Effects of inflammatory reactions on the changes in activity and expression of neuronal nitric oxide synthase in HT-1 trophoblast cells

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임신중독증(preeclampsia)에서 나타나는 산모의 고혈압유발의 주요 기전은 혈관내 nitric oxide(NO) 생성의 이상으로 설명되며, 특히 전신 및 태반내 염증매개인자인 cytokine들의 생성이 크게 증가하는 결과와 연계된다. 그러나 혈관이완 기전을 담당하는 NO는 혈관내피세포 뿐 아니라 혈관 주변에 분포하는 말초 non-adrenergic non-cholinergic(NANC) 신경계에서도 합성되며 이 또한 혈압조절에 관여한다.

그러므로, 본 연구에서는 NANC 신경계에서 작용하는 신경계 NO 합성효소(nNOS)의 발현과 활성을 태반내 trophoblast 세포주인 HT-1에서 확인하고, 염증반응에서 생성되는 주요 cytokine들(TNF-α, IL-1β, INF-γ)과 내독소 endotoxin인 lipopolysaccharide(LPS)이 그들 활성 및 발현에 미치는 영향을 분석했다. 또한, 이들 염증매개인자들로 유발되는 세포내 칼슘농도의 증가로 유발될 수 있는 단백질 인산화효소(PKC, PKA, CaM-KII)들의 활성과 이들 효소로 유도되는 nNOS의 인산화 정도를 확인하였다.

HT-1 세포주는 크기가 다른 nNOSα(~155 kDa) 및 nNOSµ (~165 kDa)을 소포체형태로 발현하였으며 대부분이 nNOSµ형이었다. HT-1 세포주에 처리된 cytokine과 LPS는 nNOSα의 인산화 정도를 증가시키고, 이 유발경로는 각 인산화 효소의 선택적 결합학자 KN-93와 H-89의 처리로 감소되는 결과에서 주로 CaM-KII와 PKA의 활성에 의해 유도됨을 확인하였다. 특히, 칼슘
ionophore의 하나인 ionomycin (10 µM)의 처리는 CaM-KII 경로를 매개로 하는 HT-1 세포내 nNOSα의 인산화를 유도했으며, 그 결과는 nNOS의 활성도 감소로 예측되었다. 따라서, 태반내 cytokine 들의 증가는 세포내 칼슘유입을 활성화시키고 결국에는 CaM-KII의 활성화로 trophoblast 세포내 nNOSα의 인산화를 초래하여 전체적으로 감소된 nNOS의 활성으로 NO 생성에 손상 결과를 제시하였다.

그러나, ionomycin이 처리된 HT-1 세포주에서 nNOS의 활성은 nNOSα의 인산화 결과에도 불구하고 현저하게 증가되어 있었다. 이 결과는 칼슘 의존적으로 측정되는 nNOS의 활성이 칼슘 유입으로 더욱 증가될 수 있으며 이는 매우 낮게 발현되고 인산화된 nNOSα형보다는 아마도 nNOSµ형에 의한 결과로 예측된다.

결론적으로, HT-1 세포주에서 나타나는 nNOSα의 인산화 기전은 전체의 nNOS 활성도에는 크게 영향을 주지 않았지만, 염증 및 세포내 칼슘농도의 증가를 초래하는 다양한 산화적 스트레스에 대하여 세포내 방어기전으로 생각된다. 그러므로, 임신중독증 유발기전에서는 trophoblast 세포주에 최소한 2가지 이상의 paradoxical 경로가 존재하는 것 같다. 즉, 염증반응으로 생성된 cytokine들은 칼슘유입을 증가시켜 세포내 nNOS 활성을 증가시키지만(아마도 nNOSµ형의 활성), nNOSα형의 인산화는 NO 생성의 감소로 세포방어기전에 작용할 수 있으므로 임신중독증의 원인 규명을 위한 NO 생성 기전은 중요하게 연구되어야 할 것으로 사료된다.
Abstract

Impairment of endothelial nitric oxide (NO) production in pregnancy has been suggested as a major cause of the hypertension of preeclampsia (PE) in the third trimester, which is also associated with an increase in placental inflammatory cytokines. However, NO synthesized by peripheral non-adrenergic non-cholinergic (NANC) nerves distributed in the blood vessels is also involved in vasodilatation.

Therefore, in the present study, the activity and expression of neuronal NO synthase (nNOS), which mediates NO release in the NANC nerves, was examined in a human placental full-term cell line (HT-1), and the effects of inflammatory cytokines (TNF-α, IL-1β and INF-γ) and/or endotoxin (lipopolysaccharide, LPS) on its expression and activity were compared. Furthermore, we analyzed the mechanism underlying the phosphorylation of nNOS, which is induced by protein kinases like PKC, PKA, or CaM-KII, with an increase in intracellular Ca$^{2+}$ concentration.

