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ENDOTHELIN-1 STIMULATES ITS OWN SYNTHESIS IN HUMAN ENDOTHELIAL CELLS

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SUMMARY We studied whether endothelin-1 (ET-1) would affect its own synthesis. Human umbilical cord vein endothelial cells in methionine-poor culture medium containing [^{35}S] methionine were treated with synthetic ET-1 or ET-3. Immunoprecipitation of ^{35}S-labeled ET-1 was performed with rabbit ET-1 antiserum. ET-1 caused an 40 ± 4 % (mean ± SEM) increase of immunoprecipitable ^{35}S-labeled ET-1 as confirmed by its elution point in reversed phase high power liquid chromatography (HPLC). ET-3 caused a 23 ± 2 % increase in ET-1 concentration. Amplification of cDNA by PCR showed both ET-1 and ET_B receptor mRNAs in human cord vein endothelial cells. We conclude that ET-1 increases its own synthesis in endothelial cells. This suggests a positive autocrine feed-back action of ET-1 on its own synthesis, an effect which is probably mediated by non-specific ET_B receptors.

Endothelin-1 (ET-1) is a powerful vasoconstrictor peptide produced by endothelial cells (1). Three distinct isoforms of endothelin; ET-1, ET-2 and ET-3 have been found by cloning human endothelin genes (2). However, ET-1 is the only isoform secreted by endothelial cells. Endothelin-1 may play an important role in modulating vascular tone. In addition to its vasoconstrictive effect on blood vessels ET-1 stimulates the release of other vasoactive substances such as EDRF and prostacyclin (3-6) from endothelial cells. The mechanism of action of ET-1 in vasculature is unclear, but it may be mediated by two distinct intracellular signal transduction systems; opening of calcium channels and activation of phospholipase C and subsequent phosphoinositol hydrolysis (7-11). Also species and tissue specific activation of phospholipase A2 by ET-1 has been described (12).
The mechanism of production of ET-1 in endothelium is not clear. Stimulated production may be coupled to phosphoinositide turnover pathway (13,14).

Abbreviations used in the text: ET-1 and ET-3 = Endothelin-1 and -3. HPLC = high power liquid chromatography.

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The production of ET-1 is stimulated by substances such as thrombin, angiotensin II, and vasopressin (14,15). Recently it has been shown, that ET-3 stimulates the release of ET-1 by human endothelial cells (16).

In the present study we show that endothelin-1 stimulates its own synthesis in human umbilical cord vein endothelial cell culture.This effect is likely to be mediated by ET_B receptors.

MATERIALS AND METHODS

Cell culture. Endothelial cells were prepared from human umbilical cord veins according to Jaffe et al (17). Veins were cannulated, washed with phosphate buffered saline and treated with 0.5 % collagenase (Sigma, St Louis, MO, USA) in phosphate buffered saline for 15 min in 37 °C water bath. Cells were grown in 0.2 % gelatin (Sigma) coated cell culture flasks (Costar, Cambridge, USA) in Medium 199 (Gibco Laboratories, Inc., Belmont, California, USA) supplemented with 20 % fetal calf serum (Gibco), 20 µg/ml endothelial cell growth supplement (Sigma), 12 U/ml heparin (Sigma), 100 U/ml G-penicillin , 100 µg/ml streptomycin (Gibco) and 2 mM glutamine (Gibco) at 37 °C in humidified 5 % carbon dioxide in air. Medium was changed every other day. Cells were tested for viability with Trypan Blue.

