Oxidative stress-induced S100B protein from placenta and amnion affects soluble Endoglin release from endothelial cells

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ABSTRACT: Oxidative stress with elevated intracellular Ca\(^{2+}\) concentration as well as endothelial dysfunction is a component of pre-eclampsia. Our aim was to investigate the oxidative stress-dependent expression of Endoglin and Ca\(^{2+}\)-binding S100B protein from villous and amniotic tissue cultures, and to assess sEng expression from S100B protein-stimulated endothelial cells. We initially examined Endoglin and Hydroxy-nonenal-(HNE)-modified proteins in the placentas and amnion obtained from women with pre-eclampsia (n = 8), and healthy controls (n = 8) by immunohistochemistry. To examine oxidative stress and the S100B protein effect on sEng expression from endothelial cells, normal villous and amniotic tissue cultures were stimulated by 4-HNE, sodium fluoride and xanthine/xanthine oxidase, whereas human umbilical vein endothelial cell cultures were treated with S100B protein in a dose- and time-dependent manner at 37°C in an environment of 95% air and 5% of CO\(_2\). Culture supernatants were assessed using ELISA. Cell viability was determined using MTS assay. The concentrations of sEng and S100B protein were significantly increased in the villous and amniotic tissue culture supernatants under oxidative stress. S100B protein-stimulated endothelial cells released sEng into conditioned media with a significantly higher expression levels at a concentration of 200 pM–20 nM S100B by 2 h, whereas treated with 200 nM of S100B endothelial cells significantly expressed sEng by 12 h and stimulated the cell proliferation by the same period of time. Our findings show that oxidative stress affects sEng and S100B protein expression from villous and amniotic tissues, and picomolar and low nanomolar concentrations of S100B protein significantly up-regulate sEng release from endothelial cells leading to endothelial dysfunction.

Key words: oxidative stress / endothelial cells / pre-eclampsia / sEng / S100B

Introduction

Pre-eclampsia is the most frequently developed medical complication of pregnancy, and is characterized by the new onset of hypertension and proteinuria, affecting 6–8% of all pregnancies. This multisystemic disease can lead to severe clinical conditions. For fetuses, these include intrauterine growth restriction, death and prematurity with attendant complications, while the mother is at risk for seizures, renal failure, pulmonary edema, stroke and death. Delivery is the only effective treatment for pre-eclampsia, which is commonly considered as a two-stage disease (Roberts and Gamill, 2005; Roberts and Hubel, 2009). Initially, the pre-eclamptic placenta is affected by oxidative stress (Hung and Burton, 2006). Reactive oxygen species (ROS) have been recognized as secondary messengers in intracellular cascades, and endoplasmic reticular (ER) stress has recently been identified as a major regulator of cell homeostasis (Zhang and Kaufmann, 2008; Cindrova-Davies, 2009). The ER serves many specialized functions in the cell, including synthesis, folding and transport of membrane and secretory proteins, and sequestration of calcium ions (Ca\(^{2+}\)). Protein folding and generation of ROS as a byproduct of protein oxidation in the ER are closely linked events, and typical targets of ROS signaling are Ca\(^{2+}\) channels which mediate both long-term and acute cellular responses to oxidative stress (Redman, 2008; Yung et al., 2008).

Endothelial cell dysfunction has been identified as the final common pathway in the pathogenesis of pre-eclampsia, but the stress response...
is wider: there are associated changes such as the acute phase response and metabolic responses triggered by systemic inflammation (Redman and Sargent, 2009). The pathogenic role of soluble Endoglin (sEng) has been suggested in the development of pre-eclampsia.

Endoglin (CD105) is a transmembrane glycoprotein of 180 kDa, expressed on placental syncytiotrophoblasts, endothelial cells and on the surface of several other cell types (Cheifetz et al., 1992; Gougos et al., 1992). It is an accessory protein of the transforming growth factor-β (TGF-β) receptor system, and its primary roles in TGF-β binding include angiogenesis, endothelial cell differentiation and migration, and regulation of vascular tone through endothelial nitric oxide synthase regulation (Bertolino et al., 2005). In vitro studies revealed that hypoxia causes an increase in Endoglin mRNA levels with maximum transcription at 13 h, and regulation of Endoglin expression by hypoxia occurs at the transcriptional level (Li et al., 2003). According to in vivo studies, hypoxia caused up-regulation of Endoglin on the endothelium of focally ischemic areas in the brain (Zhu et al., 2003).

