Endothelin-1 Induces Endoplasmic Reticulum Stress by Activating the PLC-IP₃ Pathway

Implications for Placental Pathophysiology in Preeclampsia

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Recent evidence implicates placental endoplasmic reticulum (ER) stress in the pathophysiological characteristics of preeclampsia. Herein, we investigate whether endothelin (ET)-1, which induces Ca²⁺ release from the ER, can induce placental ER stress. Loss of ER Ca²⁺ homeostasis impairs post-translational modification of proteins, triggering ER stress-response pathways. IHC confirmed the presence of both ET-1 and its receptors in the syncytiotrophoblast. Protein levels and immunoreactivity of ET-1 and the endothelin B receptor (ETBR) were increased in preeclamptic samples compared with normotensive controls. JEG-3 and BeWo choriocarcinoma cells treated with ET-1 displayed an increase in ER stress markers. ET-1 induced phospho-activation of the ETBR. Treating cells with BQ788, an ETBR antagonist, or small-interfering RNA knockdown of the receptor inhibited induction of ER stress. ET-1 also stimulated p-phospholipase C (PLC)γ1 levels. By using inhibitors of PLC activation, U73122, and the inositol 1,4,5-triphosphate (IP₃) receptor, xestospongine-C, we demonstrated that ET-1 induces ER stress via the PLC-IP₃ pathway. Furthermore, ET-1 levels increased in the syncytiotrophoblast of explants from normal placentas after hypoxia-reoxygenation *in vitro*. Conditioned medium from hypoxia-reoxygenation explants also contained higher ET-1 levels, which induced ER stress in JEG-3 cells that was abolished by an ET-1-neutralizing antibody. Collectively, the data show that ET-1 induces ER stress in trophoblasts via the ETBR and initiation of signaling through the PLC-IP₃ pathway, with the potential for autocrine

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Preeclampsia is a major cause of morbidity and mortality in pregnant women and prenatal infants,¹ affecting between 2% and 8% of all pregnancies worldwide. Most cases of preeclampsia have an onset near term, but approximately 10% of cases have an early onset before 34 weeks of gestation.² Early-onset preeclampsia that requires preterm delivery has underlying pathological features that differ and are more severe than those of late-onset preeclampsia³; they are often associated with intrauterine growth restriction (PE + IUGR). Recent evidence⁴ implicates placental endoplasmic reticulum (ER) stress in the pathophysiological characteristics of PE + IUGR. The ER is involved in synthesizing and packaging the membrane and secreted proteins,⁵ and also serves as a reservoir of intracellular Ca²⁺. In the ER lumen, Ca²⁺ is buffered by calcium-binding proteins. Many of these proteins also serve as molecular chaperones involved in folding and quality control of ER proteins,⁶ and their functional activity alters with changes in Ca²⁺ concentration. The resting Ca²⁺ concentration in the ER is maintained by a balance of Ca²⁺ uptake and leakage. Resting Ca²⁺ leakage is a slow process that can be balanced by a modest Ca²⁺ influx mediated by calcium pumps.⁷

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Table 1. Clinical Data of Tissue Used for IHC Staining

Group	Gestational age (weeks)	Fetal weight (g)	Placental weight (g)	Umbilical artery flow category	Maximum blood pressure (mmHg)		MgSO ₄ treatment
					Systolic	Diastolic	
Normal	38.9 ± 0.3	3427.7 ± 155.3	481.3 ± 28.2	Normal = 6	124.0 ± 3.5	75.0 ± 3.2	No
Early-onset preeclampsia	29.7 ± 0.5	911.5 ± 67.9	209.9 ± 46.4	Abnormal end-diastolic blood flow = 6	161.0 ± 5.8	103.2 ± 3.9	1 sample

Data are given as mean ± SEM unless otherwise indicated.

However, stimulated Ca²⁺ release is much faster⁸ and can be mediated by inositol 1,4,5-triphosphate (IP₃) receptors, ryanodine receptors, and receptors to cyclic ADP-ribose and to nicotinic acid adenine dinucleotide phosphate. Loss of ER Ca²⁺ homeostasis suppresses post-translational modification of proteins; consequently, misfolded proteins accumulate, provoking ER stress-response pathways known collectively as the unfolded protein response (UPR).⁹ The UPR aims to resolve the protein-folding defect and restore ER homeostasis. As part of the response, nonessential protein synthesis is inhibited through phosphorylation of the eukaryotic initiation factor subunit α (eIF2 α).¹⁰ This may contribute to the smaller placental phenotype seen in some complicated pregnancies, because it is associated with reduced cell proliferation.⁴ Another action is to induce transcription of genes encoding ER chaperones and enzymes that promote protein folding, maturation, and secretion, as well as ER-associated degradation of misfolded proteins.¹¹

The stimulus for ER stress in placentas from complicated pregnancies is unknown. Endothelin (ET)-1 can induce Ca²⁺ release from the ER¹² and, therefore, disrupt ER Ca²⁺ homeostasis and potentially induce ER stress. ET-1 is the most abundant member of the family of endothelins¹³ and is synthesized and secreted by a diverse range of cells, including the syncytiotrophoblast of the placenta and endothelial cells.¹⁴ ET-1 exerts its effects by binding to the endothelin A receptor (ETAR) and endothelin B receptor (ETBR), two highly homologous cell surface proteins that belong to the G-protein-coupled receptor superfamily.¹⁵

ET-1 is abundant in the placenta, where it plays a potential role in stimulating extravillous trophoblast invasion that is essential to normal placenta development.¹⁶ Preeclampsia is associated with increased maternal plasma levels of ET-1. In plasma from healthy pregnant women, the concentration of ET-1 ranges from 5 to 10 pg/mL, whereas the concentration is 20 to 50 pg/mL in the presence of preeclampsia.¹⁴ Given this increased concentration, the prevalence of ER stress in the pathophysiological features of preeclampsia, and the potential of ET-1 to disrupt ER Ca²⁺ homeostasis, this study investigated whether ET-1 could be a potential stimulus of the placental ER stress.

