We investigated the role of nitric oxide (NO) in vascular endothelial growth factor (VEGF) expression in the rat placenta. A nitric oxide synthase (NOS) inhibitor, N\textsuperscript{G}-nitro-L-arginine-methyl ester (L-NAME), was constantly infused into pregnant rats 6–24 h before sacrifice on gestational day (GD) 15.5. NO production declined to about 15% of the control level as monitored by NO trapping and electron paramagnetic resonance spectroscopy. VEGF mRNA expression was temporally decreased by L-NAME, but recovered to normal levels after 24 h of treatment, whereas hypoxia inducible factor (HIF)-1α and induced NOS (iNOS) expression increased. VEGF expression decreased significantly in placental explants after 6 h of co-treatment with L-NAME and lipopolysaccharide, an iNOS inducer. Our data indicate that NO induce VEGF expression in vivo and in vitro in the rat placenta, suggesting that peaked NO production was maintained by a reciprocal relationship between NO and VEGF via HIF-1α.

**Key words:** fetal rat placenta; nitric oxide (NO); electron paramagnetic resonance (EPR) spectroscopy; angiogenesis

During the past decade, researchers have shown tremendous interest in nitric oxide (NO), a water-soluble gaseous free radical that is highly reactive and unstable in vivo. NO is now accepted to be an important mediator of multiple cellular functions including smooth muscle relaxation, cell growth promotion, and neurotransmission in the central nervous system. In living cells, NO is produced from L-arginine and oxygen molecules via the catalytic action of nitric oxide synthase (NOS).

Elevated concentrations of circulating nitrate, a stable NO metabolite, have been reported in pregnant women and animals, and probably to result from increased production of NO by peripheral maternal tissues, the placenta, or both. We have reported that NO production in the rat placenta reached a peak on GD 15.5 and then decreased towards full term, and that the NO production in the placenta was predominantly derived from inducible NOS (iNOS), indicating that NO levels in the placenta are iNOS-dependent and differ at different gestational stages.

The embryo and the fetal placenta are known to develop rapidly in mid-gestation. In fact, the weight of the fetal placenta in rats was reported to increase dramatically from GD 13.5 to GD 15.5. Considering this together with a report that the placenta is rich in a vascular system that contributes gas and nutrients, and functions in waste exchange, which are essential for fetal growth, the peak of NO production on GD 15.5 may be related to placental function.

Vascular endothelial growth factor (VEGF) is a key regulator of placental angiogenesis and vascular functions, functioning via activation of two high-affinity tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1/flt-1) and -2 (VEGFR-2/KDR). NO has been reported to induce the VEGF gene in tumor cells and in vascular smooth muscle cells, and thus NO production may be related to VEGF expression in the placenta. The effect of NO on VEGF expression differs for the different types of cells and tissues involved. The reason is perhaps related to the differing amounts of NO production in different cell types. However, endogenously generated NO is a very short-lived gaseous free radical that reacts with many substances, including molecular oxygen and superoxide, to generate NO derivatives such as nitrogen dioxide, peroxynitrite, and nitrate.

In the present study, we investigated the effects of NO on VEGF expression in the fetal placenta to clarify the biological roles of NO in vivo and in vitro. We examined the expression of VEGF, VEGFR-1, and VEGFR-2, as well as the expression of iNOS and hypoxia inducible factor (HIF)-1α, in in vivo animal models through steady infusion of a specific NOS inhibitor, N\textsuperscript{G}-nitro-L-arginine-methyl ester (L-NAME), in which the NO production level was consistently reduced. In this animal model, we confirmed reduction of NO levels by a NO-trapping technique, using the Fe-dithiocarbamate complex combined with electron paramagnetic resonance.
administered for 24 h instead of L-NAME under the same treatment. CLEA Japan, Tokyo) and tap water were used. The rats were maintained on a commercial diet (CE-2; Japan, Yokohama, Japan) aged 10–12 weeks at the time of mating.

