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■ Original Article ■

Hypoxia Enhances Nitric Oxide Synthesis by Upregulation of Inducible Nitric Oxide Synthase in Endothelial Cells

Ki-Jong Rhee[1], Sun-Yeong Gwon[1] and Seunghyung Lee[2,†]

[1]Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University at Wonju, Wonju, Gangwon-do 220-710, Korea.

[2]Department of Anatomy & Cell Biology and The Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA 19140, USA.

Hypoxia is an integral part of the environment during luteolysis. In this study we examined whether hypoxia could directly stimulate endothelial cells to produce nitric oxide (NO). Endothelial cells were cultured in hypoxic (5% O_2) or normoxic (20% O_2) conditions and the levels of total NO, inducible NO and endothelial NO was measured. We found that hypoxia but not normoxia upregulated NO production. The increased NO levels correlated with increased inducible NO synthase (iNOS) expression whereas expression of endothelial NOS (eNOS) expression remained constant. Addition of the iNOS specific inhibitor 1400W to hypoxic cultures prevented NO production suggesting that hypoxia-induced NO production in endothelial cells was due mainly to upregulation of iNOS. We also found that prostaglandin $F_{2\alpha}$ (PGF) production was unaffected by hypoxia suggesting that upregulation of NO was not due to increased synthesis of PGF. In summary, we report that endothelial cells cultured under hypoxic conditions produce NO via the iNOS pathway. This study provides the importance of the relation between the hypoxic environment and the induction of NO by endothelial cells during regression of the corpus luteum in the ovary.

Key Words: Hypoxia, Nitric oxide, Inducible nitric oxide synthase, Endothelial cell

INTRODUCTION

Nitric oxide (NO) can function as a vascular, immunological and neuronal signaling molecule (Ignarro et al., 2001). NO plays a role in luteolysis role in cows, rats, rabbits and humans (Vega et al., 1998; Motta, 1999; Jaroszewski and Hansel, 2000; Boiti et al., 2003; Klipper et al., 2004). In addition, Prostaglandin $F_{2\alpha}$ (PGF), a luteolytic factor, is responsible for the regression of corpus luteum in mammals (Horton and Poyser, 1976; Hansel W, 1986; Silvia et al., 1991).Recently, it has been demonstrated that endothelial cells have the ability to produce NO that NO increases PGF production in endothelial (Maul et al., 2003; Lee et al., 2010). Therefore, endothelial cell derived NO may play a pivotal role regulating luteolysis in the corpus luteum.

Hypoxia is an integral part of the environment during luteolysis. During both spontaneous and PGF-induced luteolysis, a decrease in luteal blood flow occurs in parallel with systemic P_4 concentrations (Nett et al., 1976; Niswender et al., 1976; Ford and Chenault, 1981; Wise et al., 1982). Hypoxia has been suggested to induce apoptosis in many cell types, including ovarian cells (Graeber et al., 1994; Shimizu S, 1996; Saikumar et al., 1998; Matsushita H, 2000; Lee et al., 2005). Moreover, the oxygen content in the ovarian venous blood begins to decrease at the late luteal stage (Wise et al., 1982). The

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†Corresponding author: Seunghyung Lee. Department of Anatomy & Cell Biology and The Sol Sherry Thrombosis Research Center Temple University School of Medicine, Philadelphia, PA 19140, USA.
Tel: +1-215-707-8015, Fax: +1-215-707-2783
e-mail: S.Lee@temple.edu

above findings suggest that hypoxia may be a contributing factor during corpus luteum regression in the ovary. Recent studies reported that hypoxia regulates P_4 synthesis in rats and promotes endothelial apoptosis in mice and humans suggesting that hypoxia may directly promote luteolysis in corpus luteum of ovary (Lefter et al., 2007; de Jesus Perez et al., 2012).

Nitric oxide is synthesized from L-arginine via the activity of NO synthase (NOS) such as inducible NOS (iNOS) and endothelial NOS (eNOS) (Lowenstein et al., 1994). Recently, it has been shown that eNOS proteins are decreased in human term trophoblasts during hypoxic conditions but the overall production of NO is unaffected (Park et al., 2011)Also, iNOS but not eNOS enhanced NO production in endothelial cells (Lee et al., 2009). Thus, we hypothesize that hypoxia promotes luteolysis by induction of NO synthesis in endothelial cells of corpus luteum in ovary. In the present study, we determined the influence of hypoxic conditions on NO production by endothelial cells from the corpus luteum. In addition, we also investigated whether hypoxia specifically upregulates PGF production in cultured endothelial cells.

