NOTE *Anatomy*

Gestational Changes in Production of NO and Expression of NOS mRNA Isoforms in the Rat Placenta

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ABSTRACT. To monitor changes in NO production over time in the fetal placenta of rats, we used electron paramagnetic resonance spectroscopy with the Fe-N-(dithiocarboxy) sarcosine (Fe-DTCS) complex as an NO-trapping reagent. The expression of nitric oxide synthase (NOS) isoforms was examined in parallel using quantitative RT-PCR. NO production was first detected on day 13.5 of gestation. NO levels reached a peak on day 15.5, then decreased significantly during the last few days of gestation. The pattern of expression of NOS II mRNA was in good agreement with changes in NO levels, whereas NOS III mRNA expression did not change markedly during gestation. Thus, it appears that NO levels in the placenta are NOS II-dependent and differ at different gestational stages.
KEY WORDS: electron paramagnetic resonance (EPR), fetal placenta, nitric oxide.

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During the past decade, there has been tremendous interest in nitric oxide (NO), which is now accepted as an important mediator of multiple cellular functions. In living cells, NO is synthesized from L-arginine via the catalytic action of a family of enzymes known as NO synthases (NOSs) [8–10]. Elevated concentrations of circulating nitrate are likely to result from increased production of NO by peripheral maternal tissues, the placenta, or both [2]. A gestation-dependent up-regulation in NO synthesis in the rat myometrium has been reported [3], and increased NO production in the rat uterus during pregnancy was shown to result from increased iNOS expression [1, 3]. Thus, it has been proposed that 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 [19], whereas NO production is lower in the placenta under pathophysiological conditions such as preeclampsia and diabetes during pregnancy [7, 20]. However, the developmental time-course of NO production in the placenta throughout gestation has not been reported.

Endogenously generated NO is a very short-lived gaseous free radical [12, 16] that reacts with many substances, including molecular oxygen and superoxide, to generate NO derivatives, such as nitrogen dioxide, peroxynitrite, and nitrate [27]. This property makes NO difficult to detect; however, NO-trapping techniques, such as using Fe-dithiocarbamate complex [21, 28] combined with electron paramagnetic resonance (EPR) spectroscopy, have been successfully used to detect NO in biological systems [21, 22]. In the present study, we determined the developmental time-course of NO production in the rat placenta by EPR spectroscopy using the Fe-*N*-(dithiocarboxy)-sarcosine (DTCS) complex as an NO-trapping reagent. Additionally, we examined the expression of specific NOS isoforms responsible for NO production.

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Female Crj: Wistar rats (Charles River Japan, Yokohama, Japan), aged 10–12 weeks old at the time of mating, were used in the study. They were maintained on a commercial diet (CE-2, CLEA Japan, Tokyo, Japan) and tap water *ad libitum* and kept in a room at a temperature of 22 ± 3°C with relative humidity of 55% ± 10% and a constant 12L:12D schedule. Three females were placed with a male overnight, and the presence of sperm was detected the next morning from a vaginal smear. Noon of the day on which sperm was found was designated as day 0.5 of gestation, and the females were maintained individually thereafter. All procedures were performed with the guidance of the Committee for Animal Experimentation at Azabu University.

Pregnant rats from day 13.5 to day 21.5 were subjected to the study of NO production detection by spin trapping followed by EPR analysis. For NO trapping using the Fe-DTCS complex, Fe-DTCS was dissolved in PBS and injected subcutaneously at a dose of 500 mg/kg-body wt for 0.5 hr before sampling. Rats were decapitated under ether anesthesia. Blood was rapidly taken from the abdominal vein, and only the fetal placenta was then removed and minced with surgical scissors. Each sample (approximately 0.5 g) was transferred to a quartz EPR tube and frozen immediately in liquid nitrogen. To verify that the EPR spectrum resulted from the activity of placental NOS, an NOS-specific inhibitor, N^G-nitro-L-arginine-methyl ester (L-NAME, Wako Pure Chemical, Osaka, Japan), was administered subcutaneously to pregnant rats on day 15.5 of gestation at 100 mg/kg-body wt, 1 hr before sampling.

