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Expression of Endothelial NO Synthase, Inducible NO Synthase, and Estrogen Receptors Alpha and Beta in Placental Tissue of Normal, Preeclamptic, and Intrauterine Growth-restricted Pregnancies

Barbara Schiessl, Ioannis Mylonas, Peer Hantschmann, Christina Kuhn, Sandra Schulze, Susi Kunze, Klaus Friese, and Udo Jeschke

Ludwig-Maximilians-University Munich, 1st Department of Obstetrics and Gynaecology, Maistrasse 11, 80337 Munich, Germany

SUMMARY In the physiology of placental blood circulation, nitric oxide (NO) synthases seem to play important roles, although their expression in pathological placentas and their role is still unclear. In addition, NO synthase activation seems to be related to estrogen receptor expression. Therefore, the aims of this study were to investigate the expression of estrogen receptors alpha (ERα), estrogen receptor beta (ER and the endothelial NO synthase (eNOS), and inducible NO synthase (iNOS) in intrauterine growth-restricted (IUGR) placentas, preeclamptic placentas, and in normal healthy control placentas. Slides of paraffin-embedded placental tissue were obtained after delivery from patients diagnosed with IUGR, preeclampsia, and normal term placentas and analyzed for eNOS, iNOS as well as ERα and ERβ expression. Intensity of immunohistochemical reaction was analyzed using a semiquantitative score and statistical analysis was performed. In addition, Western blot experiments were performed for comparison of staining intensities obtained by immunohistochemistry and western blot. Expression of eNOS, iNOS, and ERβ is significantly reduced in trophoblast cells of placentas with IUGR. However, preeclamptic placentas demonstrated a significant elevated expression intensity of these proteins compared with normal controls. A different expression of eNOS, iNOS, ERα, and ERβ by human trophoblast cells seems to results in lower NO output and impaired trophoblast invasion. Results obtained in our study provide evidence that reduced expression of the investigated proteins is related to IUGR. **(J Histochem Cytochem 53:1441–1449, 2005)**

DURING ESTABLISHMENT OF FETOPLACENTAL CIRCULATION, uterine spiral arteries undergo remodeling: spiral artery endothelial cells are replaced by endovascular extravillous trophoblasts cells (EVT) and the arterial smooth muscle and elastic is lost and replaced by fibrinoid (Brosens et al. 1967; Pijnenborg et al. 1983). This process terminates in low-resistance, high-output vessels. Placental blood flow thereafter is dependent on humoral and endothelial derived factors because placental tissue and vessels lack autonomic innervation (Nasiell et al. 1998). A decrease in uterine impedance parallels advancing gestational age up to the 22nd week of pregnancy and remains stable until delivery (Yagel et al. 1999; Papageorghiou et al. 2001,2002; Di Paolo et al. 2003).

Failure of this process has been associated with complications of pregnancy such as preeclampsia (PE), intrauterine growth restriction (IUGR), and, in severe cases, second-trimester miscarriage (Pijnenborg et al. 1991).

IUGR is diagnosed either by intrauterine growth assessment (sonography) showing an estimated weight below the 5th centile for gestational age or postnatal showing a birth weight below the third centile (Chatelain 2000). Small fetuses resulting from IUGR are at higher risk for poor perinatal and long-term outcome (Baschat and Hecher 2004).

Preeclampsia is associated with significant maternal

and perinatal morbidity in those patients who suffer early onset of PE (Myatt and Miodovnik 1999). Patients with chronic hypertension, pregestational diabetes, or multifetal gestation are at risk for developing preeclampsia as are nulliparous women, but factors defining the risk for multiparous women are yet to be defined. Although factors such as callicrein-creatinine, coagulation, and vascular function tests and oxidant stress parameters as well as placental peptide hormones have been identified as potential markers for patients at risk for PE prospective and longitudinal studies are mandatory to verify the data (Myatt and Miodovnik 1999).

Nitric oxide (NO) as a potent vasodilator is thought to contribute to the phenomenon of decreasing vascular resistance in uterine circulation, but still its role in normal pregnancy and pregnancies complicated by PE or IUGR is controversial and remains to be clarified (Nasiell et al. 1998). Izumi and coworkers report on vasorelaxation in the human umbilical artery: their data suggest that NO synthase (NOS) is stimulated in endothelial cells and the derived NO activates guanylate cyclase to produce cyclic guanosine monophosphate in umbilical smooth muscle cells (Izumi et al. 1995). The NO systems also seems to be mainly involved in regulation of uterine quiescence during pregnancy and initiation of labor: Buhimshi et al. demonstrated an NO-cyclic guanosine monophosphate reaction pathway in the human uterus (Buhimschi et al. 1995).

The question if impaired trophoblast invasion is related to different expression of endothelial NOS (eNOS) and inducible NOS (iNOS) and results in lower NO output or if the remaining elevated impedance at the uterine and spiral arteries causes elevated compensatory NO production is topic of ongoing discussions. Purcell and colleagues showed changing concentrations of iNOS in rat placentas during the course of pregnancy with a decrease after day 16 to day 22 before labor and during delivery (Purcell et al. 1997). The reported data suggest a paracrine role for nitric oxide in regulation of uterine contractility, blood flow and immunosuppression, which are all requested for pregnancy maintenance (Purcell et al. 1997).

