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Endothelin-1 Triggers Placental Oxidative Stress Pathways: Putative Role in Preeclampsia

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Context: Preeclampsia (PE) is a disorder that occurs only during pregnancy. The placenta has a controlling role in this condition. Recent literature suggests that the oxidative stress is a component of PE and plays a main role in the link between decreased placental perfusion and the impaired function of maternal endothelium.

Objective: Because the human placenta expresses endothelin-1 (ET-1) and its circulating levels are high in pregnancies complicated with PE, the present study investigated the role of ET-1 on placental oxidative stress pathways.

Design: Human placental explants, JEG-3, and primary cytotrophoblast cells were cultured with increasing ET-1 concentrations for 6 and 24 h.

Setting: The study was conducted at tertiary clinical care centers in Siena and Padova, Italy

Interventions: Human placental explants, JEG-3, and primary cytotrophoblast cells were used to test ET-1 effect.

Main Outcome Measure(s): The main outcome measure was ET-1 mRNA and its receptor mRNAs, type A and B, detection by RT-PCR. The common markers of oxidative stress [malondialdehyde (MDA), glutathione (GSH), glutathione disulfide (GSSG), ascorbic acid (AA)] as well as cell proliferation and vitality were measured after stimulation periods.

Results: ET-1 inhibits cell proliferation and vitality and triggers oxidative stress in the human placenta by altering the balance between oxidant (increased MDA levels) and antioxidant (decreased GSH, GSSG, and AA) forces in favor of oxidation.

Conclusions: Because MDA damages endothelial cells, whereas GSH, GSSG, and AA protect them, we postulate that ET-1 may be one of the key links between primary placental disorders and the systemic endothelial dysfunction of PE. **(*J Clin Endocrinol Metab* 90: 4205–4210, 2005)**

PREECLAMPSIA (PE) IS a human pregnancy-specific disorder that adversely affects maternal vascular function and fetal intrauterine growth. It is the leading cause of maternal and perinatal mortality in developed countries (1). Its prevalence is between 3 and 10% of pregnancies, and it is diagnosed by the new development of hypertension, significant proteinuria, and remission of these signs after placental removal (1).

Although the central role of the placenta in the pathogenesis of PE is undisputed, the systemic endothelial cell dysfunction is the key event of the diverse clinical manifestations of PE (1–3). Indeed, increased maternal vascular permeability and enhanced vasoconstriction are major underlying pathophysiological events in PE and clinically, proteinuria, and interstitial edema are manifestations of the diminished endothelial barrier function that occur in the maternal circulation during PE (2, 3). However, the ill-defined links between the primary placental causes and the secondary systemic illness of PE are still debated. In this regard, it has been proposed that insufficient uteroplacental circulation leads to placental hypoxia, oxidative stress, and consequently the release of placental factors that disrupt normal endothelial barrier function and induce increased endothelial permeability (1–3).

Oxidative stress has been implicated in the pathophysiology of PE because it damages the maternal vascular endothelium, and there is incontrovertible evidence that the normal role of this cell layer is severely compromised in PE (4, 5). Indeed, free radicals released from the poorly perfused fetoplacental unit initiate lipid peroxidation by attacking polyunsaturated fatty acids in cell membranes and converting them to lipid peroxides (LPOs) and a variety of secondary metabolites (4, 5). Uncontrolled peroxidation alters membrane fluidity and permeability; therefore, the LPOs and their secondary metabolites, such as malondialdehyde (MDA; a good indicator of oxidant forces formed at a primary site), are then transported through the circulation by lipoproteins, causing damage at distant tissues (4–6). Lipid peroxidation is balanced in all cells and tissues by a variety of antioxidant mechanisms. Among these, glutathione (GSH) and ascorbic acid (AA) are important substances that belong to the systems involved in protecting against reactive oxygen compounds and free radicals, which are regarded as indi-

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Abbreviations: AA, Ascorbic acid; ET-1, endothelin-1; ETRA, ET-1 receptor type A; ETRB, ET-1 receptor type type B; FBS, fetal bovine serum; GSH, glutathione; GSSG, glutathione disulfide; LPO, lipid peroxide; MDA, malondialdehyde; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromides; PE, preeclampsia.

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cators of antioxidant forces. Upon exposure to oxidants, GSH forms glutathione disulfide (GSSG), thus changing the redox status of the GSH system, and changes in the reduced-oxidized ratio of thiol antioxidant (GSH to GSSG) reflect oxidative stress conditions (6).

