Effect of Hypoxia on Endothelial Nitric Oxide Synthase, NO Production, Intracellular Survival Signaling (p-ERK1/2 and p-AKT) and Apoptosis in Human Term Trophoblast

Mi-Hye Park1,2, Henry L. Galan1, Juan A. Arroyo1

1From the Division of Perinatal medicine in Departments of Obstetrics and Gynecology, University of Colorado at Denver Health Sciences Center, Denver, CO, USA; 2Department of Obstetrics and Gynecology, Ewha Woman’s University, Seoul, Korea

Introduction

Intrauterine growth restriction (IUGR) is an important cause of fetal morbidity and mortality affecting upwards of 8% of all pregnancies.1 In addition, it is now apparent that IUGR infants exhibit higher rates of cardiovascular disease and diabetes during adulthood.2,3 This disease is usually associated with uteroplacental insufficiency and abnormal umbilical Doppler velocimetry, suggesting abnormal blood flow resistance and fetoplacental hypoxia.4 Thus, villi are exposed to hypoperfusion and hypoxia, particularly in severe cases of IUGR. As such, it is important from a pathophysiology standpoint to understand the effects of hypoxia on the trophoblast. The underlying mechanisms by which hypoxia impacts the placenta are currently under study and remain poorly understood. Alterations in vasoactive mediators, such as nitric oxide (NO), endothelin and angiopoietin II, as well as perturbations of the process of apoptosis have been established during this disease.5–13

Keywords
Apoptosis, eNOS, hypoxia, signal transduction, trophoblast

Problem
Hypoxia is commonly associated with complicated pregnancies such as intrauterine growth restriction. We evaluated the effects of hypoxia on phospho (p)-eNOS, p-ERK, p-AKT and apoptosis in human trophoblast.

Method of study
Isolated trophoblast were cultured in 21% oxygen or 2% oxygen for 24, 48 and 72 hr. p-eNOS, p-ERK and p-AKT protein were assessed by Western blot and apoptosis by TUNEL assay. NOx was determined in the culture media.

Results
Compared to controls, hypoxia-exposed CT showed the following: (1) decreased eNOS at 48 and 72 hr, (2) increased p-eNOS at 48 hr, (3) no differences in total NOx production, (4) increased p-ERK at 24, 48 and 72 hr, (5) increased p-AKT at 24 hr (P < 0.05) and (6) increased apoptosis at 48 hr.

Conclusion
Hypoxia increases activation of p-ERK and induces apoptosis of cultured trophoblast. Hypoxia decreases overall total eNOS but increases p-eNOS, which may allow for NO production to be maintained in trophoblast cells.
(eNOS), which has been localized to both the syncytiotrophoblast and placental vascular endothelium from sheep and human pregnancies. eNOS expression and NO synthesis have been variably reported to be decreased, unchanged or increased in the placenta of human and animal pregnancies complicated by fetal growth restriction. eNOS expression has correlated poorly with NO production, suggesting that other mechanisms of regulating eNOS activity are important. The activity of eNOS is regulated primarily by post-translational mechanisms including phosphorylation at multiple sites. The two most studied sites are the activation site ser1177 and the inhibitory site Thr495. Low-oxygen conditions had been shown to decrease eNOS protein in BeWo trophoblast cells. Although the regulation of eNOS and NO production in the cultured trophoblast in response to hypoxia has been previously studied, the specific role of eNOS phosphorylation has not.

In addition to impacting eNOS protein production, hypoxia impacts apoptosis, which plays a role in the normal development, remodeling and aging of the placenta. The increased placental apoptosis reported in pregnancies with fetal growth restriction supports the assumption that apoptosis may be induced in placenta under hypoxia, distorting the balance of cellular proliferation, differentiation and death, thereby impairing placental function. However, little is known about the role of apoptosis and signal transduction in cultured trophoblast under hypoxic conditions. Hypoxia activates extracellular signal-regulated kinase (ERK)1/2 and the kinase B (AKT), negatively regulating apoptosis and facilitating cell survival. Because studies of patients with IUGR may or may not include pre-eclampsia and because the definition of IUGR varies between studies, we chose to begin with in vitro trophoblast studies of normal term placentas assessing eNOS, p(ser1177) eNOS, intracellular survival signaling (p-ERK and p-AKT) and apoptosis in human term trophoblast. The primary objective of our study was to evaluate the effects of hypoxia on the activation of these proteins, media NO production and apoptosis in human term trophoblast.