Myatt et al. examined placental villous tissue from normal, PE, and IUGR pregnancies by investigating the expression of eNOS (Eis et al. 1995; Myatt et al. 1997). They found increased eNOS expression in IUGR, which they interpreted as a possible adaptive response to increased resistance and poor perfusion in these pathological pregnancies (Eis et al. 1995; Myatt et al. 1997). Nasiell investigated expression of endothelial constitutive NOS (Nasiell et al. 1998). Total nuclei acids were prepared and a hybridization technique was used for mRNA analysis. The mRNA expression was significantly higher in pathological groups (IUGR, IUGR+PE, and PE) compared with normal placentas, which might re-

flect a compensatory mechanism in the disturbed uterine circulation seen in PE or IUGR (Nasiell et al. 1998). Within the three pathologic groups investigated, no significant differences in elevated endothelial constitutive NOS expression in IUGR, IUGR + PE, and PE alone was found. Based on these findings, elevated NO concentrations in venous umbilical blood in placentas of IUGR have also been reported, suggesting a compensatory response to improve placental circulation (Lyall et al. 1996; Macara et al. 1996). Additionally, elevated NO concentrations could also play a role in limiting platelet adhesion aggregation (Lyall et al. 1996). In contrast, Beinder and coworkers found that NOS activity from patients with preeclampsia was significantly lower in the uterine placental bed (Beinder et al. 1999).

Based on the hypothesis that heat exposure disrupts placental structure and reduces placental eNOS protein expression, Galan et al. described reduced eNOS protein content in the hyperthermic group (Galan et al. 1999,2001). Myatt et al. showed that eNOS is expressed by the syncytiotrophoblast (Myatt et al. 1993). Trophoblast differentiation is associated with expression of estrogen receptors (ER)α and ERβ (Bukovsky et al. 2003a,b). Interestingly, the ERα expression has been associated with a inhibition of angiogenesis in cancer cells (Ali et al. 2000), but if this also is the case in human pregnancy remains unknown (Ali et al. 2000). We therefore combined the immunohistochemical detection of eNOS and iNOS with investigations on ERα and ERβ expression in normal, PE, and IUGR placentas.

The aims of this study were (a) clarifying the relation between expression of iNOS/eNOS and (b) assessing the expression of ERα and ERβ in normal, preeclamptic and growth restricted human placental tissue by immunohistochemistry and Western blot experiments.

Materials and Methods

Tissue Samples

Placental tissues were obtained from 22 women who underwent delivery at the 1st Department of Obstetrics and Gynecology of the LMU Munich. Specimens were collected immediately after delivery from six patients with IUGR (mean date of delivery: 33 ± 3 weeks of gestation), eight patients with preeclampsia (mean date of delivery: 33 ± 3.2 weeks of gestation), and eight patients after a normal course of pregnancy (mean date of delivery: 38.2 ± 3 weeks of gestation) after delivery. The study had the approval of the local ethical committee of the LMU Munich, Germany (No. 158/00) and informed consent from the patients was obtained.

Immunohistochemistry

Immunohistochemistry on paraffin sections (7 μm) of the different specimens was done by incubating the slides in methanol/H$_2$O$_2$ (30 min) to inhibit endogenous peroxidase activ-

ity, followed by washing in PBS (5 min) and treating with goat serum (20 min, 22C) to reduce nonspecific background staining. Incubation with the primary antibody (Table 1) was done overnight at 4C. Sections were then thoroughly incubated with the biotinylated secondary anti-mouse or anti-rabbit antibody (1 hr, 22C) and avidin-biotinylated peroxidase (45 min, RT). Between each step, sections were washed with PBS (pH 7.4). Peroxidase staining reaction was done with diaminobenzidine/H_2O_2 (1 mg/ml; 5 min) and stopped in tap water (10 min). Sections were counterstained in hemalum (1 min) and then cover-slipped. In controls, the primary antibody was replaced with preimmune mouse serum with positive and negative controls being included. From each section, five digital images were obtained with a 3CCD color camera (JVC; Victor Company of Japan, Japan) and a Leitz (Wetzlar, Germany) microscope.

Immunochemical Detection of eNOS, iNOS, ERα, and ERβ in Villous Trophoblast Cell Lysates on Blots

Villous trophoblast cells (600 μg) of normal, PE, and IUGR placental tissue were lysed in 400 μl Laemmli sample buffer for 5 min at 100C. Lysate proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 4% BSA in TBS/Tween (20 mM Tris/HCl, pH 7.2, 1 M NaCl, 0.05% Tween 20), blots were incubated with 2 μg/ml antibodies (Table 1) with 1% BSA for 16 hr at 6C. Blots were washed and incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate (diluted 1:500 in TBS/Tween with 1% BSA) for 2 hr or with goat anti-mouse IgG-alkaline phosphatase conjugate (diluted 1:500 in TBS/Tween with 1% BSA) for 2 hr. Staining was performed with 5-bromo-4-chloro-3-indolyle phosphate/nitroblue-tetrazolium chloride in 0.1 M Tris-HCl, 0.15 M NaCl, pH 9.5.

