Endothelin-induced phosphoinositide hydrolysis in the muscular layer of stem villi vessels of human term placenta

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Endothelin-1 (ET-1) is one of the most potent vasoactive peptides synthesized by vascular endothelial cells. This 21-amino-acid peptide was cloned from a human placental cDNA library. Three distinct human endothelin-related genes have been identified. The products of their expression are ET-1, ET-2 and ET-3. Many studies have demonstrated that ET-1 produces contraction of vascular smooth muscle by interacting in a paracrine and autocrine fashion with cell surface-specific receptors (for a review, see Refs. 1 and 2). Because human placenta is a non- innervated organ (3), regulation of fetoplacental blood flow is dependent on locally released and circulating vasoactive factors. A progressive decrease in vascular resistance normally occurs in the placental bed during pregnancy, and the stem villi vessels are considered the major sites of fetal placenta vascular resistance (4). However, the mechanisms by which these changes in vascular resistance take place are poorly understood (5). It has been shown by different in vitro methods that ET-1 is a potent vasoconstrictor of the human fetal placental vasculature and has the potential to influence placental blood flow either directly or in concert with other vasoactive agents (6-10).

Specific and high-affinity binding sites for [125I]ET-1 have been identified previously in membrane fractions prepared from whole placenta (6, 11). Because the human hemomonochorial placenta is a very heterogeneous organ consisting of various cell types, it is essential to determine in which of its constitutive tissues the endothelin receptors are localized. In a previous study, we characterized abundant specific high-affinity binding sites for ET-1 on membranes of smooth muscle of fetoplacental vessels (12, 13). These results were confirmed by Rutherford et al. (14) and Wilkes et al. (15).

Two endothelin receptor subtypes have been delineated based on biochemical and physiological assays. These results have been confirmed recently with the cloning and expression of the ET_A and ET_B receptors (2). Functional characterization revealed that they are members of the G protein-coupled receptor superfamily. Classically, the ET_A subtype has a higher affinity for ET-1 than for ET-3; the ET_B subtype has almost equal affinity for ET-1 and ET-3. Our studies on placental stem villi vessels suggested the existence of at least two endothelin receptors in the muscular layer (12). Rutherford et al. (14) supported these findings and distinguished ET_A and ET_B-type binding sites localized in the media of placental stem villi vessels. Wilkes et al. (15) showed a predominance of ET_A-type receptors on small human placental arteries.

The exact mechanism whereby endothelin causes biological effects after binding to cell surface receptors has not yet been defined completely. Endothelins have been shown to mediate vascular smooth-muscle contraction, mainly through two distinct second messenger signalling pathways: Ca^{2+} influx through the activation of voltage-dependent calcium channels, and phospholipase C (PLC), which catalyzes the hydrolysis of
phosphatidyl inositol-4,5-biphosphate (IP_{2}) to give rise to
inositol-1,4,5-triphosphate (IP_{3}) and 1,2-diacylglycerol
(DAG). The IP_{3} acts as a second messenger to mobilize
Ca^{2+} from the intracellular pool, while the increase in
DAG activates protein kinase C (PKC). However, signal
transduction involved in endothelin receptor stimulation
seems to be more complex. Endothelin also stimulates
phospholipase A_{2} and phospholipase D, and modifies the
activity of adenylate cyclase (1, 2).

In this study, we demonstrated, in human term
placenta, that ET-1 vascular binding sites are coupled to
the PLC transducing system. Indeed, our results indicate
that inositol phosphates increase after ET-1 stimulation
of phosphoinositide (PI) hydrolysis. We compared the
effects of endothelin homologs ET-1, ET-3 and sarafotoxin
6b, and the precursor of ET-1, human-big ET-1, on PI
turnover in muscular explants of placental stem
villi vessels. In addition, selective ET_{A} receptor antago-
nists BO 123 (a cyclic pentapeptide) and BO 610 (a
linear peptide) (16, 17), as well as a selective ET_{B}
receptor antagonist IRL 1038 (18–20) and an ET_{B}
receptor agonist sarafotoxin 6c (21), were used as tools
to determine the involvement of endothelin receptor
subtypes in PLC activation.

Materials and methods

Placenta

Human placentae were obtained from healthy mothers
immediately after elective cesarean section. Cesarean
section was performed prior to labor under thiopental
sodium succinylcholine anesthesia in the 39th week of
pregnancy because of previously diagnosed cephalo-
pelvic disproportion.

