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Neuronal Nitric Oxide-mediated Cytotoxicity in Trophoblast Cells Induced by Increase of Intracellular Calcium

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Abstract

Cell death of trophoblast, particularly by abnormal release of physiological nitric oxide (NO) has been known to be a causative factor of pre-eclampsia. In the present study, effects of intracellular calcium increase enhancing the activity of NO synthases (neuronal NO synthase, nNOS in this trophoblast cells) on the cell death were examined in a human placental full-term cell line (HT-1). Furthermore, we analyzed the possible mechanisms underlying the augmentation of Ca^{++}-mediated NOS activity mediated by protein kinases like PKC, PKA, or CaM-KII. In experiments for cell toxicity, a calcium ionophore (ionomycin 10 μM) enhanced cell death confirmed by MTT assay, and increased significantly nNOS activity determined with a hemoglobin oxidation assay. This cell death was partially protected by pre-treatment of 7-nitroindazole (7-NI, 10 μM and 100 μM), a nNOS-specific inhibitor. Additionally, Ca^{++}-ionophore -induced increase of nNOS activity also was partially normalized by pre-treatment of specific inhibitors of protein kinases, PKC, PKA or CaM-KII. Therefore, we suggest that an increase of calcium influx, leading to the activation of nNOS activity, which in turn may result in the death of trophoblast cells by involvement of signaling mechanisms of protein kinases.

Keywords: Trophoblast, Preeclampsia, Neuronal nitric oxide synthase, Calcium

Preeclampsia 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[1,2]. 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 A_2 (TXA_2)[3-5].

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 or 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[6]. 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[7,8].

Trophoblasts distributed in the placenta also produce NO constitutively by eNOS or nNOS, or both[28]. Trophoblast-derived NO prevents platelet and leukocyte adhesion and eNOS-mediated NO release also controls vascular tone[10]. Therefore, impairment of the NO release mediated by placental trophoblasts and endothelial cells may be associated with several systems in preeclampsia. Human villous trophoblasts express eNOS in the syncytiotrophoblast, but not in the underlying cytotrophoblast of the first trimester or in

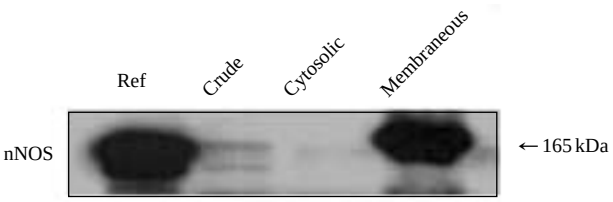

Figure 1. Protein expression in HT-1 cells determined Western blot analysis. Ref: crude sample of rat brain tissues. Crude: supernatant fraction of homogenates, Cytosolic: cytosolic fraction, Membraneous: particulate fraction of homogenates.

the full-term villous tissue[11]. In *in vitro* culture, eNOS is expressed during differentiation of the cytotrophoblast to the syncytiotrophoblast[12]. Huang *et al.*[13] and Shesely *et al.*[9] 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.*[14].

However, NO is also synthesized by peripheral non-adrenergic non-cholinergic (NANC) nerves in the blood vessels, thereby mediating vasodilatation[15,16]. In the normal rat pregnancy, renal eNOS is decreased, but iNOS and nNOS expression is increased[17]. In eNOS-null mutant mice, nNOS plays an important role in blood pressure regulation, both acutely and chronically[18].

It has also been suggested that preeclampsia is associated with increases in trophoblast cell death like apoptosis, as well as with modulations of physiologically essential gene expression[19,20]. Furthermore, inflammatory cytokines increase intracellular calcium concentrations in neonatal cardiac myocytes[21]. Moreover, Steinert *et al.*[22] showed that preeclampsia is associated with changes in calcium regulation and NO production. This calcium influx increases Ca^{++}/calmodulin-dependent nNOS activity, Ca^{++}/calmodulin kinase II, cAMP-dependent protein kinase A (PKA) and Ca^{++}-phospholipid-dependent protein kinase C (PKC) are also involved in the mechanisms controlling NOS catalytic activity.

Here, we used a human full-term trophoblast cell line (HT-1) to investigate the mechanism enhancing nNOS activity and cell toxicity, and confirmed the protective effect of physiological NO release on the calcium-induced cytotoxicity by using human trophoblast cells.

Effects of Ionomycin on the Expression and Activity of nNOS

This procedure was used to examine the effects of increased intracellular calcium on nNOS activity in human trophoblast cells, HT-1. Western blot analysis showed that HT-1 cells constitutively expressed nNOS (~165 kDa), which was nNOS in particulate forms (Figure 1). The nNOS was highly expressed in the cells.