sEng inhibits endothelial capillary tube formation and promotes vascular permeability. Moreover, Endoglin mRNA is up-regulated in the pre-eclamptic placenta (Venkatesha et al., 2006), and sEng is elevated in second-trimester maternal serum in patients destined to develop severe pre-eclampsia (Robinson and Johnson, 2007). Elevated sEng concentrations were defined in amniotic fluid with a significantly positive correlation between maternal serum and amniotic fluid sEng concentrations in pre-eclampsia (Stafl et al., 2007). Alteration of serum sEng levels after the onset of pre-eclampsia is more pronounced in women with early-onset (Hirashima et al., 2008), although sEng is increased in maternal serum 2–3 months prior to the onset of disease (Levine et al., 2006). Endoglin and sEng expression are significantly increased in the placentas of intrauterine growth restriction (IUGR) singletons compared with controls and in IUGR twin placentas relative to both control co-twin and normal twins (Yinon et al., 2008).

In endothelial cells, Endoglin expression is down-regulated by tumor necrosis factor-α (TNF-α), whereas Endoglin is up-regulated by hypoxia, irradiation and TGF-β stimulation in vitro (Lebrin et al., 2005). Endoglin modulates cellular responses to TGF-β1 (Lastres et al., 1996), and the transcription factor NF-κB, as a downstream product of receptor for advanced glycation endproducts (RAGE)-mediated cellular activation, has been shown to have a specific binding site on the promoter of TGF-β1 (Lee et al., 2006). According to our recent study, Ca^{2+}-binding S100B protein, a RAGE ligand, has been connected to pre-eclampsia (Tsikitishvili et al., 2006). The aim of the present study was to assess the expression levels of sEng and S100B protein from placentas and amnion under oxidative stress, and to define the possible relationship between Ca^{2+}-binding S100B protein and sEng as a marker of endothelial dysfunction, which might lead to pre-eclampsia.

### Materials and Methods

#### Patients

For our study, we selected 16 patients with singleton pregnancies, of which eight had pregnancies complicated by pre-eclampsia and eight healthy women matched by parity and maternal age had an elective Caesarean section at term because of a breech presentation or repeat Caesarean section. Pre-eclampsia was defined by hypertension with systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg in association with proteinuria (urinary protein exceeding 300 mg per 24 h or persistent 30 mg/dl (1 + dipstick) in random urine samples) with or without edema. Baseline, clinical characteristics and pregnancy outcome of study groups are shown in Table I.

#### Table I Baseline, clinical characteristics and pregnancy outcome of study groups

<table>
<thead>
<tr>
<th></th>
<th>Pre-eclampsia (n = 8)</th>
<th>Control (n = 8)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age</td>
<td>28 (23–34)</td>
<td>31 (26–37)</td>
<td></td>
</tr>
<tr>
<td>Parity</td>
<td>4 (50)</td>
<td>4 (50)</td>
<td></td>
</tr>
<tr>
<td>Nullipara (n, %)</td>
<td>3 (37.5)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Edema (n, %)</td>
<td>8 (100)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Proteinuria (n, %)</td>
<td>113 (100–133)</td>
<td>53 (78.5–92)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>37.6 (37.2–39.1)</td>
<td>38.2 (38.1–40)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
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<tr>
<td>Vaginal birth (n, %)</td>
<td>7 (14.3)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Birthweight</td>
<td>2837 (2618–3246)</td>
<td>3373 (2982–3664)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Umbilical cord blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.3 (7.02–7.34)</td>
<td>7.32 (7.29–7.38)</td>
<td></td>
</tr>
<tr>
<td>PO₂ (mmHg)</td>
<td>14.2 (9.7–23.2)</td>
<td>19.6 (10.8–24.4)</td>
<td></td>
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<tr>
<td>PCO₂ (mmHg)</td>
<td>47.7 (34.9–73.1)</td>
<td>46.5 (33–56.1)</td>
<td></td>
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<tr>
<td>Base excess (mM/l)</td>
<td>−3.15 (−2.4 to 14.8)</td>
<td>−4.6 (−1 to 5.8)</td>
<td></td>
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<tr>
<td>Apgar score</td>
<td></td>
<td></td>
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<tr>
<td>At 1st min</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>At 5th min</td>
<td>9</td>
<td>9</td>
<td></td>
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</table>