The placental samples were snap-frozen in liquid nitrogen and carefully removed. The fetal placenta was then removed from the cavity was opened and the fetus and embryonic membranes were 15.5 under ether anesthesia. To obtain placental samples, the uterine was removed at noon on GD 15.5 after 24 h of treatment, and the rats were maintained for a further 24 h. After the indicated durations of conditions. In some pregnant rats, the implanted osmotic-mini pump (L-NAME group; 2001D; Alzet, Los Angeles, CA) implanted under the dorsal neck skin was administered subcutaneously to pregnant rats 6 h, 12 h, or 24 h after the indicated durations of treatment, the placentas from the pregnant rats were carried out at noon on GD 15.5 or from the cultured placental tissue was placed in ice-cold PBS and processed within 2 h of collection. The tissue was first dissected aseptically to obtain smaller fragments (15–25 mg wet weight), which were placed on Millicell-CM culture dish inserts (Nihon Millipore, Tokyo). These were then placed in a 24-well culture dish (Becton, Dickinson, Tokyo). The explants were incubated in Dulbecco’s Modified Eagle’s Medium nutrient mixture F12 (Invitrogen, Tokyo) and supplemented with streptomycin and penicillin (Wako, Osaka), and 10% newborn calf serum (Gibco, Tokyo) at pH 7.4. The placental explants were incubated at 37 °C under standard tissue culture conditions (5% CO2 in 95% air). On the next morning, the culture medium was exchanged, and lipopolysaccharide (LPS, 10 μg/mL; Sigma-Aldrich) and L-NAME (1 mM) were added individually or in combination. The placental explants were incubated for a further 3 h or 6 h before sampling.

Total RNA extraction and analysis of RT-PCR. Total RNA was extracted from the fetal placenta on GD 15.5 or from the cultured placental explant using an RNA extraction kit (Isogen; NPG, Toyama, Japan) following the manufacturer’s instructions. First-strand cDNA was synthesized from total RNA (0.2 or 1 μg) with reverse transcriptase (RT, SuperScript III, Invitrogen, Tokyo) and oligo(dT)12 primers. PCR amplification from reverse-transcribed cDNA was carried out with primers designed specifically for each gene. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal control to normalize template concentrations. The primer sequences used for iNOS, VEGF, VEGFR-1 and VEGFR-2, HIF-1α, and GAPDH are shown in Table 1. Quantitative determination of the expression of these genes was carried out as previously described.5,19) The PCR cycle profile for the genes are shown in Table 1. The PCR product concentration was proportional to the starting cDNA concentration with the cycle profile indicated for each gene, based on preliminary experiments. Each round of PCR was carried out in a thermal cycler (GeneAmp 2400; Perkin Elmer Japan, Yokohama). PCR products were detected on a 1.5% agarose gel, and were stained with SYBR Green I (Takara Bio Inc., Otsu). Quantitative determination of gene expression levels was performed using scanning gels stained with SYBR Green I, using a Fluoro-Image Analyzer (FLA-2000; Fuji Film, Tokyo), followed by analysis with MacBas image software (MacBas version 2.5; Fuji Film).

Statistical analysis. Data are expressed as mean ± SE. Multiple comparisons were evaluated by Tukey–Kramer test after one-way analysis of variance (ANOVA). Values at p < 0.05 were considered statistically significant.

Table 1. Primer Sequences Used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
<th>Cycle</th>
<th>Amplicon size bp</th>
</tr>
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<tbody>
<tr>
<td>iNOS</td>
<td>F</td>
<td>5'-AACGCCCCGCTCTACTCCAT-3'</td>
<td>62 °C</td>
<td>2475</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-TGGCAAGGTGTCCTGTGG-3'</td>
<td></td>
<td>295</td>
</tr>
<tr>
<td>VEGF</td>
<td>F</td>
<td>5'-GACATGGACCTGGATCAATCGTGTA-3'</td>
<td>62.5 °C</td>
<td>295</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-CCATGGATCTGGCATATGGAAGTGC-3'</td>
<td></td>
<td>295</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>F</td>
<td>5'-GTCTTGCTTGGTACCTGTGG-3'</td>
<td>60 °C</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-GGTACGATGTCAGATCAGTC-3'</td>
<td></td>
<td>209</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>F</td>
<td>5'-GAGGGGGAAGGAGATCAGGTGA-3'</td>
<td>59 °C</td>
<td>520</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-CGAGCTTTCCAAAGTTGCA-3'</td>
<td></td>
<td>520</td>
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<tr>
<td>VEGFR-2</td>
<td>F</td>
<td>5'-CCATCACAACAGAAGGACGGACGA-3'</td>
<td>57 °C</td>
<td>720</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-AGGGCGGAGCGGGATAGGAATGA-3'</td>
<td></td>
<td>720</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F</td>
<td>5'-TGATACCGGTCTGCAACGCATTGGTG-3'</td>
<td>60 °C</td>
<td>950</td>
</tr>
</tbody>
</table>

