interleukin-2, 200 ng/ml	6	2.65 ± 0.33	1.23 ± 0.35	45 ± 9	9.18 ± 0.40
Endothelin-1					
Fresh	6	4.97 ± 0.99	8.89 ± 0.92	193 ± 22	8.31 ± 0.09
Control	6	2.87 ± 0.49	6.88 ± 1.14	241 ± 10	8.53 ± 0.11
Interleukin-1, 100 ng/ml	6	3.86 ± 0.42	9.11 ± 0.71	242 ± 16	8.59 ± 0.19
TNF-α, 100 ng/ml	6	2.19 ± 0.27	7.27 ± 0.66	347 ± 35[a]	8.48 ± 0.28
Interleukin-2, 200 ng/ml	6	2.94 ± 0.30	7.65 ± 0.93	263 ± 25	8.26 ± 0.06

[a] Denotes statistical significance [$P < 0.05$] vs. respective controls.

segment was mounted on two L-shaped prongs, one of which was attached to a Grass FT-03 transducer (Grass Instr., Quincy, USA) connected to a MacLab unit (ADInstruments, Hastings, UK) for continuous recording of isometric tension. A tension of 2 mN was applied to each segment and these were allowed to stabilise at this tension for one h. Subsequently, they were exposed to a K^+-rich (60 mM) buffer solution with the same composition as the standard solution except that NaCl was replaced by an equimolar concentration of KCl. The K^+-induced contraction was used as a reference for the contractile capacity, and the segments used only if K^+ elicited a reproducible response over 0.5 mN. Concentration–response curves for the agonists were obtained by cumulative application of the peptides. There were no differences in the response to sarafotoxin 6c or endothelin-1 when concentration-response curves obtained by cumulative application were compared to those obtained by a single dose procedure. Only one concentration–response curve was performed on each segment in order to avoid any possible tachyphylaxis. In the antagonist experiments, FR 139317 and IRL 2500 were tested at 1 μM, and were added 15–20 minutes (min) before agonists. In the dilatation experiments, the arteries were pre-contracted with 10 μM noradrenaline, and the concentration response curves were obtained once a steady state was reached.

2.3. Molecular biology

2.3.1. RNA-extraction

Vessel segments were snap-frozen in liquid nitrogen immediately after acquisition or organ culture and total cellular RNA was extracted using TRIzol reagent (Gibco BRL, Paisley, Scotland) following the supplier's instructions. The resulting RNA pellet was finally washed with 70% ice-cold ethanol, air-dried and redissolved in 10 μl diethyl-pyrocarbonate-treated water. The amount of RNA was determined by use of spectrophotometry (DU64 spectrophotometer; Beckman, Fullerton, CA, USA) considering an $OD_{260:280}$ ratio of > 1.6 as pure.

2.3.2. Reverse transcriptase–polymerase chain reaction

RT–PCR was carried out using the GeneAmp RNA PCR kit (Perkin-Elmer, Foster City, CA, USA) on a DNA Thermal cycler (Perkin-Elmer). Specific primers for the rat endothelin ET_A- (generating a 264 basepair product) and ET_B receptors (generating a 560 basepair product) were designed as follows:

ET_A-receptor	forward	5′-TACAAGGGCGAG-CAGCACAGGA-3′
	reverse	5′-CACAGGGCGAAG-ATGACAACCAA-3′
ET_B-receptor	forward	5′-TGACGCCACCCA-CTAAGAC-3′
	reverse	5′-GACAGCCAGAAC-CACAGAGA-3′

The reverse transcription of total RNA to cDNA and subsequent PCR was carried out with the GeneAmp RNA PCR kit (Perkin Elmer) in a Perkin Elmer DNA Thermal Cycler. First strand cDNA was synthesized from 1 μg total RNA in a 20-μl reaction volume using random hexamers as primers. The reaction mixture was incubated at 25°C for 10 min, 42°C for 15 min, heated to 99°C for 5 min, and chilled to 5°C for 5 min. For each primer pair a 9-μl portion from the resultant cDNA was amplified by PCR in a final volume of 50 μl. The PCR was carried out with the following profile: 5 min at 95°C for 1 cycle, followed by 30 cycles of 1 min at 95°C, 1 min at 57°C and 30 s at 72°C; final extension was done for 7 min at 72°C. A blank (water) was included in all experiments. Control experiments showed that 30 cycles were within the exponential phase of the PCR (not shown). The identity of the PCR products was verified by restriction analysis as follows: the endothelin ET_A-products (264 basepairs) were digested with SpH I (Boehringer Mannheim, Germany), generating three fragments of 27, 78 and 159 basepairs, respectively. The endothelin ET_B-products (560 basepairs) were cleaved with Eco RV (Boehringer), generating two fragments of 106 and 454 basepairs, respectively.