Values are given as median (total range); n, number; mean arterial pressure was calculated as (systolic pressure + 2 × diastolic pressure)/3.
Samples
After approval from the local ethics committee of the Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, informed consent was obtained from each patient. For this study, we collected placentas, amniotic membranes and umbilical cords. Sixteen samples of placenta and amniotic membranes were obtained from women with pre-eclampsia (n = 8) as well as 16 samples of placenta and amniotic membranes from healthy women immediately after elective Caesarean section at term (n = 8). For immunohistochemistry, a portion of the placenta and amnion from the pre-eclampsia and control groups was fixed in 10% formaldehyde neutral buffer, whereas the rest of the placental and amniotic tissues from the control group were subjected to culture. Human umbilical vein endothelial cell (HUVEC) were isolated from human umbilical cords obtained from randomly selected healthy women after delivery at term (n = 10).

Immunohistochemical staining of Endoglin and 4-hydroxy-nonenal in the placenta and amniotic membrane
To determine the localization and expression of Endoglin in the placenta and amnion, immunohistochemical staining was performed using a heat-induced antigen retrieval method with sodium citrate buffer (pH = 6.0). Sections were incubated with 10% goat serum for 1 h at room temperature, followed by overnight incubation with rabbit anti-human Endoglin polyclonal primary antibody at a concentration of 2.47 μg/ml (Proteintech Group, Inc., Chicago, IL, USA). Normal rabbit IgG at a concentration of 8 μg/ml was used as a negative control (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After washing, sections were incubated with Alexa Fluor® 555 goat anti-rabbit IgG (H + L) secondary antibody at a concentration of 2.0 μg/ml (Invitrogen, Eugene, OR, USA) for 1 h at 37°C. The slides were washed with water, and then dehydrated and counterstained with hematoxylin.

To determine the localization and expression of hydroxy-nonenal (HNE) in the placenta and amnion, sections were incubated with 0.05 M Tris-HCl buffer (pH = 7.6) containing 0.0003% H2O2 for 20 min at room temperature. After washing in PBS-T buffer (pH = 7.4) containing 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4, 0.24 g of KH2PO4, and 0.1% Tween 20 (Nacalai Tesque Inc., Kyoto, Japan), for 30 min, sections were incubated with mouse anti-4-HNE-protein monoclonal antibody (Temma et al., 2004) diluted in PBS-T buffer for 4 days at 4°C. The slides were incubated with 10% goat serum for 1 h at room temperature, followed by overnight incubation with rabbit anti-human Endoglin polyclonal primary antibody at a concentration of 2.47 μg/ml (Proteintech Group, Inc., Chicago, IL, USA). Normal rabbit IgG at a concentration of 8 μg/ml was used as a negative control (Santa Cruz Biotechnology Inc.) followed by incubation with Alexa Fluor® 555 goat anti-mouse IgG (H + L) secondary antibody in PBS-T buffer at a concentration of 2.0 μg/ml (Invitrogen) for 1 h at 37°C.

Calculation of the positively stained cell numbers was used as the quantification system (field = 1 mm² at ×100 magnification). Two examiners counted eight randomly selected fields from each section. The number of Endoglin and HNE-modified protein producing positively stained syncytiotrophoblasts were quantified as the percentage of the sum of unstained and stained syncytiotrophoblasts. Similarly, the number of Endoglin and HNE-modified protein producing positively stained amniotic cells were quantified as the percentage of the sum of unstained and stained amniotic cells.