Cytokines and/or LPS caused an increase in the phosphorylation of nNOS, probably via the CaM-KII and PKA pathways, because the phosphorylation of nNOS was inhibited by specific inhibitors of CaM-KII and PKA, KN-93 and H-89, respectively. Furthermore, a calcium ionophore (10 µM) enhanced the phosphorylation of nNOS via CaM-KII activity, which led to a decrease in NOS activity. Therefore, we suggest that an increase in placental cytokines may enhance calcium influx, leading to the activation of CaM-KII, which in turn phosphorylates nNOS, thus inactivating NOS in trophoblast cells.

However, the actual nNOS activity determined with a hemoglobin
oxidation assay increased after treatment with calcium ionophore (10 µM), although the nNOSα isoform was phosphorylated. This result may be due to the activation of Ca\textsuperscript{2+}-dependent nNOS, probably the nNOSµ isoform, in HT-1 trophoblast cells, because the phosphorylated nNOSα isoform exerts little effect and is expressed at very low levels relative to the total nNOS activity.

In summary, the mechanism that phosphorylates nNOSα in HT-1 trophoblast cells probably exerts a protective role against oxidative stress during inflammation or during increases in intracellular calcium concentrations, although it has little effect on total nNOS activity. Therefore, there are at least two paradoxical pathways in HT-1 trophoblast cells involved in the development of preeclampsia: an increase in nNOS activity (probably of the nNOSµ isoform) by the calcium influx induced by inflammatory cytokines and a decrease in NO release caused by phosphorylation of nNOSα as a possible protective mechanism.
Introduction

Preeclampsia (PE) is a multisystem disease of pregnancy with an incidence of 5–7% among the general population. It involves hypertension, proteinuria, edema, abnormal clotting, fetal growth restriction, and premature birth in the third trimester of human pregnancy, all of which can be associated with vascular endothelial dysfunction (Roberts et al., 1993; Jaramillo et al., 2001; Herrera et al., 2001). Endothelial dysfunction may induce hypertension in PE via a decrease in the release of nitric oxide (NO), because endothelial NO plays an important role in the vascular system during pregnancy, in neutrophil activation and in the increase of vasoconstrictors such as endothelin-1 and thromboxane A2 (TXA2) (Greer et al., 1991a, 1991b; Myatt et al., 1992). In this context, however, the Collaborative Low-dose Aspirin Study in Pregnancy (CLASP) Collaborative Group trials (1994) failed to demonstrate the expected effects of a low-dose specific inhibitor (aspirin) of platelets, TXA2. Recently, Herrera et al. (2001) suggested that pro-inflammatory cytokines may impair endothelial functions.

NO is a major player in controlling nearly every cellular and organ function in the body. It is an endogenous molecule that functions as a neurotransmitter, cytoprotective molecule, constitutive mediator, inducible mediator, and cytotoxic molecule. Certain physiological functions of NO, such as vasodilatation and smooth-muscle relaxation, are mediated by multiple mechanisms of NO release and NO action (Ignarro, 2000). Three isoforms of the NO synthase enzyme have been identified: neuronal nitric oxide synthase (nNOS or NOS-1), inducible NOS (iNOS or NOS-2), and
endothelial NOS (eNOS or NOS-3). Two of these enzymes (nNOS and eNOS) are constitutively expressed in neurons and endothelial cells, respectively, and iNOS is expressed after stimulation with endotoxin or cytokines in a range of cells, including macrophages, neutrophils, hepatocytes, and glial cells (Colasanti et al., 1999; Park et al., 2000; Alderton et al., 2001).

Trophoblasts distributed in the placenta also produce NO constitutively by eNOS or nNOS, or both (Shesely et al., 2001). Trophoblast-derived NO prevents platelet and leukocyte adhesion and eNO also controls vascular tone (Gabour et al., 1995). Therefore, impairment of the NO release mediated by placental trophoblasts and endothelial cells may be associated with several systems in PE.

Human trophoblasts are one of the earliest cell types to differentiate during early pregnancy. They form the outer layer of the blastocyst and, following implantation of the blastocyst in the uterine wall, they develop into the placenta. Following implantation, cytotrophoblast cells differentiate, migrate, and invade the uterine stroma in early pregnancy. Cytotrophoblast stem cells either fuse to form syncytiotrophoblasts or aggregate to form anchoring villous trophoblasts. The latter give rise to a subpopulation known as extravillous trophoblasts, which invade the uterine wall and its blood vessels. Extravillous trophoblasts remodel the maternal spiral arteries, displacing smooth muscle and endothelial cells to produce a blood vessel with a larger diameter, increased blood flow, and reduced resistance (Rockwell et al., 2003).

Human villous trophoblasts express eNOS in the syncytiotrophoblast, but not in the underlying cytotrophoblast of the first
trimester or in the full-term villous tissue (Myatt et al., 1993). In *in vitro* culture, eNOS is expressed during differentiation of the cytotrophoblast to the syncytiotrophoblast (Eis et al., 1995). Huang et al. (1995) and Shesely et al. (1996) reported that eNOS-knockout or -deficient mice showed the highest blood pressure of the animals tested. This suggests that eNOS also plays an important role in vascular function, as mentioned by Hefler et al. (2001).