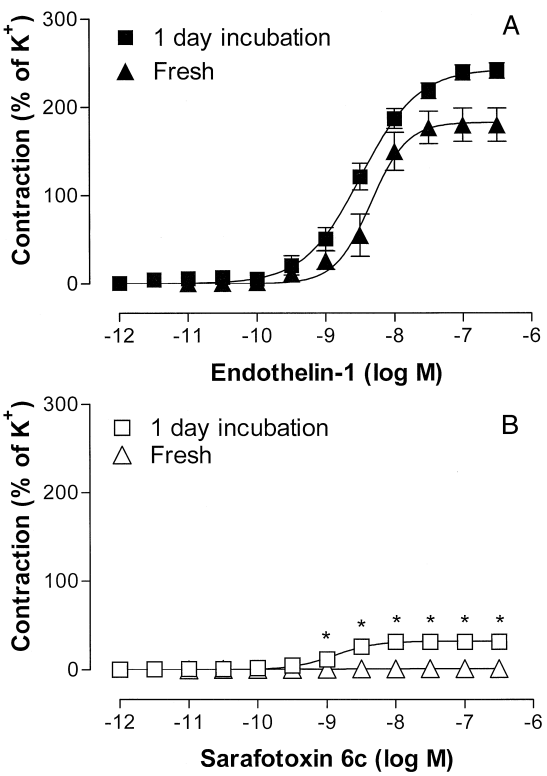

Fig. 1. Contractions induced by cumulative application of endothelin-1 (a) and sarafotoxin 6c (b) in fresh vessels and segments incubated for 24 h. The contraction of each segment was calculated as a percentage of the K^+-induced contraction in the same segment. Each point represents mean ± standard error bars, $n = 6–8$ (* denotes statistical significance [$P < 0.05$] vs. respective fresh vessels).

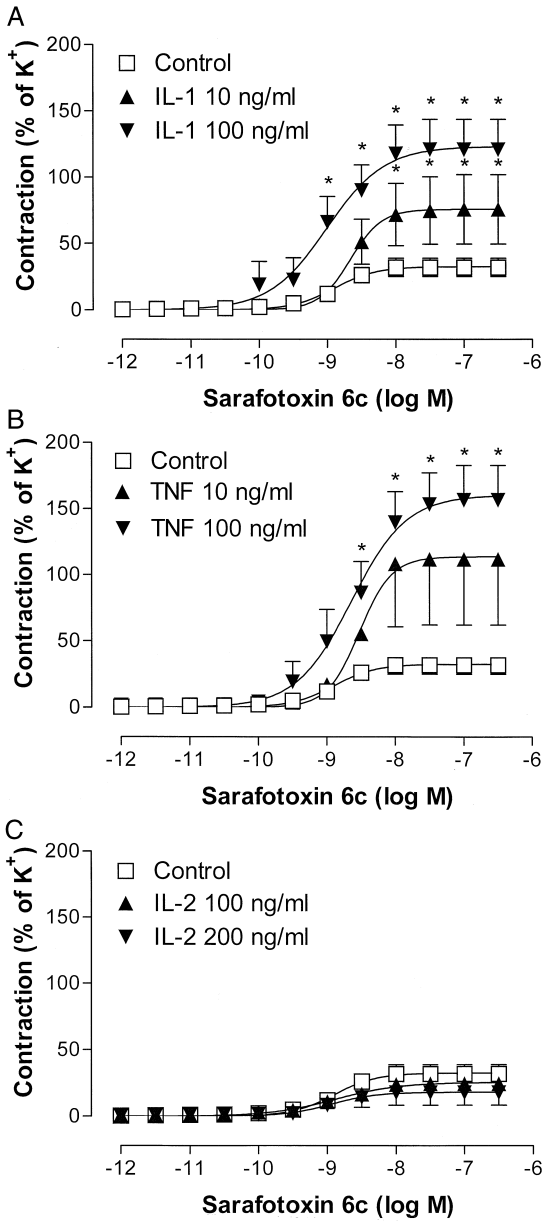

Fig. 2. Concentration–response curves for the sarafotoxin 6c-induced contraction in vessels incubated with (a) interleukin-1β (IL-1), (b) TNF-α and (c) interleukin-2 (IL-2) compared to that of the respective controls. Each point represents mean ± standard error bars, $n = 6–7$ (* denotes statistical significance [$P < 0.05$] vs. respective controls).

2.3.3. Densitometric analysis of PCR products

Densitometric analysis and calculation of the ratio of band densities was essentially performed as described previously (Möller et al., 1997). Briefly, the PCR-products were separated on a 1.5% agarose gel (Sambrook et al., 1989). Photographs of the gels were digitized and analyzed densitometrically with NIH-Image software (v1.60) (Rasband and Bright, 1995). Before calculation of the ratio between endothelin ET_A and 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 (Möller et al., 1997).

2.4. Buffer solutions and drugs

Standard buffer solution (mM): NaCl 119; $NaHCO_3$ 15; KCl 4.6; $MgCl_2$ 1.2; NaH_2PO_4 1.2; $CaCl_2$ 1.5; glucose 5.5. Analytical grade chemicals and distilled water were used for preparing all solutions.

DMEM (Gibco BRL) was supplemented with penicillin (100 U/ml and streptomycin (100 μg/ml) (Gibco BRL). Interleukin-1β, TNF-α, interleukin-2 (R&D systems, Minneapolis, USA), sarafotoxin 6c, endothelin-1 (Auspep, Parkville, Australia), were dissolved in sterile water with bovine serum albumin (0.1% w/v). IRL 1620, Suc[Glu^9, $Ala^{11,15}$]-ET-1(8–21) (Auspep) was dissolved in 0.01 M NH_4OH and further diluted in buffer solution. FR 139317 (Fujisawa Pharmaceuticals, Osaka, Japan) and IRL 2500 (Ciba-Geigy, Takarazuka, Japan) were dissolved in ethanol and methanol, respectively.