Materials and Methods

Chemicals

Tunicamycin, propidium iodide, bisbenzimidazole H 33342 trihydrochloride (Hoechst 33342), ET-1, and xesto-

spongin-C were from Sigma-Aldrich (Poole, UK). BQ788 and BQ123 were from Tocris Bioscience (Bristol, UK).

Tissue Collection

Placental samples for explant culture were collected from healthy term pregnancies delivered by elective caesarean section with approval from the Cambridge Local Ethical Committee and informed patient consent.

Placental samples from cases of early-onset severe preeclampsia delivered by elective caesarean section and from normotensive controls were collected with Local Ethical Committee and informed patient consent in Uppsala, Sweden. Preeclampsia was defined according to the International Society for the Study of Hypertension in Pregnancy guidelines. Clinical details are given in Table 1. Villous tissue was collected from the maternal side of the placenta, midway between the chorionic and basal plates. The maternal aspect of the placenta was removed, and small pieces (approximately 5×5×5 mm) were collected from four to six separate lobules. The lobules were all positioned around the center of the placenta. The samples were rinsed three times in saline, dabbed dry, and finally snap frozen in liquid nitrogen. The pieces were pooled, frozen, and stored at −80°C.

Colorimetric IHC

The immunohistochemistry (IHC) procedures were performed as previously described.¹⁷ Heat-induced epitope retrieval was used, which involved pressure-cooking samples in Tris-EDTA buffer (pH 9) for 3 minutes, before blocking the sections with 5% goat-serum and 2% bovine serum albumin in Tris-buffered saline for 1 hour at room temperature in a humid chamber. Sections were then incubated with primary antibody overnight at 4°C, followed by secondary antibody (1:200) incubation for 1 hour at room temperature in a humid chamber. Binding was detected using Vectastain Elite ABC kits (Vector Laboratories, Peterborough, UK) and SigmaFast DAB (Sigma-Aldrich, Poole, UK). Sections were lightly counterstained with hematoxylin and then mounted with DPX (Sigma-Aldrich).

Negative controls were performed by omitting the primary antibody incubation, but species-matched secondary antibody incubation was included and all remaining steps were the same.

Cell Culture

Human JEG-3 choriocarcinoma cells were maintained in RPMI 1640 medium, whereas BeWo choriocarcinoma cells were cultured in Dulbecco's modified Eagle's medium/F12 medium (Invitrogen Ltd, Paisley, UK). Both media were supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μ g/mL) at 37°C in a humidified atmosphere of 5% CO₂. Cells were cultured in 75-cm² tissue culture flasks and, before treatment, seeded in 6-well plates (Nunc, Fisher Scientific, Loughborough, UK). After the cells reached 70% confluence, the medium was aspirated, and cells were washed with serum-free media. Different treatments were then added for a 1-hour time course in serum-free media.

Western Blot Analysis

Western blot analysis of protein expression and kinase phosphorylation was performed as previously described.¹⁸ Briefly, equal amounts of protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary antibody overnight at 4°C, followed by a few hours at room temperature. Horseradish peroxidase-linked sheep anti-mouse or donkey anti-rabbit secondary antibodies (1:10,000; GE Healthcare, Buckinghamshire, UK) were used in conjugation with electrochemiluminescence to visualize the protein bands on autoradiography films. Anti-ET-1 (1 μ g/mL), anti-ETBR (0.5 μ g/mL), anti-ETAR (1 μ g/mL), and anti-KDEL (1 μ g/mL) were from Abcam (Cambridge, UK). Anti-phospho-eIF2 α (Ser51) (1 μ g/mL), anti-eIF2 α (0.5 μ g/mL), anti-phospho-phospholipase C (PLC) γ 1 (Y783) (1 μ g/mL), and anti-PLC γ 1 (1 μ g/mL) were from Cell Signaling Technology (Danvers, MA). Anti- β -actin (0.5 μ g/mL) was from Sigma-Aldrich. All data were normalized to β -actin.

RT-PCR Analysis of XBP-1 mRNA Splicing

The assay was performed as previously described.¹⁸ Forward and reverse primer sequences were as follows: 5'-CTGGAACAGCAAGTGGTAGA-3' and 5'-CTGGGTCCTTCTGGGTAGAC-3', respectively. The PCR products were resolved by 2% agarose gel electrophoresis with ethidium bromide and documented in a UVP Gel Documentation system (UVP, Cambridge, UK): 398- and 424-bp fragments represented spliced and unspliced X-box binding protein (XBP)-1 transcripts, respectively.

Immunoprecipitation

Cell lysates, 200 μ g, were pretreated with 30 μ L of uncoated agarose beads for 4 hours at 4°C. Lysates were centrifuged at 8000 $\times g$ for 2 minutes at 4°C, and the supernatants were transferred to a fresh tube. Immunoprecipitations were performed at 4°C overnight with 50 μ L of immobilized phospho-tyrosine beads (Upstate-Cell Signaling Solutions, Watford, UK). Beads were spun and washed five times in lysis buffer. Immunoprecipitates

were subjected to Western blot analysis with the ETBR antibody (Abcam, Cambridge, UK).