Immunohistochemical Evaluation and Statistical Analysis

The intensity and distribution patterns of the staining reaction was evaluated by two blinded, independent observers, including a gynecological pathologist (PH), using a semiquantitative score (graded as 0 = no, 1 = weak, 2 = moderate, and 3 = strong staining) and without knowing the pathological evaluation, the diagnosis, or the standard performed hematoxylin reaction of each specimen. The SPSS/PC software package, version 6.01 (SPSS; Munich, Germany), was used for collection, processing, and statistical analysis of all data. Statistical analysis was performed using the nonparametrical Wilcoxon's signed rank tests for comparison of the means. The Spearman rho coefficient was used to assess any significant correlations between the analyzed substances within the distinct groups. $p < 0.05$ values were considered statistically significant.

Table 1 Antibodies used for the study

Antigen	Antibody	Isotype	Dilution	Source
iNOS	PA3-030A	Rabbit IgG	1:1500	Dianova
eNOS	PA1-037	Rabbit IgG	1:300	Dianova
ERα	ER 1D5	Mouse IgG1	1:150	Immunotech
ERβ	PPG5/10	Mouse IgG2a	1:700	Serotec

Results

Immunohistochemical eNOS/iNOS Expression

We found a strong immunohistochemical expression of eNOS in normal syncytiotrophoblast cells (Figure 1A) and preeclamptic syncytiotrophoblast cells (Figure 1B). In IUGR placentas, the intensity of the eNOS expression was reduced (Figure 1C). However, a strong expression of eNOS in extravillous trophoblast cells of normal-term placentas (Figure 1D) and preeclamptic placentas (Figure 1E) could be demonstrated. In IUGR placentas, eNOS expression in extravillous trophoblasts cells is reduced (Figure 1F). The expression of iNOS is similar to eNOS. We found a strong expression of iNOS in normal syncytiotrophoblast cells (Figure 2A) and preeclamptic syncytiotrophoblast cells (Figure 2B). In IUGR placentas, expression of iNOS in the syncytiotrophoblast cells is reduced (Figure 2C). In addition, we found a strong expression of iNOS in extravillous trophoblast cells of normal term placentas (Figure 2D) and preeclamptic placentas (Figure 2E). In IUGR-placentas iNOS expression is reduced (Figure 2F) in extravillous trophoblast cells. The semiquantitative score of the eNOS/iNOS staining intensity is summarized in Figure 5. Differences in eNOS expression in the syncytiotrophoblast of normal and IUGR placentas and preeclamptic and IUGR placentas are statistically significant ($p = 0.039$ and $p = 0.038$).

ERα/ERβ Expression

A moderate expression of ERα in normal syncytiotrophoblast cells (Figure 3A), PE syncytiotrophoblast (Figure 3B) and IUGR syncytiotrophoblast cells (Figure 3C) was noted. Additionally, we also demonstrated a moderate expression of ERα in extravillous trophoblast cells of normal-term placentas (Figure 3D), PE placentas (Figure 3E), and IUGR-placentas (Figure 3F). The semiquantitative score of the ERα staining intensity showed no significant differences in all groups investigated. We found a low expression of ERβ in normal syncytiotrophoblast cells (Figure 4A) and a moderate expression in PE syncytiotrophoblasts (Figure 4B). In IUGR placentas, expression of ERβ in the syncytiotrophoblast cells is reduced (Figure 4C). In addition, we found a moderate expression of ERβ in extravillous trophoblast cells of normal-term placentas (Figure 4D) and PE placentas (Figure 4E). In IUGR placentas, ERβ expression in extravillous trophoblasts cells is almost not detectable (Figure 4F). The semiquantitative score of the eNOS/iNOS staining intensity is summarized in Figure 5, whereas the semiquantitative score of the ERα/ERβ staining intensity is summarized in Figure 6. Interestingly, a strong correlation between ERα expression and iNOS/eNOS could be demonstrated ($p < 0.05$ and $p < 0.005$, respectively) in the syncytiotrophoblast of normal placentas. However, no correlation between these factors was observed in placentas of IUGR and preeclamptic patients.
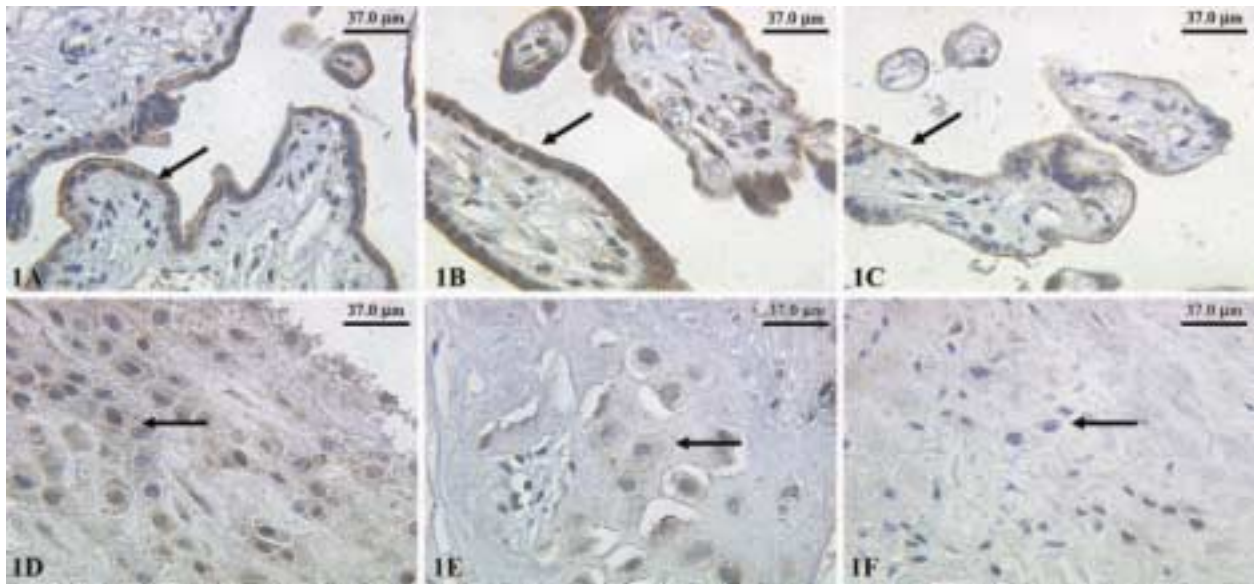
Figure 1 Expression of endothelial nitric oxide synthase (eNOS) in normal syncytiotrophoblasts indicated by arrows (**A**). Preeclamptic syncytiotrophoblast (**B**) and intrauterine growth restriction (**C**) detected with polyclonal rabbit antibodies of the IgG subtype and staining with diaminobenzidine; ×25 lens. Expression of eNOS was also detected in extravillous trophoblast cells indicated by arrows of normal-term placentas (**D**), preeclamptic placentas (**E**), and intrauterine growth–restricted placentas (**F**); ×25 lens.