However, NO is also synthesized by peripheral non-adrenergic non-cholinergic (NANC) nerves in the blood vessels, thereby mediating vasodilatation (Bult et al., 1990; Li et al., 1991). In the normal rat pregnancy, renal eNOS is decreased, but iNOS and nNOS expression is increased (Alexander et al., 1999). In eNOS-null mutant mice, nNOS plays an important role in blood pressure regulation, both acutely and chronically (Kurihara et al., 1998).

It has also been suggested that PE is associated with increases in plasma levels of cytokines known to contribute to endothelial dysfunction, as well as with modulations of physiologically essential gene expression (Knackstedt et al., 2001; Alexander et al., 2002). In particular, tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interferon γ (INF-γ) are well-known inflammatory cytokines (Terranova et al., 1995; Williams et al., 1999). Lipopolysaccharide (LPS), an endotoxin, affects pregnancy by inducing the release of inflammatory cytokines (Colasanti et al., 1999).

Furthermore, much evidence indicates that cytokines cause an increase in intracellular calcium concentrations. In macrophage cells, cytokines or LPS cause long-term changes in calcium metabolism (Lowry et al., 1999; Chen et al., 2001), and IL-1β evokes intracellular calcium
release in human astrocytoma cells and brain striatal slices (Meini et al., 2000). Cytokines also increase calcium concentrations in neonatal cardiac myocytes (Bick et al., 1999). Moreover, Steinert et al. (2002) showed that preeclampsia is associated with changes in calcium regulation and NO production.

This calcium influx activates calcium/calmodulin-dependent protein kinases. These kinases can induce the phosphorylation of nNOS, resulting in the suppression of NOS activity (Bredt et al., 1992). Neuronal NOS, especially type alpha (α), is directly phosphorylated at Ser-847 by CaM-KII, leading to a reduction in its enzyme activity in vivo and in vitro (Hayashi et al., 1999; Komeima et al., 2000; Osuka et al., 2002). Aronowski and Grotta (1996) reported that CaM-KII plays a major role in the phosphorylation of nNOS. Furthermore, cAMP-dependent protein kinase A (PKA) and Ca\(^{2+}\)- phospholipid-dependent protein kinase C (PKC) are also involved in the mechanisms controlling NOS phosphorylation and its catalytic activity. Such phosphorylation results in nearly stoichiometric phosphate/protein incorporation (Brune and Lapetina, 1991).

Here, we used a human full-term trophoblast cell line (HT-1) to investigate the mechanism modulating nNOS activity. We hypothesized that NOS activity in full-term trophoblasts is modulated by interactions between trophoblasts and cytokines, and thus may induce preeclampsia.
Methods

1. Cell culture and treatment

Human trophoblast cells (HT-1) and RAW 264.7 cells were cultured in Dulbecco’s modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS) and 100 U/µL penicillin–streptomycin (Gibco, Grand Island, NY). The cells were incubated in humidified atmospheric air in a 5% CO₂ incubator at 37 °C. Trypsin–EDTA (0.25% trypsin, 1 mM EDTA, Gibco) was used to dissociate cells from 100 mm culture dishes (TPP, Trasadingen). Subcultured HT-1 and RAW 264.7 cells were seeded at $1 \times 10^6$ cells/cm² into 60 mm culture dishes (TPP) one day before treatment.

1-1. Treatment with calcium ionomycin and kinase inhibitors

To induce an increase in intracellular calcium concentrations, we used calcium ionomycin (Sigma, St. Louis, MO), a calcium ionophore, at a final concentration of 10 µmol/L. Before treatment with calcium ionomycin, each protein kinase inhibitor (100 nM Ro-31-8220 as the PKC inhibitor, 10 µM H-89 as the PKA inhibitor, and 10 µM KN-93 as the CaM-KII inhibitor) was administered for 30 min.

1-2. Treatment with cytokine and/or LPS

HT-1 cells were treated directly with rat INF-γ (1000 U/mL; R&D, Minneapolis, MN), rat IL-1β (150 U/mL; R&D), recombinant human
tumor necrosis factor-α (TNF-α, 5 ng/mL; Invitrogen, San Diego, CA) or LPS (10 µg/mL; Sigma), and each cytokine plus LPS and three cytokines plus LPS. As positive control, the cells also were treated with medium conditioned by RAW 264.7 cells pretreated for 9 h with LPS.

2. MTT assay

The MTT assay was used to examine cell viability. This is one of the most frequently used methods with which to measure cell proliferation and cytotoxicity. Cells were seeded at $1 \times 10^4$ cells/cm$^2$ in 96-well culture plates. After treatment, the medium was changed to MTT, a tetrazolium dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, or thiazolyl blue; Sigma). Plates were incubated in the presence of MTT dye for 5 h at 37 °C. After removal of the medium and MTT solution from the wells, the remaining MTT-formazan crystals were dissolved by the addition of 200 µL of dimethyl sulfoxide (DMSO, Sigma), and incubation at 37 °C for 5 min. The absorbance was read with an ELISA reader (Power Wave, Bio-Tek Instruments, Inc., Winooski, VT) at 550 nm.