Small RNA Interference

The small-interfering RNA (siRNA) duplexes used were from Dharmacon (Thermo Scientific, Loughborough, UK). For ETBR, either the siGENOME SMARTpool ETBR (M-005490-02) or four individual siRNA duplexes from the siGENOME ETBR were used, including the following: sequence 1, 5'-UCAACGAGAUACCAAGCA-3' (D-005490-02); sequence 2, 5'-GCACAGCUACUACCUAGAAG-3' (D-005490-03); sequence 3, 5'-GAACUGAGGGGCAUCUGA-3' (D-005490-04); and sequence 4, 5'-CAGCAGAGCCGAUCCAAGA-3' (D-005490-05). For nontargeting siRNA control, the siGENOME nontargeting siRNA 4 (D-001210-04-05) (Dharmacon) was used. SiPORTamine transfection reagent was obtained from Applied Biosystems (Warrington, UK). Transfection of siRNA was performed according to the manufacturer's instructions. One day before transfection, cells were seeded at a density that would reach approximately 70% confluency the next day. In brief, 7.5 μ L of siPORTamine transfection reagent was diluted with 100 μ L of OPTIMEM (Invitrogen Ltd) and incubated at room temperature for 10 minutes. A total of 7.5 μ L of 10 μ mol/L siRNA was diluted with 100 μ L of OPTIMEM, and the two mixtures were mixed together and incubated at room temperature for 10 minutes before being applied to the cells. After 72 hours of incubation, the efficiency of the different ETBR siRNA sequences was determined by using Western blot analysis with an anti-ETBR-specific antibody.

Individual siRNA duplexes from the siGENOME ETBR were used as controls to show that a significant reduction in ETBR, compared with siControl samples, was obtained in three of the four individual siRNA duplexes, eliminating the possibility of nonspecific targeting of the siGENOME SMARTpool ETBR (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

Explant Culture

Placental samples from normotensive pregnancies delivered by elective caesarean section were dissected into 2-mm explants on ice in large vessel endothelial cell basal (LVEB) medium (TCS CellWorks Ltd, Botolph Claydon, UK), equilibrated to 10% O₂/5% CO₂, balanced with N₂. Tissue was trypsinized [25 mL of PBS plus 25 mL of 0.05% trypsin-EDTA (TE)] for 7 minutes at 37°C to remove the original layer of syncytiotrophoblast, rinsed in LVEB medium to neutralize TE, and incubated in 4 mL of medium at 10% O₂/5% CO₂ for 3 days to allow the syncytiotrophoblast to regenerate.¹⁹

Conditioned Medium

For collection of conditioned medium, regenerated tissue was incubated on Costar Netwell inserts (24-mm diameter, 500- μ m mesh) (Corning, Appleton Woods, UK) in LVEB medium under the following conditions: 10% O₂/5% CO₂ for 16 hours or 10% O₂/5% CO₂ for 8

Figure 1. Increased levels of ET-1 and ETBR, but not ETAR, in pathological placentas. **A:** IHC staining of normotensive controls and PE + IUGR sections revealed that ET-1 and its receptors are expressed in the syncytiotrophoblast and on endothelial cells. Scale bar = 50 μ m. **B:** Semiquantitative scoring of the IHC staining was performed by random sampling of fields for each specimen, with the observer blinded to the specimen source during analysis. A score of + or ++ was used to designate the staining intensity. Three control and three preeclamptic sections were analyzed for each of the proteins stained for. For both ET-1 and the ETBR there was approximately a twofold increase in staining intensity between control and preeclamptic sections, which was maintained even with the $MgSO_4$ -treated sample. **C:** Measurement of ET-1 and ET receptor protein levels in normotensive and early-onset preeclamptic placental tissue. Western blot analyses revealed a significant increase in ET-1 and the ETBR, but not the ETAR, in early-onset preeclamptic tissue compared with normotensive controls. The densitometry of bands was expressed as the mean of three samples after the data were normalized to β -actin. * $P < 0.05$.

hours, followed by 1% O_2 /5% CO_2 for 8 hours. Media were collected and centrifuged at $2860 \times g$ for 30 minutes at $4^\circ C$, and concentrated for ET-1 using Viva-spin 2 30000 MWCO and 3000 MWCO centrifugal concentrators (Sartorius Stedim Biotech, Epsom, UK). The levels of ET-1 were measured using a Quantikine ELISA kit (R&D Systems, Abingdon, UK). All samples and standards were assayed in duplicate, according to the manufacturer's instructions. Results were then normalized per gram of placental tissue.

Statistics

Statistical analysis was performed by analysis of variance (Fisher's Protected Least Significant Difference test) or nonparametric Mann-Whitney U -test, with $P < 0.05$ being considered significant.

Results

ET-1 and Its Receptors Are Expressed in the Syncytiotrophoblast

Sections of normotensive and early-onset preeclamptic (PE + IUGR) placentas immunostained for ET-1, ETAR, and ETBR revealed the presence of all three in the placental syncytiotrophoblast and on endothelial cells (Figure 1A). Semiquantitative scoring of the IHC staining revealed that, in the case of ET-1 and the ETBR, the immunoreactivity was increased in the pathological samples compared with the normotensive controls (Figure 1B). The increase was pronounced in the syncytiotrophoblast, with endothelial cells displaying less change between control and pathological samples. The ETAR did not exhibit any change in expression in either the syncytiotrophoblast or endothelial cells in sections from normotensive and PE + IUGR placentas. The results were

Figure 2. Dose-response study of ET-1 on induction of ER stress. Western blot analyses of ER stress markers after treatment with different doses of ET-1. The densitometry of bands was expressed relative to controls (100%) after data were normalized to β -actin. JEG-3 cells treated with ET-1 over a 1-hour time course displayed increased levels of ER stress markers, GRP78, GRP94, and p-eIF2 α , in a bell-shaped dose-response. Data are given as mean $\pm$ SEM ($n \geq 3$). * $P < 0.05$. A 15.6 nmol/L dose of ET-1 gave a significant increase in expression of ER stress markers compared with control untreated samples.

confirmed by using Western blot analysis, in which both ET-1 and ETBR levels were significantly higher in preeclamptic tissue compared with normotensive controls, but there was no change in ETAR levels (Figure 1C). The existence of both ET-1 and its receptors in the syncytiotrophoblast suggests a possible autocrine mode of action of ET-1.