PE is also associated with increased plasma levels of endothelin-1 (ET-1) (7, 8), a potent endogenous vasoconstrictor peptide thought to participate in the regulation of vascular tone in PE. Indeed, long-term (4 h) systemic infusions of ET-1 in pregnant sheep increase arterial pressure, reduce uteroplacental blood flow, and produce hemoconcentration and proteinuria (9), all of which are typical features of PE. ET-1 is known to be produced by endothelial cells (10), and its mRNA has been also found in the human placenta (11). Trophoblastic ET-1 mRNA expression was higher in PE than in control subjects (11), suggesting that increased production of ET-1 from placental tissue in PE might explain the elevated levels found in PE.

The aim of the present study was to investigate the effects of increasing the dose of ET-1 on oxidative stress in placental explant cultures, JEG-3 cells (a placental choriocarcinoma cell line), and primary cultured human cytotrophoblast cells and evaluate whether ET-1 is involved in the event cascade linking the human placenta and the endothelium through placental oxidative stress activation.

Materials and Methods

Placental explant culture

Placental villi obtained from late first-trimester (10–12 postmenstrual wk) legal terminations of pregnancy were dissected under sterile conditions in ice-cold PBS. Maternal consent was obtained according to the guidelines of the ethics committee, and fetuses from first-trimester terminations were normal. Small clusters of placental villi (50 mg/wet weight) were placed in 24-well plastic plates and cultured in Ham's F10 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/liter L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 C under 5% CO_2-95% air. The culture medium was removed after 2 d of culture, and the explants were treated with different concentrations of ET-1 (0–100 pg/ml) and dissolved in fresh Ham's F10 medium for 6 and 24 h. We chose these concentrations of ET-1 because the circulating concentrations of ET-1 in plasma from healthy pregnant women range from 5 to 10 pg/ml, whereas they range from 20 to 50 pg/ml in presence of PE (7, 8).

Experiments were performed at least six times, using a single placenta for each one.

Purification and isolation of cytotrophoblast cells

Placentae were obtained under sterile conditions from normal pregnancies undergoing elective cesarean section at term. Maternal consent was obtained according to the guidelines of the ethics committee. Trophoblast-enriched cell cultures were purified according to the method of Kliman *et al.* (12). Briefly, pieces of placental cotyledons weighing approximately 30 g were thoroughly rinsed in calcium- and magnesium-free Hanks' solution; villous tissue was identified and isolated from membranes, large vessels, decidua, and connective tissue under a dissection microscope and then coarsely minced with scissors and transferred to 150 ml Hanks' solution containing 0.25% trypsin, 25 mM HEPES, and 0.4 mg/ml type I DNase. The tissue was incubated in a water-shaking bath at 37 C for 30 min. The resultant cell suspensions were layered over FBS and centrifuged at 1000 $\times$ *g* for 5 min at room temperature, whereas the remaining placental tissue was digested two more times with the addition of fresh digestion solution.

The pellets collected after three digestions were resuspended in DMEM containing 25 mM HEPES, 25 mM glucose, and 2 mM glutamine, pooled, centrifuged at 1000 $\times$ *g* for 10 min and resuspended in 4 ml DMEM. This suspension was placed on a discontinuous Percoll gradient (5–70% Percoll in 5% steps), and centrifuged at 1200 $\times$ *g* for 30 min. A population of mononuclear cells that resulted as a band at a density from 1.051 to 1.065 g/ml was removed, washed with DMEM, and resuspended in cold PBS/BSA 0.1%. The nontrophoblast cells expressing common leukocyte antigen were removed by immunodepletion using a monoclonal antibody against CD45RB (clone PD7/26; Dako, Trappes, France). After the depletion the cells were resuspended in DMEM with 4 mM glutamine and 50 μg/ml gentamicin. This process of digestion, isolation, and depletion yields a trophoblast-enriched preparation containing approximately 95% trophoblast cells and 5% stromal contaminants, as ascertained by immunocytochemical analyses with cytokeratin 7 and vimentin.

RNA isolation and RT-PCR

Total RNA was isolated from JEG-3 cells using the Trizol reagent and quantified by UV absorption at 260 nm. One microgram of total RNA was reverse transcribed at the following conditions: 50 mM Tris acetate (pH 8.4), 75 mM potassium acetate, 8 mM magnesium acetate, 1 mM deoxynucleotide triphosphate mix, 15 U avian RNase H-minus reverse transcriptase (Invitrogen, Milan, Italy), 40 U RNase out, and 2.5 μM oligo d(T) primers in a final volume of 20 μl. The reaction was run at 65 C for 5 min and 50 C for 40 min, and then the enzyme was heat inactivated at 85 C for 5 min. Two microliters of reaction product were used for PCR. PCR conditions were: 20 mM Tris-Cl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphates, 1 U *Taq* DNA polymerase, recombinant (*Thermus aquaticus*, Invitrogen), and 0.4 μM (final concentration) primers (see Table 1 for their sequences) in 50 μl of total volume. The PCR profile included an initial denaturation at 94 C for 5 min, followed by 40 cycles of 94 C for 30 sec, 53–54 C (depending on the primer set) for 30 sec, and 72 C for 30 sec, and a final extension of 5 min at 72 C. For each reaction, a blank in which the cDNA sample was replaced by water was included to test the possibility of cross-contamination.