3. Isolation of total cellular proteins

Treated cells were scraped from the plates and sonicated in 30% (w/v) lysis buffer (320 mM sucrose, 200 mM HEPES, and 1 mM EDTA) containing 1 mM dithiothreitol (DTT), 10 µg/mL leupeptin, 2 µg/mL trypsin inhibitor, 2 µg/mL aprotonin, 1 mM PMSF, and 1 µg/mL pepstatin. Total protein concentrations were determined using a Pierce BCA Protein assay kit (Pierce, Rockford, IL).
4. Western blot analysis

Equivalent amounts of total protein from each sample were separated by electrophoresis on an 8–16% Tris–glycine gel (Invitrogen). The positive controls used were rat or mouse brain proteins. Gels were loaded in running buffer containing 24.9 mM Tris, 194 mM glycine, and 1.156 mM sodium dodecyl sulfate (SDS) at room temperature and run at 125 V. After the proteins had been separated, they were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Miliford, MA) by incubation in transfer buffer containing 25 mM Tris, 192 mM glycine, and 10% methanol for 3 h at room temperature at 25 V. Membranes were blocked in blocking buffer containing 5% non-fat dry milk in 2 M Tris-HCl (pH 7.4), 5 M NaCl, and 0.1% Tween 20 at room temperature for one hour. The membrane was then incubated at room temperature for 2 h in fresh blocking buffer containing a monoclonal antibody (1:1000 dilution) directed against nNOS (Transduction Laboratories, Lexington, KY) or a Ser-847-specific rat polyclonal antibody directed against phospho-nNOS. Anti-actin monoclonal antibody (Sigma), an internal control, was used to normalize protein levels. Membranes were then incubated for 2 h with 0.141 μg/mL goat anti-mouse or horseradish-peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL) as the secondary antibody. Proteins were visualized by chemiluminescence (ECL kit, Amersham, Aylesbury, UK) and quantified with a densitometry program (Bio1D, Ver. 97; Vilber Lourmat, France). To compare the expression levels, we determined nNOS expression as a ratio to actin and phospho-nNOS as a ratio to nNOS.
5. Measurement of nitric oxide synthase activity

NOS activity was determined by measuring NO synthesis, calculated as the oxidation of oxyhemoglobin to methemoglobin by NO. Before measurements were made, methemoglobin was converted to oxyhemoglobin through a Sephadex G-25 column. The protein (250 µg) samples were mixed with 250 µM potassium phosphate buffer (pH 7.4), 20 µM L-arginine, 100 µM NADPH, 1 µM FAD, 1 µM FMN, 10 µM BH₄, 100 units calmodulin, and 40 µL hemoglobin (10 mg/mL). The conversion of oxyhemoglobin to methemoglobin by NO was measured by spectrophotometer at 401 nm and 411 nm.

6. Measurement of nitrite in media

The concentration of nitrite (NO₂⁻) in the cell-culture media was determined by the Griess reaction, as a measure of nNOS activity. Culture medium (100 µL) was mixed with the same volume 100 µL of Griess reagent and then incubated for 10 min in a 37 °C incubator. Sodium nitrite (Sigma) was used as the standard. Griess reagent was composed of 1% sulfonic acid (Sigma), 0.1% N-1-naphthylenediamine dihydrochloride (ICN, Aurora, Ohio), and 5% o-phosphoric acid (Sigma). After incubation, the absorbance was measured at 546 nm and the nitrite concentration was determined using a calibration curve with a sodium nitrite standard.

7. Statistical analysis

Data are presented as means ± standard deviations. A one-tailed t test was used to determine significance. Statistical significance was assumed when P < 0.05.
Results

Effects of ionomycin on the expression of nNOS and phosphorylated nNOS

This procedure was used to examine the effects of increased intracellular calcium on nNOS expression and modification in human trophoblast cells, HT-1, because intracellular calcium concentrations are increased under inflammatory conditions such as preeclampsia.

Western blot analysis showed that HT-1 cells constitutively expressed two different sizes of nNOS (165 kDa and 155 kDa), which were probably nNOSµ and nNOSα in particulate forms, respectively (Fig. 1). The larger nNOSµ was highly expressed in the cells, whereas nNOSα was expressed at very low levels.

A western blot probed with an monoclonal anti-nNOS antibody designed to recognize the C-terminal region of human nNOS (residues 1095–1289) indicated that nNOSµ expression decreased slightly after treatment with ionomycin (10 μM) but not significantly, whereas no nNOSα was detectable (Fig. 2). The blot was probed with a phospho-nNOS-specific polyclonal antibody directed only against nNOSα, and indicated that phosphorylation levels were significantly increased by ionomycin treatment, as shown in Fig. 2A. Thus, the increase in intracellular calcium concentration induced by ionomycin treatment caused an increase in the phosphorylation of nNOSα only compared with that of the control, but not in the phosphorylation of nNOSµ. This modification of phospho-nNOSα did not change after NO release was inhibited by the
nNOS-specific inhibitor, 7-nitroindazole (7-NI). This increase in the phosphorylation of nNOSα may constitute a protective mechanism against the overproduction of physiological NO induced by increases in Ca^{2+}-dependent nNOS activity in trophoblast cells.

