RESEARCH ARTICLE

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Roles of Cytokines and Progesterone in the Regulation of the Nitric Oxide Generating System in Bovine Luteal Endothelial Cells

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SUMMARY

Nitric oxide (NO) produced by luteal endothelial cells (LECs) plays important roles in regulating corpus luteum (CL) function, yet the local mechanism regulating NO generation in bovine CL remains unclear. The purpose of the present study was to elucidate if tumor necrosis factor-α (TNF), interferon γ (IFNG), and/or progesterone (P4) play roles in regulating NO generating system in LECs. Cultured bovine LECs obtained from the CL at the mid-luteal stage (Days 8–12 of the cycle) were treated for 24 hr with TNF (2.9 nM), IFNG (2.5 nM), or P4 (0.032–32 μM). NO production was increased by TNF and IFNG, but decreased by P4 ($P < 0.05$). TNF and IFNG stimulated the relative steady-state amounts of inducible nitric oxide synthase (*iNOS*) mRNA and iNOS protein expression ($P < 0.05$), whereas P4 inhibited relative steady-state amounts of *iNOS* mRNA and iNOS protein expression ($P < 0.05$). In contrast, endothelial nitric oxide synthase (eNOS) expression was not affected by any treatment. TNF and IFNG stimulated NOS activity ($P < 0.05$) and 1400W, a specific inhibitor of iNOS, reduced NO production stimulated by TNF and IFNG in LECs ($P < 0.05$). Onapristone, a specific P4 receptor antagonist, blocked the inhibitory effect of P4 on NO production in LECs ($P < 0.05$). The overall findings suggest that TNF and IFNG accelerate luteolysis by increasing NO production via stimulation of iNOS expression and NOS activity in bovine LECs. P4, on the other hand, may act in maintaining CL function by suppressing iNOS expression in bovine LECs.

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INTRODUCTION

The corpus luteum (CL) is a transient endocrine gland in mammals that is formed from a ruptured follicle after ovulation. The CL produces progesterone (P4), which is required for the establishment and maintenance of pregnancy (Hansel and Blair, 1996; Meidan et al., 1999). Formation of the CL is associated with rapid angiogenesis, making the CL one of the most highly vascularized organs in the body (Suzuki et al., 1998; Hazzard and Stouffer, 2000; Sugino et al., 2005). As a result, luteal endothelial cells (LECs) compose more than 50% of the CL (O'Shea et al., 1989; Lei et al., 1991). In addition, several lines of experimental evidence indicate that LECs are involved in the regulation of luteal function (Girsh et al., 1995; Hojo et al., 2009).

The formation of nitric oxide (NO) in the endothelium is controlled by inducible NO synthase (iNOS) and endothelial NOS (eNOS). Recent studies demonstrated that NO produced by endothelial cells directly inhibits P4 secretion

Abbreviations: CL, corpus luteum; e/iNOS, endothelial or inducible nitric oxide synthase; IFNG, interferon γ; LEC, luteal endothelial cell; NO, nitric oxide; OP, onapristone; P4, progesterone; TNF, tumor necrosis factor-α.

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(Girsh et al., 1995) and induces apoptosis (Korzekwa et al., 2006) in bovine luteal steroidogenic cells. On the other hand, NO inhibits apoptosis in the trophoblast (Dash et al., 2003ab, 2005). These findings suggest that NO regulates the CL lifespan, although it is still unclear how the NO generating system, including iNOS and eNOS enzymes, is regulated in the bovine CL.

Immune cells invade the CL at the late luteal stage (Penny et al., 1999), and can produce pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF) and interferon γ (IFNG). Both cytokines have been found to have luteolytic effects (Fukuoka et al., 1992; Wang et al., 1992; Davis and Rueda, 2002; Taniguchi et al., 2002). Previous studies showed that NO production can be stimulated by TNF and IFNG in mammary adenocarcinoma cells, and that NO production increased synergistically with combinations of these cytokines (Amber et al., 1988). NO production has been postulated to arise in conjunction with the pro-inflammatory events associated with ovulation (Ben-Shlomo et al., 1994). In addition, LECs express receptors for TNF and respond to IFNG treatment (Okuda et al., 1999; Hojo et al., 2010), suggesting that TNF and IFNG act as local modulators of NO synthesis in bovine LECs.