MATERIALS AND METHIDS

Materials

Hypoxia-inducible factor 1α (HIF1α), β-actin (ACTB), iNOS, and eNOS antibodies were purchased from Santa Cruz Biotechology (Dallas, Texas, USA). N - ([3-(Aminomethyl)phenyl]methyl) ethanimidamide dihydrochloride (1,400 W) and Lipopolysaccharide (LPS) were from Sigma-Aldrich (St. Louis, MO, USA). Nitric oxide assay kit was Abcam (Cambridge, MA, USA).

Isolation of endothelial cells and cell culture

Endothelial cells were isolated from the corpus luteum during the mid-luteal phase in cows (days 8-12 of the estrous cycle) and cultured in vitro. Endothelial cells were seeded at a concentration of 1×10^5/ml/well in 24-well plates (BD Biosciences) or at a concentration of 1×10^6/ml in 75-cm^2 culture flasks (BD Biosciences). Cells were cultured in DMEM/F-12 (Cellgro) containing 20% fetal bovine serum (Cellgro), 5% microvascular growth supplement (Gibco) and 0.1% gentamicin/amphotericin B. When the cells became confluent the medium was replaced with fresh media supplemented with 5 μM holo-transferrin, 500 μM ascorbic acid, 5 nM sodium selenite and 0.1% (w/v) bovine serum albumin (Thermo Scientific). Cell cultures were then subjected to normoxia (20% O_2, 5% CO_2, 75% N_2) or hypoxia (5% O_2, 5% CO_2, 90% N_2) for different times depending on the experiment. Experiments were performed on confluent cultures during passages 1-5.

Determination of NO production

Endothelial cells were cultured to confluence in 24-well plates and then subjected to normoxia (20% O_2) or hypoxia (5% O_2) for 2 h, 6 h or 24 h. For iNOS inhibition studies, N-([3-(Aminomethyl)phenyl]methyl) ethanimidamide dihydrochloride (1 μM 1400W, Sigma-Aldrich) was dissolved in sterile water and added prior to culture under normoxia or hypoxia. At the end the of the incubation period, 500 μl of conditioned media was collected and immediately used for determination of nitrite/nitrate concentration by the Griess method [26]. The assay sensitivity was 0.07 μg/ml and the standard curve ranged from 0.05 μg/ml to 6.9 μg/ml. The intra- and inter-assay coefficients of variation were on average 8.5% and 13.5%, respectively.

NOS mRNA and protein sample preparation

Endothelial cells were cultured in 24-well plates for mRNA isolation or cultured in 75-cm^2 culture flasks for protein isolation. Confluent cells were subjected to normoxia (20% O_2) or hypoxia (5% O_2) for 6 h. Lipopolysaccharide (LPS)-treated cells (1 μg/ml LPS, Sigma-Aldrich) were used as a positive control.

PGF production

Endothelial cells were cultured to confluence and then subjected to normoxia (20% O_2) or hypoxia (5% O_2) for 2 h, 6 h or 24 h. Conditioned media was collected in microfuge tubes containing 5 μl of a stabilizer solution (0.2 M EDTA, 1% acid acetyl salicylic, pH 7.4) and

stored at -80℃. The concentration of PGF in the culture medium was determined by enzyme immunoassay. The PGF standard curve ranged from 15.625 to 4,000 pg/ml, and the median effective dose (ED_{50}) of the assay was 250 pg/ml. The intra- and inter-assay coefficients of variation were 7.4 and 11.4%, respectively.