ET-1 Induces ER Stress in Human JEG-3 Choriocarcinoma Cells

After having established the presence of both ET-1 and its receptors in the placental syncytiotrophoblast, the next step was to test the ability of ET-1 to induce ER stress. Therefore, *in vitro* models used human choriocarcinoma JEG-3 cells, which serve as a model for trophoblast and were treated with different doses of ET-1. The ET-1 induced an increase in the level of three different ER stress markers: phosphorylation of eIF2 α , glucose regulated protein 78 (GRP78), and GRP94 (Figure 2). All increased in the same bell-shaped dose-response, with a maximum response obtained at a concentration of 15.6 nmol/L ET-1. There were no changes in the total protein level of eIF2 α .

These results show that ET-1 is able to stimulate ER stress in JEG-3 cells. However, splicing of *XBP-1* mRNA, via the action of Ire1 ribonuclease, was not stimulated by ET-1 (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). There was also no significant increase in apoptosis under ET-1 treatment (see Supplemental Figure S3 at <http://ajp.amjpathol.org>).

These results are consistent with a study by Cervar-Zivkovic et al,²⁰ which demonstrated that ET-1 attenuates apoptosis in trophoblast cells cultured from term human placentas. We have previously demonstrated that a sublethal dosage of ER stress inducer, tunicamycin, elevates GRP78, GRP94, and p-eIF2 α levels, with neither induction of *XBP-1* mRNA splicing nor apoptosis in JEG-3 cells.⁴ Overall, the results suggest that ET-1

induces relatively mild sublethal levels of ER stress in JEG-3 cells.

Increased Phosphorylation of ETBR in JEG-3 Cells in Response to ET-1

Given that only the ETBR showed increased levels in the pathological samples, the expression and activation of the ETBR was examined under different treatments with ET-1. Western blot analysis revealed that the levels of total ETBR remain unchanged under the different doses of ET-1 tested before (Figure 3A), suggesting that the changes in ER stress previously described were the result of the action of ET-1, rather than the availability of its receptor.

No commercial antibody is available for the phosphorylated ETBR. Therefore, to investigate whether ET-1 activates the ETBR, immunoprecipitation using an anti-phospho-tyrosine antibody was used to pull down all proteins phosphorylated at tyrosine residues, which were then probed for the ETBR using immunoblotting. Results illustrated in Figure 3B revealed that ET-1 induces phosphorylation of the ETBR, confirming that this is a potential mechanism of action of ET-1 in initiating ER stress signaling. In the absence of ET-1, no phosphorylation of the ETBR was detected, excluding the possibility of another agent acting via this receptor to induce ER stress.

The fact that ET-1 acts via the ETBR was confirmed by treating the JEG-3 cells with BQ788, an ETBR antagonist (Figure 3C), which inhibited the induction of ER stress. The levels of all three ER stress markers showed a significant decrease after a 50 μ mol/L dose of BQ788 treatment to levels comparable to untreated control samples. To further substantiate the action of ET-1 via the ETBR, siRNA was used to knock down the ETBR. A 75% reduction in ETBR levels was achieved, and this almost completely abolished the induction of ER stress by ET-1

Figure 3. ET-1 acts via the ETBR to induce ER stress. **A:** ETBR expression in JEG-3 cells remained unchanged in a dose-response study with ET-1. **B:** IP data revealed that ET-1 induces phospho-activation of the ETBR. An anti-phospho-tyrosine antibody was used to pull down all tyrosine residue phosphorylated proteins, followed by immunoblotting analysis with an anti-ETBR antibody. **C:** Western blot analyses of ER stress markers after cotreatment with 10 nmol/L ET-1 and increasing concentrations of BQ788 during a 1-hour time course. The densitometry of bands was expressed relative to controls (100%) after data were normalized to β-actin. Data are given as mean ± SEM (*n* ≥ 3). A 50 μmol/L dose of BQ788 gave a significant decrease in expression of ER stress markers, GRP78, GRP94, and p-eIF2α, compared with control untreated samples. **D:** siRNA knockdown data confirmed action of ET-1 specifically through the ETBR. JEG-3 cells were transfected with siETBR pool or a nontargeting siRNA sequence as a control. After a 72-hour incubation, after treatment with or without 10 nmol/L ET-1 for 1 hour, proteins were extracted and immunoblotted for the ETBR and GRP78. **P* < 0.05.

(Figure 3D). Three more siRNA sequences specific for ETBR were tested, and similar results were obtained by eliminating any off-target effect (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). There was a significant decrease in expression of GRP78 in small-interfering ETBR (siETBR) samples compared with siControl sam-

ples treated with the same dose of 10 nmol/L ET-1. Furthermore, there was no significant increase in ER stress in siETBR samples treated with ET-1 compared with siETBR controls with no ET-1 treatment. However, siControl samples treated with ET-1 produced a significant increase in expression of GRP78 compared with siControl samples

with no ET-1 treatment. These results suggest that ET-1 is acting exclusively through the ETBR when inducing ER stress. The ETAR is, however, expressed in JEG-3 cells (see Supplemental Figure S4 at <http://ajp.amjpathol.org>);

to confirm that ET-1 is not acting via this receptor, JEG-3 cells were treated with BQ123 (see Supplemental Figure S5 at <http://ajp.amjpathol.org>), an ETAR-specific antagonist. The data revealed that there is no reduction in

Figure 4. ET-1 activates the PLC pathway. **A:** Western blot analyses of p-PLCγ1 and total-PLCγ1 after treatment with given doses of ET-1, with densitometry of bands expressed relative to controls (100%) after data were normalized to β-actin. JEG-3 cells treated with ET-1 during a 1-hour time course displayed increased phosphorylation of PLCγ1 in a bell-shaped dose-response. **B:** Cotreating JEG-3 cells with U73122 reduced the induction of p-PLCγ1, with no change in total-PLCγ1 levels in a dose-dependent manner. A 2.5 μmol/L dose of U73122 gave a significant decrease in expression of p-PLCγ1/total-PLCγ1 compared with control untreated samples. **C:** U73122 also decreased the expression of ER stress markers, GRP78, GRP94, and p-eIF2α, in a dose-dependent manner. A 2.5 μmol/L dose of U73122 produced a significant decrease in expression of all three ER stress markers compared with control untreated samples. Data are given as mean ± SEM ($n \geq 3$). * $P < 0.05$.

