Expression of nitric oxide synthase isoforms and detection of nitric oxide in rat placenta

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Takizawa, Tatsuya, Hiroshi Yoshikawa, Miho Yamada, and Hidetoshi Morita. Expression of nitric oxide synthase isoforms and detection of nitric oxide in rat placenta. Am J Physiol Cell Physiol 282: C762–C767, 2002.—Nitric oxide (NO) production in the rat placenta was monitored and quantified by electron paramagnetic resonance (EPR) spectroscopy with hemoglobin and an Fe-N-(dithiocarboxy)sarcosine (DTCS) complex as NO-trapping reagents. Expression of nitric oxide synthase (NOS) isoforms was also examined by quantitative RT-PCR analysis. The EPR spectrum of the placenta with hemoglobin trapping showed a three-line hyperfine structure ($g = 2.008$ and $a = 1.66$ mT). The EPR signal was diminished after the placenta was homogenized or the NOS inhibitor L-NAME was administered to pregnant rats. Therefore, the specific signal was definitely identified as being derived from endogenous NO spin-trapped by hemoglobin, and the EPR spectrum showed that the NO adduct existed as a pentacoordinate α-NO heme species. The EPR spectrum of the placenta with Fe-DTCS trapping showed a triplet signal ($g = 2.038$) derived from an NO-Fe-DTCS complex. The height of the triplet signal did not vary significantly with gestational stage during the last few days of gestation. At the gestational stages examined, the level of NOS II mRNA expression was significantly higher than that of NOS III mRNA. NOS II expression in term (day 21.5) placenta was significantly increased compared with that in preterm (day 19.5) placenta ($P < 0.01$, $n = 4$ or 5). These results suggest that NOS II is the predominant producer of NO in the placenta and that NOS II-generated NO plays significant roles in the maintenance of placental functions immediately before birth.

IT HAS BEEN PROPOSED THAT nitric oxide (NO) plays a decisive role in the maintenance of normal pregnancy and that increased NO formation in a variety of tissues is involved in the normal physiological changes that occur during gestation (24). Several lines of evidence indicate that nitric oxide synthase (NOS) is expressed in the placenta (3, 16, 17, 27) and suggest that NO can regulate placental functions such as maintenance of blood flow or suppression of immune response (2, 7, 15, 22). In mammals, three isoforms of NOS, namely, NOS I (neuronal NOS, nNOS), NOS II (inducible NOS, iNOS), and NOS III (endothelial NOS, eNOS), have been identified. Expression of NOS II and NOS III has been observed in human (4) and rat (1) placenta, a noninnervated organ (23). NOS II is a high-output NOS whose expression is induced by cytokines (and other agents) and whose activity is largely or completely Ca2+ independent. NOS III is a low-output, constitutively expressed NOS whose activity is regulated by Ca2+ and calmodulin (6, 13, 28).

To clarify the physiological role of NO, in vivo detection of NO and determination of the distribution and level of NO are very important. However, endogenously generated NO is a very short-lived gaseous free radical (14, 20) that reacts with many substances such as molecular oxygen and superoxide to generate NO derivatives such as nitrogen dioxide, peroxynitrite, and nitrate (29). To overcome these difficulties, an NO-trapping technique combined with electron paramagnetic resonance (EPR) spectroscopy has been applied to detect the NO in biological systems (25). Hemoglobin and Fe-dithiocarbamate complex have been applied as NO-trapping reagents to detect NO production in the tissues of cytokine-treated animals or in a model of reperfusion after ischemia (9, 10, 25, 30).

In the present study, we monitored and quantified endogenous NO generation in the rat placenta by EPR spectroscopy with hemoglobin and Fe-N-(dithiocarboxy)sarcosine (DTCS) complex as NO-trapping reagents and examined the expression of NOS isoforms by quantitative reverse transcriptase-mediated polymerase chain reaction (RT-PCR) analysis.

MATERIALS AND METHODS

Animals. Female Wistar rats (Charles River Japan, Tokyo, Japan), 10–12 wk old at the time of mating, were used. They were maintained on a commercial diet (CE-2; Clea Japan, Tokyo, Japan) and tap water ad libitum and were kept in a room at a temperature of 22 ± 3°C with relative humidity of 50–60%.

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55 ± 10%. Three females were placed with a male overnight and examined the next morning for the presence of sperm in a vaginal smear. Noon of the day on which sperm was found was designated as day 0.5 of gestation, and the females were caged individually thereafter.