Amplification products were visualized on 2% agarose gel and stained with ethidium bromide. All primer sets were designed to span introns to rule out genomic DNA contamination.

JEG-3 choriocarcinoma cell culture

JEG-3 cells were obtained from the American Type Culture Collection (Manassas, VA; passage number 125) and were used within seven passages. JEG-3 cells were maintained routinely in RPMI 1640 medium supplemented with 10% FBS, 2 mmol/liter L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 C under 5% CO_2-95% air.

Cells were plated at a density of 6×10^5 cells/well in 6-well plastic plates, containing culture medium. JEG-3 cells were cultured for 2 d. When they had reached a subconfluent state, the culture medium was removed, and the cells were treated with different concentrations of ET-1 (0–100 pg/ml), dissolved in 2 ml of fresh RPMI 1640 medium for 6 and 24 h. The incubation was performed in a humidified 5% CO_2-95% air at 37 C.

Determination of proliferation and vitality

The trophoblast vitality and proliferation rate were determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromides (MTT) assay at the end of the exposure period with and without ET-1. Cells were plated at a density of 6×10^4 cells/well in 96-well plastic plates, and they have been treated as previously described in experimental protocol.

TABLE 1. Sequences of PCR primers

Primer	Sequence (5′–3′)	Size (bp)
ET-1	CCAAGGAGCTCCAGAAACAG GATGTCCAGGTGGCAGAAGT	169
ETRA	TATCCTGGCCATTCCTGAAG TTCTCAAGCTGCCATTCCTT	233
ETRB	TCCCGTTCAGAAGACAGCTT CAGAGGGCAAAGACAAGGAC	231

After 6 and 24 h of exposure with and without ET-1, MTT stock solution (5 mg/ml) was added to each well being assayed to equal one tenth the original culture volume and incubated for 4 h. At the end of the incubation period, converted dye was solubilized with acidic isopropanol (0.04–0.1 N HCl in absolute isopropanol). Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630 nm.

Measurement of GSH, GSSG, AA, and LPOs

The GSH, GSSG, AA, and LPOs levels were determined in placental explants, trophoblast cells after some steps of the protocol of purification, and JEG-3 cells.

For GSH, GSSG, and AA measurement in the placental explants, tissues were homogenized using Mixer Mill MM 300 (QIAGEN, Milan, Italy) in EDTA-K^+ phosphate buffer (pH 7.4) and 10% (wt/vol) metaphosphoric acid (1:1) at 4 C and centrifuged at 2000 × *g* for 10 min; supernatants were stored at −80 C until assayed.

LPO content was measured by the stable metabolite MDA using HPLC. Placental explants were homogenized in mixture (1:1) of 0.04 mol/liter Tris-HCl buffer (pH 7.4) and a solution of acetonitrile containing 0.1% butyl hydroxytoluene (4 C), to prevent the artificial oxidation of polyunsaturated free fatty acid during the assay, and centrifuged at 3000 × *g* for 15 min at 4 C. The supernatants were stored at −80 C until the assay.

JEG-3 cells were washed, trypsinized, and counted. For GSH, GSSG, and AA assay, cells were lysed and suspended in EDTA-K^+ phosphate buffer (pH 7.4) and 10% (wt/vol) metaphosphoric acid (1:1) at 4 C, immediately centrifuged at 2000 × *g* and 4 C for 10 min, and stored at −80 C until assay. Cells for MDA assay were conversely centrifuged, and the pellet was lysed with 3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (20 μM; Sigma, Milan, Italy). Then lysed cells were suspended in a mixture (1:1) of 0.04 mol/liter Tris-HCl buffer (pH 7.4), and a solution of acetonitrile containing 0.1% butyl hydroxytoluene (4 C) was centrifuged at 3000 × *g* for 15 min at 4 C, and the supernatants were stored at −80 C until assay.

Placental cells recovered during the purification protocol were treated like JEG-3 cells for the following measure of GSH and MDA levels. The concentrations of GSH and GSSG were measured by using a colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI). The concentrations of AA and MDA were measured by HPLC as previously described (13, 14), respectively.

Statistical analysis

Explant data were expressed as nanomoles per milligram protein for GSH and GSSG, and as nanomoles per gram tissue for MDA. The data are reported as mean ± SD for six experiments, each one performed on a single placenta. JEG-3 data were expressed as percent variation with respect to control (each point represents six experiments, performed in duplicate) to better evidence the differences between the various treatments with ET-1.