**Effect of ionomycin on cell viability**

The effect of ionomycin on HT-1 cell viability was determined using the MTT assay (Fig. 3). Ionomycin was administered at concentrations of 0.1, 1, 5, and 10 µM. There was no difference in cell viability between HT-1 cells treated with DMSO (control) and those treated with a low concentration of ionomycin (0.1 µM). However, cell viability decreased significantly at high concentrations (1–10 µM) of ionomycin after a short time (30 min). The viability of cells treated with 1 µM ionomycin was later restored to normal (24 h), whereas there was no restoration of cells treated with 5 or 10 µM ionomycin.

Therefore, a 10 µM concentration of ionomycin was used in later experiments to assay the effects of a significant increase in intracellular calcium concentration.

**Effect of 7-NI on ionomycin -induced cell death**

Death of the HT-1 cells was accelerated by high concentrations of ionomycin (10 µM) administered for 24 h, with viability reduced to about 20% of the control value (Fig. 4). This cell death was partly ameliorated by co-treatment with a specific inhibitor of nNOS, 7-NI. This result suggests that cell death is caused by the NO overproduction induced by increased
Ca²⁺-dependent nNOS activity. Therefore, the phosphorylation of the minor isoform, nNOSα, which was increased by ionomycin treatment, did not seem not sufficient to protect cells against cell death.

Effect of protein kinase inhibitors on ionomycin-mediated phosphorylation of nNOSα

As previously shown, phospho-nNOSα protein levels were increased by ionomycin treatment (Fig. 2). Phospho-nNOSα protein levels were significantly increased 2 h after ionomycin treatment (Fig. 5), whereas nNOSµ protein levels decreased, although not significantly. Furthermore, the decrease in phospho-nNOSα protein levels detected time-dependently in controls was delayed after ionomycin treatment. However, these delayed decreases in phospho-nNOSα levels caused by ionomycin were inhibited by co-treatment with PKA or CaM-KII inhibitors. This suggests that the ionomycin-induced phosphorylation of nNOSα involves the PKA or CaM-KII pathways.

Effect of protein kinase inhibitors on ionomycin-mediated NOS activity

NOS activity was increased by ionomycin treatment, but co-treatment with protein kinases prevented the increase in NOS activity induced by ionomycin (Fig. 6). In a previously described result (above), we suggested that the increased phosphorylation of nNOSα might constitute a protective mechanism against the overproduction of physiological NO in trophoblast cells. Therefore, this result may indicate that the increase in
nNOS activity caused by ionomycin is mediated independently by nNOS\(\alpha\) phosphorylation at Ser-847 via the PKC, PKA, or CaM-KII pathways.

**Phosphorylation of nNOS\(\alpha\) in cells treated with conditioned medium**

To determine the effects of the pro-inflammatory mediators produced by endotoxins like LPS on the expression and phosphorylation of nNOS\(\alpha\), the medium conditioned by LPS-treated RAW 264.7 cells was used to culture HT-1 cells. The expression and phosphorylation of nNOS\(\alpha\) were observed over time (Fig. 7). The levels of phospho-nNOS\(\alpha\) increased early (10 min) in all groups. In HT-1 cells cultured with conditioned medium, the time-dependent decrease in nNOS\(\alpha\) phosphorylation was delayed, but in LPS-untreated conditioned medium, the decreased in phosphorylation was normal.

Maximum levels of nNOS\(\alpha\) phosphorylation were observed at the 30 min time point. Therefore, we used this time point to compare the differences in nNOS\(\alpha\) phosphorylation modified by protein kinases between the early (30 min) and late time points (6 h).

**Effects of protein kinase inhibitors on nNOS\(\alpha\) phosphorylation in cells treated and not treated with conditioned medium**

At 30 min after treatment with LPS-mediated conditioned medium, the phosphorylation of nNOS\(\alpha\) increased in HT-1 cells, but this increase was prevented in cells co-treated with PKA inhibitor (Fig. 8). However, the CaM-KII inhibitor did not affect the levels of LPS-induced nNOS\(\alpha\)
phosphorylation at the early time point.

Later (at 6 h), the LPS-mediated increase in nNOSα phosphorylation was not significant, but a decrease in the phosphorylation of nNOSα was clear when co-treated with the PKA inhibitor, H-89 (Fig. 8). The CaM-KII inhibitor also reduced phosphorylation to low levels.

**Direct effects of cytokines or LPS on the expression and phosphorylation of nNOS**

The LPS or single cytokines used in this study did not affect nNOSα phosphorylation, and only TNF-α increased its phosphorylation (Fig. 9). Interestingly, its phosphorylation was increased by treatment with LPS plus INF-γ, IL-1β, or TNF-α, and was slightly increased by treatment with LPS plus all three cytokines. This means that, whereas LPS-treated medium conditioned by RAW 264.7 cells in a mixture of LPS plus cytokines (especially TNF-α) was effective in increasing the phosphorylation of nNOSα, direct treatment with cytokines or LPS did not significantly affect NO concentrations in the culture medium (Fig. 10).