F, Forward; R, Reverse.
Results

Nitric oxide production levels in the placenta were monitored by the NO-trapping technique, followed by EPR analysis. The EPR spectra of the NO-Fe-DTCS complex in the placenta on GD 15.5 showed a typical triplet signal ($g = 2.038$), previously identified as NO-Fe-DTCS by Yoshimura [18] (Fig. 1A). The typical triplet signal of NO-Fe-DTCS was almost completely abolished following treatment with l-NAME (Fig. 1B). NO levels were significantly decreased by the 6-h, 12-h, and 24-h treatments with l-NAME, and decreased to about 15% of the control level. The expression of VEGF mRNA was significantly decreased after 6h of treatment with l-NAME, but was restored to the normal level following 24h of treatment, and the expression level did not change after removal of the osmotic-mini pump (Fig. 2A). VEGFR-1 and VEGFR-2 mRNA expression levels were not affected by l-NAME treatment. On the other hand, $i\text{NOS}$ and HIF-1α mRNA expression levels increased significantly under 12h, and 24h of treatment with l-NAME, and $i\text{NOS}$ expression significantly declined, to the control levels after removal of the osmotic-mini pump (Fig. 2B).

Using explant cultures of fetal placenta obtained from pregnant rats on GD 15.5, we examined the decrease in the expression of $\text{VEGF}$ caused by the NOS inhibitor in vivo. We used explant culture of fetal placenta obtained from pregnant rats on GD 15.5 (Fig. 3). The $i\text{NOS}$ mRNA expression was increased under 3 h of stimulation with LPS, and the increment reached significance treatment. VEGF expression was not affected by LPS treatment, but showed a slight incremental trend treatment. Co-treatment with l-NAME and LPS for 6h did not affect the expression of $i\text{NOS}$ mRNA as compared to expression in the LPS-alone group, whereas expression of $\text{VEGF}$ mRNA significantly decreased under co-treatment under the same conditions. HIF-1α mRNA expression in the explant culture also increased in the co-treatment group as compared to the l-NAME-alone group, but the difference was not significant, while $i\text{NOS}$ mRNA expression in the co-treatment group increased significantly as compared to the l-NAME-alone group.

Discussion

In mammals, three isoforms of NOS, neuronal NOS or nNOS (NOS 1) or inducible NOS or iNOS (NOS 2), and endothelial NOS or eNOS (NOS 3), have been identified. NOS 1 expression has not observed in the placentas of humans [24] or rats [25]. Inducible NOS is capable of producing large amounts of NO, whereas eNOS and nNOS produce low amounts of NO [26,27]. Thus NO is produced predominantly by $i\text{NOS}$ in the rat placenta. [9] On the basis of the results of previous studies, eNOS and nNOS isoforms make a negligible contribution to NO production in the placentas, although l-NAME inhibits all NOS isoforms.

In the current study, using a constant infusion model and monitoring by NO-trapping followed by EPR spectroscopy, we found that $\text{VEGF}$ mRNA expression was temporarily decreased following 6h of treatment with NOS inhibitor l-NAME, indicating that NO induces $\text{VEGF}$ mRNA expression in the placenta, but $\text{VEGF}$ mRNA expression was restored to a normal level following treatment with the inhibitor, although decreased NO production continued throughout the experimental period. Our data suggest that other factors can induce $\text{VEGF}$ expression in the placenta.

Using our NO inhibitory model, we confirmed that the NO production level decreased to approximately 15% of the control level. The expression of $i\text{NOS}$ and HIF-1α mRNAs in the placenta was also significantly induced following treatment with the NOS inhibitor. HIF-1 is a heterodimer that consists of a constitutively expressed...
HIF-1β subunit and an HIF-1α subunit. HIF-1α is a known oxygen sensor that is ubiquitinated and subjected to proteasome degradation under non-hypoxic conditions. HIF-1 binds directly to the hypoxia response element on DNA, which is located within the 5′-promoter of VEGF, iNOS, and other genes, promoting transcription of these genes. NO physiologically regulates numerous cellular responses through S-nitrosylation of protein cysteine residues. It has been found to cause S-nitrosylation at Cys533 of HIF-1α, preventing the degradation of HIF-1α, and leading to accumulation of it. NOC 18, an NO donor, also stabilizes HIF-1α and increases protein synthesis of it to induce VEGF mRNA expression under normal oxygen tension. Additionally, accumulation of S-nitrosylated transcription factor reportedly inhibits nuclear translocation of it. In view of these studies, our results suggest that a low amount of NO influences the stabilization of HIF-1α or increases the nuclear translocation of HIF-1, leading to VEGF and iNOS mRNA expression. This is supported by our data showing that iNOS expression levels can be reduced by removing the osmotic-mini pump. iNOS mRNA induction preceded the expression of HIF-1α mRNA, what appeared to result from a different timing of VEGF expression.