Fe-DTCS complex was prepared by anaerobically dissolving DTCS disodium salt (Dojin, Kumamoto, Japan) together with FeCl₃ (Wako, Osaka, Japan) in N₂-bubbled phosphate buffered saline (PBS; Fe concentration [Fe] = 68 mM; [DTCS]/[Fe] = 3). The NO donor 4-methyl-2-[hydroxynitroimino]-5-nitro-6-methoxy-3-hexanamide (NOR 1; Dojin) was added to the solution of Fe-DTCS complex to prepare the standard solution of NO-Fe-DTCS complex used for EPR spectroscopy. For NO trapping by Fe-DTCS complex, Fe-DTCS solution (500 mg/kg body wt as DTCS) was injected subcutaneously 0.5 h before sampling.

To verify that the EPR spectrum resulted from the activity of NOS, the NOS-specific inhibitor N⁵-nitro-1-arginine methyl ester (l-NAME; Biomol) was administered subcutaneously to pregnant rats at various doses (3, 10, 30, 100, or 300 mg/kg body wt) 1 or 3 h before the placenta was removed. All chemicals were dissolved in sterile PBS.

**Sample preparation for EPR spectroscopy.**  Pregnant rats were used from day 19.5 to day 21.5 of gestation. Rats were decapitated under light ether anesthesia. Blood was rapidly taken from the abdominal vein, and the placenta was then rapidly removed and minced with surgical scissors. Each sample (0.5 ml of blood or ~0.5 g of minced placenta) was transferred to a quartz EPR tube and frozen immediately in liquid nitrogen. When hemoglobin was used as an NO-trapping agent, to verify that the EPR spectrum was derived from NO, placental samples were homogenized before EPR analysis. The homogenized placental pellet and supernatant were prepared as follows. Frozen placenta was homogenized in an equal volume of chilled PBS (pH 7.4) and then centrifuged (3,000 rpm, 5 min, 4°C) to separate the pellet and supernatant. The placental pellet was resuspended in PBS and centrifuged again. This washing procedure was repeated several times, resulting in a finely homogenized placental pellet.

**EPR spectroscopy measurement.**  EPR spectra were recorded with an EPR spectrometer (JES-TE3X, JEOL, Tokyo, Japan) under the following conditions: microwave power, 10 mW; center field and width, 330 ± 20 mT for hemoglobin trapping, 320 ± 10 mT for Fe-DTCS trapping; temperature, 77 K; measurement time, 4 min; time constant, 0.3 s. In some experiments, MnO powder was used as a standard and the heights of NO-Fe-DTCS and MnO signals were simultaneously measured to calculate the ratio of these signal heights for use in the quantification of NO production levels.

**RT-PCR.**  Total RNA was extracted from each placenta from rats on days 19.5 and 21.5 of gestation with an RNA extraction kit (Isgen; NPG, Toyama, Japan) according to the manufacturer’s instructions. First-strand cDNA was synthesized with reverse transcriptase (RT; SuperScript II, Gibco BRL, Tokyo, Japan), 1 µg of each total RNA preparation, and an oligo d(T)₁₂ primer.

PCR amplification from reverse-transcribed cDNA was carried out with primers specifically designed for NOS II [sense: 5'-TTCAACGACACCTCCTCACCAAA-3' (5' position: 298, GenBank D14051); antisense: 5'-CCATCCTCCTGCCCACCTTC-CTC-3' (3' position: 1188)] and NOS III [sense: 5'-TGGGAGACATACCTACAGA-3' (5' position: 231, GenBank AP085195); antisense: 5'-TCCCGGACATAACTACGT-3' (3' position: 5885)]. The above primers were synthesized by Sawaday Technology, (Tokyo, Japan). RT-PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was carried out with the same sample as a positive control. The primers used for RT-PCR of rat GAPDH were purchased from Clontech.

For the PCR analysis, 1 µl of reverse-transcribed cDNA was added to a 20-µl reaction mixture containing each dNTP at 250 µM, 1 unit of Taq polymerase (rTaq; Takara, Kyoto, Japan), and each primer at 0.2 µM. Each reaction cycle consisted of an initial denaturation step for 1 min at 94°C, an annealing step for 1 min at 62°C (NOS II) or 60°C (GAPDH, NOS III), and an elongation step for 1 min at 72°C carried out by using a thermocycler (Perkin Elmer). A 10-µl aliquot of the PCR product of each reaction was electrophoresed on a 1.5% agarose gel and stained with SYBR Green I (Takara).