This suggests that phospho-nNOSα is induced by pro-inflammatory cytokines, particularly by TNF-α in HT-1 cells.
Discussion

In the present study, we examined whether changes in nNOS activity in full-term trophoblasts are related to the causative mechanisms of preeclampsia. We used HT-1 cells derived from villous cytotrophoblasts of a normal-term human placenta to identify the changes in major factors affecting enzyme activity, like gene expression and post-translational modification, with especial attention to the phosphorylation of nNOS.

Human villous trophoblasts express eNOS in the syncytiotrophoblast, but not in the underlying cytotrophoblast of the first-trimester and full-term villous tissue (Myatt et al., 1993). In culture, eNOS is expressed during differentiation of the cytotrophoblast to the syncytiotrophoblast (Eis et al., 1995). Therefore, impairment of NO release mediated by placental trophoblast and endothelial cells may be associated with the causative mechanisms of PE. Huang et al. (1995) and Shesely et al. (1996) reported that eNOS-knockout or -deficient mice showed the highest blood pressure of the animals examined. This suggests that eNOS also plays an important role in vascular function, as mentioned by Hefler et al. (2001).

However, several reports suggest that, in early pregnancy, the trophoblasts have important activities such as proliferation and differentiation (Benirschke and Kaufmann, 1995) that are related to physiological NO release. However, many of these studies are controversial. For examples, Ramsay et al. (1996) detected cytosolic Ca\textsuperscript{2+}-dependent NOS activity in villous trophoblasts in each of the three trimesters of pregnancy, whereas Al-Hijji et al. (2003) reported both
particulate and cytosolic Ca$^{2+}$-dependent NOS activities in trophoblasts in the first trimester. However, their studies were mainly directed towards the involvement of eNOS in physiological NO function, with no evidence of specific nNOS expression.

NO is also synthesized by peripheral non-adrenergic non-cholinergic (NANC) nerves in the blood vessels, where it mediates vasodilatation (Bult et al., 1990; Li et al., 1991). In eNOS-null mutant mice, nNOS plays an important role in blood pressure regulation, both acutely and chronically (Kurihara et al., 1998). The HT-1 cells used in this study express nNOS only as a particulate fraction, with no expression of other NOS isoforms such as eNOS or iNOS (Fig. 1). Furthermore, this cell line should express very low NOS activity, because HT-1 trophoblast cells are a full-term cell line. Generally, NOS activity in the placenta at term is very much lower than it is in tissues from the first trimester, as shown in several reports (Buttery et al., 1994; Al-Hijji et al., 2003). Recently, Sanyal et al. (2000) found, for the first time, that only nNOS immunoreactivity occurs in the human trophoblast cells of the normal-term placenta and discussed the role of NO in trophoblast proliferation and differentiation.

Therefore, we tried to identify the role of NO in terms of nNOS, particularly in the context of the proliferation and death of the full-term trophoblasts induced by changes in intracellular calcium concentrations. As shown in Fig. 2, the calcium ionophore ionomycin (10 µM) caused a slight decrease in nNOS expression and a strong elevation of nNOS phosphorylation. However, the size of nNOS detected in the trophoblasts with an nNOS-specific monoclonal antibody (nNOS-mAb) was larger (~165 kDa) than the nNOS$\alpha$ isoform found in the rat brain (~155 kDa).
The large nNOS was similar in size to the nNOSµ isoform of nNOS (which has five isoforms: α, β, γ, δ and µ) reported in previous studies (reviewed by Forstermann et al., 1998). However, there is no evidence supporting this identity, except that partial nNOS sequences identified by RT-PCR in this trophoblast cell line were identical to those previously reported (data not shown). Interestingly, the size of the phosphorylated nNOS detected with phospho-nNOS-specific antibody (phospho-nNOS-pAb) was identical to that of nNOSα, as found in the brain. In other words, nNOSα is probably expressed in HT-1 trophoblast cells at very low levels, although nNOSα was not detected by western blot analysis, even though it is specifically targeted by phospho-nNOS-pAb. Trophoblastic nNOSα was also slightly phosphorylated, even in the absence of the calcium ionophore (Fig. 2). These results suggest that the phosphorylation of nNOSα results in the loss of enzyme activity, as reported in other studies (Bredt et al., 1992; Hayashi et al., 1999; Komeima et al., 2000; Osuka et al., 2002), even though the increase in intracellular calcium concentrations increases Ca\(^{2+}\)-dependent NOS activity. However, total Ca\(^{2+}\)-dependent NOS activity measured as the major form(nNOSµ) in HT-1 cells increased markedly, probably because of the low level of nNOSα expression (Figs. 2 and 6). Therefore, we suggest that the phosphorylation of the nNOSα is a protective mechanism against the overproduction of physiological NO in HT-1 trophoblast cells.

When the viability of HT-1 trophoblast cells was examined by MTT assay (Fig. 3), almost all the cells (about 90%) died at high concentrations of ionomycin (5 and 10 µM), but were resistant to or later
recovered from low concentrations (0.1 or 1 µM, respectively). In particular, the cell death caused by 10 µM ionomycin was significantly and dose-dependently ameliorated by co-treatment with an nNOS-specific inhibitor, 7-NI (Fig. 4). These results suggest that cell viability is mediated essentially by an optimum concentration of physiological NO.