2.5. Calculation and statistics

Data are expressed as mean values ± S.E.M. Contractile responses in each segment are expressed as a percentage of the K^+-induced contraction. E_{max} represents the maximal contraction induced by an agonist, and given either in absolute values or expressed as a percentage of the K^+-induced response. The pEC_{50} value was calculated from the line between the concentrations above and below the midpoint of the concentration–response curve. pK_B was calculated using the equation $pK_B = -\log([B]/(r-1))$ in which $[B]$ denotes the concentration of the antagonist and r denotes the ratio of the EC_{50} value of the antagonist and the EC_{50} value of the control experiment. The analysis of

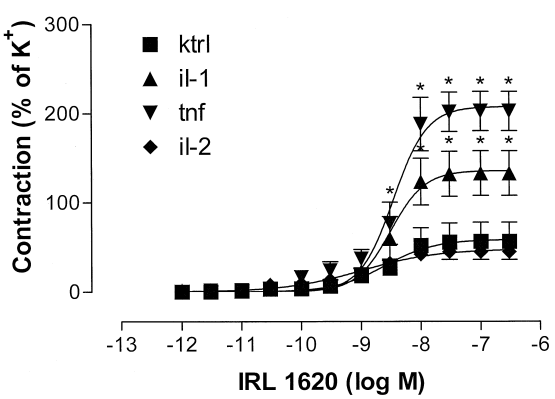

Fig. 3. Concentration–response curves for the IRL 1620-induced contraction in vessels incubated with (a) interleukin-1β (IL-1), (b) TNF-α and (c) interleukin-2 (IL-2) compared to that of the respective controls. Each point represents mean ± standard error bars, $n = 6–7$ (* denotes statistical significance [$P < 0.05$] vs. respective controls).

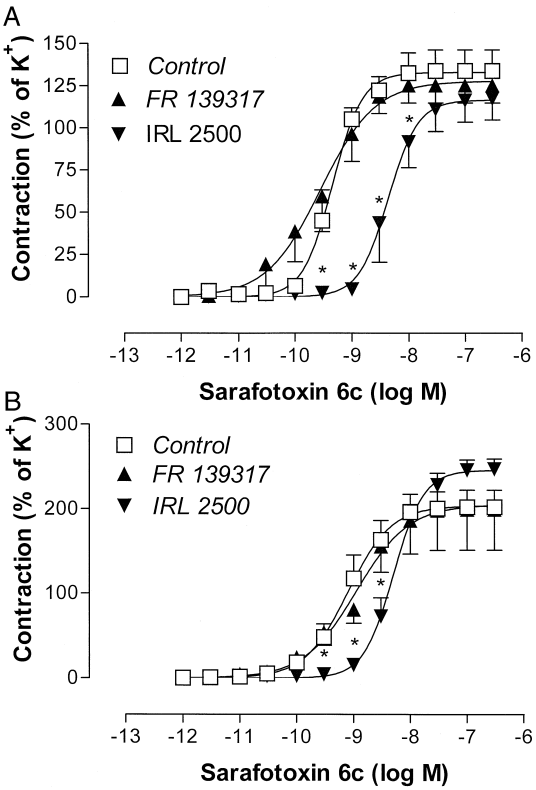

Fig. 4. Concentration–response curves for the sarafotoxin 6c-induced contraction in vessels incubated with interleukin-1β (a) and TNF-α (b) and with either the endothelin ET_A receptor antagonist FR 139317 or the endothelin ET_B receptor antagonist IRL 2500. Each antagonist was added to the bath 15–20 min before the agonists for a concentration of 1 μM. Each point represents mean ± standard error bars, $n = 5–6$ (* denotes statistical significance [$P < 0.05$] vs. respective controls).

variance (ANOVA) factorial test for unpaired data was used in all analyses. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Cytokine effects on sarafotoxin 6c response

In control experiments on fresh vessel segments, sarafotoxin 6c induced a negligible contraction (2 ± 1% of K^+-response). After 1 day of organ culture, there was a significant reduction in the K^+ response (Table 1). In incubated segments, sarafotoxin 6c induced a contraction of 22 ± 6% compared to the K^+ response (Fig. 1b).

Rat mesenteric arteries incubated for 24 h with 10 ng/ml of interleukin-1β revealed an increase in E_{max} response to sarafotoxin 6c, whereas vessels incubated with 100 ng/ml interleukin-1β exhibited an even stronger increase in E_{max} (Fig. 2a, Table 1). There were no significant shifts of the pEC_{50} values of the concentration–response curves following organ culture with interleukin-1β.

Segments treated with 100 ng/ml TNF-α showed an increase in the contractile effect (E_{max}) for sarafotoxin 6c (Fig. 2b), without any increase in the pEC_{50} value. The lower concentration of TNF-α (10 ng/ml) did not significantly alter the sarafotoxin 6c concentration response curve. However, TNF-α also caused a significant decrease in the response to 60 mM K^+ (Table 1). The increased contractile response to sarafotoxin 6c in the presence of 100 ng/ml TNF-α was significant both when analysed as a percentage of the K^+-induced response and in absolute mN values.

Arteries treated with 200 ng/ml of interleukin-2, but not with 100 ng/ml showed a nonsignificant lower E_{max} as compared to control (Table 1). There was also a reduction in the 60 mM K^+ response, and thus no significant difference when the results were compared as a percentage of the K^+ response (Fig. 2c). There was no change in the pEC_{50} of sarafotoxin 6c in interleukin-2-treated segments.