The DNA sequences of the PCR products were determined and were identified by comparison with sequences available in the GenBank database as the sequences of rat NOS II (GenBank D14051) and NOS III (GenBank AF085195) cDNAs. To check for the contamination of genomic DNA in the total RNA samples, the first cDNA synthesis was performed without RT, and the product was used as the template for an RT-negative PCR control.

For each target gene, cycle profiles were carried out on pooled PCR product samples to determine the exponential phase of amplification, in which the product concentration is proportional to the starting cDNA concentration. A total of 5–40 cycles were carried out, and the products were examined at 2- to 5-cycle intervals. With 1 µg as the initial amount of RNA template, quantitation could be achieved during the exponential phase with 20 cycles for GAPDH or 30 cycles for NOS II and NOS III. Quantitative analysis of the expression of rat NOS II and NOS III was performed by scanning gels stained with SYBR Green I with a Fluoro-Image Analyzer (FLA-2000; Fuji Film, Tokyo, Japan) and analysis with MacBas image software (MacBas version 2.5; Fuji Film).

**Data analysis.**  Data are expressed as means ± SE. The differences among groups of rats were assessed by ANOVA. If a significant difference among the groups was demonstrated, Scheffe’s test was applied to assess which groups were different. A P value <0.05 was considered significant.

**RESULTS**

**EPR spectra of rat placenta with hemoglobin trapping.**  The EPR spectrum of the rat placenta showed a three-line hyperfine structure at g = 2.008 (Fig. 1A), where g is the spectroscopic splitting constant. The
EPR signal \((g = 2.008;\) hyperfine coupling constant = 1.66 mT) was diminished in the placental pellet and supernatant after homogenization, whereas the EPR signal of methemoglobin at \(g = 6.00\) was markedly increased in the homogenized placental pellets and the supernatant (Fig. 1, \(B\) and \(C\); Fig. 2). No specific three-line hyperfine structure of the EPR spectrum was detected in blood obtained from the abdominal vein of anesthetized pregnant rats (data not shown).

To verify that the EPR spectrum resulted from the activity of NOS, pregnant rats were injected subcutaneously with \(N^G\)-nitro-\(L\)-arginine methyl ester (\(L\)-NAME) (3, 10, 30, 100, or 300 mg/kg body wt). After 3 h, the placenta was removed from each rat, transferred to an EPR tube, and frozen in liquid nitrogen. The three-line hyperfine structure of the EPR signal \((g = 2.008)\) was clearly observed in the untreated placenta (Fig. 3). When pregnant rats were injected subcutaneously with \(L\)-NAME doses of \(\geq 30\) mg/kg, the EPR signal was not detected, whereas at \(L\)-NAME doses of \(\leq 10\) mg/kg, this EPR signal was observed.

**EPR spectra of rat placenta with \(Fe\)-DTCS trapping and quantification of NO formation.** The EPR spectra of the standard samples of NO-\(Fe\)-DTCS complex in fresh blood showed a typical triplet signal \((g = 2.038)\).
that was previously identified as NO-Fe-DTCS by Yoshimura (Ref. 29; Fig. 4A). The EPR spectrum with Fe-DTCS trapping in the placenta of rats pretreated with L-NAME (100 mg/kg) was identified as the $g_z$ signal of the Cu-dithiocarbamate complex (Ref. 25; Fig. 4B). The EPR spectrum in the placenta of preterm (day 19.5) rats was identified as the signal of NO-Fe-DTCS superimposed on the $g_z$ signal of the Cu-dithiocarbamate complex (25). The NO-Fe-DTCS and MnO signal heights were measured simultaneously, and the ratio of these heights was calculated and used for quantification of the NO levels (Fig. 5) because the signal height of the NO-Fe-dithiocarbamate complex can be used as an index for NO formation (25). The ratio of these two signal heights did not vary significantly in the placenta during the last few days of gestation; however, the ratio in the placentas of untreated rats was significantly higher than that in the placentas of rats pretreated with L-NAME (Fig. 5).

Quantitation of the expression of NOS isoforms by RT-PCR analysis. To examine the expression of NOS isoforms responsible for NO production in the placenta, quantitative RT-PCR analysis was performed on the total RNA extracted from the placenta. The level of expression of NOS II was significantly higher than that of NOS III in the placenta from the same stage (Fig. 6; $P < 0.01, n = 4$ or $5$), and the NOS II mRNA expression in term (day 21.5) placenta was significantly higher than that in preterm (day 19.5) placenta.