P4 has a considerable influence on the vascular function of the female reproductive system throughout the estrous cycle (Reynolds and Ford, 1984). In fact, P4 inhibits apoptosis in bovine luteal cells (Pate, 1988; Okuda et al., 2004b). Moreover, ovarian steroids have been shown to regulate NO production by modulating NOS mRNA and protein expression and NOS enzyme activity in the endometrium (Figueroa and Massmann, 1995; Batra and Al-Hijji, 1998; Ferreira-Dias et al., 2001). We hypothesized that P4 is one of the key regulators of NO generating system in LECs.

To elucidate if NO production in bovine LECs is regulated by cytokines and an ovarian steroid, we examined the effects of TNF, IFNG, and P4 on the relative steady-state amounts of *NOS* (*iNOS* and *eNOS*) mRNA and NOS protein expression, NOS activity, and NO production.

RESULTS

Effects of P4 on NO Production

LECs were cultured with or without P4 (0.032–32 μM) to evaluate the effects of this steroid on NO production, measured by proxy as nitrite and nitrate. All doses significantly decreased NO concentrations in the medium of cultured LECs compared with that of the untreated control (Fig. 1; $P < 0.05$). A concentration of 3.2 μM P4 was used to examine the effect of P4 on NOS expression and its activity since this concentration of P4 is close to the levels of P4 within the mid CL (Ohtani et al., 1998; Hayashi et al., 2003).

Effects of TNF, IFNG, and P4 on NOS Expression and NOS Activity

Changes in the relative steady-state mRNA and protein levels of eNOS and iNOS in LECs treated with TNF, IFNG, and P4 were determined by semi-quantitative RT-PCR using specific primers and Western blot, respectively.

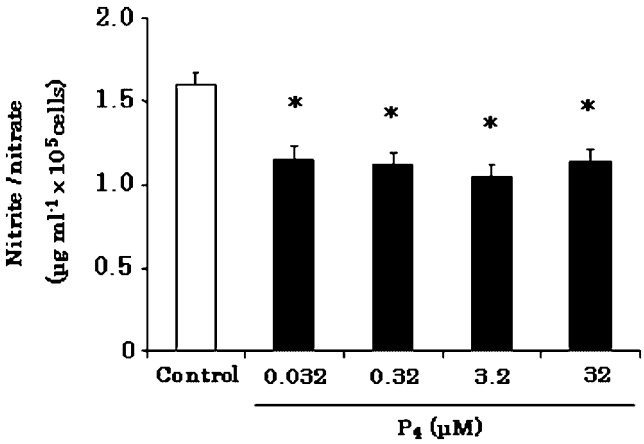

Figure 1. Effect of P4 on NO production in cultured bovine LECs. The cells were culture with P4 (0.032–32 μM) for 24 hr. Data are expressed as the means $\pm$ SEM of three separate experiments, each performed in triplicate. Asterisks indicate significant differences between untreated cells (control) and P4-treated cells ($P < 0.05$).

Figure 2 shows representative gels demonstrating the relative steady-state amounts of eNOS, iNOS, and beta-actin (ACTB) levels resulting from LEC stimulation. TNF and IFNG increased both relative steady-state amounts of *iNOS* mRNA and iNOS protein expression (Fig. 2C,F; $P < 0.05$), but did not affect those of eNOS compared with untreated control (Fig. 2B,E). In addition, P4 decreased the relative steady-state amounts of *iNOS* mRNA and iNOS protein expression (Fig. 2C,F; $P < 0.05$), but did not affect the relative steady-state amounts of *eNOS* mRNA or eNOS protein expression (Fig. 2B,E).

To gain better insight into the NO generating system, we examined the effect of TNF, IFNG, and P4 on NOS activity in cultured LECs. A single treatment with TNF or INFG stimulated the NOS activity ($P < 0.05$), whereas P4 did not affect the NOS activity in cultured LECs compared with that of untreated control (Fig. 3).