Immunochemical Detection of eNOS, iNOS, ERα, and ERβ in Villous Trophoblast Cell Lysates on Blots

Results of immunochemical detection (Western blots) of eNOS in villous trophoblast cell lysates are shown in Figure 7A. The eNOS polyclonal antibody generates a main protein band in the 140 kDa molecular mass range. Normal villous trophoblast tissue (Lane 1) and PE trophoblast tissue (Lane 2) showed almost
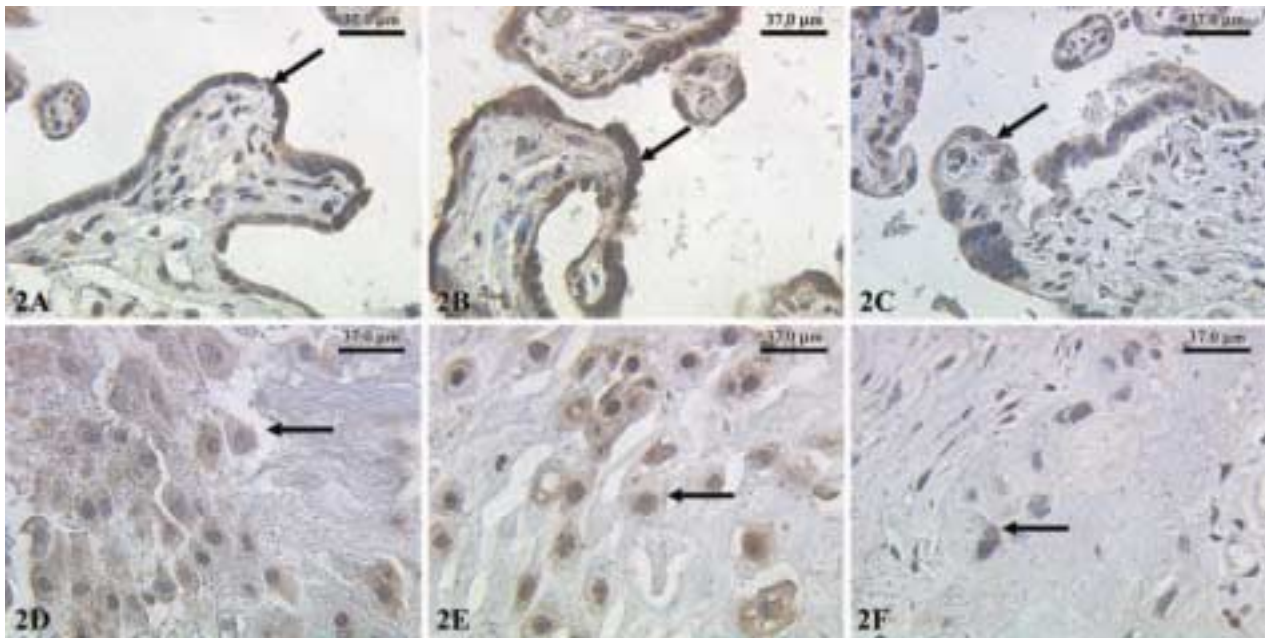