3.2. Pharmacological characterisation of contractile responses

The effects of cytokines was also studied using another endothelin ET_B receptor agonist, IRL 1620. The cytokines interleukin-1β and TNF-α (100 ng/ml) both induced significant increases in the IRL 1620-induced contraction. Neither of the cytokines altered the pEC_{50} values of IRL 1620 (Fig. 3, Table 1).

To further examine the identity of the receptors mediating the sarafotoxin 6c-induced contraction, experiments were made with the selective endothelin ET_A receptor

Table 2
Contractile responses for sarafotoxin 6c in segments cultured 1 day in interleukin-1β, TNF-α, with either FR 139317 or IRL 2500 added 20 min before the experiment

Sarafotoxin 6c	*n*	K^+ (mN) ± S.E.M.	E_{max} (mN) ± S.E.M.	E_{max} (% of K^+) ± S.E.M.	pEC_{50} ± S.E.M.
Interleukin-1 control	5	4.00 ± 0.33	5.33 ± 0.58	134 ± 12	9.40 ± 0.12
FR 139317, 6 M	5	3.89 ± 0.56	4.98 ± 1.01	125 ± 10	9.55 ± 0.36
IRL 2500, 6 M	5	4.40 ± 0.75	4.93 ± 0.47	117 ± 13	8.33 ± 0.13[a]
TNF-α Control	6	2.70 ± 0.24	5.40 ± 0.55	203 ± 20	9.06 ± 0.20
FR 139317, 6 M	6	2.41 ± 0.52	4.82 ± 1.31	200 ± 48	9.21 ± 0.24
IRL 2500, 6 M	6	2.43 ± 0.26	6.92 ± 0.54	247 ± 12	8.28 ± 0.08[a]

[a] Denotes statistical significance [$P < 0.05$] vs. respective controls.

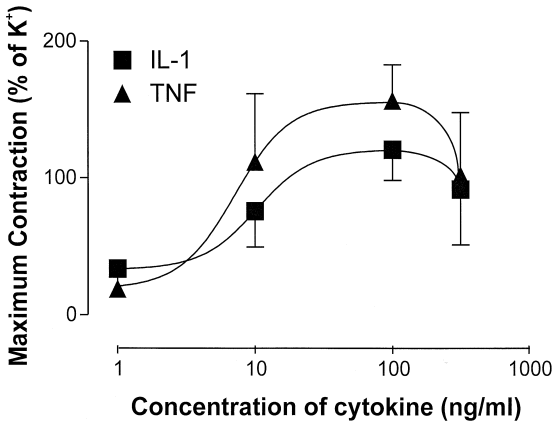

Fig. 5. Concentration–response curves for the maximal contractions induced by sarafotoxin 6c on vessels incubated with 1–300 n/ml interleukin-1β and TNF-α. Each point represents mean ± standard error bars, $n = 3–6$.

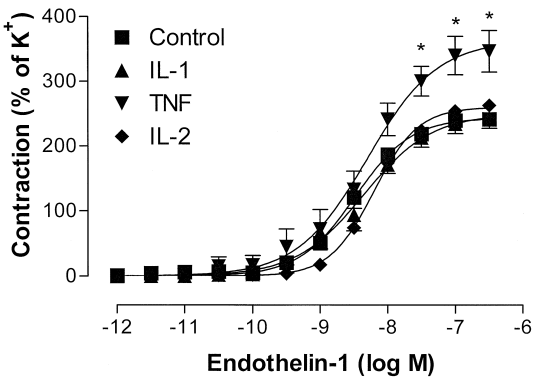

Fig. 6. Concentration–response curves for the endothelin-1-induced contraction in vessels incubated with interleukin-1β, TNF-α and interleukin-2 compared to that of controls. Each point represents mean ± standard error bars, $n = 6$.

antagonist FR 139317 and the selective endothelin ET_B receptor antagonist IRL 2500, on vessels incubated with interleukin-1β and TNF-α. Neither of the antagonists showed any contraction by themselves, in the concentrations used, nor did they affect the E_{max} of the concentration–response curves. Both the arteries incubated with interleukin-1β and TNF-α showed a parallel rightward-shift in their concentration–response curves, when blocked with IRL 2500 (Fig. 4, Table 2), compared to controls. FR 139317 did not cause any shift of the sarafotoxin 6c concentration–response curve. The calculated pK_B for IRL 2500 was 7.01 ± 0.22 for arteries incubated with interleukin-1β and 6.59 ± 0.31 for arteries incubated with TNF-α.

3.3. Concentration–response curves for interleukin-1β and TNF-α

Experiments were made with 1 ng/ml to 300 ng/ml ($n = 3–6$) of interleukin-1β and TNF-α to create concentration–response curves for the E_{max} values induced by sarafotoxin 6c (Fig. 5). Both concentration–response curves reached a maximum at 100 ng/ml and then the sarafotoxin 6c E_{max} decreased at the highest concentration studied. EC_{50} values were calculated to 8.79 ± 0.41 ng/ml (0.51 $\pm$ 0.024 nM) and 8.38 ± 0.69 ng/ml (0.49 $\pm$ 0.04 nM) for interleukin-1β and TNF-α, respectively. The difference was not significant.