Western blotting analysis

Endothelial cells were cultured to confluence in 75-cm^2 flasks and then subjected to normoxia (20% O_2) or hypoxia (5% O_2). Cells were scraped and placed in ice-cold homogenization buffer (25 mM Tris-HCl, 300 mM sucrose, 2 mM EDTA, cOmplete Mini, EDTA-free protease inhibitor cocktail, pH 7.5) and then frozen in liquid nitrogen and stored at -80℃. HIF1α, iNOS, and eNOS protein expression in cultured endothelial cells were assessed by Western blotting analysis. Cells were lysed in 200 μl lysis buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Triton X-100, cOmplete protease inhibitor cocktail). Protein concentrations in the lysates were determined by Bicinchoninic acid (BCA) protein assay reagent (Theromo Scientific). Cell lysates were solubilized in SDS sample buffer (10% SDS, 20% glycerol, 0.2M Tris-HCl, pH 6.8, 0.5% bromophenolblue) and heated at 95℃ for 5 min. Samples (10 μg protein) were electrophoresed on a 10% SDS-PAGE gel (Life technologies) for 2 h at 95 V. The separated proteins were transferred to a 0.2-μM nitrocellulose membrane at 45 V for 3 h in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). The membrane was washed in TBS-T (10 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.1% Tween 20) and incubated in blocking buffer for 1 h at room temperature. Membranes were incubated with the primary antibody at 4℃ overnight. The primary antibodies are HIF1α, iNOS, eNOS, and β-actin (all from Santa Cruz Biotechnology). The membranes were washed three times for 5 min each in TBS-T and then incubated for 1 h at room temperature with fluorophore-conjugated secondary antibodies, and were detected by Li-COR (Li-COR Biosciences). Relative protein levels were determined by densitometry using NIH Image/J software (National Institutes of Health).

RT-PCR

Total RNA was extracted from cells using TRIzol® according to the manufacturer's instructions. cDNA was synthesized by reverse transcription with 1 μg total RNA, 0.25 μg of random hexamer (Invitrogen) and 200 unit of murine Moloney leukemia virus reverse transcriptase (MMLV-RT; Invitrogen) for 10 min at 25℃, 50 min at 37℃ and 15 min at 70℃. cDNA was PCR amplified using Prime *Taq* premix PCR kit (Genet Bio, Chungnam, Korea) for 25-40 cycles using specific primers. cDNA was amplified using specific primers for the *iNOS* (forward 5`GCTTAAATCCAGGCAAACGA3` & reverse 3`TTCTGGTGAAGCGTGTCTTG5`), *eNOS* (5`GATCAGCAACGCTATCACGA3` & 3`GGACAG CGGTAGAGCCATAG5`) and *ACTB* (5`GAAGAT CTGGCACCACAC3` & 3`AGAGGCATACAGGGAC AGC5`). The PCR conditions were as follows: activation of DNA polymerase for 20 sec at 95℃ followed by n cycles (see below) of denaturation for 30 sec at 95℃, annealing for 1 min at 60℃, and extension for 1 min at 75℃, followed by final extension for 5 min at 75℃. The number of cycles was 29 for *iNOS* and *eNOS*, and *19 for ACTB*. PCR products were electrophoresed on 2.0% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide and the product size determined by comparison to 100bp DNA ladder marker (Intron, Gyeonggi, Korea). Gel images were taken using Gel Doc™ XR+ system (Bio-Rad, Hercules, CA, USA). The PCR product band intensity was measured and normalized against *ACTB* (β-actin) using Image Lab™ software (version 2.0, Bio-Rad).

Statistical analysis

One-way ANOVA followed by Fisher's protected least-significant difference (PLSD) procedure as multiple comparison tests was used to determine the statistical significance. Data are expressed as the mean ± SEM of three separate experiments. $P<0.05$ was considered to be statistically significant.

RESULTS

Hypoxia induces NO production in endothelial cells

First we determined whether endothelial cells produced NO when cultured under hypoxic conditions. Endothelial cells were cultured under hypoxia (5% O_2) or normoxia (20% O_2) for 2 h, 6 h and 24 h and NO production was assessed using the Griess method. We found that cells cultured under hypoxia produced more NO at 6 h and 24 h compared to endothelial cells cultured under normoxia (Fig. 1; $P<0.05$). There was no significant increase in NO production in endothelial cells cultured under normoxic conditions for up to 24 h. This result demonstrates that hypoxia stimulates NO production in endothelial cells.