ANOVA for repeated-measures test followed by Bonferroni *post hoc* test was applied for statistical comparison. Differences were considered significant at a value of $P < 0.05$.

Results

Explants of placenta villi cultured with different ET-1 concentrations showed a significant ($P < 0.001$) decrease of GSH and a significant ($P < 0.01$) increase of MDA levels after both 6 and 24 h of treatment (Fig. 1, A and B). Indeed, the effects of ET-1 after 6 h were dose dependent on both MDA and GSH, and the different treatments did not show any significant difference among them after 24 h. After ET-1 addition, GSSG levels, reported as GSH to GSSG ratio to better show the changes induced by ET-1 on the GSH system, significantly ($P < 0.001$) decreased (Fig. 1C). This reduction was dose dependent after 6 h of treatment, but after the 24-h maximum, a decrease was observed in presence of the lowest

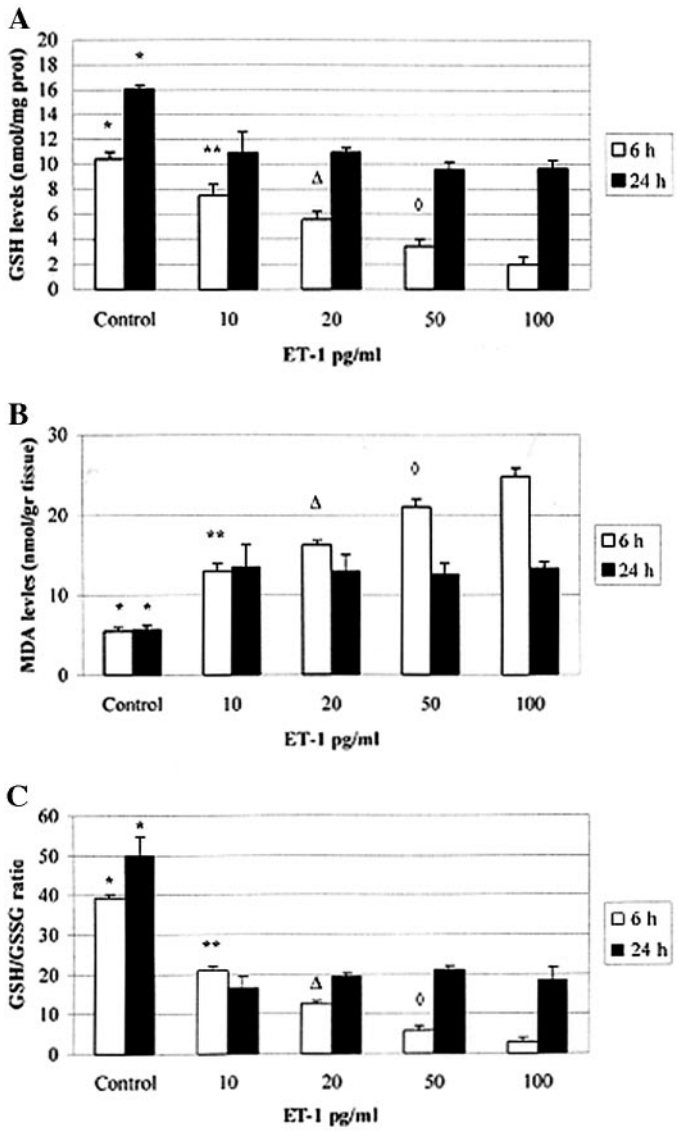

FIG. 1. Effect of increasing ET-1 concentrations on oxidative stress markers in placental explants. Each *bar* represents the mean ± SD of six experiments. A, GSH changes after 6 and 24 h of ET-1 of stimulation. After 6 h: *, $P < 0.001$ *vs.* 10, 20, 50, and 100 pg/ml ET-1; **, $P < 0.01$ *vs.* 20 and 50 pg/ml ET-1 and $P < 0.001$ *vs.* 100 pg/ml ET-1; Δ, $P < 0.01$ *vs.* 50 and 100 pg/ml ET-1; ▽, $P < 0.01$ *vs.* 100 pg/ml ET-1. After 24 h: *, $P < 0.001$ *vs.* 10, 20, 50, and 100 pg/ml ET-1. B, MDA changes after 6 and 24 h of ET-1 stimulation. After 6 h: *, $P < 0.001$ *vs.* 10, 20, 50, and 100 pg/ml ET-1; **, $P < 0.001$ *vs.* 20, 50, and 100 pg/ml ET-1; Δ, $P < 0.01$ *vs.* 50 and 100 pg/ml ET-1; ▽, $P < 0.01$ *vs.* 100 pg/ml ET-1. After 24 h: *, $P < 0.01$ *vs.* 10, 20, 50, and 100 pg/ml ET-1. C, GSSG, reported as GSH to GSSG ratio, changes after 6 and 24 h of ET-1 stimulation. After 6 h: *, $P < 0.001$ *vs.* 10, 20, 50, and 100 pg/ml ET-1; **, $P < 0.001$ *vs.* 20, 50, and 100 pg/ml ET-1; Δ, $P < 0.001$ *vs.* 50 and 100 pg/ml ET-1; ▽, $P < 0.01$ *vs.* 100 pg/ml ET-1. After 24 h: *, $P < 0.001$ *vs.* 10, 20, 50, and 100 pg/ml ET-1.