Materials and methods

Trophoblast Isolation and Culture

This study was approved by the Colorado Multiple Institutional Review Board at the University of Colorado Denver at Denver Health Sciences Center. Human placentas (6) were collected immediately following normal vaginal or cesarean deliveries from uncomplicated term pregnancies. Cytotrophoblast cells (CT) were isolated as previously described by Arroyo et al. Briefly, 50 g of villous tissue were digested in 250 mL of Hanks balanced salt solution media (HBSS; Sigma, St. Louis, MO, USA) containing 40 mg of DNAse (Roche Diagnostics, Indianapolis, IN, USA), 100 mg of trypsin (Sigma) and 500 units of dispase (BD Biosciences, Bedford, MA, USA). Digestion was performed at 37°C for 60 min in an orbital shaking incubator at 350 x g. Digest media was first strained to remove tissue fragments and then centrifuged for 5 min at 500 x g. The cell pellet was resuspended in Dulbecco’s modified Eagle media containing 10% fetal calf serum (FCS; Sigma). Cell suspensions (2.5 mL) were layered on a continuous 10–70% Percoll gradient (Sigma) and spun for 20 min at 1200 x g. The CT band was removed from the continuous percoll gradient at a density of 1.05–1.06 g/mL as a determined by density gradient beads (Amersham, Uppala, Sweden). Purity of the trophoblast preparations was estimated from the proportion of cytokeratin-positive and vimentin-negative cell (both form Santa Cruz Biotechnology, Santa Cruz, CA, USA). Only cultures containing >98% trophoblast were used in these studies (see Fig. 1). CT were plated at a density of 2 x 10⁶ cells in a 100 mm² plate (n = 5) and 1 x 10⁶ cells (n = 4) in chamber slide in keratinocyte basal medium 2 (KBM-2; Biowithaker, Walkersville, MD, USA) with keratinocyte growth medium (KGM) Bullet kit (Biowithaker) together with 10% FCS. After 48 hr in culture, cells were incubated for 24, 48 or 72 hr in either 21% oxygen (normoxia) or 2% oxygen (hypoxia), and proteins extracted at each time point were studied.

Western Blot Analysis

Cultured CT cells were assessed for p-eNOS, eNOS, p-ERK, ERK, p-AKT or AKT proteins by Western blot. Protein tissue lysates (25 µg) were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane and incubated overnight with a horseradish peroxidase-conjugated mouse monoclonal antibody against eNOS (1:500; Santa Cruz Biotechnology), rabbit p-eNOS, p-ERK, ERK, p-AKT or AKT (all at a dilution of 1:500; Cell Signaling, Danvers MA, USA). A secondary anti-rabbit Ig-HRP antibody (1:5000; Cell Signaling) was incubated for 1 hr at room temperature.
After rinsing, the membranes were incubated with chemiluminescent substrate (Pierce, Rockford, IL, USA) for 5 min, and the emission of light was digitally recorded using a charged couple device camera. To determine loading consistencies, each membrane was stripped of antibodies and reprobed using antibodies against mouse β-actin (1:4000 for 1 hr; MP Biomedicals, Aurora, OH, USA). Changes in these proteins were quantified by densitometry, normalized by actin, and comparisons were made between normal and hypoxic conditions. eNOS phosphorylated ratios were normalized by actin.