**DISCUSSION**

The present study directly demonstrated NO formation in the intact rat placenta by EPR spectroscopy with hemoglobin and Fe-DTCS as NO trapping reagents. With hemoglobin trapping, a specific EPR signal ($g = 2.008$, a 3-line hyperfine structure) was observed that coincided with that of a pentacoordinated α-NO heme species present in the blood of rats treated with cytokines (9). When the placental sample was homogenized to allow oxygenation before the EPR measurement, the three-line hyperfine structure of the EPR spectrum was completely lost, whereas the EPR signal of methemoglobin at $g = 6.00$ was markedly increased (Fig. 2). Thus the three-line hyperfine structure of the EPR spectrum was inferred to be derived from nitrosylhemoglobin (NOHb). When the rats were pretreated with L-NAME, the EPR signal obtained with hemoglobin trapping completely disappeared.

The EPR spectrum obtained from the placenta with Fe-DTCS trapping was identical to the reported EPR spectrum of NO-Fe-dithiocarbamate (25), indicating that the triplet EPR signal ($g = 2.038$) observed in the present study was derived from the NO-Fe-DTCS com-
plex. In addition, the EPR signal observed in the placenta with Fe-DTCS trapping after administration of L-NAME showed loss of the triplet signal only, indicating that the NO observed was synthesized by placental NOS and was not derived from elsewhere. To our knowledge, the present study is the first to demonstrate endogenous NO production by NO trapping after EPR spectroscopy in the placenta, although several investigators have reported the detection of elevated NO production induced by lipopolysaccharide (LPS) treatment or by reperfusion after occlusion (9, 10).

Expression of NOS II has been shown to be regulated at the transcriptional level (21), whereas NOS III is constitutively expressed. The enzyme activity of NOS III was shown to be required for an increase of intracellular calcium, and many factors have been reported to stimulate NOS III activity (5, 8, 12). As shown in Fig. 6, quantitative RT-PCR analysis revealed that the level of expression of NOS II was significantly higher than that of NOS III at the same gestational stage and that NOS II expression in term (day 21.5) placenta was significantly increased compared with that in preterm (day 19.5) placenta. Together with the fact that NOS II is a high-output NOS whereas NOS III is a low-output NOS (6, 13), the present results suggest that NO is produced predominantly by NOS II at this stage in the placenta. As shown in Fig. 5, quantification of the NO production level in the placenta revealed that NO formation in the placentas of rats did not vary significantly with gestational stage during the last few days of gestation.

In the placenta, NO has been reported to play significant roles in maintenance of local blood flow, inhibition of the immune response, and platelet aggregation in the vessels (2, 7, 15, 22). Although the level of NOS II mRNA expression in the term rat placenta was significantly higher than that in the preterm rat placenta (Fig. 6), NO production in the term rat placenta was not increased compared with that in the preterm rat placenta (Fig. 5). It is well known that the rat fetus grows extensively in the last few days of gestation, which causes a decline in the oxygen tension in the placenta, and thus the placenta is hypoxic at term. A hypoxia-responsive element has been reported in the NOS II promoter (11), and a hypoxia-inducible factor is involved in the induction of NOS II in pulmonary artery endothelial cells (19). The presence of these promoter elements predicts that low oxygen would increase NOS II gene expression, which would cause increased NO production at low oxygen tension. However, Otto and Baumgardner (18) recently showed that NO production due to NOS II activity of macrophages was markedly reduced at lower oxygen tension ranges, representative of typical physiological and pathophysiological ranges, than at higher oxygen tension ranges, including those typically used in cell culture. Thus the present results suggest that NOS II mRNA expression may increase to generate sufficient NO production in the hypoxic conditions of the placenta to maintain placental functions, e.g., the local blood flow, which are essential immediately before birth.

In summary, NO was detected in the intact placenta using NO trapping by hemoglobin and Fe-DTCS combined with EPR spectroscopy. The NO production level in the placenta did not vary with the gestational stage during the last few days of gestation; however, NOS II mRNA expression was increased in the term placenta, indicating that NOS II may be the predominant producer of NO in the placenta and suggesting that NOS II mRNA expression may increase to generate sufficient NO in the hypoxic conditions of the term rat placenta to maintain placental functions, such as local blood flow, that are required immediately before birth.

REFERENCES