Isolation of placental stem villi vessels

Placental tissue (1–2 cm^{3}) was excised rapidly between
the decidual and chorionic plates and immersed in
Medium-199 containing 50 IU/ml penicillin and 100 μg/
ml streptomycin. Villi vessels (approximately 200–
300 μmol/l mean internal diameter) were dissected as
reported previously (12) by fine mechanical dissection
and scratching, under an operational microscope, so that
virtually all of the surrounding trophoblast, stroma,
adventitia and endothelial cells were removed. Stem villi
vessels were cut into 1–3 mm^{3} pieces, then washed
thoroughly in fresh medium under gentle magnetic
agitation to eliminate residual endothelial cells and used
immediately. The absence of adherent endothelial cells
was confirmed by histological study (data not shown).

Measurement of inositol phosphate formation

Explants of stem villi vessel smooth muscle were incubated
at 37°C for 24 h in the same medium containing insulin
(10 μmol/l) and transferrin (10 μmol/l) as growth factors
and 20 μCi/ml myo-[2-{3H}]inositol as described previously
(22). Briefly, after washing, tissue pieces were preincu-
bated in Medium-199 at 37°C for 5 min and then
20 mmol/l LiCl, which prevents the conversion of IP_{1}
into inositol, was added and left for 30 min. When present,
antagonists were added and preincubated at the same
time as LiCl. After incubation with the indicated peptide,
the reaction was stopped at a selected time with chloroform–
methanol (1 : 2, v/v) and the aqueous phase was separated
from the organic phase by sequential addition of chloro-
form and distilled water and centrifugation (23). The
water-soluble products of PI hydrolysis were extracted and
separated by ion exchange chromatography (24). Briefly,
total [3H]inositol phosphates were eluted with 6 ml of
1 mol/l ammonium formate and 0.1 mol/l formic acid.
In some experiments, individual [3H]inositol phosphates
were eluted sequentially with a buffer containing
0.1 mol/l formic acid and increasing concentrations
of ammonium formate (0.2, 0.4 and 1 mol/l). Aliquots of
the eluate were counted for radioactivity in a scintillation
mixture in a Tri-Carb 4000 scintillation counter
(Packard). Samples of the chloroform phase containing
inositol lipids were dried under vacuum and the
[3H]inositol associated with the PIs was counted in
parallel with the fractions eluted from the columns.

Results are expressed as percentage total radio-
activity incorporated in the inositol phospholipids. The
8% values were calculated by non-linear regression
using “graph PAD software” (San Diego, CA).

Data analysis

The unpaired Student’s t-test was used to determine
statistical significance; p values less than 0.05 were
considered to be significant.

Chemicals

Myo-[2-{3H}]inositol (specific activity 10–20 Ci/mol) was
purchased from Amersham International (Buckingham-
shire, UK) and ET-1, ET-3, human-big ET-1,
sarafotoxin 6b (S6b), sarafotoxin 6c (S6c), angiotensin
II, BO 123 (cyclo-(μ-Trp-δ-Asp-Pro-δ-Val-Leu)), BO 610
((N,N-hexamethylene)carbamoyle-Leu-μ-Trp(CHO)-μ-Trp-
OMe) and IRL 1038 ((Cys^{11}-Cys^{15})endothelin-1(11-21))
were obtained from Neosystem (Strasbourg, France).
Dowex AG1-X8 (100–200 mesh) was purchased from
Bio-Rad Laboratories (Richmond, CA). Medium-199 was
purchased from Gibco BRL (Ergany, France). All other
reagents were of analytical grade and were obtained from
Sigma Chemical Co (St Louis, MO).

Results

Formation of inositol phosphates by endothelin-related
peptide receptor stimulation

The accumulation of [3H]inositol phosphates was
examined in stem villi vessel explants after administration of 1 µmol/l ET-1 in the presence of 20 mmol/l LiCl. Figure 1 shows the time course of ET-1 on IP₁, IP₂ and IP₃ formation. The ET-1-induced accumulation of IP₁ was detected 3 min after addition of the peptide and continued to increase for at least 30 min. Both IP₂ and IP₃ reached a maximum within 3 min after stimulation and decreased thereafter; IP₃ was detected as early as 30 s after stimulation. Stimulation by ET-1 at these short times was substantial for IP₃ and IP₂, whereas IP₁ increased more slowly, but reached much higher levels than IP₃ and IP₂ at 30 min. We verified that in the absence of ET-1, basal inositol phosphate accumulation remained unaltered throughout the incubation period.

Fig. 1. Time course of accumulation of [¹H]inositol-4-phosphate (A), [¹H]inositol-1,4-biphosphate (B) and [¹H]inositol-1,4,5-triphosphate (C) induced by endothelin 1 (µmol/l) in muscle explants of placental stem villi vessels prelabeled with myo[²H]inositol, in the presence of 20 mmol/l LiCl. Values presented are means ± SEM of three experiments performed in triplicate with different placentae.

To characterize further the endothelin-induced inositol phosphate turnover in these explants, all subsequent experiments were performed with a standard 30-min incubation period.