Nitric Oxide Assays

CT culture media were collected and assessed for NO per protocol as suggested by the manufacturer, Assay Designs, Ann Arbor, MI, USA. This is based on the determination of the total NO in the samples by the conversion of NO into nitrate and nitrite. Briefly, culture media were ultrafiltrated through a 10,000 MWCO filter. After filtration, a solution of NADH was added to the samples followed by the addition of the nitrate reductase solution. Samples were incubated at 37°C for 30 min. After incubation, the Griess reagents were added, and samples were incubated for 10 min at room temperature. The optical densities of the sample assessments were determined at 540–570 nm in unknown and calibration curve samples.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL)

TUNEL was performed on cultured chamber slides. The TUNEL protocol was followed as suggested by the manufacturer (Chemicon, Inc., Danvers, MA, USA). Briefly, slides were post-fixed using a solution of ethanol:acetic acid (2:1) for 5 min. The equilibration buffer was added directly to the tissue slide for 10 s followed by incubation with the TdT enzyme for 1 hr at 37°C. Following the enzyme treatment, the anti-digoxigenin conjugate was incubated on the slide for 30 min. 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI) was used for nuclear staining in our slides followed by mounting with a glass coverslip. Slides were viewed using fluorescein excitation and emission filters. The number of TUNEL-stained and unstained nuclei were in 10 randomly selected microscopic fields at 400× magnification providing a minimum of 200 cells. An apoptotic index was defined as the percentage of cells that were stained by the TUNEL method divided by the total number of cells counted.

Statistical Analysis

Data are shown as mean ± S.E. Comparisons of the following end points were made between normoxia and hypoxia: p-eNOS, p-ERK, p-AKT Western blot analysis, NO and apoptotic index. Hypoxic effects were determined using Student’s t-test or ANOVA as appropriate with P < 0.05 considered significant.

Results

Effect of Hypoxia on eNOS and p-eNOS Expression

Western blot was performed to determine the effects of hypoxia in trophoblast eNOS. Compared to CT in control conditions, CT in hypoxia conditions showed
reduced eNOS expression at both 48 and 72 hr (1.5-fold; $P < 0.002$ and 1.2-fold; $P < 0.02$, respectively; Fig. 2). In contrast, hypoxia significantly increased CT expression of p-eNOS (Ser1177) at 48 hr (2.7-fold; $P < 0.003$) with a trend for significance at 72 hr (1.7-fold; $P = 0.074$; Fig. 3).

**Effect of Hypoxia on NO Production in Media**

Next, we asked whether these changes in eNOS affect the release of NO into the CT culture media. For this, NO production was analyzed in the cultured media of term control trophoblast and trophoblast under hypoxic conditions. Compared to controls, there were no significant changes in total NO production with hypoxia treatment at any time point studied (Fig. 4).

**Effect of Hypoxia on p-ERK and p-AKT in Cultured Term Trophoblast**

Knowing that ERK and AKT signaling are associated with the expression of eNOS, we assessed the activation of these signaling proteins in cultured term trophoblast using Western blotting. Compared to controls conditions, CT cells exposed to hypoxia showed a significant increase in p-ERK expression at 24, 48 and 72 hr of culture (3.8-fold; $P < 0.02$, *P < 0.02*).
1.3-fold; \(P < 0.04\) and 1.8-fold; \(P < 0.04\) respectively, Fig. 5). A significant increase in p-AKT expression was observed only at 24 hr (2.5-fold; \(P < 0.05\)) in CT cultured under hypoxia (Fig. 6).

**Effect of Hypoxia on Apoptosis in Cultured Term Trophoblast**

In addition to the effects of hypoxia in eNOS, we next investigated the effects of this treatment in trophoblast apoptosis. The effect of hypoxia on apoptosis in the cultured term CT was assessed with TUNEL methodology. A significant increase in apoptosis was detected in trophoblast cells exposed to hypoxia at 48 hr (1.6-fold; \(P < 0.02\)), while a trend for a significant increase (1.3-fold; \(P = 0.079\)) was seen at 72 hr (Fig. 7).