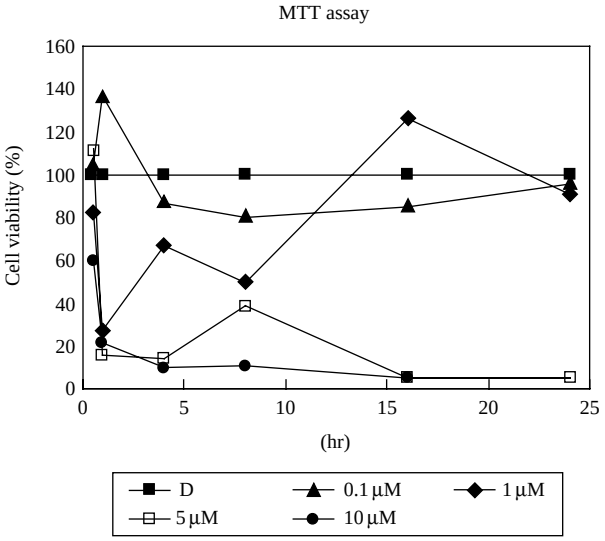

Figure 2. Viability of HT-1 cells determined by MTT assay after treatment of ionomycin. D: Control (DMSO). Cell death was induced dose-dependently in Ionomycin-treated cells.

The effect of ionomycin on HT-1 cell viability was determined using the MTT assay (Figure 2). 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 cytotoxic effects of a significant increase in intracellular calcium concentration.

Effects of nNOS-specific Inhibitor 7-NI on the Ionomycin-induced Cell Death

As shown in Figure 3, death of the HT-1 cells was accelerated by high concentrations of ionomycin (10 μM) administered for 24 h, with viability reduced to about 10% of the control value. This cell death was partially ameliorated by pre-treatment with a specific inhibitor of nNOS, 7-NI. This result suggests that cell death is caused by the NO overproduction induced by increased Ca^{++}-dependent nNOS activity.

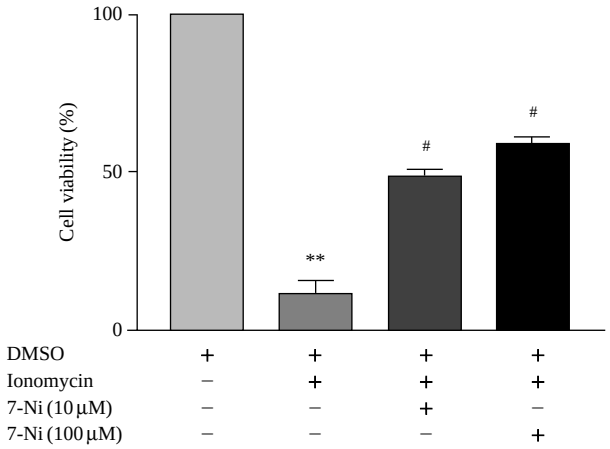

Figure 3. Effect of 7-NI on ionomycin-induced cell death. Cell death induced by ionomycin treatment (10 μM, $P < 0.01$ compared with DMSO-treated control) was significantly ameliorated by pre-treatment with 7-NI ($P < 0.05$ compared with Ionomycin-treated group).

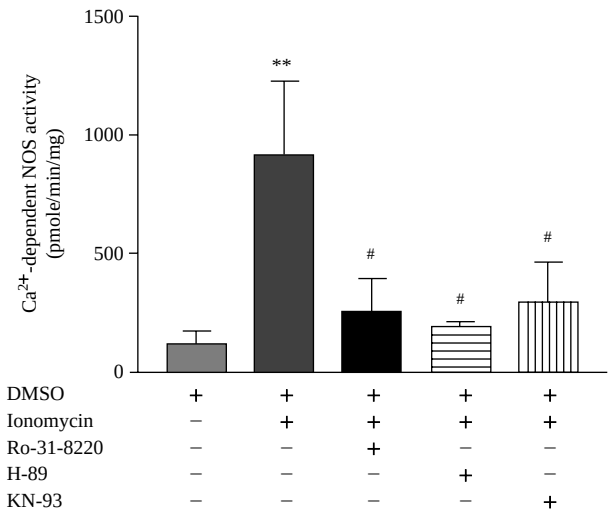

Figure 4. Effect of protein kinases on the Ca^{++}-dependent NOS activity determined by hemoglobin oxidation assay. The increase of Ca^{++}-dependent NOS activity by ionomycin treatment (10 μM, $P < 0.01$ compared with DMSO-treated control) was prevent by pre-treatment with PKC (100 nM)-, PKA (10 μM)-, or CaM-KII (10 μM)-selective inhibitors ($P <$ 0.05 for each group compared with Ionomycin-treated group).