The ability of increasing concentrations of ET-1 to stimulate total inositol phosphate production (IP₁ plus

Fig. 2. Concentration–response curves for the accumulation of inositol phosphate induced by endothelin-1 (△) and human-big endothelin-1 (○) in muscle explants of placental stem villi vessels. Endothelin-1 and human-big endothelin-1 were allowed to act for 30 min in the presence of 20 mmol/l LiCl. Values presented are means ± SEM of five and three experiments performed in triplicate with different placentae for endothelin-1 and human-big endothelin-1, respectively.

Fig. 3. Concentration–response curves of accumulation of [¹H]inositol phosphate induced by endothelin homologs in muscle explants of placental stem villi vessels. Endothelin homologs were allowed to act for 30 min in the presence of 20 mmol/l LiCl. Values presented are means ± SEM. The number of experiments, each performed in triplicate with different placentae, is shown in parentheses as follows: endothelin-1 (△, N = 5); endothelin 3 (○, N = 3); sarafotoxin 6b (●, N = 3) and sarafotoxin 6c (□, N = 3).
Table 1. Total inositol phosphate formation induced by endothelin homologs in muscle explants of placental stem villi vessels.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$c_{50}$</th>
<th>Maximal response (% of control)</th>
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<tbody>
<tr>
<td>Endothelin 1</td>
<td>44 ± 16 pmol/l</td>
<td>549 ± 30</td>
</tr>
<tr>
<td>Sarafotoxin 6b</td>
<td>18 ± 13 nmol/l</td>
<td>452 ± 53</td>
</tr>
<tr>
<td>Endothelin 3</td>
<td>33 ± 24 nmol/l</td>
<td>381 ± 29**</td>
</tr>
<tr>
<td>Sarafotoxin 6c*</td>
<td>Not determined</td>
<td>170 ± 27**</td>
</tr>
</tbody>
</table>

Values are means ± SD of three to five experiments performed in triplicate with different placentae. *Dose response curves obtained with S6c could not be analyzed by non-linear regression. **p < 0.01 compared with endothelin 1. Maximal responses were obtained during 30 min of incubation in the presence of 20 mmol/l LiCl with the indicated peptide. $c_{50}$ is the concentration eliciting 50% of the maximal stimulating response.

IP$_2$ plus IP$_3$) in the presence of LiCl during a 30-min incubation is shown in Fig. 2. Dose-dependent stimulation was observed from a concentration of 1 pmol/l ET-1 and the maximal response (550% of stimulation over basal values) was reached at 0.1 µmol/l. The concentration of ET-1 causing a half-maximal accumulation of IP$_3$ ($c_{50}$ value) was 44 ± 16 pmol/l. The ET-1 precursor human-big ET-1 also induced an increase in inositol phosphates. While the maximal effect was the same as that of ET-1 at 10 µmol/l, the $c_{50}$ values were 1000-fold higher for human-big ET-1 (60 ± 12 nmol/l) than for ET-1. Data in Fig. 3 illustrate the comparative effect of ET-1, S6b, ET-3 and S6c in causing increased generation of total inositol phosphates. The rank order of potency was ET-1 ≫ S6b ≫ ET-3. Compared with ET-1, S6b and ET-3 appeared less potent ($c_{50}$ = 18 ± 13 and 33 ± 24 nmol/l, respectively) and displayed a weaker effect (78% and 62% of ET-1 response) at a concentration of 1 µmol/l (Table 1). The concentration–response curve obtained with S6c showed that the maximal response at 1 µmol/l did not exceed 15% of the response caused by ET-1 (Fig. 3).

In contrast, angiotensin II (1 pmol/l–10 µmol/l) had no effect on PLC activation (data not shown).

**Fig. 5.** Differential abilities of an ET$_A$ receptor antagonist (BQ 123) and an ET$_B$ receptor antagonist (IRL 1038) to inhibit inositol phosphate release induced by endothelin homologs in muscular explants of placental stem villi vessels. Explants were preincubated without (■) or with the antagonists BQ 123 (■); 100 µmol/l and IRL 1038 (■); 100 µmol/l for 30 min in the presence of 20 mmol/l LiCl and then incubated with the indicated peptides (10 nmol/l) for an additional 30 min. Values presented are means ± SD of three experiments performed in triplicate with different placentae. *p < 0.05 and **p < 0.01: significant differences between the values obtained in the absence or in the presence of antagonists. ET-1 = endothelin 1; S6b = sarafotoxin 6b; S6c = sarafotoxin 6c.

**Fig. 4.** Effect of selective ET$_A$ receptor antagonists BQ 123 (■) and BQ 610 (○) and ET$_B$ receptor antagonist IRL 1038 (▲) on endothelin (ET-1)-induced inositol phosphate release in muscle explants of placental stem villi vessels. Explants were preincubated with increasing concentrations of antagonists in the presence of 20 mmol/l LiCl for 30 min and then incubated with ET-1 (10 nmol/l) for an additional 30 min. The data shown represent a typical experiment performed in triplicate.