Figure 5. Inhibition of IP₃R blocks ET-1-induced ER stress. Western blot analyses of ER stress markers after cotreatment with 10 nmol/L ET-1 and different doses of xestospongin-C during a 1-hour time course. Xestospongin-C reduced the expression of ER stress markers, GRP78 and p-eIF2α, in a dose-dependent manner. The densitometry of bands was expressed relative to controls (100%) after data were normalized to β-actin. Data are given as mean ± SEM (*n* ≥ 3). **P* < 0.05.

expression of ER stress markers under the doses of BQ123 tested.

ET-1 Activates the PLC Pathway

To identify the downstream signaling pathways stimulated by activation of the ETBR, components of the PLC pathway were examined by using Western blot analysis. The dose-dependent study indicates that ET-1 stimulates p-PLCγ1 (Y783) levels, whereas the level of total-PLC remains unchanged (Figure 4A). Phosphorylation by Syk at Y783 activates the enzymatic activity of PLCγ1.²¹ The p-PLCγ1 can stimulate the release of Ca²⁺ from the ER, and disruptions in ER calcium

homeostasis can affect protein folding and induce ER stress. Therefore, the ET-1-induced increase in p-PLCγ1 levels provides a potential mechanism by which ET-1 can cause ER stress.

To confirm this hypothesis, U73122, an inhibitor of PLC activation, was used to block the induction of PLCγ1 phosphorylation after administration of ET-1 and the resulting levels of the ER stress markers were examined. There was a significant decrease in p-PLCγ1 (Y783) under a 2.5 μmol/L dose of U73122 treatment to levels comparable to ET-1-untreated control samples, and this correlated with reduced expression of all three ER stress markers (Figure 4, B and C). These data confirm that ET-1 acts via the PLC pathway to induce ER stress.

Figure 6. Increased ET-1 expression and release by the syncytiotrophoblast under H/R can act in an autocrine manner to induce ER stress. **A:** IHC staining of explants from term (aged 38 to 40 weeks) placentas exposed to H/R or 10% O₂ revealed that ET-1 expression is increased in the syncytiotrophoblast under H/R. Higher-power images of the boxed areas are included below each main image. **B:** An enzyme-linked immunosorbent assay (ELISA) revealed increased levels of ET-1 in conditioned medium from H/R-treated explants. **C:** Western blot analysis of ER stress marker, GRP78, under given conditions during a 1-hour time course, with the densitometry of bands expressed relative to controls (100%) after data were normalized to β-actin. Data are given as mean ± SEM (*n* = 4). **P* < 0.05. Ab, antibody.

Inhibition of IP_3R Inhibits ER Stress

IP_3 produced from the p-PLC γ 1-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate binds and activates the inositol 1,4,5-triphosphate receptor (IP_3R) to induce Ca^{2+} release from the ER. This final point of action in the pathway by which ET-1 can induce ER stress was confirmed using the xestospongine-C inhibitor, which blocks IP_3R -mediated Ca^{2+} release. Xestospongine-C caused a significant decrease in expression of GRP78 and p-eIF2 α /total-eIF2 α under a 2 μ mol/L dose of inhibitor treatment (Figure 5). There was no significant decrease in p-PLC γ 1/total-PLC γ 1 levels at any concentration of xestospongine-C (see Supplemental Figure S6 at <http://ajp.amjpathol.org>). This is consistent with the fact that xestospongine-C acts downstream of PLC γ 1.

Collectively, these data show that ET-1 is able to induce ER stress via the ETBR by initiating signaling through the PLC- IP_3 pathway. The question, then, was what is the source of the increased ET-1 in pathological pregnancies?

ET-1 Potentially Acts in an Autocrine Manner to Induce ER Stress

Preeclampsia is associated with deficient conversion of the spiral arteries supplying the placenta, which contributes to hypoxia-reoxygenation (H/R).^{22,23} Hence, explants from elective caesarean-delivered normotensive placentas were exposed to H/R *in vitro*, whereas control samples were maintained in a constant 10% O_2 atmosphere. Immunoreactivity for ET-1 was increased in the syncytiotrophoblast of samples treated with H/R compared with control samples (Figure 6A); furthermore, ELISA data confirm that conditioned medium from H/R-treated explants contains a higher concentration of ET-1 than conditioned medium from explants incubated at 10% O_2 (Figure 6B).

JEG-3 cells treated with conditioned medium from H/R-treated explants displayed increased levels of GRP78 compared with cells treated with conditioned medium from explants incubated at 10% O_2 (Figure 6C). Preincubating the medium with a neutralizing antibody to ET-1 abolished the induction of ER stress in both conditioned medium from H/R-treated samples and the positive controls, confirming that the increased ER stress observed was specific to the action of ET-1. These results suggest that ET-1 released by the syncytiotrophoblast under H/R might potentially act in an autocrine manner to induce placental ER stress.