Effects of Protein Kinase Inhibitors on Ionomycin-mediated Augmentation of NOS Activity

NOS activity was increased by ionomycin treatment, but pre-treatment with inhibitors (Ro-31-8220, H-89 or KN-93) of protein kinases like PKC, PKA or CaM-KII, respectively, prevented the increase in NOS activity (Figure 4). Thus, this result indicates that the increase in nNOS activity caused by ionomycin is mediated dependently by PKC, PKA, or CaM-KII pathways, and results in cell death.

Discussion

In the present study, we examined whether changes in nNOS and/or eNOS activity and NO release induced calcium influx are associated with death of trophoblast cells known as a causative mechanism of preeclampsia. We used HT-1 cells derived from villous cytotrophoblasts of a normal-term human placenta to identify the changes in major factor affecting enzyme activity, like gene expression and post-translational modification, with especial attention to the nNOS and/or eNOS.

Human villous trophoblasts express eNOS in the syncytiotrophoblast, but not in the underlying cytotrophoblast of the first-trimester and full-term villous tissue[11]. In culture, eNOS is expressed during differentiation of the cytotrophoblast to the syncytiotrophoblast[12]. Therefore, impairment of NO release mediated by placental trophoblast and endothelial cells may be associated with a causative mechanism of pre-eclampsia. Huang *et al.*[13] and Shesely *et al.*[9] 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.*[14].

However, several reports suggest that, in early pregnancy, the trophoblasts have important activities such as proliferation and differentiation[23] that are related to physiological NO release. However, many of these studies are controversial. For examples, Ramsay *et al.*[24] detected cytosolic Ca^{2+}-dependent NOS activity in villous trophoblasts in each of the three trimesters of pregnancy, whereas Al-Hijji *et al.*[25] 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[15,16]. In eNOS-null mutant mice, nNOS plays an important role in blood pressure regulation, both acutely and chronically[18]. 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 as shown in Figure 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[25,26]. Recently, Sanyal *et al*.[27] 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 death of the full-term trophoblasts induced by changes in intracellular calcium concentrations. As shown in Figure 2, treatment of calcium ionophore ionomycin (0.1-10 μM) caused dose-dependent cell death. When the viability of HT-1 trophoblast cells was examined by MTT assay (Figure 2), 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 (Figure 3). 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[28]. Therefore, changes in intracellular Ca^{2+} concentration in the trophoblast directly affect Ca^{2+}-dependent NOS activity. Additionally, 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 mechanisms that stimulate or inhibit NOS activity. The calcium ionomycin-mediated increase in trophoblastic nNOS activity was prevented by pretreatment with each protein kinase inhibitor (Figure 4). 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 in HT-1 cells. In contrast with our results, Hayashi *et al*.[29], Komeima *et al*.[30] and Osuka *et al*.[31] reported that nNOS is directly phosphorylated at Ser-847 by CaM-KII leading to a reduction in its enzyme activity *in vivo* and *in vitro*. However, we could not explain clearly the effects of protein kinase inhibitors on the activity of nNOS increased by the calcium ionophore, ionomycin (Figure 4).

In the connections with another speculation, it is relevant that Kupferminc *et al*.[32] 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[33,34]. Therefore, we suggest in the present study that inflammatory TNF-α and some other cytokines can increase intracellular calcium concentrations and some protein kinases can be induced to increase nNOS activity in HT-1 cells.

In summary, the viability of trophoblast cells is mediated essentially by physiological NO release. Thus, we suggest that intracellular calcium concentrations increase in human full-term trophoblasts stimulated probably by inflammatory cytokines, and nNOS activity in these cells is increased by the relevant PKC, PKA and/or CaM-KII, resulting cell death as a causative factor of preeclampsia.

Methods

Cell Culture and Treatment

Human trophoblast cells (HT-1) was 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% CO_2 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 cells were seeded at 1×10^6 cells/cm^2 into 60 mm culture dishes (TPP) one day before treatment.

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 1-10 μM. 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.

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 1×10^4 cells/cm^2 in 96-well culture plates. After treatment, the medium was changed to MTT, a tetrazolium dye (3-[4,5-dimethylthiazol-2-y1]-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 0.2 mL of dimethyl sulfoxide (DMSO, Sigma), and incubation at 37˚C for 5 min. The absorbance was read with an ELISA reader (Power $Wave_x$ 340, Bio-Tek Instruments, Inc., Winooski, VT) at 550 nm.

Isolation of Total Cellular Proteins and Westernblo Analysis

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). 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 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 : 1,000 dilution) directed against nNOS (Transduction Laboratories, Lexington, KY). 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.

Measurement of Nitric Oxide Synthase Activity

Microarray 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 BH_4, 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.

Statistical Analysis

All data are presented as means ± S.D. Statistical comparisons between groups were determined by Student's *t* test. Differences among means were assumed significant when $P < 0.05$.

Acknowledgements

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