Figure 2 Expression of inducible nitric oxide synthase (iNOS) (indicated by arrows) in normal syncytiotrophoblasts (**A**), preeclamptic syncytiotrophoblast (**B**), and intrauterine growth restriction (**C**) detected with polyclonal rabbit antibodies of the IgG subtype and staining with diaminobenzidine; ×25 lens. Expression of iNOS was also detected in extravillous trophoblast cells of normal-term placentas (**D**), preeclamptic placentas (**E**), and intrauterine growth–restricted placentas (**F**); ×25 lens.
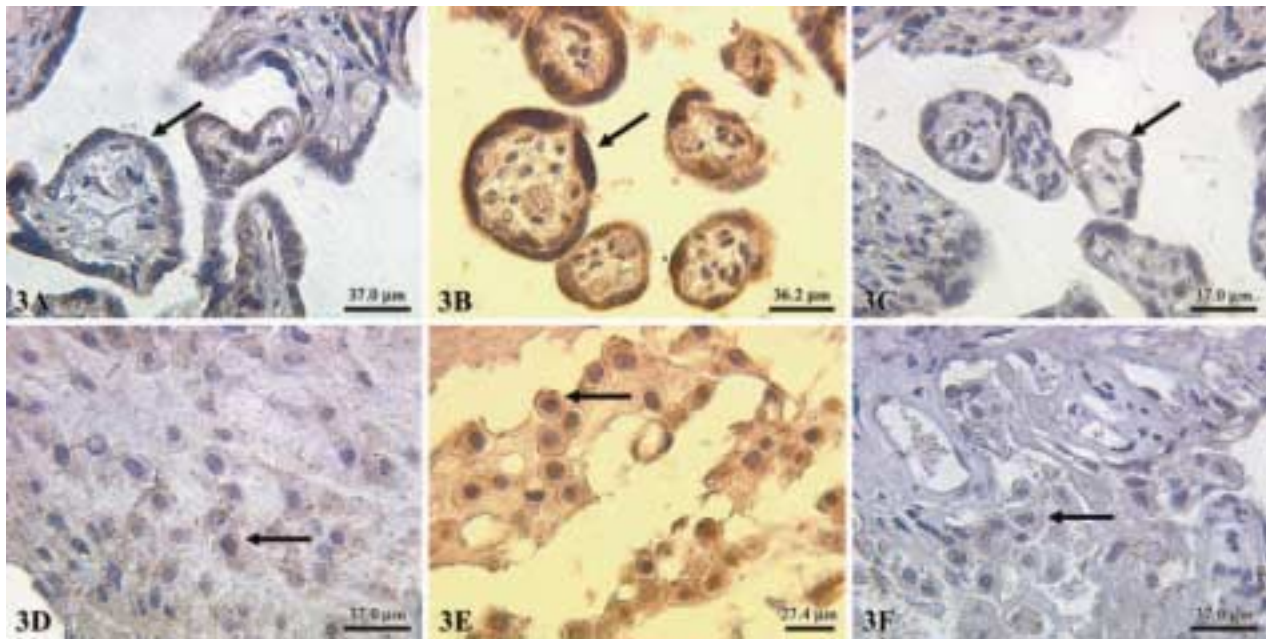
Figure 3 Expression of estrogen receptor (ER)α (indicated by arrows) in normal syncytiotrophoblasts (**A**), preeclamptic syncytiotrophoblast (**B**), and intrauterine growth restriction (**C**). Expression of ERα was also detected in extravillous trophoblast cells of normal term placentas (**D**) preeclamptic placentas (**E**) and intrauterine growth–restricted placentas (**F**); ×25 lens.

the same staining intensity, whereas, in IUGR tissue (Lane 3), eNOS expression is reduced.