Confirmation of ET-1 Mode of Action in the Induction of ER Stress in the BeWo Cell Line

The BeWo cell line was used to investigate if ET-1 can induce ER stress in an alternate model system, which would reduce the chance of a cell type-specific effect and give greater significance to the action of ET-1 in the induction of ER stress. Indeed, ET-1 induced a significant increase in ER stress compared with untreated controls (Figure 7). This stress could be blocked by both the

Figure 7. ET-1 induced the ER stress response in the BeWo cell line. Western blot analysis of ER stress markers, GRP78 and p-eIF2 α , under given conditions during a 1-hour time course, with densitometry of bands expressed relative to controls (100%) after the data were normalized to β -actin. Data are given as mean $\pm$ SEM ($n = 3$). * $P < 0.05$ compared with untreated controls; ** $P < 0.05$ compared with 10 nmol/L ET-1-treated samples.

Figure 8. Potential mechanism by which ET-1 induces ER stress in pathological pregnancies. DAG, diacylglycerol; PIP, phosphatidylinositol 4,5-bisphosphate.

ETBR-specific antagonist, BQ788, and the IP₃R inhibitor, xestospongin-C, which gave a significant reduction in GRP78 levels. These results confirm that ET-1 also acts via the ETBR in BeWo cells to activate the PLC-IP₃ pathway to induce ER stress. Tunicamycin was used as a positive control for this experiment. Neither BQ788 nor xestospongin-C had any effect on the tunicamycin-induced stress response, which confirmed that their effects were specific to the action of ET-1.

Discussion

These findings demonstrate that application of ET-1 to trophoblast-like cell lines causes activation of the UPR, as evidenced by increased levels of ER stress molecular markers: phosphorylation of eIF2 α and the ER chaperone proteins, GRP78 and GRP94. This effect is mediated via the ETBR, activation of the PLC-IP₃ pathway, and the release of calcium ions from intracellular stores. Furthermore, we demonstrate that concentrations of ET-1 increase in the supernatants of placental explants exposed to oxidative stress, and that the conditioned medium induces ER stress when applied to trophoblast-like cell lines. Thus, we conclude that, in cases of severe preeclampsia (PE + IUGR), oxidative stress may cause increased release of ET-1 by the placenta, which may act in an autocrine manner to induce syncytiotrophoblastic ER stress. A potential mechanism by which ET-1 may induce ER stress via the ETBR by initiating signaling through the PLC-IP₃ pathway is illustrated in Figure 8.

Although it would have been ideal to confirm the ET-1-induced stress response in primary cultures of trophoblast cells, preliminary work in our laboratory has demonstrated that the process of isolation and culture of trophoblast cells generates high levels of ER stress. The different markers of ER stress, GRP78, p-eIF2 α , and

C/EBP homologous protein, are all activated (data not shown). Hence, the ER stress induced by ET-1 would not be detectable against the high basal levels of stress. Furthermore, levels of AKT decline rapidly, indicating impending cell death. These changes may also explain why proliferative cytotrophoblast cells have not been isolated successfully, and why the cells have a limited life span in culture.

Recent studies⁴ have suggested that ER stress contributes to the pathogenesis of pregnancy complications, such as IUGR and PE + IUGR. One consequence of ER stress is the inhibition of nonessential protein synthesis, which can explain the smaller placental phenotype seen in complicated pregnancies. Activation of pro-inflammatory pathways may also occur under ER stress through phosphorylation of TRAF2 by Ire1, stimulating both the p38 mitogen-activated protein kinase and NF- κ B pathways.²⁴ The pro-inflammatory environment is thought to lead to maternal endothelial cell dysfunction, resulting in the syndrome of hypertension and proteinuria.²⁵ Because preeclampsia is associated with increased plasma levels of ET-1, a vasoactive peptide that has the potential to disrupt ER Ca²⁺ homeostasis, this study explored the role of ET-1 in the induction of ER stress.

ET-1 is involved in various physiological functions, including modulation of vascular tone, differentiation, development, cell proliferation, and hormone production.²⁶ ET-1 exerts its effects by binding to the ETAR and ETBR, two highly homologous cell surface proteins that belong to the G-protein-coupled receptor superfamily. IHC confirmed the presence of both ET-1 and its receptors in the syncytiotrophoblast. Immunoreactivity in both IHC and Western blot analyses for ET-1 and the ETBR, but not the ETAR, was increased in PE + IUGR sections compared with normotensive controls, consistent with previous clinical data that showed plasma ET-1 levels are elevated in preeclamptic patients compared with healthy pregnant women.¹⁴

The chaperone proteins, GRP78 and GRP94, and p-eIF2 α are all increased during the UPR and, therefore, serve as markers for ER stress. JEG-3 and BeWo chorionic carcinoma cells treated with ET-1 displayed an increase in the levels of these ER stress markers, indicating that ET-1 induces ER stress. This occurred via the ETBR, which is consistent with the fact that only ETBR levels are increased on the syncytiotrophoblast in pathological pregnancies. The IP data show that the ETBR is phospho-activated only under ET-1 treatment. The fact that ET-1 is acting via the ETBR was confirmed by treating cells with BQ788, an ETBR antagonist, and siRNA knockdown of the receptor, both of which inhibited the induction of ER stress (Figure 3).

Our data suggest that ET-1 stimulates ER calcium release via the PLC-IP₃ pathway, because ET-1 stimulates p-PLC γ 1 levels. Phosphorylation by Syk at Y783 activates the enzymatic activity of PLC γ 1.²¹ After activation, subsequent production of IP₃ leads to activation of the IP₃R in the ER membrane to induce Ca²⁺ release from the ER. Because ER Ca²⁺ regulates the formation of chaperone complexes in the ER, the folding and maturation of several proteins will be affected by depletion of Ca²⁺.⁵ Fur-

thermore, loss of Ca^{2+} homeostasis affects Ca^{2+} -dependent post-translational modifications, such as N-linked glycosylation, disulphide bond formation, and controlled proteolysis.⁹ Hence, we hypothesized that ET-1-stimulated disruption in ER Ca^{2+} homeostasis leads to an accumulation of misfolded proteins, provoking ER stress response pathways or the UPR. We found that the ET-1-induced increase in p-PLC levels could be inhibited by U73122, a membrane-permeable aminosteroid inhibitor that blocks activation of PLC by interfering with G-protein coupling.²⁷ This inhibitor also reduced the expression of all three ER stress markers previously used (Figure 4), confirming that p-PLC γ 1 activation is the mechanism by which ET-1 induces ER stress. Fluo-4 Ca^{2+} imaging was used to capture the actual release of Ca^{2+} from the ER induced by ET-1 (see Supplemental Figure S7 at <http://ajp.amjpathol.org>). This was inhibited by pretreating JEG-3 cells with the ETBR inhibitor, BQ788, which showed the effect was specific to the action of ET-1 via the ETBR.