Villous and amniotic tissue cultures and stimulation with 4-HNE, natrium fluoride and xanthine/xanthine oxidase
Normal placentas (n = 8) and amnion (n = 8) obtained after Caesarean section from healthy women at term were thoroughly washed, separated from the connective tissue and decidua, and minced on ice to produce tissue blocks 3 mm in diameter (100 mg of weight). For each experimental condition, six explants from the same sample of placenta and amnion were cultured separately with 1 ml medium (RPMI 1640 containing 10% heat-inactivated fetal bovine serum and 1% penicillin) at 37°C in an environment of 95% air and 5% of CO2 in 24-well flat-bottomed microplates (Corning Incorporated, Corning, NY, USA) for 24 h. Cell viability was examined by trypan blue exclusion. The viability of the cells was over 96%. The tissues were stimulated with HNE (10, 25 and 50 μM) (Cayman Chemicals Company, Ann Arbor, MI, USA), natrium fluoride (NaF) (2.5, 50 and 500 μM) (Sigma-Aldrich Inc., St Louis, MO, USA) and xanthine/xanthine oxidase (X/XO) (0.5 mM/0.005 U/ml, 2.3 mM/ 0.015 U/ml) (Sigma-Aldrich Inc.) as previously described by others (Chiulek et al., 1999; Walsh et al., 2000; Malek et al., 2001; Temma et al., 2004), and incubated for 2 and 6 h. Cell viability was examined by trypan blue exclusion. The viability of the cells was over 93% after incubation for 2 h with different concentrations of 4-HNE, NaF and X/XO, whereas after incubation for 6 h, the cell viability was over 80%. The supernatants were collected, centrifuged to remove cell debris at 12,000g for 15 min and stored at −80°C until use.

HUVEC culture and stimulation with S100B protein
Human umbilical cords were obtained from healthy women after delivery at term (n = 10). Endothelial cells were harvested enzymatically according to the method of Jaffe et al. (1973), and were maintained in HUMEDIA EG-2, containing 2% fetal calf serum, 1 mg/ml of hydrocortisone, 5 μg/ml of fibroblast growth factor-B, 10 μg/ml of epidermal growth factor, 10 mg/ml of hepacin, 50 mg/ml of gentamicin and 50 μg/ml of amphotericin B as supplied by the manufacturer (Kurabo, Osaka, Japan). Endothelial cell cultures underwent three passages (once in every 48 h) at 37°C in an environment of 95% air and 5% CO2. Before stimulation, endothelial cells were plated onto 24-well flat-bottomed plates (Corning Incorporated) at 1.5 × 10⁵ cells per well in 1 ml of Medium 199 (Invitrogen Corporation, Carlsbad, CA, USA). Stimulation of HUVEC cultures was performed with different concentrations (200 pM, 2, 20 and 200 nM) of human recombinant S100B protein (ProSpecBio, Rehovot, Israel) and incubated for 30 min, 2, 4, 8, 12 and 24 h at 37°C in an environment of 95% air and 5% of CO2. For each experimental condition, eight cultures harvested from the same sample of umbilical cord were used. The equal amount of supernatants (900 μl) were collected, centrifuged at 12,000g for 15 min and stored at −80°C until use. The rest of the treated HUVEC cultures (100 μl in each well) were subjected to the cell viability assay.

Cell viability assay
To determine the cell viability in HUVEC cultures stimulated by different concentrations of S100B protein in a dose- and time-dependent manner, a commercial CellTiter 96® aqueous one solution cell proliferation assay kit was used (Promega Corporation, Madison, WI, USA). This is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays by converting a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] into formazan product, which is directly proportional to the number of living cells in culture. Thus, the increased number of viable cells denotes cell proliferation. All procedures were performed according to the manufacturer’s protocol.

Enzyme-linked immunosorbent assay to determine 8-isoprostane, sEng and S100B concentrations
The 8-isoprostane concentrations in the tissue cultures alone as well as sEng and S100B concentrations in the tissue culture and HUVEC culture...
supernatants were measured using 8-isoprostan EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA), QuantiKine Human Endoglin/CD105 enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN, USA) and S100B ELISA kit (Sandtech® 100ELISA, DiaSorin Inc., USA) respectively according to the manufacturer’s protocol.

Statistical analysis
The obtained data were not normally distributed. The data of baseline, clinical characteristics and pregnancy outcome of study groups were expressed as median and total range, whereas the rest of the expressed as median and inter-quartile range (IQR). The data were subjected to Mann–Whitney U-test and Kruskal–Wallis test with Dunn’s post-test using the Statview statistics package (Abacus Concepts, Inc., Berkeley, CA, USA). P < 0.05 was considered significant.