Results of immunochemical detection (Western blots) of iNOS in villous trophoblast cell lysates are shown in Figure 7B. The iNOS polyclonal antibody generates a main protein band in the 130-kDa molecular mass range. Normal villous trophoblast tissue (Lane 1) and preeclamptic trophoblast tissue (Lane 2) showed al-
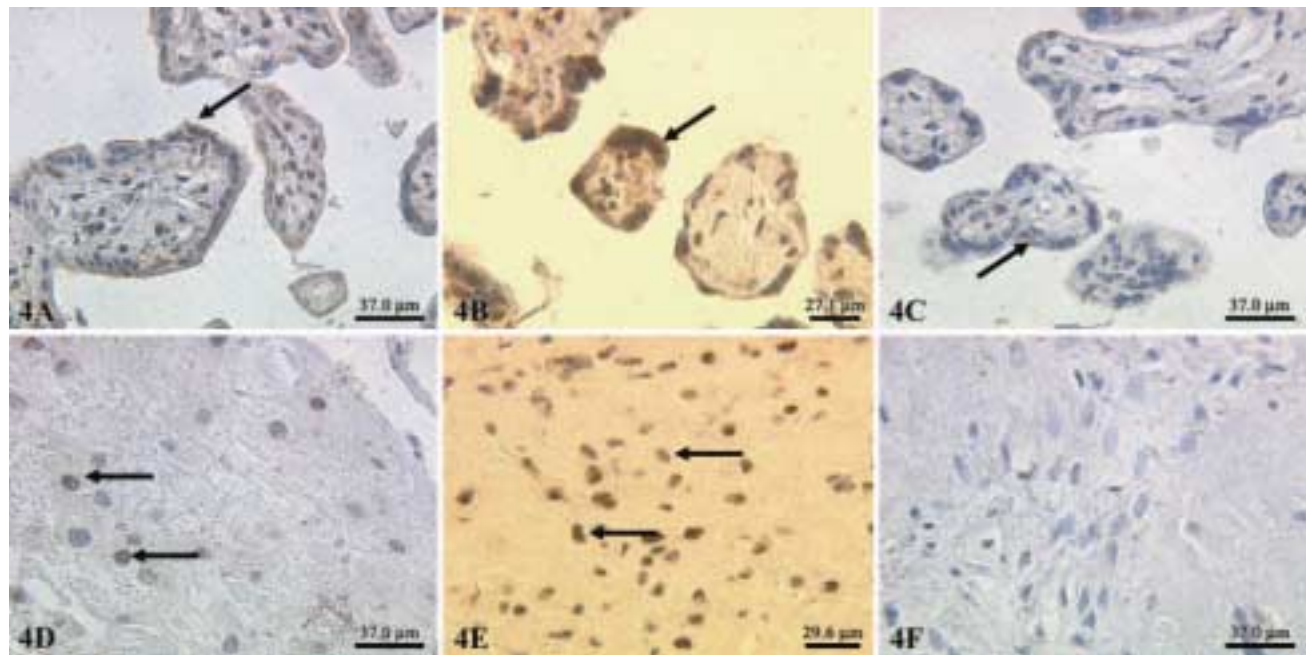

Figure 4 Expression of estrogen receptor (ER)β (indicated by arrows) in normal syncytiotrophoblasts preeclamptic (**A**), syncytiotrophoblast (**B**), and intrauterine growth restriction (**C**). Expression of ERβ was also detected in extravillous trophoblast cells of normal-term placentas (**D**), preeclamptic placentas (**E**), and intrauterine growth–restricted placentas (**F**); ×25 lens.
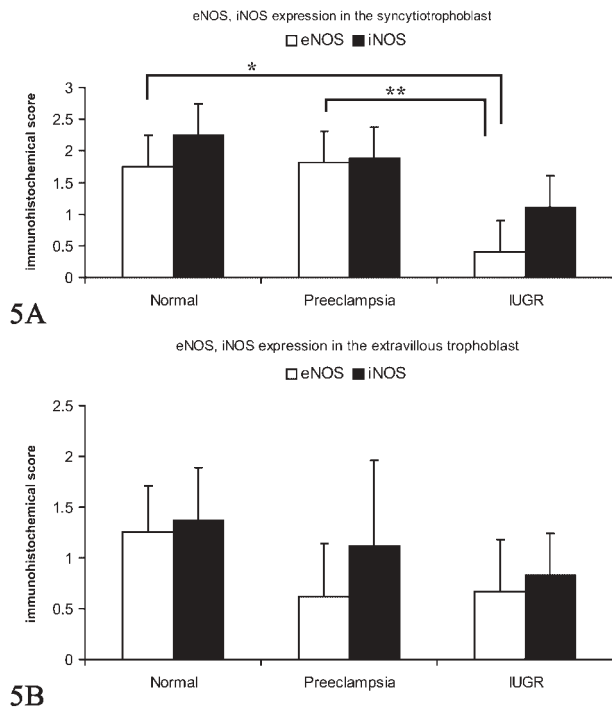
Figure 5 (**A**) Staining intensity of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) in normal, preeclamptic, and intrauterine growth–restricted (IUGR) syncytiotrophoblast determined by a semiquantitative immunohistochemical score on the different tissue slides. Data shown represent mean ± SEM. Differences in eNOS expression in normal and IUGR syncytiotrophoblast (*) and preeclamptic and IUGR syncytiotrophoblast (**) are statistically significant (p=0.039 and p=0.038). (**B**) Staining intensity of eNOS and iNOS in normal, preeclamptic, and IUGR extravillous trophoblast determined by a semiquantitative immunohistochemical score on the different tissue slides. Data shown represent mean ± SEM.
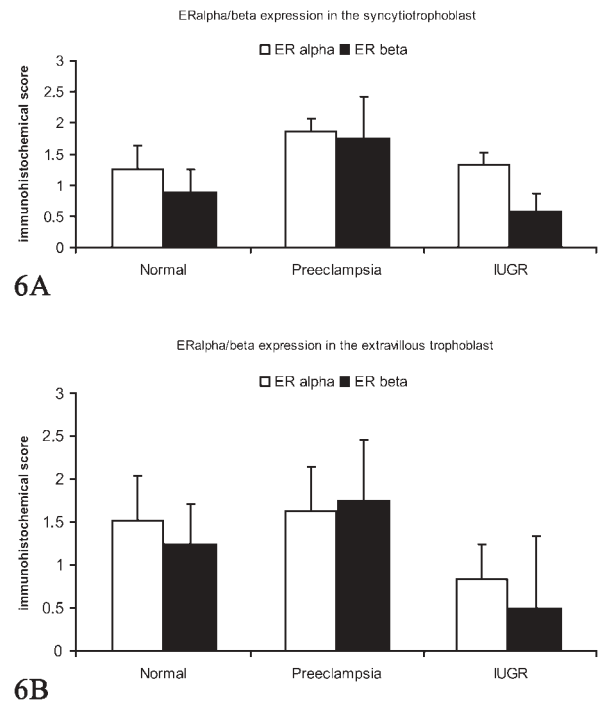