Effect of NO Synthesis Inhibitor on NO Production Stimulated by Cytokines

To elucidate whether or not TNF and IFNG stimulate NO production via iNOS, we examined the effects of TNF or IFNG in the presence or absence of 1400W, a specific inhibitor of iNOS. A single treatment of TNF or IFNG increased NO concentrations in the medium of cultured LECs compared with that of untreated control (Fig. 4; $P < 0.05$). Treatment of cells with 1400W, however, reduced the nitrite and nitrate concentrations both in TNF- and IFNG-treated LECs (Fig. 4; $P < 0.05$).

Effects of P4 Receptor Antagonists on NO Production

To examine whether or not the inhibitory effect of P4 on NO production is mediated by the P4-receptor, LECs were

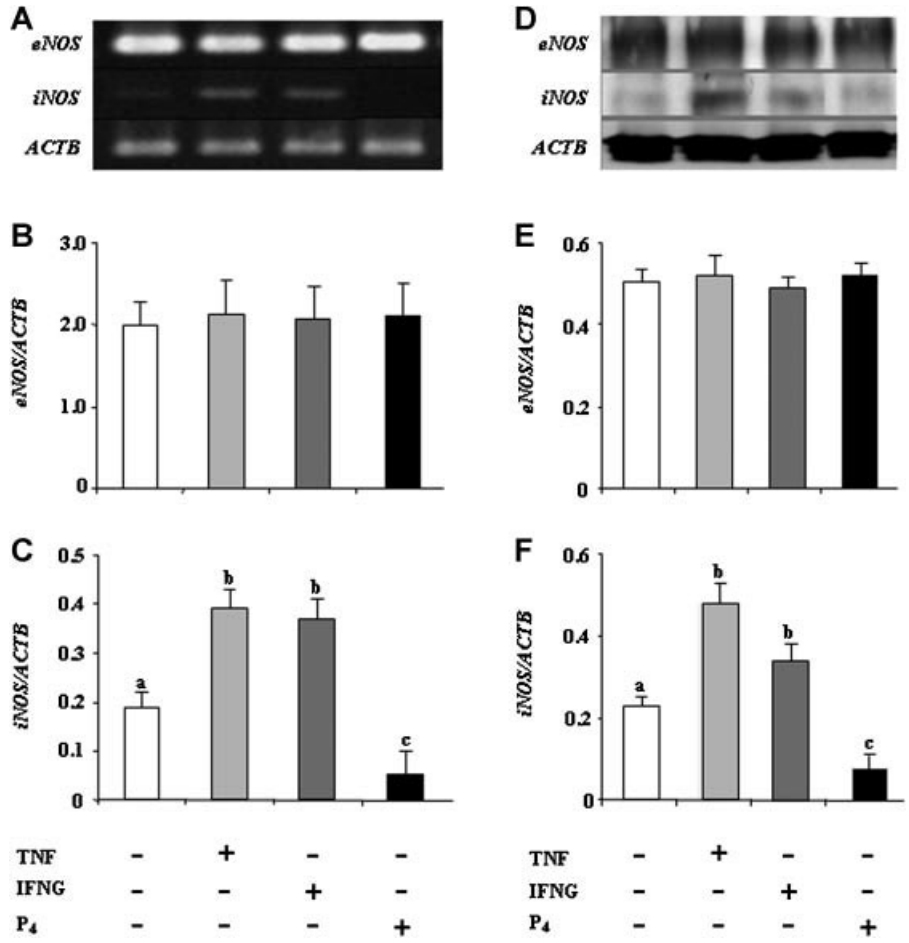

Figure 2. Effects of TNF, IFNG, and P4 on steady-state amounts of eNOS and iNOS mRNA (**A**–**C**) and protein expression (**D**–**F**) in cultured bovine LECs. The cells were exposed to TNF (2.9 nM), IFNG (2.5 nM), or P4 (3.2 μM) for 24 hr. Panel A shows the relative steady-state amounts of *eNOS* (233 bp), *iNOS* (350 bp), and *ACTB* (187 bp) mRNA. The amounts of *eNOS* (B) and *iNOS* (C) mRNA are expressed relative to the amount of *ACTB* mRNA. Panel D shows the expression of eNOS (135 kDa), iNOS (130 kDa), and ACTB (42 kDa) protein. The resultant signal was detected by chemiluminescence and quantitated by computer-assisted densitometry. The eNOS and iNOS protein levels are expressed relative to the amounts of ACTB protein (E,F). Superscripts indicate significant differences between untreated cells (control) and TNF-, IFNG-, or P4-treated cells ($P < 0.05$). All values represent means ± SEM of three separate experiments.

treated with P4 alone or in combination with onapristone (OP), a P4-receptor antagonist. P4 reduced NO concentrations in the medium of cultured LECs (Fig. 5; $P < 0.05$). Treatment of LECs with OP reduced the inhibitory effect of P4 on NO concentrations in the medium of cultured LECs (Fig. 5; $P < 0.05$).