Hypoxia induces upregulation of HIF1α protein synthesis

Hypoxia-inducible factor 1α (HIF1α) mediates adaptive molecular responses to low oxygen availability and is known to accumulate in cells specifically under hypoxic conditions (Semenza, 2007). To confirm that 5% O_2 was indeed a hypoxic environment, we examined HIF1α induction in endothelial cells grown under normoxia and hypoxia. Cells were cultured for 2, 4, and 24 h and HIF1 α protein levels were determined by Western blot analysis. HIF1α was significantly increased at 6 h under hypoxia (Fig. 2; $P<0.05$) compared to cells cultured under normoxia. As expected HIF1α levels remained constant when endothelial cells were cultured under normoxia. Based on this result, all subsequent experiments were performed at 6 h of culture.

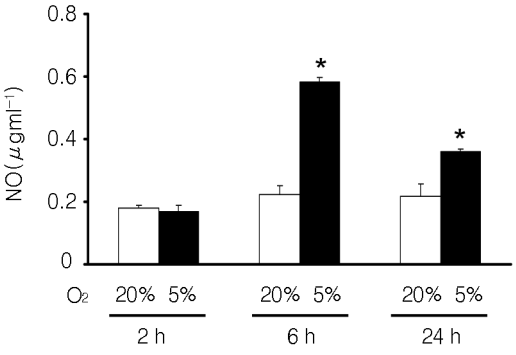

Fig. 1. Hypoxia induces NO production by endothelial cells. Endothelial cells were cultured under normoxia (20% O_2) and hypoxia (5% O_2) for 2 h, 6 h and 24 h. Cell culture supernatant was collected and assessed for NO levels by the Griess method. All values represent mean ± SEM of three separate experiments. Asterisks indicate significant differences compared with incubated cells under normoxia ($P<0.05$, n=3).

Hypoxia induces upregulation of NOS mRNA and NOS protein synthesis

The increase in NO under hypoxic conditions may occur through the iNOS or eNOS pathway. To determine which of these pathways were involved, endothelial cells were grown under normoxia or hypoxia for 6 h and levels of iNOS and eNOS examined at the mRNA and protein level. Lipopolysaccharide (LPS), a potent inducer of iNOS but not eNOS in most cells, was added to endothelial cells cultured under normoxia as a positive control. Endothelial cells cultured under hypoxia showed increased expression of both *iNOS* mRNA and iNOS protein comparable to LPS stimulated controls (Fig. 3, $P<0.05$). In contrast, hypoxia did not induce either *eNOS* mRNA and eNOS protein expression. This result suggests that hypoxia enhances NO synthesis in endothelial cells

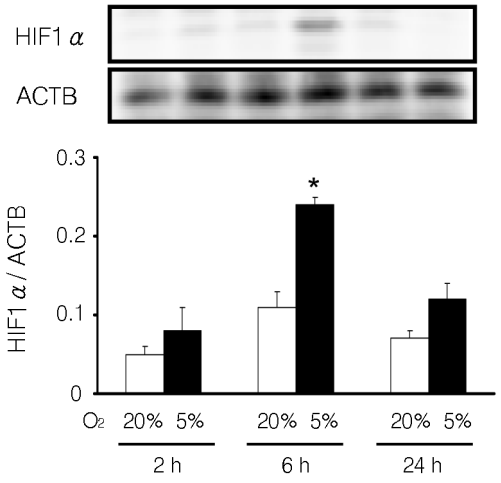

Fig. 2. Hypoxia upregulates HIF1α protein synthesis in endothelial cells. Endothelial cells were cultured under normoxia (20% O_2) or hypoxia (5% O_2) for 2 h, 6 h and 24 h. Cell lysates were harvested and examined for HIF1α levels by Western blot analysis. Upper panel; a representative Western blot of HIF1α and ACTB. Lower panel; densitometric analysis. The HIF1α protein levels are shown relative to levels of ACTB protein. All values represent mean ± SEM of three separate experiments. Asterisk indicates significant differences compared with incubated cells under normoxia ($P<0.05$, n=3).