ET-1 dose because the GSH to GSSG ratio was not further affected by increasing ET-1 concentrations. The GSH/GSSG decrease induced by ET-1 suggested that the balance of the GSH system changed in favor of oxidation. Regarding AA, its levels did not change after both 6 and 24 h of treatment with ET-1 (data not shown). Taken together, our data led us to conclude that ET-1 does alter the balance between the

prooxidant and antioxidant systems in placental explants. However, this system cannot evaluate the net effect of ET-1 on trophoblast cells because placental villi are a complex structure, composed of terminal arterioles and postcapillary venules and surrounded by cytotrophoblast cells that are covered by the syncytiotrophoblast layer.

Thus, to investigate whether the trophoblast cells are a target of ET-1 and whether such a peptide could alter their redox balance, we directly isolated and purified trophoblast cells in placentas (n = 6) obtained from elective cesarean section at term. As reported in Table 2, the purification protocol significantly altered, step by step, GSH and MDA levels; the trypsinization step caused a high increase of MDA and a decrease of GSH levels, respectively; Percoll and immunodepletion steps decreased MDA levels and completely depleted GSH to undetectable concentrations.

Therefore, we decided to study the effects of ET-1 on JEG-3 cells, a human choriocarcinoma cell line widely used as a trophoblast cell model to understand placental functions.

First, we verified whether JEG-3 cells express ET-1 and its receptor type A (ETRA) and type B (ETRB). Specific primers were used in PCR to amplify cDNA prepared from JEG-3 cells, and RT-PCR resulted in the generation of DNA fragments with the expected sizes (Fig. 2). Thus, the effects of ET-1 on the vitality and proliferation of JEG-3 cells were considered through the MTT test, a precise indicator of mitochondrial function that measures directly the succinate dehydrogenase mitochondrial activity of living cells because the ODs obtained are directly proportional to the number of living cells. MTT evaluation showed that ET-1 significantly ($P < 0.001$) decreased the vitality and proliferation rate of the JEG-3 cells after both 6 and 24 h of incubation (Fig. 3A).

With respect to LPOs on JEG-3 lysates, ET-1 affected its concentrations: after 6 h of stimulation, higher ET-1 doses (50–100 pg/ml) induced a significant ($P < 0.001$) increase of MDA content, whereas after 24 h of stimulation MDA content was significantly ($P < 0.001$) increased by lower ET-1 concentrations (10–20 pg/ml) and then significantly ($P < 0.05$) decreased in presence of higher ET-1 levels (Fig. 3B). This decrease of LPO levels observed after 24 h of stimulation may be explained by GSH data. Indeed, the addition of ET-1 significantly ($P < 0.05$) decreased GSH levels to 20 pg/ml concentration, followed by a significant ($P < 0.01$) dose-dependent GSH increase in presence of higher ET-1 concentrations after both 6 and 24 h stimulation (Fig. 3C). Taken together, these findings would suggest that the intracellular GSH content after short and low ET-1 stimulation may be enough to protect the cells from the LPO production, whereas in presence of higher ET-1 doses, the increase of GSH levels, as already described (15), may be the compensating mechanism to reduce excessive lipid peroxidation.

This interpretation was further reinforced by data obtained on GSSG, reported as GSH to GSSG ratio. After 6 h of stimulation, ET-1 increased GSH oxidation to GSSG, resulting in a significant ($P < 0.01$) decrease of GSH to GSSG ratio, with a maximal effect at 20 pg/ml. After 24 h of stimulation, the redox status of the GSH system was in favor of GSSG to 20 pg/ml ET-1 concentrations and changed in favor of GSH at higher concentration (Fig. 4A). This ET-1-induced increase in the intracellular GSH levels may be associated with an increase in GSH synthesis and/or a reduction in GSH efflux out of the cell, which may protect cells against the oxidative stress induced by ET-1. In fact, this cellular reaction is essential to stop the lipid peroxidation progression.