DISCUSSION

The present study demonstrated that TNF and IFNG stimulate NO production by increasing iNOS expression and NOS activity, whereas P4 inhibits NO generation by reducing iNOS expression in bovine LECs. These results suggest that TNF, IFNG, and P4 play important roles in regulating CL function by controlling NO generation.

We previously demonstrated that LECs express prostaglandin F2α (PGF) receptor, and that PGF stimulates NO production in LECs (Lee et al., 2009). Furthermore, NO decreased P4 production (Korzekwa et al., 2004) and induced apoptosis in bovine luteal steroidogenic cells (Korzekwa et al., 2006), indicating that NO produced by LECs is one mediator of PGF-induced luteolysis. The ability of LECs to generate NO depends on the expression of NOS isoforms and NOS activity. Cells expressing eNOS generally produce a small amount of NO, which may have

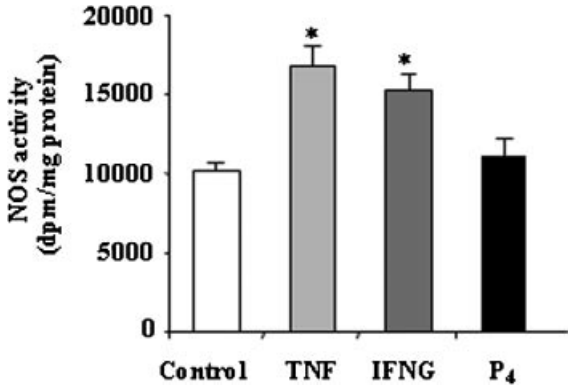

Figure 3. NOS activity in bovine LECs treated with TNF (2.9 nM), IFNG (2.5 nM), or P4 (3.2 μM) for 24 hr. NOS activity was determined by conversion of [^{3}H] L-arginine to [^{3}H] L-citrulline. The results represent means ± SEM of three different experiments, each performed in triplicate, and are expressed as dpm/mg protein. Asterisks above the bars indicate significantly different values compared with the control ($P < 0.05$).

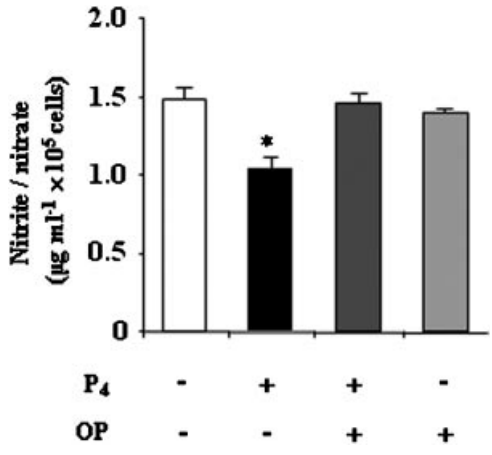

Figure 5. Effects of P4 and P4 receptor antagonists (onapristone; OP) on NO production in cultured bovine LECs. The cells were preincubated for 1 hr with OP (100 μM), and then treated with P4 (3.2 μM) for 24 hr. All values are the means ± SEM concentrations of nitrite/nitrate in the culture media. Asterisks indicate significant differences ($P < 0.05$).

anti-apoptotic effects (Dimmeler and Zeiher, 1997; Nakatsubo et al., 1998; Vega et al., 2000). When iNOS is induced in cells stimulated with endotoxins, however, a large amount of NO is produced (Nakatsubo et al., 1998). Excessive synthesis of NO by iNOS induces apoptosis (Nakatsubo et al., 1998). Therefore, an understanding of the mechanisms regulating NO generation in LECs is needed to elucidate the mechanisms controlling CL maintenance and regression. In the present study, TNF and IFNG did not affect eNOS protein expression, but did stimulate iNOS protein expression and NOS activity, thereby enhancing NO production. In addition, a specific iNOS inhibitor (1400W) decreased cytokines-stimulated NO production. These findings suggest that TNF and IFNG induce luteolysis not only via the FAS/FAS ligand system (Taniguchi et al., 2002) but also by inducing a large amount of intraluteal NO production via increasing iNOS and NOS activity.