Results

Expression of Endoglin and HNE-modified proteins in the placentas and amnion in normal and complicated by pre-eclampsia pregnancies
To examine Endoglin expression and the presence of HNE-modified proteins as a marker of oxidative stress in placentas and amniotic membranes, we immunohistochemically stained the samples. As shown in Figs 1 and 2, the placentas (Figs 1 A, B and 2A, B) and amniotic membranes (Figs 1 C, D and 2C, D) were positively stained. The intensity of immunostaining by anti-Endoglin protein of placental syncytiotrophoblasts and amniotic epithelial cells of patients with pre-eclampsia (Fig. 1A and C) tended to be stronger than that of healthy controls (Fig. 1B and D). Similarly, the immunostaining intensity of HNE-modified proteins of placental syncytiotrophoblasts and amniotic epithelial cells of patients with pre-eclampsia (Fig. 2A and C) tended to be stronger than in healthy controls (Fig. 2B and D).

Figure 3 demonstrates proportion of positively stained Endoglin (Fig. 3A and B) and HNE-modified protein producing cells (Fig. 3C and D) in the placenta (Fig. 3A and C) and amnion (Fig. 3B and D). The percentage of positively stained cells was significantly higher in pre-eclamptic patients, than in healthy controls.

8-Isoprostane, sEng and S100B protein expression from villous and amniotic tissues under oxidative stress in vitro
To evaluate the effect of oxidative stress on villous and amniotic tissues, we treated human placental and amniotic tissue cultures with 4-HNE, NaF and X/XO in a dose- and time-dependent manner. Figure 4 shows the 8-isoprostane levels in the conditioned media of villous (Fig. 4A, C and E) and amniotic tissues (Fig. 4B, D and F) after treatment with 4-HNE (Fig. 4A and B), NaF (Fig. 4C and D) and X/XO (Fig. 4E and F) chemicals. Significant difference in 8-isoprostane concentrations between treated and untreated controls were observed in all supernatants. The 8-isoprostane expression levels were significantly higher in placental and amniotic tissue cultures stimulated with 25 μM of 4-HNE (Fig. 4A and B), 50 μM of NaF (Fig. 4C and D) and 2.3 mM of X/XO (Fig. 4E and F) by 2 and 6 h, respectively. However, a time-dependent increase of 8-isoprostane concentrations was not significant.

As shown in Fig. 5, the sEng expression level was significantly higher in placentual tissue culture conditioned media treated with 25 μM of 4-HNE by 2 h (Fig. 5A), whereas in amniotic tissue culture supernatants, the sEng expression level was significantly higher at 25 μM by 6 h with a decrease at 50 μM. Interestingly, placental and amniotic tissue culture treatment with other doses of 4-HNE did not show significant difference of the sEng expression level in comparison with controls. Tissue cultures treated with NaF showed a significantly higher expression level of sEng from villous tissues at 50 μM by 2 h with the time-dependent decrease by 6 h (Fig. 5C). sEng expression from amniotic tissue cultures was significantly higher in all treated cultures than in controls (Fig. 5D). As shown in Fig. 5F, the sEng expression levels from amniotic tissues were significantly higher at 0.5 mM and 2.3 mM of X/XO by 2 and 6 h than in untreated controls (Fig. 5F), whereas the villous tissue sEng expression was not significantly different between treated and untreated culture supernatants (Fig. 5E).

Figure 6 shows the S100B protein expression level in conditioned media of villous (Fig. 6A, C and E) and amniotic tissues (Fig. 6B, D and F) treated with 4-HNE (Fig. 6A and B), NaF (Fig. 6C and D) and X/XO (Fig. 6E and F) in a dose- and time-dependent manner. We found that S100B dose-dependent expression was significantly higher in villous tissue cultures at 25 μM of 4-HNE by 2 h, and S100B protein expressed by villous and amniotic tissue cultures at 25 μM was significantly higher than in controls by 2 and 6 h, respectively (Fig. 6A and B). Moreover, treatment of villous and amniotic tissue cultures with different concentrations of NaF (Fig. 3C and D) and X/XO (Fig. 3E and F) by 2 and 6 h showed significantly higher expression levels of S100B protein in comparison with untreated controls.

Effect of different concentrations of S100B protein on sEng expression from HUVEC cultures
To study the effect of S100B protein on sEng expression from endothelial cells, HUVEC cultures were treated with 200 pM, 2 or 20 nM of S100B protein by 30 min, 2, 4, 8, 12 and 24 h or with 200 nM of S100B protein by 4, 8, 12 and 24 h. As shown in Fig. 7, a significantly different expression of sEng between treated and untreated conditioned media of endothelial cells was observed at 200 pM, 2 and 20 nM of S100B protein by 2 h (Fig. 7A, C and E). Moreover, the sEng expression levels were significantly higher in cultures treated with 200 μM of S100B protein by 2 h than in cultures treated by 30 min, 4, 8 and 24 h as well as cultures treated with 200 pM of S100B protein by 12 and 4, 24 h (Fig. 7A). Furthermore, a significant difference of sEng expression has been observed between cultures treated with 2 nM of S100B protein by 2 and 4, 8, 12 and 24 h as well as between samples treated by 12 h and 30 min, 4, 8 and 24 h (Fig. 7C).