3.4. Dilatation experiments

There was no dilatory effect of sarafotoxin 6c in noradrenaline pre-contracted vessel segments neither in controls nor after incubation with cytokines ($n = 5$, not shown). None of the cytokines affected the noradrenaline precontraction.

3.5. Cytokine effects on de-endothelialized arteries

While the procedure caused somewhat lower contractile responses to K^+, there was still an increase in E_{max} after incubation with interleukin-1β and TNF-α in de-endothelialized arteries, which was only significant for interleukin-1β (Table 3). As before, a decreased K^+ response was seen in arteries incubated with TNF-α. There was no significant change in the pEC_{50} of the de-endothelialized arteries.

3.6. Cytokine effects on endothelin-1 response

In fresh arterial segments, endothelin-1 induced a strong contraction. Following organ culture there was a signifi-

Table 3
Contractile responses for sarafotoxin 6c and endothelin-1 in de-endothelialized segments cultured 1 day in interleukin-1β, TNF-α and interleukin-2

Sarafotoxin 6c	n	K^+ (mN) ± S.E.M.	E_{max} (mN) ± S.E.M.	E_{max} (% of K^+) ± S.E.M.	pEC_{50} ± S.E.M.	Endothelin-1 (% of K^+) ± S.E.M.
Control	6	2.22 ± 0.37	2.17 ± 0.99	150 ± 98	9.45 ± 0.29	399 ± 137
Interleukin-1, 100 ng/ml	6	2.06 ± 0.37	4.79 ± 0.60[a]	252 ± 37	8.74 ± 0.11	395 ± 48
TNF-α, 100 ng/ml	6	1.16 ± 0.28[a]	3.35 ± 1.00	267 ± 33	8.91 ± 0.21	529 ± 66
Interleukin-2, 200 ng/ml	6	1.33 ± 0.29	1.42 ± 0.28	128 ± 38	9.21 ± 0.36	383 ± 40

[a] Denotes statistical significance [$P < 0.05$] vs. respective controls.

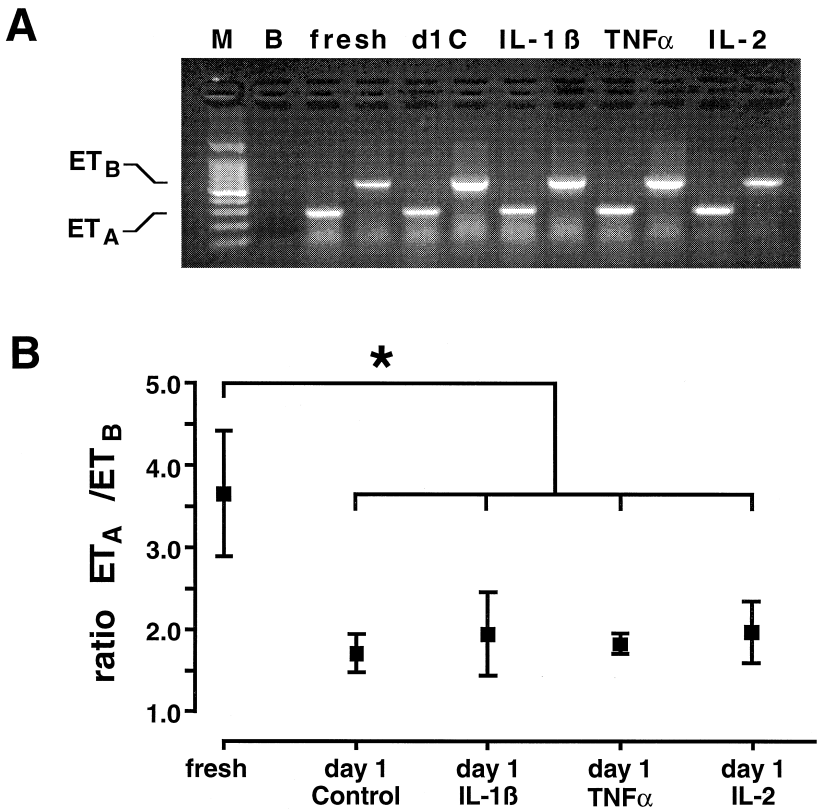

Fig. 7. RT–PCR analysis from RNA which was isolated from fresh rat mesenteric arteries and arteries incubated with cytokines. (a) Gel electrophoresis of RT–PCR products. The sizes of the products were 264 basepairs for endothelin ET_A and 560 basepairs for endothelin ET_B. Lane M: 100 basepairs ladder; lane B: blank (H_2O); lanes d0C: fresh artery; lanes d1C: artery cultured for 1 day; lanes IL-1β: artery cultured for 1 day with 100 ng/ml interleukin-1β; lanes TNF-α: artery cultured for 1 day with 100 ng/ml TNF-α; lanes IL-2: artery cultured for 1 day with 200 ng/ml interleukin-2. (b) Compiled ratios of mRNA for endothelin ET_A and endothelin ET_B receptors. Ratios are calculated as the densitometrically analysed signal for the endothelin ETA receptor product divided by the signal of the endothelin ET_B receptor product. Each point represents mean ± standard error bars, $n = 3$ (* denotes statistical significance [$P < 0.05$] vs. fresh controls).

cantly increased response at two concentrations of endothelin-1, but the E_{max} and pEC_{50} values were not affected (Fig. 1a). Incubation with TNF-α caused a significant increase in the E_{max} of endothelin-1, from 241 ± 10% in controls to 347 ± 35% (Fig. 6). However, the K^+-induced response was further decreased at the same time and hence the absolute (mN) values were not significantly altered ($P > 0.05$). Interleukin-1β did not induce any significant increase when compared to the K^+-induced contraction, but the mN-values were higher (Table 1). A single dose experiment was also performed on the de-endothelialised arteries after the sarafotoxin 6c concentration–response curves was performed. In all these experiments there were no significant change in E_{max} or pEC_{50} after incubation with cytokines.