ET-1 synthesis. The synthesis of ET-1 was studied by incorporation of [^{35}S] methionine (Amersham, Buckinghamshire, UK) into ET-1. Endothelial cells were subcultured on 6-well dishes (Nunc, Roskilde, Denmark). At confluence medium was removed, the cells were rinsed with Minimum Essential Medium (MEM, Gibco) and 2 ml of 80 % methionine free MEM supplemented with 30 µCi [^{35} S] methionine/ml were added and the incubation was continued for 24 h. Control, ET-1 (1.25 ng/ml, Peptide Institute, Barnet, UK), or ET-3 (1.25 ng/ml, Peptide Institute), treated cultures were run in parallel. Thrombin (2 U/ml, Sigma), and nitroglycerine (100 µg/ml, Orion, Espoo, Finland) treated cultures were used as a positive and negative controls, respectively. After incubation period the peptides in the media were extracted with Bondelut C18 OH columns (Analytichem International, Harbor City, USA) as described earlier (18) and the eluates were lyophilized. For immunoprecipitation of [^{35}S] ET-1 the lyophilized samples were dissolved in 200 µl of 50 mM phosphate buffer pH 7 containing 1 mM Na_2 EDTA, 0,2 mM cystine, 0,1 g/l merthiolate, 1 g/l Triton X-100 and 1 g/l BSA and incubated at 4 °C overnight with 5 µl of ET-1 antiserum (18) or with 5 µl of normal rabbit serum (nonspecific binding). The ET antiserum used showed 100 % cross-reaction with ET-2 and ET-3, and <0.1% cross reaction with big ET-1 sequences 1-38 and 22-38 (human; Peninsula). The antigen-antibody complexes were precipitated by adding 200 µl of Goat Anti-Rabbit IgG serum (Peninsula). The precipitates were then washed three times, dissolved in phosphate buffer, boiled at 100 °C for 5 min and centrifuged. The supernatant was acidified with trifluoroacetic acid (Fluka, Basel, Switzerland) and subjected to reversed phase HPLC using a spherisorb ODS-2 column (Pharmacia, Upsala, Sweden) and an acetonitrile gradient as described earlier (18). The HPLC fractions were counted using a liquid scintillation counter (LKB, Bromma,Sweden). Counts of nonspecific binding experiments were subtracted.

ET-1 and ET_B receptor mRNA. Reverse transcription (RT) and polymerase chain reaction (PCR): All reagents used for total RNA isolation were molecular biology reagents from Sigma Chem. Co. and for reverse transcription and polymerase chain reaction from Perkin Elmer Cetus (Emeryville, CA, USA). Total RNA from human umbilical cord vein endothelial cells was isolated by a guanidium thiocyanate method (19). For

generation of cDNA 1.0 µg of total RNA template was incubated for 60 min at 37 °C in 20 µl reaction volume containing PCR buffer (50 mM KCl, 10 mM Tris, pH 8.3), 3.5 mM $MgCl_2$, 1 mM of each deoxynucleotide, 2.5 µM oligo d(T) primer, 20 U human placental ribonuclease inhibitor and 50 U murine Maloney leukemia virus reverse transcriptase. The reaction was stopped by incubating at 95 °C for 5 min and was then quick-chilled on ice. For cDNA amplification PCR was performed for 30 cycles. The concentration of specific primers was 0.5 µM and Ampli Taq polymerase 1.25 U, in 50 µl PCR buffer. The specific primers were as follows. ET-1: 5'S primer, nucleotides 157-186, and 3'AS primer nucleotides 592-619 (20). PCR product 463 base pairs (bp). ETB-receptor: 5'S primer, nucleotides 308-327 and 3'AS primer, nucleotides 818-837 (21). PCR product 530 bp. The PCR products were separated by 2 % agarose gel electrophoresis and visualized with ethidium bromide. The identity of the bands was confirmed by Southern blotting using specific [32p] 5' end labeled 27-mer oligonucleotides occurring between the two primers used in the PCR (results not shown).

Statistical evaluation. Student's t-test for grouped observations was used to evaluate statistical signifance of differences.

RESULTS

Synthesis of ET-1. Human umbilical cord vein endothelial cells were incubated with [^{35} S] methionine and cell culture medium then subjected to immunoprecipitation using ET-1 antiserum and to reversed phase HPLC analysis. In HPLC a peak of radioactivity was found with a retention time identical to that of synthetic ET-1 indicating that [^{35} S] methionine had been incorporated into ET-1 (Fig.1).

After incubation of endothelial cells with ET-1 the amount of immunoprecipitable [^{35} S] ET-1 in culture medium was 140 ± 4 % (mean ± SEM) as compared to control ($p < 0.01$)and 123 ± 2 % when the cells were incubated with ET-3 ($p < 0.05$)Table 1. Thrombin increased and

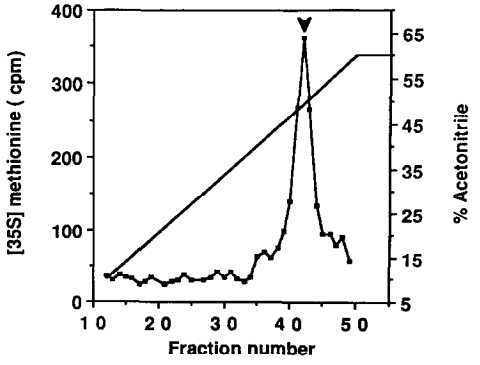

Fig. 1. Reversed phase HPLC of immunoprecipitated [^{35}S] ET-1 synthesized by human umbilical cord vein endothelial cells. Counts of nonspecific binding experiments were subtracted. Elution point of synthetic human ET-1 is indicated by an arrowhead.