Figure 6 (**A**) Staining intensity of estrogen receptor (ER)α and ERβ in normal, preeclamptic, and intrauterine growth–restricted (IUGR) syncytiotrophoblast determined by a semiquantitative immunohistochemical score on the different tissue slides. Data shown represent mean ± SEM. (**B**) Staining intensity of ERα and ERβ in normal, preeclamptic, and IUGR extravillous trophoblast determined by a semiquantitative immunohistochemical score on the different tissue slides. Data shown represent mean ± SEM.

most the same staining intensity, whereas in IUGR tissue (Lane 3) iNOS expression was reduced.

Results of immunochemical detection (Western blots) of ERα in villous trophoblast cell lysates are shown in Figure 8A. The ERα monoclonal antibody generates a main protein band in the 67-kDa molecular mass range. Normal villous trophoblast tissue (Lane 1), preeclamptic trophoblast tissue (Lane 2), and IUGR tissue (Lane 3) showed almost the same staining intensity.

Results of immunochemical detection (Western blots) of ERβ in villous trophoblast cell lysates are shown in Figure 8B. The ERβ monoclonal antibody generates a main protein band in the 57-kDa molecular mass range. Normal villous trophoblast tissue (Lane 1) and PE trophoblast tissue (Lane 2) showed almost the same staining intensity, whereas in IUGR tissue (Lane 3), ERβ expression is reduced.

Discussion

We investigated the expression of ERα, ERβ, eNOS, and iNOS in IUGR, PE, and normal control placentas.
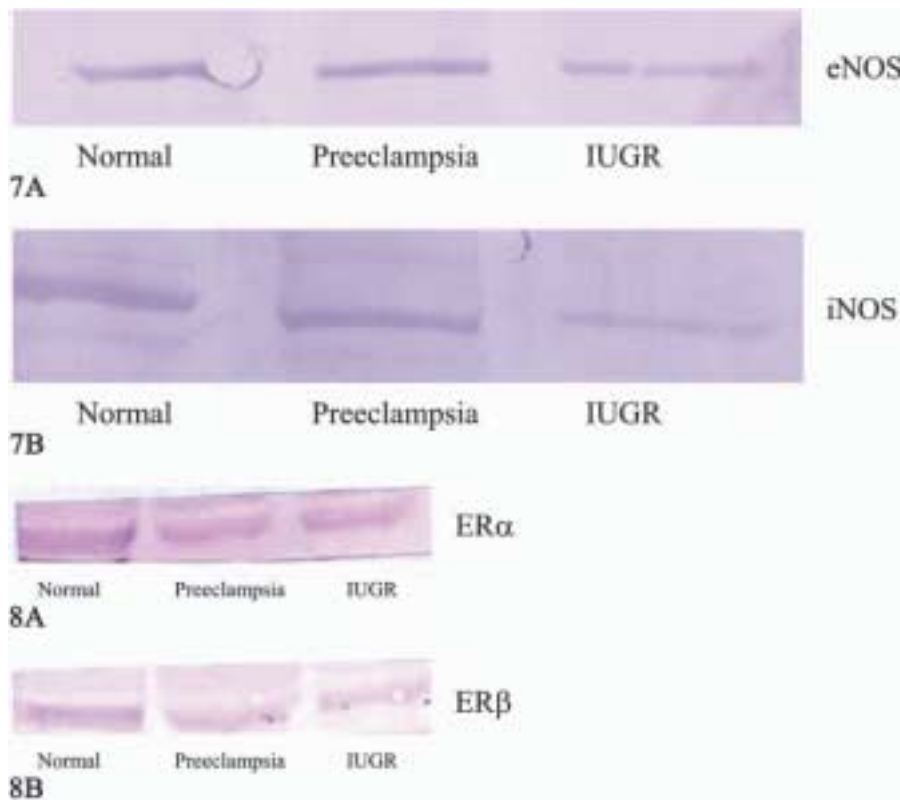
In addition to previously published data, we found a significant lowered expression of iNOS and eNOS in placentas of growth-restricted fetuses, but not in PE placentas. We also identified a lowered expression of ERβ in IUGR placentas compared with normal and an elevated expression of ERβ in PE placentas compared with IUGR and normal placentas. To our best knowledge, there is no previous report on the interaction of these systems in the placenta in normal and pathologic pregnancy.