3.7. mRNA for the endothelin ET_A and ET_B receptors

RT–PCR was performed on RNA extracted from fresh arteries, and arteries incubated with, and without cytokines. After 30 cycles of amplification, products of the expected sizes (264 basepairs for endothelin ET_A and 560 basepairs for ET_B receptors) were detected (Fig. 7). In fresh arteries the endothelin ET_A/ET_B ratio was 3.66 ± 0.77. After 1 day of organ culture, this ratio was changed to 1.71 ± 0.23, indicating a relative increase of endothelin ET_B receptor mRNA compared to ET_A. The endothelin ET_A/ET_B ratio for arteries incubated with 100 ng/ml of interleukin-1β, TNF-α and 200 ng/ml interleukin-2 was 1.95 ± 0.51, 1.83 ± 0.12 and 1.97 ± 0.38, respectively. Thus, neither of the cytokines caused any further alteration in the endothelin ET_A/ET_B ratio.

4. Discussion

Arterial segments of rat superior mesenteric artery incubated for 24 h in DMEM demonstrated a de novo contractility to the endothelin ET_B receptor selective agonist sarafotoxin 6c, while there was no increase in endothelin-1-induced contractility. The cytokines, interleukin-1β and TNF-α, caused a significant increase in the E_{max} responses to sarafotoxin 6c and IRL 1620, both in absolute values (mN) and relative to K^+-induced contractions. The identity of the ET_B receptor was confirmed by the antagonist study with IRL 2500 and FR 139317. While organ

culture itself did not alter the endothelin-1 responses, incubation with TNF-α, but not interleukin-1β, caused an increased response, which may be due to the decreased K^+ contraction. Segments incubated with interleukin-2 did not change in responsiveness to endothelin-1 or sarafotoxin 6c. With RT–PCR it was found that organ culture caused an increase of endothelin ET_B mRNA relative to ET_A, but none of the cytokines affected the endothelin ET_A/ET_B mRNA ratio.

The effect of sarafotoxin 6c in control segments after 1 day of organ culture was similar to that reported earlier (Adner et al., 1998b). Thus, after 1 day of organ culture there was a moderate spontaneous increase in the endothelin ET_B receptor-response, with a concomitant relative increase in endothelin ET_B receptor mRNA, suggesting a de novo synthesis of receptors. The spontaneous increase in the endothelin ET_B response and the receptor mRNA after incubation have been shown to be blocked by actinomycin D and cyclohexamide, which block transcription and translation of the receptor, respectively (Möller et al., 1997). It was therefore considered that the endothelin ET_B receptor upregulation is mediated via increased transcription and subsequent translation of endothelin ET_B mRNA. The endothelin ET_B receptor-induced contraction was also studied with IRL 1620. IRL 1620 is believed to have a higher selectivity for the endothelin ET_B receptor than sarafotoxin 6c, having 120 000-fold and 30 000-fold selectivity for binding at the endothelin ET_B receptor compared to the endothelin ET_A receptor, respectively (Williams et al., 1991; Takai et al., 1992). However, sarafotoxin 6c was twice as potent as IRL 1620 in the rat mesenteric artery, comparable to previous findings (Adner et al., 1998a). With IRL 1620 there was a similar increased endothelin ET_B receptor response after incubation with interleukin-1β and TNF-α, as was found with sarafotoxin 6c. Interleukin-2 did not affect the IRL 1620-induced contraction. Furthermore, the characterisation with IRL 2500 resulted in pK_B values in agreement with previous studies (Balwierzak et al., 1995; Adner et al., 1998b).

Incubation with the cytokines interleukin-1β and TNF-α resulted in enhanced maximum responses to sarafotoxin 6c, thus suggesting either upregulation of the endothelin ET_B-receptor or enhanced efficacy. If the increased response was due to an increase in the number of receptors, a leftward-shift in the concentration–response curves after incubation with cytokines would be expected (Black and Leff, 1983). However, since there was no significant increase in potency following culture with either of the cytokines, an increase in the number of receptors is not likely. In addition, there was no change in the endothelin ET_A/ET_B mRNA ratio in arteries incubated with cytokines compared to controls. This agrees well with preliminary results from studies of endothelin receptors in human temporal artery after cytokine administration (unpublished results). Interestingly, the endothelin ET_A/ET_B ratios for both fresh and cultured arteries were considerably higher than those found in human omental arteries (Möller et al., 1997), which is in accordance with a lower endothelin ET_B contractility in rat mesenteric artery.

In contrast, it is likely that incubation with interleukin-1β or TNF-α induces increased endothelin ET_B-receptor responses putatively via increased intracellular mechanisms. Both endothelin ET_A and ET_B receptors have been shown to be linked to more than one G-protein (Eguchi et al., 1993), and mediate contractions via both receptor-coupled and voltage-gated calcium (Ca^{2+}) channels, in addition to an intracellular Ca^{2+} release via inositol triphosphate (Van Renterghem et al., 1988). Thus, one or more of these intracellular pathways may be affected by the cytokines. This phenomenon has to be elucidated by further studies.