The concentrations of inhibitors used in the study are based on previously used concentrations.^{26,28,29} A significant effect was also obtained at concentrations lower than the maximal dose of treatment. For example, there was a significant reduction in the levels of all ER stress markers at 25 $\mu\text{mol/L}$ BQ788. Furthermore, cell death assays showed no significant increase, except at 50 $\mu\text{mol/L}$ BQ788, during a 24-hour time course, which would be sufficient time for any possible toxic effects of these compounds to manifest (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). Hence, the doses used were nontoxic to the cells.

ET-1 induced ER stress in a bell-shaped dose-response, and there are two possible explanations for this phenomenon. Previous studies³⁰ have shown that ET-1 is able to activate protein kinase C after PLC-induced conversion of phosphatidylinositol 4,5-bisphosphate to diacylglycerol. The activation of protein kinase C can induce extracellular Ca^{2+} uptake, and we speculate that this may be a secondary effect occurring at the higher concentrations of ET-1. Consequently, release of Ca^{2+} from the ER may be reduced because of the lower ER-cytoplasm ionic gradient. Alternatively, excessive ET-1 could cause ETBR desensitization, thereby diminishing the efficacy of ET-1 in the induction of ER stress. Repetitive application of ET-1 to rat aortic rings results in diminished ET-1-induced nitric oxide production because of ETBR desensitization.³¹ More studies will be required to test these two possibilities.

The cause for the increased concentrations of ET-1 in the maternal plasma in preeclampsia¹⁴ is unclear, but early-onset disease is associated with deficient spiral artery conversion,²² causing decreased uteroplacental blood flow and either hypoxia or ischemia-reperfusion. Under hypoxic conditions, endothelial cells produce more ET-1³² and, thus, may contribute to the increased levels observed. Equally, our data show that H/R stimulates ET-1 release by the syncytiotrophoblast. H/R increased the levels of ET-1 in conditioned medium by approximately 8% when the H/R treatment was 16 hours. Longer exposure to H/R would most likely induce higher

ET-1 secretion into the medium, but we did not want to risk compromising the viability of the tissue and causing nonspecific release.

The fact that ET-1 and the ETBR are co-expressed in the syncytiotrophoblast suggests a possible autocrine mode of action of ET-1. This hypothesis is supported by our finding that conditioned medium from H/R-treated explants generated higher ER stress when applied to JEG-3 cells (a model for the trophoblast) compared with medium from controls maintained at 10% O_2 . The effect was abolished by a neutralizing antibody and, thus, was specific to ET-1. It is notable that ET-1 released by the placental tissue is able to induce ER stress in JEG-3 cells, even at a concentration lower than the synthetic ET-1 applied to cells. This could be because the conditioned medium from the explants contains other factors released under H/R that can sensitize the cells to the effects of ET-1. For instance, a recent study³³ from our group has shown that soluble fms-related tyrosine kinase 1, which is elevated in preeclampsia and after H/R *in vitro*, sensitizes endothelial cells to inflammatory cytokines, such as tumor necrosis factor α , by antagonizing vascular endothelial growth factor receptor-mediated signaling. Further studies are required to test this hypothesis.

In conclusion, this study indicates that ET-1 is a potential inducer of placental ER stress in early-onset preeclampsia. Because ETAR antagonists have proved beneficial for gestational hypertension,³⁴ co-application with ETB antagonists might provide additional improvement by reduction of placental ER stress.

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References

1. Roberts JM, Hubel CA: Is oxidative stress the link in the two-stage model of pre-eclampsia? *Lancet* 1999, 354:788–789
2. Lain KY, Roberts JM: Contemporary concepts of the pathogenesis and management of preeclampsia. *JAMA* 2002, 287:3183–3186
3. Moldenhauer JS, Stanek J, Warshak C, Khoury J, Sibai B: The frequency and severity of placental findings in women with preeclampsia are gestational age dependent. *Am J Obstet Gynecol* 2003, 189:1173–1177
4. Yung HW, Calabrese S, Hynx D, Hemmings BA, Cetin I, Charnock-Jones DS, Burton GJ: Evidence of placental translation inhibition and endoplasmic reticulum stress in the etiology of human intrauterine growth restriction. *Am J Pathol* 2008, 173:451–462
5. Stevens FJ, Argon Y: Protein folding in the ER. *Semin Cell Dev Biol* 1999, 10:443–454
6. Michalak M, Mariani P, Opas M: Calreticulin, a multifunctional Ca^{2+} -binding chaperone of the endoplasmic reticulum. *Biochem Cell Biol* 1998, 76:779–785
7. Ashby MC, Tepikin AV: ER calcium and the functions of intracellular organelles. *Semin Cell Dev Biol* 2001, 12:11–17
8. Mogami H, Tepikin AV, Petersen OH: Termination of cytosolic Ca^{2+} signals: Ca^{2+} reuptake into intracellular stores is regulated by the free Ca^{2+} concentration in the store lumen. *EMBO J* 1998, 17:435–442