Indeed, Ca\(^{2+}\)-dependent NOS activity in villi and non-villous cells of the trophoblast, both in early and full-term placentas, is detected in placental tissues and the trophoblast, and is mediated by constitutively expressed eNOS or nNOS, or both (Shesely et al., 2001). Therefore, changes in intracellular Ca\(^{2+}\) concentration in the trophoblast directly affect Ca\(^{2+}\)-dependent NOS activity, although nNOS\(\alpha\) has little effect on the total NOS activity. In this study, however, we examined whether increased intracellular calcium concentrations activate calcium/calmodulin-dependent protein kinases such as PKC and CaM-KII, as well as cAMP-dependent PKA, using a specific inhibitor of each, because of the importance of the mechanism that inhibits the phosphorylation of nNOS. Recently, Hayashi et al. (1999), Komeima et al. (2000), and Osuka et al. (2002) reported that nNOS is directly phosphorylated at Ser-847 by CaM-KII, especially in nNOS\(\alpha\), leading to a reduction in its enzyme activity in vivo and in vitro. We found that CaM-II\(\alpha\) plays a major role in the phosphorylation of nNOS\(\alpha\) (Fig. 5), and that PKA also mediates the phosphorylation pathways in these cells. However, we could not explain clearly the effects of protein kinase inhibitors on the expression of nNOS\(\mu\) induced by the calcium ionophore, ionomycin (Fig. 5). The calcium ionomycin-mediated increase in trophoblastic nNOS activity was prevented by co-treatment with each protein kinase inhibitor (Fig. 6). This
result suggests that each of the PKC, PKA, and CaM-KII pathways or an interaction between them, is involved positively in the increase in total NOS activity, but not in the direct phosphorylation of the major isoform, nNOSµ, in these HT-1 cells.

Not only are calcium influx and the calcium-mediated activation or modulation of nNOS and the three kinases associated with endothelial dysfunction in preeclampsia; increases in the plasma levels of inflammatory cytokines, like TNF-α, IL-1β and INF-γ, and the endotoxin LPS are also involved in this dysfunction (Colasanti et al., 1999; Knackstedt et al., 2001; Alexander et al., 2002). In particular, LPS affects pregnancy in its role as an endotoxin by inducing the release of pro-inflammatory cytokines, and these cytokines increase intracellular calcium concentrations. Therefore, inflammatory cytokine-mediated increase in intracellular calcium concentrations in the trophoblast can cause the phosphorylation of nNOSα. For this experiment, we used LPS to induce the release of cytokines in RAW264.7 macrophage cells, and the conditioned medium was applied to HT-1 trophoblast cells. As is apparent in Figs. 7–9, the LPS-treated conditioned medium induced a delay in the phosphorylation of nNOSα in HT-1 cells, but LPS-untreated conditioned medium did not. This nNOSα phosphorylation was reduced by co-treatment with a PKA inhibitor (H-89) both early (0.5 h) and late (6 h), but only slightly by a CaM-KII inhibitor (KN-93) and only at a late time point (Fig. 8). This result suggests that the activation of autophosphorylated CaM-KII or PKA is responsible for nNOSα phosphorylation, which results in a decrease in NOS activity, although it has little effect on total NOS activity in HT-1 cells.
When the cytokines were administered directly to HT-1 trophoblast cells, phosphorylation levels were differentially increased in the LPS plus INF-γ-, IL-1β-, or TNF-α-treated cells, and particularly in TNF-α-treated cells, and in the LPS plus three cytokines, but did not decrease in response to a single cytokine (Fig. 9). This suggests that TNF-α or LPS plus each cytokine used can modulate the phosphorylation of nNOSα. It is relevant in this context that Kupferminc et al. (1994) observed an increase in the circulating levels of TNF-α in women with preeclampsia in the third trimester of pregnancy. Furthermore, in several reports, serum concentrations of cytokines like IL-2, IL-6, and TNF-α were significantly higher in patients than in controls (Sacks et al., 1998; Williams et al., 1999). Therefore, we suggest in the present study that TNF-α and some other cytokines can increase intracellular calcium concentrations and some protein kinases can be induced to phosphorylate nNOS-α in HT-1 cells.

This phosphorylation mechanism for nNOSα in HT-1 trophoblast cells can be seen as a protective device against the oxidative stress produced in inflammation or increases in intracellular calcium concentrations, although there is little effect on total nNOS activity. Therefore, we suggest that intracellular calcium concentrations increase in human full-term trophoblasts stimulated by inflammatory cytokines, and nNOS activity in these cells is increased. We also report that the nNOSα isoform, although expressed at very low levels, can be the target of phosphorylation induced by the activation of the relevant PKA or CaM-KII, leading to a decrease in enzyme activity.