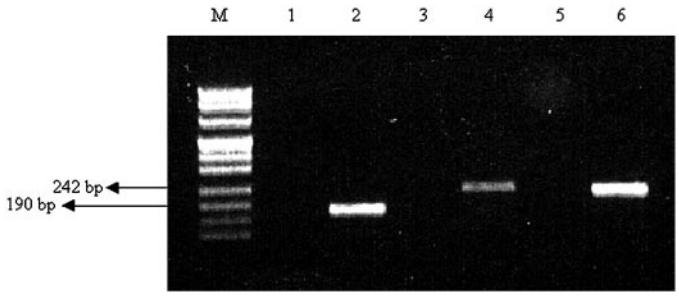

FIG. 2. Detection of ET-1 mRNA and its receptors mRNAs (ETRA, ETRB) in JEG-3 cells by RT-PCR. Lanes 1, 3, and 5, Blanks for each reaction; lane 2, ET-1; lane 4, ETRA; lane 6, ETRB. M, Molecular weight (marker VIII, Roche Diagnostic, Basel, Switzerland).

With respect to AA, the addition of exogenous ET-1 induced a significant ($P < 0.001$) dose-dependent increase of AA content in cell lysates only after 24 h of stimulation (Fig. 4B), suggesting that AA takes part in the cellular reply to compensate LPO excess occurring in presence of ET-1.

Discussion

Oxidative stress, defined as an imbalance between oxidant and antioxidant forces in favor of the former, is thought to be the link between the poor perfused placenta and the impaired maternal vascular function occurring in PE. Indeed, in PE the markers of oxidative stress are increased in maternal plasma (16) and the levels of antioxidant are decreased (17, 18); oxidative stress damages vascular endothelium (1–3). In the present study, we first found that ET-1, at concentrations corresponding to values in plasma from women with PE, stimulates the secretion of oxidative stress molecules, such as MDA, and simultaneously reduces the production of antioxidant molecules, such as GSH and GSSG, by trophoblast cells. The findings that after 6 h, but not 24 h, of treatment, ET-1 dose-dependently affects the balance between prooxidant and antioxidant system in placental explants lead us to suggest that the duration of ET-1 stimuli is also a critical factor and has great relevance in triggering the secretion of stress molecules. In addition, we also have to

TABLE 2. GSH and MDA levels during the protocol of purification and isolation of cytotrofoblast cells

Protocol steps	GSH levels	MDA levels
Fresh tissue	20.04 nmol/mg protein	4.91 nmol/g tissue
After trypsinization	0.015 nmol/mg protein	1.17 nmol/10^6 cells
After Percoll purification	0.0001 nmol/mg protein	0.18 nmol/10^6 cells
After immunodepletion	Undetected	0.13 nmol/10^6 cells