There was no dilatory effect of sarafotoxin 6c in the incubated vessels. Furthermore, de-endothelialized arteries did not differ in the sarafotoxin 6c response from their untreated counterparts, indicating that the endothelium does not play a role in the potentiation of endothelin ET_B receptor responses by cytokines. Furthermore, it has been shown that removal of the endothelium does not affect the sarafotoxin 6c upregulation after organ culture (Adner et al., 1998a).

When the concentration–response curves for interleukin-1β and TNF-α were analysed, we noted that the strongest effects of interleukin-1β and TNF-α were observed at a concentration of 100 ng/ml (corresponding to 5800 U/ml, 5.8 nM), and that the response then decreased. Kuo et al. (1997) found a similar peak potency in the ability of TNF-α to attract neutrophils and to induce interleukin-8 production in broncoalveolar lavage after intratracheal administration in vivo.

In the second series of experiments, the effect of incubation with cytokines was examined on the endothelin-1-induced contraction. There was a increase in the endothelin-1 responses relative to K^+ contractions after incubation with TNF-α, probably an effect of the decreased K^+ contraction. Despite the increased sarafotoxin 6c response, we saw neither any increase in E_{max} of endothelin-1, nor a leftward-shift of the concentration–response curve. This may indicate that endothelin-1 at high concentrations evokes maximum possible contraction for the vessel segments through endothelin ET_A receptors, and that coexisting endothelin ET_A receptors mask the effect of endothelin ET_B receptors (Mickley et al., 1997).

It is considered that although interleukin-1β and TNF-α act on different receptors, they trigger biologically indistinguishable effects by activating the same set of transcription factors through a system of mitogen-activated protein kinase kinase kinases (Eder, 1997). Interleukin-2 does not activate this system, which may explain why incubation with interleukin-2 did not result in an increased contractile effect. Furthermore, there was a decrease in the K^+-induced response in all arteries incubated with TNF-α. This decrease does not seem to affect the maximum contraction

induced by endothelin-1, and may be due to that TNF-α is able to impair both resting and hormone-stimulated Ca^{2+} levels in vitro (Yang et al., 1995).

Cytokines have in several studies been shown to induce expression of receptors (Audet et al., 1994; Hakonarson et al., 1996). The best documented case is that of the bradykinin B_1 receptor which has been shown to be upregulated following incubation with interleukin-1β, interferon γ and lipopolysacharides (Donaldsson et al., 1997). Furthermore, interleukin-1β and TNF-α are known to increase the secretion of endothelin-1 from vascular endothelium and renal epithelial cells (Tasaka and Kitazumi, 1994). The combined increment in vascular tone due to the increased endothelin-1 secretion together with an increased effect of the contractile ET_B receptor suggests a functional significance in pathological conditions. Even though less enhancement of the endothelin-1 contraction was seen, there are several other possible actions of additional ET_B receptors, such as mitogenesis (Eguchi et al., 1994) and potentiation of noradrenaline-induced contractions (Kita et al., 1997). This would suggest that treatment with combined endothelin ET_A and ET_B receptor blockers may be of benefit in patients with subarachnoid hemorrhage and coronary artery disease as have been indicated in novel studies (Douglas, 1997).

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References

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.

Adner, M., Geary, G.G., Edvinsson, L., 1998a. Appearance of contractile endothelin-B receptors in rat mesenteric arterial segments following organ culture. Acta Physiol. Scand. 163, 121–129.

Adner, M., Uddman, E., Cardell, L.O., Edvinsson, L., 1998b. Regional variation in appearance of vascular contractile endothelin-B receptors following organ culture. Cardiovasc. Res. 37, 254–262.

Arai, H., Hori, S., Aramori, I., Ohkubo, H., Nakanaishi, S., 1990. Cloning and expression of a cDNA encoding an endothelin receptor. Nature 248, 730–732.

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

Audet, R., Petitclerc, E., Drapeau, G., Rioux, F., Marceau, F., 1994. Further analysis of the upregulation of bradykinin B_1 receptors in isolated rabbit aorta by using metabolic inhibitors. Eur. J. Pharmacol. 271, 551–555.

Balwierzak, J.L., Bruseo, C.V., DelGrande, D., Jeng, A.Y., Savage, P., Shetty, S.S., 1995. Characterization of a potent and selective endothelin-B receptor antagonist, IRL 2500. J. Cardiovasc. Pharmacol 26 (Suppl. 3), S393–S396.

Bigaud, M., Pelton, J.T., 1992. Discrimination between ET_A- and ET_B-receptor-mediated effects of endothelin-1 and [$Ala^{1,3,11,15}$]endothelin-1 by BQ-123 in the anaesthetized rat. Br. J. Pharmacol. 107, 912–918.

Black, J.W., Leff, P., 1983. Operational models of pharmacological agonism. Proc. R. Soc. London 220, 141–162.

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

DeNucci, G., Thomas, R., Dorleans-Juste, P., Antunes, E., Walder, C., Warner, T.D., Vane, J.R., 1988. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. Proc. Natl. Acad. Sci. 85, 9797–9800.