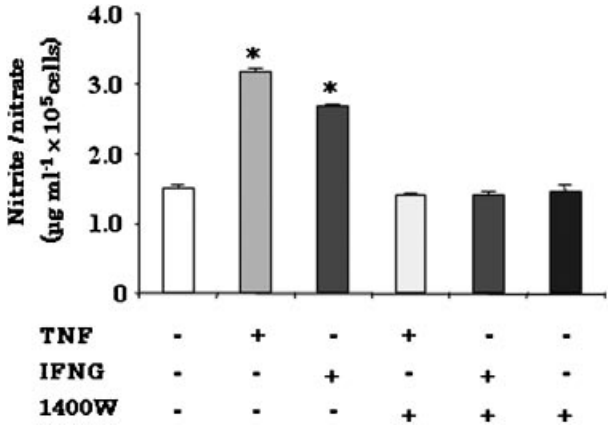

Figure 4. Effect of a NO synthesis inhibitor (1400W, a specific iNOS inhibitor) on NO production stimulated by cytokines (TNF and IFNG) in cultured bovine LECs. The cells were exposed to TNF (2.9 nM) or IFNG (2.5 nM) with or without 1400W (1 μM) for 24 hr. All values are the means ± SEM concentrations of nitrite/nitrate in the culture media. Asterisks indicate significant differences compared with the control ($P < 0.05$).

The CL mainly synthesizes P4, which is necessary to establish and maintain pregnancy (Okuda et al., 2001), resulting in differential exposure of LECs to P4 throughout the estrous cycle. Several studies have demonstrated that P4 regulates NO synthesis in the uterus (Figueroa and Massmann, 1995; Batra and Al-Hijji, 1998; Roberto da Costa et al., 2007). These findings suggest that P4 may also play one or more roles in controlling LEC function. Consistent with this hypothesis, suppression of P4 action by OP accelerates apoptosis of luteal steroidogenic cells, suggesting that P4 is a luteotropic factor (Okuda et al., 2004b). In the present study, P4 decreased iNOS protein expression and NO production. Moreover, a P4 receptor antagonist (OP) blocked the suppressive effect of P4 on NO generation, indicating that the inhibition is a P4 receptor-mediated process. Interestingly, eNOS and its activity are unaffected by P4. Together, these results suggest that P4 maintains CL function by decreasing intraluteal NO production during the functional CL phase. Further studies are needed to clarify the exact mechanisms of NO down-regulation by P4.

The overall results indicate that TNF and IFNG accelerate NO production by stimulating iNOS expression as well as NOS activity. On the other hand, P4 suppressed NO synthesis in bovine LECs. Thus, tight control of luteal NO production may be essential for luteal maintenance, whereas excessive NO production may result in luteolysis.