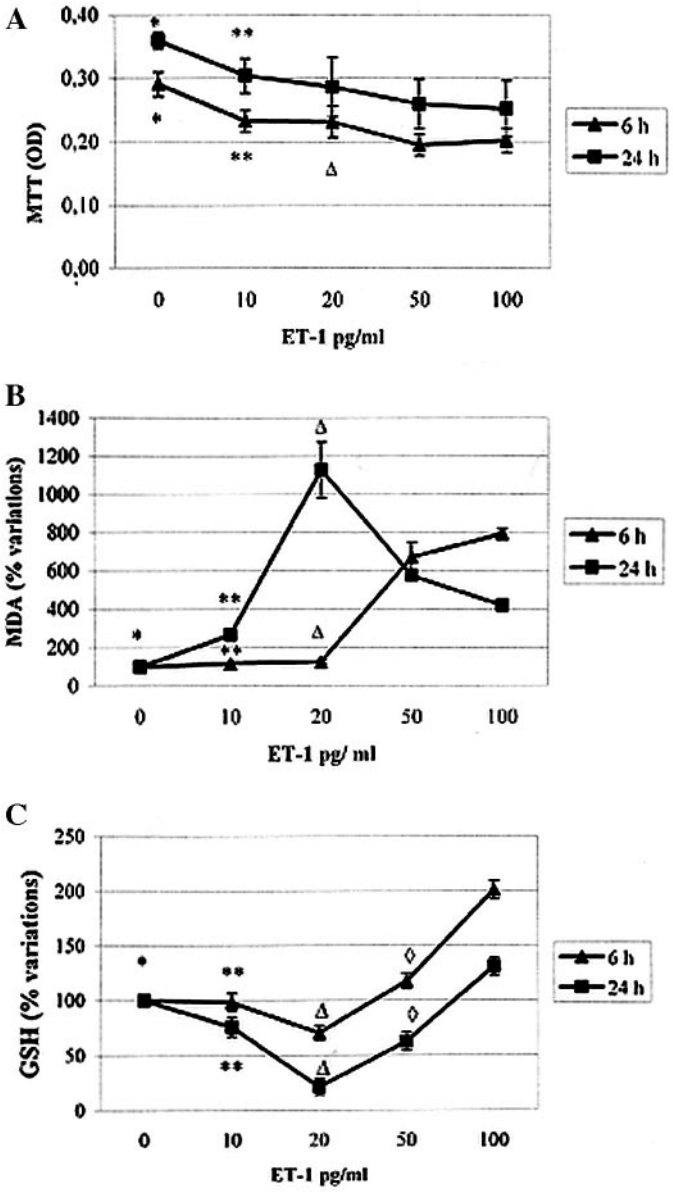

FIG. 3. Effect of increasing ET-1 concentrations on oxidative stress markers in JEG-3 cells. Each *point* represents the mean ± SD of six experiments, each performed in duplicate. A, Effect on the vitality and proliferation rate of JEG-3 cells as evaluated by MTT OD after 6 and 24 h of ET-1 incubation. After 6 h: *, $P < 0.001$ *vs.* 10, 20, 50, and 100 pg/ml ET-1; **, $P < 0.05$ *vs.* 50 pg/ml ET-1; Δ, $P < 0.05$ *vs.* 50 pg/ml ET-1. After 24 h: *, $P < 0.05$ *vs.* 10 pg/ml ET-1, $P < 0.01$ *vs.* 20 pg/ml ET-1, and $P < 0.001$ *vs.* 50 and 100 pg/ml ET-1; **, $P < 0.05$ *vs.* 100 pg/ml ET-1. B, MDA changes after 6 and 24 h of ET-1 incubation. After 6 h: *, $P < 0.001$ *vs.* 50 and 100 pg/ml ET-1; **, $P < 0.001$ *vs.* 50 and 100 pg/ml ET-1; Δ, $P < 0.001$ *vs.* 50 and 100 pg/ml ET-1. After 24 h: *, $P < 0.05$ *vs.* 100 pg/ml ET-1, $P < 0.01$ *vs.* 50 pg/ml ET-1, and $P < 0.001$ *vs.* 20 pg/ml ET-1; **, $P < 0.05$ *vs.* 50 pg/ml ET-1, $P < 0.001$ *vs.* 20 pg/ml ET-1; Δ, $P < 0.001$ *vs.* 50 and 100 pg/ml ET-1. C, GSH changes after 6 and 24 h of ET-1 incubation. After 6 h: *, $P < 0.05$ *vs.* 20 pg/ml ET-1 and $P < 0.001$ *vs.* 100 pg/ml ET-1; **, $P < 0.05$ *vs.* 20 pg/ml ET-1 and $P < 0.001$ *vs.* 100 pg/ml ET-1; Δ, $P < 0.01$ *vs.* 50 and 100 pg/ml ET-1; ▽, $P < 0.001$ *vs.* 100 pg/ml ET-1. After 24 h: *, $P < 0.001$ *vs.* 10, 20, 50, and 100 pg/ml ET-1; **, $P < 0.001$ *vs.* 20, 50, and 100 pg/ml ET-1; Δ, $P < 0.001$ *vs.* 50 and 100 pg/ml ET-1; ▽, $P < 0.001$ *vs.* 100 pg/ml ET-1.