As shown in Fig 7E, sEng was significantly over-expressed in cultures treated with 20 nM of S100B protein by 2 h compared with those treated by 30 min, 4, 8 and 24 h as well as in cultures treated by 12 and 4, 8 and 24 h. Moreover, a significant difference of sEng expression was detected between untreated and treated cultures by 24 h. Interestingly, sEng expression has been significantly increased in a dose-dependent manner from 200 pM to 2 nM of S100B protein by 12 h with a significant decrease from 2 to 20 nM of
S100B at the same time point, whereas further significant increase of sEng concentration was observed from 2 to 20 nM of S100B by 24 h. Figure 7G demonstrates sEng expression levels from endothelial cells treated with 200 nM of S100B protein by 4, 8, 12 and 24 h. sEng expression was significantly higher by 12 h in comparison with untreated control and a time-dependent significant decrease by 24 h was observed.

**Figure 1** Immunohistochemical staining of Endoglin-producing cells in the placenta and amnion. Syncytiotrophoblast cells (Sc) of the placenta (A, B, E and F) and epithelial amniotic cells (Am) of the amnion (C, D, G and H) were stained as described in the text. (A, C, E and G) Pre-eclampsia cases with hematoxylin counterstaining and negative controls (N); (B, D, F and H) control cases with hematoxylin counterstaining and negative controls (N). The intensity of immunostaining by anti-Endoglin protein of syncytiotrophoblasts and epithelial amniotic cells of patients with pre-eclampsia (A and C) tended to be stronger than in control patients (B and D). Scale bar: 200 μm.

Effect of different concentrations of S100B protein on cell viability in HUVEC cultures

As it has been defined by the cell viability assay, cell proliferation was significantly up-regulated in untreated cultures by 8 and 24 h (Fig. 7B, D, F and H) as well as in cultures treated with 200 pM, 2 and 20 nM of S100B protein at 4 h compared with untreated controls by 4 h (Fig. 7B, D and F). Furthermore, the number of viable cells was...
significantly increased in cultures treated with 200 pM of S100B protein at 24 h compared with cultures by 8 and 12 h as well as in cultures at 8 h compared with untreated controls by 8 h (Fig. 7B).

Figure 7H shows that the cell viability of endothelial cells treated with 200 nM of S100B protein was significantly increased by incubation for 12 h compared with the untreated control with a subsequent decrease by 24 h. Interestingly, a number of viable cells were significantly increased in a dose-dependent manner from 200 pM to 200 nM of S100B by 12 h.

**Discussion**

Oxidative stress is a key element in the pathogenesis of pre-eclampsia. Placental oxidative stress could lead to maternal endothelial cell...
Figure 3  Proportion of positively stained Endoglin and HNE-modified protein producing cells in the placenta and amnion. Percentage of positively stained syncytiotrophoblasts and amniotic cells was defined as described in the text. The number of positively stained Endoglin (A and B) and HNE-modified protein producing cells (C and D) in the placenta (A and C) and amnion (B and D) is significantly higher in pre-eclamptic patients, than in healthy controls. The data are presented as median and IQR. *P < 0.05.

Figure 4  8-Isoprostane concentration in tissue cultures stimulated with different concentrations of 4-HNE, NaF and X/XO. Villous and amniotic tissue cultures were stimulated with different concentrations of 4-HNE (A and B), NaF (C and D) and X/XO (E and F) by 2 and 6 h as described in the text. Significant difference in 8-isoprostane concentrations between treated and untreated controls were observed in all supernatants. The 8-isoprostane expression levels were significantly higher in placental and amniotic tissue cultures stimulated with 25 μM of 4-HNE (A and B), 50 μM of NaF (C and D) and 2.3 mM of X/XO (E and F) by 2 and 6 h. The data are presented as median and IQR. *P < 0.05.
activation through the release of toxic products or the activation of signaling pathways and secretion of soluble factors (Cindrova-Davies, 2009). One of these is Endoglin, which could be up-regulated by hypoxia (Yinon et al., 2008), and its release is also stimulated by inflammatory cytokines under normoxic conditions (Cudmore et al., 2007). Our present study has focused on Endoglin expression in oxidative stress as a primary source for the development of pre-eclampsia. We showed the presence of Endoglin in placental trophoblast and amniotic membranes in pre-eclampsia samples. Our results are in accordance with the previous study (Gu et al., 2008). Furthermore, we confirmed sEng expression in the placenta and amnion under experimental oxidative stress conditions.