MATERIALS AND METHODS

Bovine Luteal Endothelial Cell Isolation and Cell Culture

Ovaries were collected from nonpregnant Holstein cows at a local abattoir within 10–20 min after exsanguination. The stages of the estrous cycle were identified by macroscopic observation of the ovary and uterus, as described previously (Miyamoto et al., 2000). LECs were isolated from the CL at the mid-luteal phase (Days 8–12 of the estrous cycle) and cultured as previously described (Acosta et al., 2007). The isolated LECs were seeded in culture medium [10% calf serum DMEM/F-12 (D/F; D8900; Sigma–Aldrich, St. Louis, MO) with 20 μM gentamicin (15750-060; Invitrogen, Carlsbad, CA) and 2 μM amphotericin B (A9528; Sigma–Aldrich)] at a concentration of 1×10^5/ml into 24-well plates (662160; Greiner Bio-One, Frickenhausen, Germany) for NOS mRNA and NO production assays, and 1×10^6/ml into 75 cm^2 culture flasks (658175; Greiner Bio-One) for NOS protein and NOS activity assays. After the cells reached confluence, the medium was replaced with fresh D/F supplemented with 5 μM holo-transferrin (T3400; Sigma–Aldrich), 500 μM ascorbic acid (013-12061; Wako Pure Chemical Industries, Ltd., Osaka, Japan), 5 nM sodium selenite (S5261; Sigma–Aldrich), and 0.1% (w/v) BSA (10 735 078 001; Roche Diagnostics, Mannheim, Germany), and were cultured with or without stimulants [TNF (2.9 nM, HF-13; kindly donated by Dainippon pharmaceutical, Osaka, Japan), IFNG (2.5 nM, kindly donated by Dr. Inumaru, National Institute of Animal Health, Ibaraki, Japan), P4 (0.032–32 μM, P8783; Sigma–Aldrich), 1400W (a specific iNOS inhibitor, 1 μM, 81520; Cayman Chemical, Ann Arbor, MI), or OP (a specific P4 receptor antagonist, 10 μM, ZK98299; Schering AG, Berlin, Germany)] for 24 hr according to the protocol for each experiment.

Experiment 1: Effects of P4 on NO Production

LECs were seeded into 24-well plates in culture medium. The cells were then incubated at 37.5°C in a humidified atmosphere of 5% CO_2 in air until the cells reached confluence. The P4 concentration used for LECs treatment was based on the intraluteal P4 concentration estimated in previous microdialysis studies of bovine CL (Ohtani et al., 1998; Hayashi et al., 2003). The cells were incubated with P4 (0, 0.032, 0.32, 3.2, and 32 μM) for 24 hr. At the end of 24-hr incubation, 500 μl of conditioned media was collected and immediately used for determination of nitrite and nitrate concentrations by the Griess method (Green et al., 1982). The DNA content, estimated by the spectrophotometric method of Labarca and Paigen (1980), was used to standardize the results (n = 3).

Experiment 2: Effects of TNF, IFNG, and P4 on Relative Steady-State Amounts of eNOS and iNOS mRNA and Protein Expression

For mRNA determination, LECs were seeded at a concentration of 1×10^5/ml into 24-well plates in culture medium. After reaching confluence, the cells were incubated for 24 hr with 2.9 nM TNF, 2.5 nM INFG, or 3.2 μM P4 added to the culture media (n = 3). The cultured LECs were disrupted with 1 ml TRIZOL Reagent, and stored at −80°C until assayed for eNOS and iNOS mRNA by semi-quantitative RT-PCR.

For protein determination, LECs were seeded at a concentration of 1×10^6/ml into 75 cm^2 culture flasks in culture medium. After reaching confluence, the cells were incubated for 24 hr with or without 2.9 nM TNF, 2.5 nM INFG, or 3.2 μM P4 added to the culture media. Culture flasks (75 cm^2) were used for the analysis of iNOS or eNOS protein expression (n = 3).

Experiment 3: Effects of TNF, IFNG, and P4 on NOS Activity

LECs were seeded into 75 cm^2 culture flasks for determination of NOS activity. After reaching confluence, the cells were incubated with or without 2.9 nM TNF, 2.5 nM INFG, or 3.2 μM P4 added to the culture media. The samples were then used for analysis of NOS activity (n = 3).

Experiment 4: Effect of NO Synthesis Inhibitor on NO Production Stimulated by Cytokines

LECs were seeded into 24-well plates in culture medium, and then incubated as described in Experiment 1. After reaching confluence, the cells were incubated with or without 2.9 nM TNF or 2.5 μM IFNG, in the presence or absence of 1 μM 1400W (a specific iNOS inhibitor) for 24 hr. After 24 hr incubation, culture plates were used for determination of NO, as described in Experiment 1 (n = 3).

Experiment 5: Effects of P4 Receptor Antagonist on NO Production

LECs were seeded into 24-well plates in culture medium, and then incubated as described in Experiment 1. After reaching confluence, the cells were incubated with 3.2 μM P4, in the presence or absence of 10 μM OP (a specific P4 receptor antagonist) for 24 hr. After 24 hr incubation, culture plates were used for determination of NO as described in Experiment 1 (n = 3).