9. Brostrom MA, Brostrom CO: Calcium dynamics and endoplasmic reticular function in the regulation of protein synthesis: implications for cell growth and adaptability. *Cell Calcium* 2003, 34:345–363
10. Zhang K, Kaufman RJ: From endoplasmic-reticulum stress to the inflammatory response. *Nature* 2008, 454:455–462
11. Yamamoto K, Sato T, Matsui T, Sato M, Okada T, Yoshida H, Harada A, Mori K: Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1. *Dev Cell* 2007, 13:365–376
12. Sharma OP, Flores JA, Leong DA, Veldhuis JD: Mechanisms by which endothelin-1 stimulates increased cytosolic-free calcium-ion concentrations in single rat Sertoli cells. *Endocrinology* 1994, 135:127–134
13. Struck J, Morgenthaler NG, Bergmann A: Proteolytic processing pattern of the endothelin-1 precursor in vivo. *Peptides* 2005, 26:2482–2486
14. Fiore G, Florio P, Micheli L, Nencini C, Rossi M, Cerretani D, Ambrosini G, Giorgi G, Petraglia F: Endothelin-1 triggers placental oxidative stress pathways: putative role in preeclampsia. *J Clin Endocrinol Metab* 2005, 90:4205–4210
15. Karet FE, Daveport AP: Endothelin and the human kidney: a potential target for new drugs. *Nephrol Dial Transplant* 1994, 9:465–468
16. Chakraborty C, Barbin YP, Chakrabarti S, Chidiac P, Dixon SJ, Lala PK: Endothelin-1 promotes migration and induces elevation of [Ca²⁺]_i and phosphorylation of MAP kinase of a human extravillous trophoblast cell line. *Mol Cell Endocrinol* 2003, 201:63–73
17. Cindrova-Davies T, Yung HW, Johns J, Spasic-Boskovic O, Korolchuk S, Jauniaux E, Burton GJ, Charnock-Jones DS: Oxidative stress, gene expression, and protein changes induced in the human placenta during labor. *Am J Pathol* 2007, 171:1168–1179
18. Yung HW, Korolchuk S, Tolkovsky AM, Charnock-Jones DS, Burton GJ: Endoplasmic reticulum stress exacerbates ischemia-reperfusion-induced apoptosis through attenuation of Akt protein synthesis in human choriocarcinoma cells. *FASEB J* 2007, 21:872–884
19. Forbes K, Westwood M, Baker PN, Aplin JD: Insulin-like growth factor I and II regulate the life cycle of trophoblast in the developing human placenta. *Am J Physiol Cell Physiol* 2008, 294:C1313–C1322
20. Cervar-Zivkovic M, Hu C, Barton A, Sadovsky Y, Desoye G, Lang U, Nelson DM: Endothelin-1 attenuates apoptosis in cultured trophoblasts from term human placentas. *Reprod Sci* 2007, 14:430–439
21. Wang Z, Gluck S, Zhang L, Moran MF: Requirement for phospholipase C-gamma1 enzymatic activity in growth factor-induced mitogenesis. *Mol Cell Biol* 1998, 18:590–597
22. Brosens IA, Robertso WB, Dixon HG: The role of the spiral arteries in the pathogenesis of pre-eclampsia. *J Pathol* 1970, 101:Pvi
23. Burton GJ, Jauniaux E: Placental oxidative stress: from miscarriage to preeclampsia. *J Soc Gynecol Investig* 2004, 11:342–352
24. Xu C, Bailly-Maitre B, Reed JC: Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 2005, 115:2656–2664
25. Redman CW, Sargent IL: Latest advances in understanding preeclampsia. *Science* 2005, 308:1592–1594
26. Rauh A, Windischhofer W, Kovacevic A, DeVaney T, Huber E, Semlitsch M, Leis HJ, Sattler W, Malle E: Endothelin (ET)-1 and ET-3 promote expression of c-fos and c-jun in human choriocarcinoma via ET(B) receptor-mediated G (i) - and G (q) -pathways and MAP kinase activation. *Br J Pharmacol* 2008, 154:13–24
27. Evdonin AL, Guzova IV, Margulis BA, Medvedeva ND: Phospholipase c inhibitor, u73122, stimulates release of hsp-70 stress protein from A431 human carcinoma cells. *Cancer Cell Int* 2004, 4:2
28. Jan CR, Tseng CJ, Chou KJ, Chiang HT: Novel effects of clotrimazole on Ca²⁺ signaling in Madin Darby canine kidney cells. *Life Sci* 2000, 66:2289–2296
29. Ozaki H, Hori M, Kim YS, Kwon SC, Ahn DS, Nakazawa H, Kobayashi M, Karaki H: Inhibitory mechanism of xestospongine-C on contraction and ion channels in the intestinal smooth muscle. *Br J Pharmacol* 2002, 137:1207–1212
30. He JQ, Pi Y, Walker JW, Kamp TJ: Endothelin-1 and photoreleased diacylglycerol increase L-type Ca²⁺ current by activation of protein kinase C in rat ventricular myocytes. *J Physiol* 2000, 524(Pt 3):807–820
31. Magazine HI, Srivastava KD: Thrombin-induced vascular reactivity is modulated by ETB receptor-coupled nitric oxide release in rat aorta. *Am J Physiol* 1996, 271:C923–C928
32. Heida HS, Gomes-Sanches CE: Hypoxia increases endothelin release in bovine endothelial cells in culture, but epinephrine, norepinephrine, serotonin, and angiotensin II do not. *Life Sci* 1990, 47:247–251
33. Cindrova-Davies T, Sanders DA, Burton GJ, Charnock-Jones DS: Soluble FLT1 sensitizes endothelial cells to inflammatory cytokines by antagonizing VEGF receptor-mediated signalling. *Cardiovasc Res* 2011, 89:671–679
34. George EM, Granger JP: Endothelin: key mediator of hypertension in preeclampsia. *Am J Hypertens* 2011, 24:964–969