There are several reports on NO and its fetoplacental synthases (NOS) distribution in normal and preeclamptic placentas. PE is characterized by hypertension, edema, and proteinuria and affects ~5–10% of all pregnancies (Bartl and Muller-Tyl 1985). PE is associated with IUGR and impaired uterine blood flow (Schonfelder et al. 2004; Takagi et al. 2004; Torry et al. 2004). IUGR, on the other hand, is not necessarily associated with PE symptoms such as hypertension and proteinuria. In PE, fetoplacental NOS activity and NO concentrations in the umbilical circulation are altered. Some studies have described a decreased or unchanged placental NOS activity in preeclampsia (Lee et al. 1997; Nasiell et al. 1998; Faxen et al. 2001;

Figures 7 and 8

Figure 7 **(A)** Western blot of endothelial nitric oxide synthase detection in villous trophoblast cell lysate. Lane 1: normal placenta; Lane 2: preeclamptic placenta; Lane 3: intrauterine growth–restricted (IUGR) placenta. **(B)** Western blot of inducible nitric oxide synthase detection in villous trophoblast cell lysate. Lane 1: normal placenta; Lane 2: preeclamptic placenta; Lane 3: IUGR placenta.

Figure 8 **(A)** Western blot of estrogen receptor (ER)α detection in villous trophoblast cell lysate. Lane 1: normal placenta; Lane 2: preeclamptic placenta; Lane 3: intrauterine growth–restricted (IUGR) placenta. **(B)** Western blot of ERβ detection in villous trophoblast cell lysate. Lane 1: normal placenta; Lane 2: preeclamptic placenta; Lane 3: IUGR placenta.

eNOS

Normal   Preeclampsia   IUGR

7A

iNOS

Normal   Preeclampsia   IUGR

7B

ERα

Normal   Preeclampsia   IUGR

8A

ERβ

Normal   Preeclampsia   IUGR

8B

Schonfelder et al. 2004). Elevated nitrite/nitrate concentrations in umbilical vein blood from PE patients compared with control patients have also been demonstrated (Lyall et al. 1995; Norris et al. 1999), suggesting an increase of NO production in the fetoplacental unit in PE. NO in the fetoplacental circulation is derived from eNOS activity, found predominantly in the syncytiotrophoblast (Lyall et al. 1995; Norris et al. 1999; Ayuk et al. 2002). Ayuk and coworkers described investigations on ʟ-arginine as precursor for NO synthesis. They found no differences of the transport systems of ʟ-arginine in relation to NO production in normal-term pregnancies or those complicated by IUGR or PE (Ayuk et al. 2002; Speake et al. 2003). Their findings lead to speculate that decreased or lowered NO concentration is not the result of a reduced substrate concentration in PE or IUGR. In this study, we identified only moderate elevated eNOS expression in the syncytiotrophoblast of PE placentas, although differences were not significantly different from normal controls. Differences in eNOS expression were significant between IUGR placentas and normal controls and IUGR placentas and PE placentas. Both iNOS and eNOS expression is reduced in IUGR placentas, especially in the extravillous trophoblast (Figures 1F and Figure 2F). In addition, Witlin et al. demonstrated that L-NAME–treated rats show increased decidual necrosis and deficient fetal vessel development. Appli-

cation of adrenomedullin did not ameliorate hypertension or growth restriction (Witlin et al. 2003).

Bukovsky and coworkers recently demonstrated both in vivo and in vitro that ERα+/ERβ− trophoblast cells differentiate into ERα+/ERβ+ in the first trimester of pregnancy, changing into ERα−/ERβ+ in mature states. They demonstrated a dynamic and maturity-dependent expression of ERα and ERβ (Bukovsky et al. 2003a,b). In the present study, we identified a raised expression of ERα/ERβ in PE placentas but a reduced ERβ expression in IUGR placentas compared with normal controls, but without statistical significance. ERα is known to play an important role in the proliferation and so does ERβ in the maturation of estrogen-dependent cells (Bukovsky et al. 2003a,b). Bukovsky et al. also stated that significantly enhanced expression of ERβ in differentiating trophoblast cells and stimulation of trophoblast differentiation by estrogens, indicates a unique role of the ERβ hormone-binding domain in the regulation of placental function (Bukovsky et al. 2003a,b). Because the trophoblast is a major source of placental hormones, ERβ expression by trophoblast cells may be involved in stimulation of placental hormonal production by estrogens.

In summary, we demonstrated reduced ERβ, iNOS, and eNOS expression in trophoblast cells in placentas of growth-restricted pregnancies. Regarding these two findings, one may speculate that ER trophoblast differ-

entiation in pathological pregnancies is altered. Whether reduced iNOS/eNOS expression results in altered differentiation of ERα to ERβ or if an unchanged status of ER expression results in reduced expression of iNOS/eNOS merits further investigation. The significant association between ERα and NOS in normal pregnancies suggest an important role in the establishment of the fetoplacental unit and the physiological ongoing of pregnancy. This is underlined by the lack of association between IUGR and PE placentas and might suggest that during pathogenesis of IUGR and PE, the normal relation between ER and NOS is disrupted, although additional data are still needed.

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