**Discussion**

In our studies, hypoxia reduced eNOS expression in cultured human trophoblast; however, the anticipated concomitant decrease in the NO in the media of these cells was not seen. In contrast, hypoxia increased p-eNOS (Ser 1179) expression significantly in cultured human trophoblast. This latter finding provides a possible explanation for the lack of change in NO. More specifically, hypoxia had opposite effects on p-eNOS and total eNOS, which may have negated any possible decrease on NOx production, related to the decrease in the non-phosphorylated eNOS protein. Thus, activation of eNOS phosphorylation at Ser 1179 is likely to be an important determinant of NO synthesis in cultured human CT under hypoxia and further remains to be evaluated in vivo. The effects of hypoxia on eNOS expression appear to be both species and tissue specific. Kiss et al.\(^{10}\) examined eNOS mRNA and protein production in different cytotrophoblastic cell lines cultured under hypoxia. They found increased eNOS mRNA and decreased eNOS protein in trophoblast at low-oxygen tension. No evaluation of phosphorylated eNOS was performed. In addition, we showed a similar decrease in total eNOS protein concentration. We further report an increase in p-eNOS expression with hypoxia exposure in trophoblast cells, which has not been previously reported. Chen and Meyrick\(^{26}\) did report that hypoxia stimulates phosphorylation of eNOS at Ser 1177 and increases eNOS activity and NO production in porcine coronary artery endothelial cells. Our findings are consistent with their findings of an increased...
Fig. 6 Effect of hypoxia on p-AKT protein expression in cultured trophoblast. p-AKT was only increased at 24 hr in cultured trophoblast treated with hypoxia.

Fig. 7 Trophoblast apoptosis under hypoxia. Apoptosis was significantly increased at 48 hr in trophoblast treated with hypoxia. A trend for a significant increase was observed in hypoxia-treated trophoblast at 72 hr.
p-eNOS (Ser 1179) expression with hypoxic exposure; however, changes in NOx production was not seen, which may have been because of a decrease in total protein observed in our studies.

Hypoxia is known to be a significant trigger for apoptosis in the placenta. Increased apoptosis has been reported in placenta from pregnancies complicated with IUGR and/or pre-eclampsia, which are conditions associated with placental hypoxia. The pathway of hypoxia-induced apoptosis in the trophoblast is not well defined. ERK and AKT modulate the function of numerous proteins involved in cell survival. Several pro-apoptotic molecules, such as Bad, ASK1 and caspase-9, are inactivated through phosphorylation of ERK and AKT. ERK and AKT are, in turn, phosphorylated under hypoxic conditions. Studies have also shown that inhibition of AKT during hypoxia leads to increased apoptosis in trophoblast cells. In the present study, hypoxia increased apoptosis in cultured human trophoblast at 48 and 72 hr. Interestingly, Kilini et al. showed opposite results for apoptosis in primary trophoblast cultured with hypoxia. These differences could be because of the fact that their results were calculated 5 hr after isolation, while in the current studies the trophoblast were differentiated in cultures to syncytiotrophoblast before hypoxia treatment. These results suggest a different response maybe present to hypoxia stress in the different types of trophoblast. This increase in apoptosis occurred in spite of activation of p-ERK at all time points and activation of p-AKT (cell survival) only at 24 hr. These findings suggest that hypoxia-induced apoptosis could be the result of insufficient p-AKT expression in cultured human trophoblast. Although with different cell lines and time points in gestation, Perkins et al. observed that inhibition of the PI3K/AKT pathway sensitized first-trimester trophoblast into hypoxia-induced cell death by 48 hr of culture, a phenomenon that may be relegated to early pregnancy.

In conclusion, hypoxia decreases eNOS, but increases phospho-eNOS (Ser1177) expression and likely helps to maintain NO production in cultured human trophoblast. These studies support eNOS phosphorylation as an important determinant of NO synthesis in cultured human CT under hypoxia. We also conclude that although p-ERK is activated with hypoxic conditions, the hypoxia-induced apoptosis in the cultured human trophoblast may be secondary to a lack of cell survival capacity as indicated by insufficient AKT activation. Further studies are needed to investigate the following: (i) the role of eNOS phosphorylation, more specifically whether phosphorylation of Thr 495 site is impacted by hypoxia and (ii) whether other signal transduction molecules such as JNK or p38 and apoptotic molecules are associated with apoptosis in cultured human trophoblast under hypoxia. These studies may provide depth of the understanding of the pathophysiologic mechanisms associated with hypoxia that is commonplace in human IUGR and pre-eclampsia.

References


26 Chen JX, Meyrick B: Hypoxia increases Hsp90 binding to eNOS via PI3K-Akt in porcine coronary artery endothelium. Lab Invest 2004; 84:182–190.