**Effect of BQ 123, BQ 610 and IRL 1038 on PI hydrolysis induced by endothelin peptides.**

The differential abilities of selective ET$_A$ (BQ 123, BQ 610) and ET$_B$ (IRL 1038) receptor antagonists to affect PI hydrolysis induced by a maximal dose of ET-1 (10 nmol/l) were tested. As shown in Fig. 4, preincubation of muscle explants of stem villi vessels with increasing concentrations of BQ 123 and BQ 610 resulted in dose-dependent inhibition of inositol phosphate accumulation induced by the subsequent addition of 10 nmol/l ET-1. These inhibitions were not abolished totally by the 100 µmol/l antagonists. Indeed, it should be noted that about 12% of PI hydrolysis elicited by ET-1 was resistant to the action of the two ET$_A$ antagonists. Preincubation of explants with increasing concentrations of the ET$_B$ antagonist IRL 1038 partially inhibited the stimulatory effect of 10 nmol/l ET-1, but this inhibition was observed only at a very high concentration (10 µmol/l) and did not exceed 30% at 100 µmol/l IRL 1038. In these
inhibited ET-1 vessels studied effect experiments, by however, lins muscular and and phosphoinositide 1038 both of subtypes establish hydrolysis order 400 smooth-muscle and antagonists, (100/xmol/l), potency of and ETA induced is not inhibition by these ETA antagonists. In contrast, IRL 1038 (100 μmol/l) inhibited S6c-induced isositol phosphate accumulation by more than 80%, whereas the ETB antagonist reduced the ET-1 and S6b stimulation by about 30%.

Discussion
The present study shows for the first time that, in the muscular layer of placental stem villi vessels, endothe¬lins are coupled to the PLC transducing system. Phosphoinositide hydrolysis has been found to be an important signal transduction event linked to the activation of endothelin receptors in vascular smooth muscle as well as in a multitude of tissues (1, 2).

Endothelin 1, ET-3, S6b and S6c induced total isositol phosphate accumulation in a dose-dependent manner. However, there were marked differences in the efficacy and potency of the induced stimulations. Isositol phosphate maximal accumulation induced by S6b and ET-3 differed significantly from the responses produced by ET-1 (respectively, 78% and 62% of the ET-1 response). Stimulation induced by S6c was very slight and did not exceed 15% of the response caused by ET-1. Endothelin 1 was the most potent endothelin, with an EC50 of 44 ± 16 pmol/l. This EC50 value was in the same range as the Kd value (26 ± 4 pmol/l) we had determined previously for binding [125I]ET-1 to smooth-muscle membranes of these stem villi vessels (12). In contrast, the EC50 values for S6b and ET-3 were 400 and 750 times higher than for ET-1. The rank order of potency (ET-1 > ET-3) for activation of PI hydrolysis was in agreement with the ET peptide selectivity expected for ETA receptors.

In previous studies, we had suggested the existence of at least two subtypes of endothelin receptors on the muscular layer of placental stem villi vessels (12). To establish the involvement of both endothelin receptor subtypes in the stimulation of PI hydrolysis in explants of placental vessels, two ETA and ETB receptor antagonists, BQ 123 and IRL 1038, respectively, were used as pharmaceutical tools. At a high concentration (100 μmol/l), BQ 123 failed to block completely the ET-1- and S6b-induced isositol phosphate production. However, its capacity to produce 90% inhibition of both ET-1- and S6b-induced isositol phosphate formation indicates that these peptides act predominantly through the activation of ETA receptors. In contrast, as reported previously (25), the cyclic pentapeptide did not affect the weak response elicited by S6c, confirming that
and/or vasoconstriction of fetoplacental vessels could account for fetomaternal disorders such as fetal growth retardation, pre-eclampsia and prematurity.

In summary, we have demonstrated that, in the muscular layer of placental stem villi, both endothelin receptor subtypes (ET$_A$ and ET$_B$), but predominantly ET$_A$, were linked to PI hydrolysis. The function of each of these receptors in the vascular response remains to be elucidated. Most evidence indicates that ET$_A$ is the predominant endothelin receptor on vascular smooth-muscle cells and that it may be responsible for the vasoconstrictor effect of endothelin, whereas the ET$_B$ receptor is more prevalent on endothelial cells and appears to be involved in the vasoconstriction action of endothelin (2). However, each endothelin receptor type has now been localized on both endothelial and smooth-muscle cells (36, 37). To confirm the possibility that placental vascular tone is regulated by the ratio of ET$_A$ to ET$_B$ receptors in these different vascular cells, in vitro stem villi vessel contractile assays using highly selective agonists are necessary.

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