In summary, the viability of trophoblast cells is mediated essentially by physiological NO release. Therefore, we suggest that the
phosphorylation of nNOSα constitutes a protective mechanism against the overproduction of physiological NO by increased nNOS activity in HT-1 trophoblast cells. Furthermore, each of the PKC, PKA, and CaM-KII pathways or an interaction between them is involved positively in the increase in total NOS activity, but not in the direct phosphorylation of the major nNOS isoform, nNOSµ, in these HT-1 cells. We also suggest that TNF-α and some other cytokines can increase intracellular calcium concentrations and that some protein kinases are thus induced to phosphorylate nNOSα in HT-1 cells. This phosphorylation mechanism for nNOSα in HT-1 trophoblast cells probably serves a protective role against oxidative stress during inflammation or increases in intracellular calcium concentrations, although it has little effect on total nNOS activity. Thus, at least two paradoxical pathways act in HT-1 trophoblast cells that are likely to be involved in the development of preeclampsia: an increase in nNOS activity caused by the calcium influx induced by inflammatory cytokines (probably involving the nNOSµ isoform) and a decrease in NO release caused by phosphorylation of nNOSα as a possible protective mechanism.
References


Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, Van Maercke YM


Fig. 1. Protein expression in HT-1 cells determined western blot analysis. Ref: crude sample of rat brain tissues. Crude: supernatant fraction of homogenates centrifuged at $500 \times g$ for 5 min. Cytosolic: cytosolic fraction of homogenates centrifuged at $100,000 \times g$ for 1 h. Membraneous: particulate fraction of homogenates centrifuged at $100,000 \times g$ for 1 h. The membraneous fraction has two differently sized nNOSs, 165 kDa, and 155 kDa.
A.

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B.

Fig. 2. Effect of ionomycin and 7-nitroindazole (7-NI) on the expression of nNOS µ and phos-nNOSα in HT-1 cells. Ref: rat brain. The expression of phos-nNOSα was greatly increased by ionomycin treatment.
Fig. 3. Viability of HT-1 cells determined by MTT assay after various dose of ionomycin. D: Control, treated with DMSO only. Cell viability was detected at five different time points (0.5, 4, 8, 16 and 24 h) with four different concentrations of ionomycin (0.1, 1, 5 and 10 µM).
Fig. 4. Effect of 7-NI on ionomycin-induced cell death. Cell death induced by ionomycin treatment was significantly ameliorated by co-treatment with 7-NI.
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**10 μM calcium ionomycin**

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**10 μM calcium ionomycin + 10 μM PKA inhibitor**

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**10 μM calcium ionomycin + 100 nM PKC inhibitor + 10 μM CaM-KII inhibitor**

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Fig. 5. Effect of protein kinase inhibitors on the nNOSμ and phospho-nNOSα expression levels (A) and a quantitative comparisons (B) of HT-1 cells treated time-dependently with 10μM ionomycin. C: control treated with DMSO only; PKC inhibitor: Ro-31-8220, PKA inhibitor: H-89, CaM-KII inhibitor: KN-93.
Fig. 6. Effect of protein kinases on the Ca\(^{2+}\)-dependent NOS activity determined by hemoglobin oxidation assay. The increase of Ca\(^{2+}\)-dependent NOS activity by ionomycin treatment was prevent by co-treatment with PKC-, PKA-, or CaM-KII-selective inhibitors.
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nNOS

Phospho-nNOS

Actin

165 kDa

155 kDa

42 kDa

B.

Fig. 7. Effect of LPS-mediated conditioned medium on the expression of the expression of nNOSµ and phospho-nNOSα(A) and a quantitative comparisons of nNOSµ and phospho-nNOSα in HT-1 cells over time. RAW 264.7 cells were treated with LPS (10 μg/mL) for 9 h, and the culture medium was then transferred to HT-1 cells.
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B.  

Fig. 8. Effect of LPS-mediated conditioned medium on the expression levels of nNOSμ and phospho-nNOSα (A) and a quantitative comparisons of nNOSμ and phospho-nNOSα in HT-1 cells at two different time points. Ref: mouse brain.
Fig. 9. Effect of cytokines or LPS-mediated conditioned media in HT-1 cells. 1: Control untreated with LPS, 2: LPS-mediated conditioned media, 3: LPS, 4: INF-γ, 5: IL-1β, 6: TNF-α, 7: INF-γ + LPS, 8: IL-1β + LPS, 9: TNF-α + LPS, 10: All (INF-γ + IL-1β + TNF-α + LPS).
Fig. 10. Effect of cytokines on the nitrite concentration released to the cell culture medium. 1: Control untreated with LPS, 2: LPS-mediated conditioned media, 3: LPS, 4: INF-γ, 5: IL-1β, 6: TNF-α, 7: INF-γ + LPS, 8: IL-1β + LPS, 9: TNF-α + LPS, 10: All (INF-γ + IL-1β + TNF-α + LPS).
감사의 글

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- 39 -
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그리고, 유치원부터 현재까지 길긴 연을 맺고 있는 세택 경은, 꿈을 이루기 위해 열심히 노력하고 있는 수정, 미원, 선영, 직업 전선에서 열심히 뛰고 있는 희정. 우리 우정 변치말자. 너희들의 존재만으로 난 충분히 복받은 사람이라고 생각한다.

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