The previous study demonstrated that no difference in the levels of 4-hydroxy-2-nonenal, a product of lipid peroxidation was detected immunohistochemically between IUGR and pre-eclampsia placentas (Takagi et al., 2004). On the other hand, trophoblast cells of pre-eclamptic placentas showed increased mitochondrial damage by lipid peroxidation (Morikawa et al., 1997), and the accumulation of HNE-modified proteins was enhanced in the trophoblasts of the floating villi (Shibata et al., 2001). In the present study, we demonstrated that placentas and amniotic membranes of patients with pre-eclampsia appeared to have been exposed to oxidative stress and to have accumulated HNE-modified proteins. HNE-modified proteins in the placenta and amniotic membranes can be used as markers that indicate damage under oxidative stress condition.

The placenta secretes many bioactive factors that are significantly increased in pre-eclampsia. According to our recent study, Ca$^{2+}$-binding S100B protein expression is increased in the amnion and amniotic fluid in pregnancies complicated by pre-eclampsia (Tskitishvili et al., 2006). Ca$^{2+}$-binding S100B protein is a proinflammatory ligand of the RAGE, which is elevated in women with pre-eclampsia and may contribute to vascular dysfunction in pre-eclampsia (Cooke et al., 2003). In our present study, we demonstrated Ca$^{2+}$-binding S100B protein expression in villous and amniotic tissues under oxidative stress. These observations are in accordance with previous studies. Moreover, in the present study, we have demonstrated sEng expression in HUVEC cultures treated with different concentrations of S100B protein. Interestingly, S100B protein has been shown to activate endothelium, vascular smooth muscle cells, monocytes and T cells via RAGE, resulting in the generation of cytokines and proinflammatory adhesion molecules (Hofmann et al., 1999; Yan et al., 2003). Moreover, NF-κB, a downstream product of RAGE-mediated cellular activation, has been shown to have a specific binding site on the promoter of TGF-β1 (Lee et al., 2006). On the other hand, Endoglin acts as a co-receptor for TGF-β1, a potent pro-angiogenic molecule, signaling in endothelial cells (Kopcow and Karumanchi, 2007).

**Figure 5** Effect of 4-HNE, NaF and X/XO on sEng concentration in the villous and amniotic tissue culture supernatants.

Villous and amniotic tissue cultures were stimulated with different concentrations of 4-HNE (A), (B), NaF (C and D) and X/XO (E and F) by 2 and 6 h as described in the text. Maximal concentration of sEng in the tissue culture supernatants was observed from villous culture at 25 μM of HNE by 2 h (A), and from amniotic tissues at 25 μM of HNE (B). Tissue cultures treated with NaF showed significantly higher expression levels of sEng from villous tissues at 50 μM by 2 h (C), and from amniotic tissues at any dose and any time-point (D). No effect on sEng expression from villous tissue was observed in cultures treated by X/XO (E). The sEng expression levels from amniotic tissues were significantly higher at 0.5 mM X/XO and 2.3 mM X/XO by 2 and 6 h than in untreated controls (F). The data are presented as median and IQR. *P < 0.05.
ROS activate NF-κB activating protein-1 signal transduction pathways, which promote the transcription of genes involved in cell growth, immunity, inflammation, apoptosis and the stress response (Cindrova-Davies, 2009). Endoplasmic reticulum (ER) stress has recently been identified as a major regulator of cell homeostasis. A link has been found between increased oxidative stress, ER stress and inflammation, suggesting that NF-κB activation could be a result of the oxidative stress arising from excessive protein folding and/or stress mediated Ca²⁺ leakage from ER (Zhang and Kaufmann, 2008). Taken together, the pre-eclampsia production of Endoglin from endothelial cells might be caused by oxidative stress through Ca²⁺-binding S100B protein expression from the placenta and amnion.