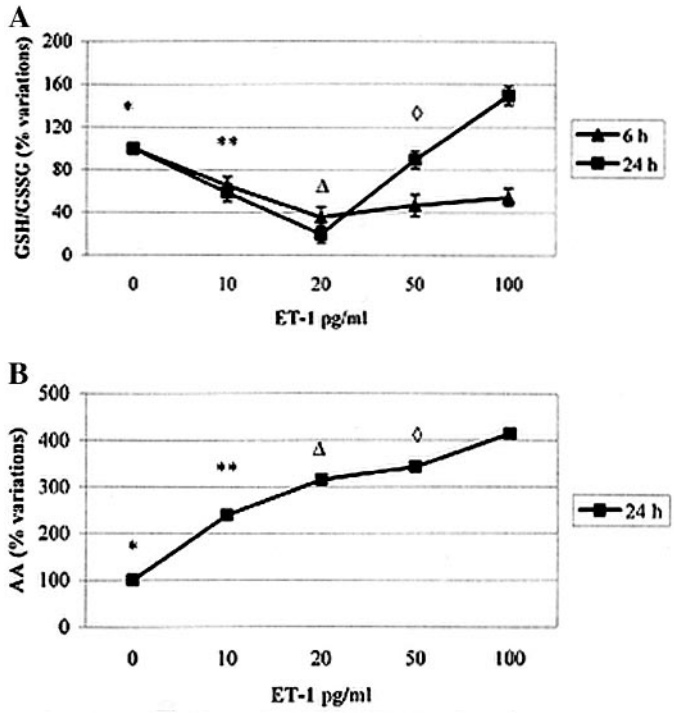

FIG. 4. Effect of increasing ET-1 concentrations on oxidative stress markers in JEG-3 cells. Each *point* represents the mean ± SD of six experiments, each performed in duplicate. A, Changes of GSH/GSSG system after 6 and 24 h of ET-1 incubation. After 6 h: *, $P < 0.05$ *vs.* 10 pg/ml ET-1 and $P < 0.001$ *vs.* 20, 50, and 100 pg/ml ET-1; **, $P < 0.05$ *vs.* 20 pg/ml ET-1. After 24 h: *, $P < 0.05$ *vs.* 10 pg/ml ET-1 and $P < 0.01$ *vs.* 20 and 100 pg/ml ET-1; **, $P < 0.05$ *vs.* 20 pg/ml ET-1 and $P < 0.001$ *vs.* 100 pg/ml ET-1; Δ, $P < 0.001$ *vs.* 50 and 100 pg/ml ET-1; ▽, $P < 0.001$ *vs.* 100 pg/ml ET-1. B, AA changes after 24 h of ET-1 incubation. After 24 h: *, $P < 0.001$ *vs.* 10, 20, 50, and 100 pg/ml ET-1; **, $P < 0.001$ *vs.* 20, 50, and 100 pg/ml ET-1; Δ, $P < 0.001$ *vs.* 100 pg/ml ET-1; ▽, $P < 0.001$ *vs.* 100 pg/ml ET-1.

consider that placental explants are a complex structure, composed not only of trophoblasts but also other cell types that may contribute to the balance between prooxidant and antioxidant molecules.

Because trophoblast cells are directly exposed to maternal blood in the intervillous space of the placenta, it is highly likely that these molecules will enter the maternal circulation during PE and disturb endothelial functions. This interpretation is reinforced by evidence showing that: 1) MDA is one of the secondary metabolites of lipid peroxidation, a mechanism that plays a central role in oxidative damage (19); 2) its levels are significantly increased in association with PE (1, 2, 16); and 3) exposure of endothelial cells in culture to pathophysiologic concentrations of MDA has a toxic effect (10), disorganizing oxidative phosphorylation in mitochondria and reacting with proteins and DNA bars (5).

With respect to GSH and AA, it is noteworthy that these molecules are nonenzymatic compounds involved in the defense mechanisms operating in the cells to protect themselves from reactive oxygen species. Indeed, GSH protects against reactive oxygen compounds and is also a key component of the antioxidant system because it is the substrate for glutathione peroxidase, an enzyme involved in the detoxification of lipid peroxides (6, 20). With respect to AA, it is a com-

ponent of the cellular antioxidant system, with similar antioxidant effects as GSH, and both AA and GSH are primary antioxidants able to protect the cells from free radicals and, under appropriate experimental conditions, to influence each other by enzymatic reaction (21). Our findings that long exposure to ET-1 decreased placental GSH secretion and the GSH to GSSG ratio, even in presence of lowest ET-1 concentrations, lead us to support the notion that ET-1 may alter the placental defenses against oxidative stress by reducing the antioxidant forces (GSH, GSSG) and stimulating oxidant forces (MDA) simultaneously, with a time-dependent effect on the placental equilibrium between oxidant and antioxidant forces in favor of the former.

In the present study, we also found that ET-1 significantly decreased the vitality and proliferation rate of trophoblast cells. Proliferation, function, and viability of trophoblasts are affected in PE, thus supporting the notion that degeneration of syncytiotrophoblasts, as well as infarction and necrosis, is a representative pathological findings in PE (22, 23). However, during PE the placental ischemia ensuing from the reduction in uterine perfusion pressure is thought to lead to enhanced formation of ET-1 that, in turn, plays a major role in mediating the hypertension as a rescue mechanism to enhance the flow of maternal blood through the placenta by increasing maternal blood pressure (2). Thus, it could be argued that there is an advantage for the fetus that placenta secretes higher amounts of ET-1 into the maternal circulation, with the aim to enhance the flow of maternal blood to and through the placenta (2, 3, 24). However, persisting an inadequate placental perfusion, the sustained secretion of ET-1 may result in a high-risk strategy because its elevated amounts may have cytotoxic effects on trophoblast and trigger oxidant dangerous substance release from human placenta that enter into the maternal circulation and contribute to cellular membrane damage and widespread endothelial dysfunction.

In conclusion, the findings that at concentrations corresponding to values in plasma from PE women, ET-1 induces oxidative stress in human endothelial (15) and placental cells and had cytotoxic effects on trophoblast lead us to suggest that ET-1 may be the key link between the primary placental causes and the secondary systemic illness of PE, supporting the toxemia theory, which proposes that the compromised placenta produces substances leading to the maternal syndrome of PE (1).

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