Fig. 2. Effects of Continuous Infusion of the NOS Inhibitor L-NAME on VEGF mRNA (A), iNOS mRNA (B), and HIF-1α (C) mRNA Expression in Fetal Rat Placentas on GD 15.5. The 24+/− groups were subjected to 24 h of infusion of L-NAME, followed by removal of the osmotic-mini pump. The rats were maintained for an additional 24 h before sampling. Fetal placentas were removed at noon on GD 15.5, and total RNAs were extracted. RT-PCR was performed using 1 μg of total RNA, and the expression of the gene was examined as described in "Materials and Methods." The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as internal standard. Data are expressed as mean ± SE for five independent experiments. Representative separation by 1.5% agarose gel electrophoresis of the RT-PCR products is shown in D. Significantly different among the indicated groups, *, p < 0.05. VEGF, vascular endothelial growth factor; iNOS, inducible nitric oxide synthase; HIF-1α, hypoxia inducible factor 1-alpha.

VEGF, vascular endothelial growth factor; iNOS, inducible nitric oxide synthase; HIF-1α, hypoxia inducible factor 1-alpha.
TNF-α. Furthermore, the LPS plus L-NAME group showed the same level of VEGF expression as the control group (Fig. 3B). Thus our data indicate that VEGF expression was induced primarily by NO generated by iNOS induction following LPS stimulation. HIF-1α mRNA expression in the explant culture also showed an incremental trend in the co-treatment group, suggesting that HIF-1α participates in the expression of iNOS mRNA, although the underlying mechanism, perhaps stabilization or transcriptional activity of HIF-1α, should be examined in vitro.

In mammals, the placenta is formed by invasion of the myometrium by trophoblasts, where the complex vascular system is well developed, and this is essential for fetal growth.10) Vessel formations occurs mainly through two sequential mechanisms,42) vasculogenesis and angiogenesis. Vasculogenesis is the de novo formation of blood vessels during embryogenesis, and angiogenesis is the formation of new capillaries from pre-existing vessels. VEGF is a potent and critical inducer of angiogenesis. On the basis our results and those of previous studies, the maximum NO production, observed at GD 15.5, may be related to the developmental function of the placenta. The NOS inhibitor did not affect expression of VEGFR-1 or VEGFR-2 mRNA throughout the experimental period, but these genes were expressed consistently in the placenta following treatment with L-NAME for 6–24 h. Further studies are necessary to evaluate the effect of NO on VEGFR-1 and VEGFR-2.

In conclusion, using a NOS-inhibiting model in the placentas of GD 15.5 rats, VEGF mRNA expression was temporarily decreased following treatment with NOS inhibitor L-NAME, but recovered to a normal level after treatment for 24 h. In contrast, the expression of both iNOS and HIF-1α increased following treatment. Additionally, with a placental explant culture, an incremental trend in VEGF expression was observed after 6 h of treatment with LPS, an iNOS inducer, and iNOS expression was simultaneously and significantly increased. Furthermore, VEGF expression decreased significantly during 6 h of LPS plus L-NAME co-treatment. We found that NO induced VEGF expression in vivo and in vitro in the placentas of GD 15.5 rats, suggesting that peak NO production in the placenta is maintained by a reciprocal relationship between NO and VEGF via HIF-1α.

Acknowledgments

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Fig. 3. Effects of Lipopolysaccharide (LPS) Stimulation on iNOS mRNA (A), VEGF mRNA (B), and HIF-1α (C) Expression in Rat Placental Explant Cultures.

Explant culturing of fetal placentas from rats on GD 15.5 was carried out as previously described (Canigia et al., 2000). Placental explants were maintained in culture for 3 h or 6 h under normal oxygen tension, and were treated with LPS (10 μg/mL), L-NAME (1 mM), or LPS plus L-NAME. Expression of the genes was examined by RT-PCR, as described in Fig. 2, except that the amount of total RNA used was 0.2 μg. Data are expressed as mean ± SE at least four independent experiments. Representative separation by 1.5% agarose gel electrophoresis of the RT-PCR products is shown in D. Significantly different among the indicated groups, *p < 0.05.
References