Previous in vitro studies demonstrated a negative correlation between Endoglin expression and TGF-β dose-dependent stimulation (Gajdusek et al., 1993; Pepper et al., 1993; Goumans et al., 2002). These findings are in accordance with our present study; we observed a negative correlation between sEng expression levels from endothelial cells and the S100B protein dose in connection with the incubation time. Small picomolar and some low nanomolar doses of S100B protein might significantly increase sEng production levels from endothelial cells with a short incubation time.

According to recent investigations, while nanomolar concentrations of S100B provides a pro-survival effect on neurons and stimulates the neurite outgrowth, at higher micromolar concentrations, the protein promotes neuroinflammatory processes and neuronal apoptosis (Van Eldik and Wainwright, 2003). On the other hand, at micromolar concentration, S100B might increase cellular proliferation and cell survival. However, a chronic exposure to a high level of S100B could change S100B function to the pathologic one. The observed effect of S100B on cell survival involves the formation of ROS, and the activation of PI3-Kinase/AKT as well as that of NF-κB (Leclerc et al., 2007). These studies led us to investigate the effects of different concentrations of S100B protein on HUVEC cultures. In the present study, the lowest stimulating dose of S100B protein (200 pM) denotes one of the highest concentrations of S100B protein expressed under oxidative stress from placental and amniotic tissues. Our present study showed that the lower doses (200 pM–20 nM) of S100B protein increased sEng by 2 h, whereas high nanomolar concentrations of S100B protein significantly increased sEng expression from endothelial cells by 12 h, and also up-regulated cell proliferation at this time. One of the limitations of our study is that we did not perform cell viability assays at early incubation time points. Thus, sEng expression from endothelial cells might be affected by low doses of S100B protein rather than by cell proliferation itself, whereas high doses of S100B protein can affect sEng production from endothelial cells and cell proliferation at the same time. Typically low doses of ROS favor cell proliferation, whereas severe oxidative stress causes cell death (Martindale and Holbrrok, 2002). Further investigations

Figure 6 Effect of 4-HNE, NaF and X/XO on S100B protein concentration in the villous and amniotic tissue culture supernatants.

Villous and amniotic tissue cultures were stimulated with different concentrations of 4-HNE (A and B), NaF (C and D) and X/XO (E and F) by 2 and 6 h, as described in the text. Maximal concentration of S100B protein in the tissue culture supernatants was observed at 25 μM by 2 and 6 h (A and B). Tissue cultures treated with NaF showed a significantly higher expression level of S100B from villous and amniotic tissues by 2 and 6 h (C and D). Effect of X/XO on S100B expression from villous tissues was significantly higher than observed in untreated controls by 2 h (E) as well as in amniotic tissue cultures by 2 h and 6 h (F). The data are presented as median and IQR. *P < 0.05.
are necessary to determine the possible relationship between Endoglin and S100B protein in connection with pre-eclampsia.

Endoglin is highly expressed in syncytiotrophoblasts similar to endothelial cells. Endoglin expression in mouse embryogenesis was first detected at 6.5 dpc in the amniotic fold and developing allantois, and from 9.5 to 13.5 dpc in endothelial cells throughout the developing vasculature (Jonker and Arthur, 2002). Mutations of the gene encoding Endoglin are associated with arteriovenous malformations and focal loss of capillaries in hereditary hemorrhagic telangiectasia type 1 (McAllister et al., 1994). Endoglin −/− knock-out mice die in mid-gestation due to defective angiogenesis and cardiovascular development (Bourdeau et al., 1999). In pregnant rats, sEng appeared to exacerbate the vascular damage mediated by sFlt-1, resulting in severe pre-eclampsia-like disease, including the development of HELLP syndrome and fetal growth retardation (Venkatesha et al., 2006).

In the present study, we demonstrated sEng and S100B protein expression from cultured placental and amniotic tissues under oxidative stress, suggesting that the villous and amniotic tissues may be responsible for the elevated sEng concentration in pre-eclampsia. Moreover, we showed that S100B protein-stimulated endothelial cells express sEng under normoxic conditions leading to the speculation that sEng expression may be involved in an inflammatory pathway. Further investigations will be necessary to examine the relationship between sEng and S100B protein in connection with endothelial dysfunction, systemic inflammation and pre-eclampsia.

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