Determination of Nitrite and Nitrate Concentrations

Concentrations of nitrite/nitrate, the stable metabolites of NO in culture media were measured by a colorimetric method using the Griess reaction, and validated for the culture media (Jaroszewski et al., 2003). The assay sensitivity was 0.07 μg/ml, the standard curve ranged from 0.05 to 6.9 μg/ml, and inter-assay coefficients of variation were on average 8.3% and 13.4%, respectively.

Reverse Transcription-PCR

Total RNA was prepared from the LECs using TRIZOL Reagent according to the manufacturer's directions. One microgram of each sample of total RNA was reverse

TABLE 1. Primers Used in Semi-Quantitative RT-PCR

Gene	Primer	Sequence (5′–3′)	Accession no.	Product (bp)
iNOS	Forward	GCTTAAATCCAGGCAAACGA	AF340236	350
	Reverse	TTCTGGTGAAGCGTGTCTTG		
eNOS	Forward	GATCAGCAACGCTATCACGA	M89952	233
	Reverse	GGACAGCGGTAGAGCCATAG		
ACTB	Forward	GAAGATCTGGCACCACAC	AY141970	187
	Reverse	AGAGGCATACAGGGACAGC		

transcribed using a SuperScript First-Strand Synthesis System for RT-PCR (11904-018; Invitrogen), and the reaction mixture was used in each PCR together with the appropriate oligonucleotide primer pairs. The primers for the *eNOS*, *iNOS*, and *ACTB* were designed and characterized as described previously (Table 1). The RT-PCRs were conducted with the housekeeping gene *ACTB* as an internal standard. *ACTB* primer was added at the appropriate cycle number by the ''primer-dropping method'' as described by Wong et al. (1994) with our own modification (Okuda et al., 2004a). The PCRs were carried out using TaKaRa Taq (R001A; Takara Bio Inc., Shiga, Japan) and a thermal cycler (iCycler; Bio-Rad Laboratories, Hercules, CA). The PCR conditions were as follows: activation of DNA polymerase for 20 sec at 95°C followed by n cycles (see below) of denaturation for 30 sec at 95°C; annealing for 1 min at 60°C; and extension for 1 min at 70°C, followed by final extension for 5 min at 72°C. The number of cycles was 33 for *eNOS*, 35 for *iNOS*, and 23 for *ACTB*. A portion (40%) of each reaction mixture was electrophoresed on a 1.5% agarose gel with a known standard (100 bp ladder, N3231S; New England Bio-Labs, Inc., Beverly, MA), stained with ethidium bromide, and photographed under ultraviolet illumination. The relative band intensities were analyzed by computerized densitometry using NIH image software (National Institutes of Health, Bethesda, MD).

iNOS and eNOS Protein Levels

LECs were seeded in a cell culture flask and exposed to 2.9 nM TNF, 2.5 nM INFG, or 3.2 μM P4 for 24 hr. Cells were harvested and total protein extracts were prepared after lysis of the cells in 25 mM Tris–HCl, 300 mM sucrose, 2 mM EDTA with complete protease inhibitor cocktail (11 697 498 001; Roche Diagnostics, Basel, Switzerland). Total protein concentration was determined by a bicinchoninic acid assay (Osnes et al., 1993). iNOS and eNOS protein levels were determined by Western blotting, as described previously (Lee et al., 2009).

NOS Activity Assay

NOS activity was determined in the LECs at the end of a 24-hr incubation period, by monitoring the conversion of [^{3}H] L-arginine (Amersham, Arlington Heights, IL, #TRK698) into [^{3}H] L-citrulline, with the NOS detect Assay Kit (Alexis Corp. Lavsen, Switzerland), according to the manufacturer's directions and as previously reported by Boiti et al. (2000). Data on NOS activity was normalized based on protein concentrations.

Statistical Analysis

Data are shown as the mean ± standard error mean of values obtained in three separate experiments, each performed in triplicate. The statistical significance of differences in the amounts of iNOS and eNOS mRNA, protein, and NO production were analyzed by ANOVA followed by Fisher's protected least significant difference procedure (PLSD) as multiple comparison test. Means were considered different when $P < 0.05$.

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