Dinarello, C.A., Cannon, J.G., Mier, J.W., Bernheim, H.A., LoPreste, G., Lynn, D.L., Love, R.N., Webb, A.C., Auron, P.E., Reuben, R.C., Rich, A., Wolff, S.M., Putney, S.D., 1996. Multiple biological activities of human recombinant interleukin 1. J. Clin. Invest. 77, 1734–1739.

Donaldsson, L.F., Hanley, M.R., Villablanca, A.C., 1997. Inducible receptors. Trends Pharmacol. Sci. 18, 171–181.

Douglas, S.A., 1997. Clinical development of endothelin receptor antagonists. Trends Pharmacol. Sci. 18, 408–412.

Eder, J., 1997. Tumor necrosis factor α and interleukin 1 signalling: do MAPPK kinases connect it all?. Trends Pharmacol. Sci. 18, 319–322.

Eguchi, S., Hirata, Y., Imai, T., Marumo, F., 1993. Endothelin receptor subtypes are coupled to adenylate cyclase via different guanyl nucleotide-binding proteins in vasculature. Endocrine 132, 524–529.

Eguchi, S., Hirata, Y., Imai, T., Kanno, K., Marumo, F., 1994. Phenotypic change of endothelin receptor subtype in cultured rat vascular smooth muscle cells. Endocrine 134, 222–228.

Gardiner, S.M., Kemp, P.A., March, J.E., Bennet, T., Davenport, A.P., Edvinsson, L., 1994. Effects of an ET_1-receptor antagonist, FR 139317, on regional haemodynamic responses to endothelin-1 and [$Ala^{11,15}$]Ac-endothelin-1 (6–21) in conscious rats. Br. J. Pharmacol. 112, 477–486.

Hakonarson, H., Herrick, D.J., Serrano, P.G., Grunstein, M.M., 1996. Mechanism of cytokine-induced modulation of β-adrenoceptor responsiveness in airway smooth muscle. J. Clin. Invest. 97 (11), 2593–2600.

Haynes, G.H., Webb, D.J., 1994. Contribution of endogenous generation of endothelin-1 to basal vascular tone. Lancet 344, 852–854.

Kita, S., Matsumara, Y., Tanida, Y., Kusono, T., Chatani, S., Taguchi, Y., Takaoka, M., Morimoto, S., 1997. Platelets enhance contractility in perfused rat mesenteric arteries: involvement of endothelin-1. Eur. J. Pharmacol. 340, 209–215.

Kuo, H.-P., Hwang, K.-H., Lin, H.-C., Wang, C.-H., Lu, L.-C., 1997. Effect of endogenous nitric oxide on tumor necrosis factor-α-induced leukosequestration and IL-8 release in guinea pigs airways in vivo. Br. J. Pharmacol. 122, 103–111.

Lodge, N.J., Zhang, R., Halaka, N.N., Moreland, S., 1995. Functional role of endothelin ET_A and ET_B receptors in venous and arterial smooth muscle. Eur. J. Pharmacol. 287, 279–285.

Mickley, E.J., Gray, G.A., Webb, D.J., 1997. Activation of endothelin ET_A receptors masks constrictor role of endothelin ET_B receptors in rat isolated small mesenteric arteries. Br. J. Pharmacol. 120, 1376–1582.

Möller, S., Edvinsson, L., Adner, M., 1997. Transcriptional regulated plasticity of vascular contractile endothelin ET_B receptors after organ culture. Eur. J. Pharmacol. 329, 69–77.

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, 78–86.

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 receptors. Nature 348, 732–735.

Sambrook, J., Fritsch, E., Maniatis, T. (Eds.), 1989. Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbour Laboratory Press, NY.

Takai, M., Umemura, I., Yamasaki, K., Watakabe, T., Fujitani, Y., Oda, K., Urade, Y., Inui, T., Yamamura, T., Okada, T., 1992. A potent and specific agonist, Suc[Glu^{9}, Ala^{11,15}]-endothelin-1(8–21), IRL 1620, for the ET_B receptor. Biochem. Biophys. Res. Commun. 184, 953–959.

Tasaka, K., Kitazumi, K., 1994. The control of endothelin-1 secretion. Gen. Pharmacol. 25, 1059–1069.

Van Renterghem, C., Vigne, P., Barhanin, J., Schmid-Alliana, A., Frelin, C., Ladzunski, M., 1988. Molecular mechanism of action of the vasoconstrictor peptide endothelin. Biochem. Biophys. Res. Commun. 157, 977–985.

Wenzel, R.R., Noll, G., Lüscher, T.F., 1994. Endothelin receptor antagonists inhibit endothelin in human skin microcirculation. Hypertension 23, 581–586.

Wenzel, R., Noll, G., 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.

Williams, D.L., Jones, K.L., Pettibone, D.J., Lis, E.V., Clineschmidt, B.V., 1991. Sarafotoxin 6c: an agonist which distinguishes between endothelin receptor subtypes. Biochem. Biophys. Res. Commun. 175, 556–561.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yasaki, Y., Goto, K., Masaki, T., 1998. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411–415.

Yang, K.D., Chuen, H.L., Shaio, M.F., 1995. Pentoxifylline augments but does not antagonize TNF-α-mediated neuroblastoma cell differentiation: modulation of calcium mobilization but not cAMP. Biochem. Biophys. Res. Commun. 211, 1006–1014, abstract.