Table 1. Effect of treatment with ET-1 and ET-3 on synthesis of immunoprecipitable [^{35}S] methionine labeled ET-1 in cultured human cord vein endothelial cells (mean ± SEM of 4 experiments)

Treatment	Concentration	[^{35}S] labeled ET-1 (% of control)	p-value*
Control	0	100 ± 4	
ET-1	1.25 ng/ml	140 ± 4	< 0.01
ET-3	1.25 ng/ml	123 ± 2	< 0.05

* when compared with control.

nitroglycerine decreased significantly the amount of immunoprecipitable [^{35}S] ET-1 (data not shown).

ET-1 and ET_B-receptor mRNAs. Amplification of cDNA by PCR demonstrated both ET-1 and ET_B-receptor mRNAs in human umbilical cord vein endothelial cells (Fig. 2). The sizes of the amplification bands were as predicted from the genomic maps; 463 bp for ET-1 and 530 for ET_B receptor, respectively.

DISCUSSION

The present study indicates that ET-1 stimulates its own synthesis in human umbilical cord vein endothelial cell culture as detected by incorporation of

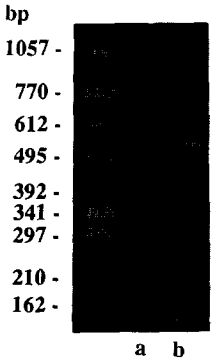

Fig.2. Expression of ET-1 mRNA and ET_B receptor mRNA in primary human umbilical cord vein endothelial cells. mRNA from endothelial cells was amplified by RT-PCR using primers for ET-1 (a) and for ET_B receptors (b). The predicted sizes for the amplification bands were: ET-1, 463 bp; ET_B receptor, 530 bp. The PCR products were separated through agarose gel electrophoresis and visualized by ethidium bromide. The base pair marker is also included in ethidium bromide agarose gel.

the labeled aminoacid precursor, [^{35}S] methionine into ET-1. This implies an autocrine positive feed-back regulation for ET-1 on its own synthesis, a property not ascribed to peptide hormones before. The synthesis of ET-1 was also stimulated by ET-3 and by thrombin and decreased by nitroglycerine as shown earlier (15, 16). We found ET-1 more potent than ET-3 in stimulating ET-1 synthesis by these human endothelial cells. Yokokawa and coworkers (16) have recently shown that ET-3 stimulates ET-1 production in human umbilical cord vein endothelial cells by a mechanism involving phosphoinositide pathway. These authors suggest that endothelin receptors existing in endothelial cells may be designed mainly for ET-3. However, it is unlikely that ET-3 has a physiological role in regulating vascular tonus, because it is not produced by vasculature and it is not a circulating hormone.

Two distinct human endothelin receptors have been cloned (21-23). The receptor expressed in human endothelial cells is a non- selective ET_B receptor, which has an equal affinity for all three of the isopeptides (21,23). In contrast, no appreciable amount of ET-1 selective ET_A receptor mRNA is present in cultured endothelial cells (22). We here confirm the expression of ET_B receptor mRNA in primary human umbilical cord endothelial cells.

Recently, ET_B receptors were reported to exist in cultured endothelial cells of human umbilical vein and binding of ^{125}I-labeled endothelin-1 to these cells was shown (24). The authors speculated that ET_B receptors may mediate autocrine effects of endothelin-1 in endothelial cells, in agreement with our findings reported here.

ET-1 has been shown to cause phospoinositide hydrolysis and increase of intracellular calcium in human umbilical vein endothelial cells (10). The mechanism of stimulation of ET-1 synthesis by endothelial cells is still unclear but may be coupled at least partly to the phoshoinositide turnover pathway and subsequent increase in intracellular calcium(13,14). Therefore, phosphoinositide breakdown and subsequent mobilization of intracellular calcium and activation of protein kinase C may be important in mediating the endothelin action on its own synthesis in endothelial cells.

In conclusion, the present study shows that ET-1 is a stimulator of its own synthesis in human endothelial cells and that this effect of ET-1 is probably mediated by ET_B receptors.

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