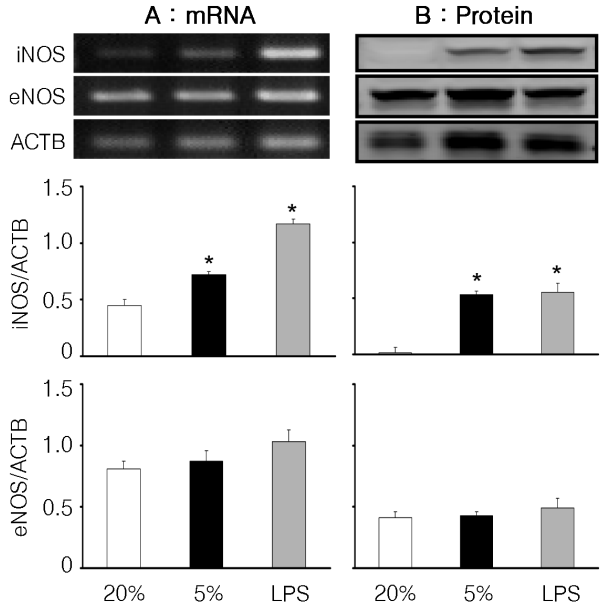

Fig. 3. Hypoxia upregulates NOS mRNA and NOS protein in endothelial cells. Endothelial cells were cultured under normoxia (20% O_2) with or without 1 μg/ml LPS (positive control) and hypoxia (5% O_2) for 6 h. Panel A; a representative RT-PCR result of iNOS, eNOS and ACTB is shown. Densitometric analysis of the iNOS and eNOS PCR products are expressed relative to ACTB levels. Panel B; a representative Western blot result is shown of protein levels of iNOS, eNOS and ACTB. Densitometric analysis of the iNOS and eNOS Western blot are normalized to ACTB levels. All values represent mean ± SEM of three separate experiments. Asterisks indicate significant differences compared with untreated cells under normoxia (P<0.05, n=3).

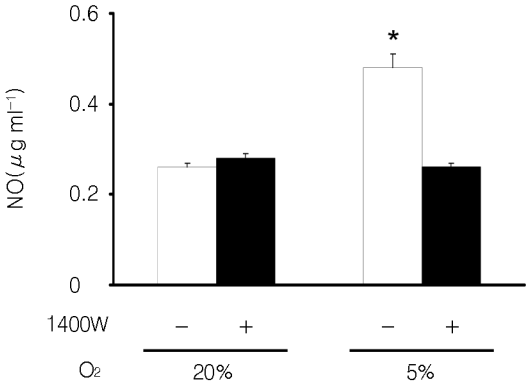

Fig. 4. Effect of iNOS synthesis inhibitor on NO production. Endothelial cells were cultured under normoxia (20% O_2) or hypoxia (5% O_2) for 6 h with or without the iNOS inhibitor 1400W (1 μM). The cell culture supernatant was collected and NO production assessed using the Griess method. All values represent mean ± SEM of three separate experiments. Asterisk indicates a significant difference compared with treated cells under normoxia and hypoxia (P<0.05, n=3).

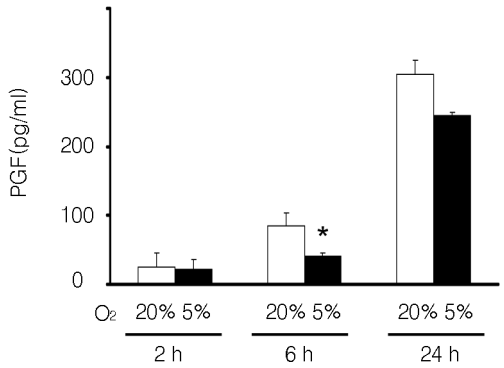

Fig. 5. Hypoxia does not induce PGF production in endothelial cells. Endothelial cells were cultured under normoxia (20% O_2) and hypoxia (5% O_2) for 2 h, 6 h and 24 h. Cell culture supernatant was collected and PGF concentration determined by enzyme immunoassay. All values represent mean ± SEM of three separate experiments. Asterisks indicate significant differences compared with incubated cells under normoxia (P<0.05, n=3).

of the corpus luteum by specific induction of iNOS. To confirm that hypoxia-induced NO production in endothelial cells was due to iNOS, endothelial cells were cultured under hypoxia in the presence of the iNOS-specific inhibitor 1,400 W (Garvey et al., 1997). Cells cultured with 1,400 W prevented hypoxia-induced NO production demonstrating that hypoxia-induced NO production was mainly due to iNOS activity.

Hypoxia does not induce PGF production

Thus far, our data suggest that hypoxia but not normoxia induces NO production in endothelial cells of corpus luteum. However, PGF has also been shown to induce NO production in endothelial cells (Lee et al., 2009). Therefore, we determined whether the NO synthesis during hypoxia was due to PGF production in endothelial cells. Endothelial cells were cultured under normoxia or hypoxia and secreted PGF levels assayed by enzyme immunoassay. There was no increased secretion of PGF in cells cultured under hypoxia compared to cells cultured under normoxia at 2 h, 6 h and 24 h (Fig. 5; P<0.05). In fact, there was a statistically significant decrease in PGF levels at 6 h in cells cultured in hypoxic conditions. This result suggests that the increase in NO during hypoxia is not due to PGF secretion.

DISCUSSION

NO plays a luteolytic role in cows, rats, rabbits and humans (Vega et al., 1998; Motta, 1999; Jaroszewski and Hansel, 2000). NO also inhibits P_4 secretion in vivo and induces apoptosis of human corpus luteum in vitro (Vega et al., 2000; Keator et al., 2008). Furthermore, the NO/NOS system is a potential mediator of lipid peroxidation induced in corpus luteum by PGF during luteolysis (Motta et al., 1996). Taken together, these observations provide a strong rationale for elucidating the mechanism of NO mediated luteolysis. PGF increases NO production by cultured endothelial cells from corpus luteum through stimulation of iNOS (Lee et al., 2010). In another study, it was found that the environment during luteolysis was rendered hypoxic after PGF injection (Wise et al., 1982). This raises the possibility that hypoxia may play a key role for NO production in endothelial cells. In the present study, we found that hypoxia increased NO production in cultured endothelial cells through upregulation of iNOS but not eNOS. In addition, we found that hypoxia alone did not enhance PGF production in endothelial cells. These results suggest that hypoxia may play a physiological role in regulating iNOS-derived NO by endothelial cells during luteolysis in ovary.

Under hypoxic conditions, HIF1α is known to rapidly accumulate in cells and enhance transcription of hypoxia-inducible genes (Semenza, 1999; Wenger, 2002). In this report, we showed that HIF1α protein levels increased in endothelial cells when cultured at 5% O_2 suggesting that the 5% O_2 partial pressure was sufficiently hypoxic. In preliminary experiments (data not shown), we cultured endothelial cells under varying concentrations of oxygen (3%, 5% 10% and 20%) and found that HIF1α was elevated when O_2 levels were at 3% and 5% but not at 10% and 20%. Therefore, we cultured cells at 5% O_2 to mimic hypoxic conditions.

In the present study, we demonstrated that hypoxia stimulates the expression of *iNOS* mRNA and synthesis of iNOS protein in cultured endothelial cells. Studies in rats also suggest that hypoxia increases iNOS mRNA and iNOS protein levels (Le Cras TD, 1996; Xue and Johns, 1996). Our findings suggests that hypoxia during luteolysis promotes regression of corpus luteum by iNOS-induced NO in endothelial cells. In our study, eNOS levels were unaffected by hypoxia in endothelial cells. Other studies have shown that human trophoblasts cultured under hypoxia decreases eNOS protein levels (Park et al., 2011). This discrepancy may be due to the differences in the length of the culture or that cells from the human trophoblasts respond differently to hypoxia than endothelial cells of corpus luteum. Hypoxia induces apoptosis in luteal cells by activating the BCL2 family of proteins and caspases. NO also induces apoptosis in luteal cells (Nakamura T, 2001). There was no increase in apoptosis in endothelial cells when cultured at 5% O_2 suggesting that hypoxia does not induce apoptosis in endothelial cells *in vivo* which then contributes to regression of the corpus luteum.

We show in the current study that hypoxia does not enhance PGF production in endothelial cells. PGF is an important factor in luteolysis and can be produced by endothelial cells (Hansel W, 1986; Silvia et al., 1991; Garvey et al., 1997). Although, PGF can increase NO production in endothelial cells of corpus luteum (Lee et al., 2009), we did not observe an increase in PGF production under the hypoxic conditions used in this study. This result suggests that the increase in NO production in endothelial cells of corpus luteum during hypoxic culture is not due to increase of PGF. Although the mechanism is unclear, we suggest that hypoxia may directly increase NO production possibly by activation of HIF1α. This possibility is currently under investigation. In conclusion, hypoxia enhances NO production in endothelial cells by activating the iNOS pathway suggesting a role of hypoxia during luteolysis in corpus luteum of ovary.

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