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, 320 mT; temperature, –196°C; measurement time, 4 min; time constant, 0.3 sec. In some experiments, MnO powder was used as a standard, and the heights of the NO-Fe-DTCS and MnO signals were simultaneously measured. The ratio of these signal heights was used to quantify NO production levels, as it has been demonstrated that the signal height of the NO-Fe-dithiocarbamate complex can be used as an index for NO formation [21].

Total RNA was extracted from the fetal placenta of rats on day 13.5 to day 21.5 of gestation with an RNA extraction kit (Isogen; NPG, Toyama, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA (1 μg) using reverse transcriptase (RT; SuperScript III, GIBCO BBL, Tokyo, Japan) and an oligo $d(T)_{12}$ primer. PCR amplification from reverse-transcribed cDNA was carried out with primers specifically designed for NOS II [forward: 5'-TTCACGACACCCTTCACCA-CAA-3' (nt: 298–320) and reverse: 5'-CCATCCTCCTGC-CCACTTCCTC-3' (nt: 1188–1210)] based on the rat NOS II gene (GenBank accession no. D14051) and for NOS III [forward: 5'-TGGGCAGCATCACCTACGA-3' (nt: 231–249) and reverse: 5'-TCCCG- AGCATCAAATCCT-3' (nt: 585–603)] based on the rat NOS III gene (GenBank accession no. AF085195). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize template concentrations. Quantitative determination of expression of NOSs was done as described previously [22]. Briefly, for PCR, 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. Thermal cycling was performed under the following conditions: 20 cycles for GAPDH or 30 cycles for NOS II and NOS III at 94°C for 1 min, at 62°C (NOS II), or 60°C (GAPDH, NOS III) for 1 min, and 72°C for 1 min (GeneAmp 2400, Perkin Elmer Japan, Yokohama, Japan). The PCR product concentration is proportional to the starting cDNA concentration with the above cycle profile for each gene. PCR products were detected on a 1.5% agarose gel and stained with SYBR Green I (Takara, Kyoto, Japan). Quantitative analysis of rat NOS II and NOS III levels was performed using 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 are expressed as means ± SEM. The differences among groups of rats were assessed by ANOVA or Student's *t*-test. If a significant difference among the groups was demonstrated, Scheffe's test was used to determine which groups were different. A p-value less than 0.05 was considered to be statistically significant.

The EPR spectra of the standard samples of the 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 [27] (Fig. 1A). The EPR spectrum with Fe-DTCS trapping in the placenta of rats pretreated with L-NAME (100 mg/kg) was identified as the $g \perp$ signal of the Cu-dithiocarbamate complex [21] (Fig. 1B). The EPR spectrum in the fetal placenta of rats from day 15.5 of gestation was identified as the signal of the NO-Fe-DTCS superimposed on the $g \perp$ signal of the Cu-dithiocarbamate complex [21] (Fig. 1C). The NO-Fe-DTCS and MnO signal heights were measured simultaneously, and the ratio of these heights was used to quantify NO production (Fig. 2).

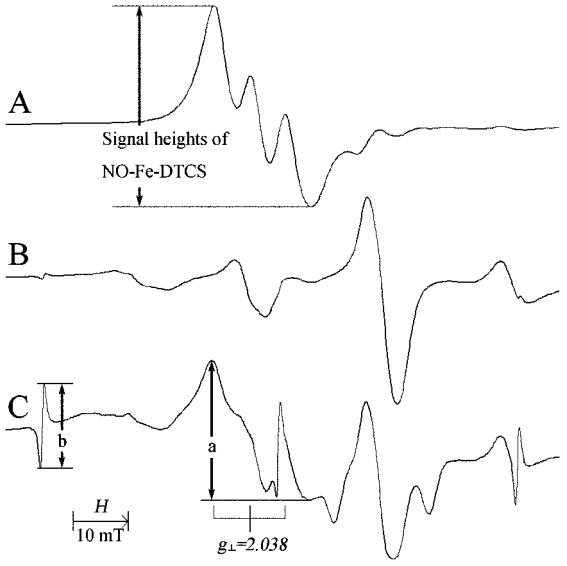

Fig. 1. Typical EPR spectra of the NO-Fe-DTCS complex. A: EPR spectrum of a standard sample of the NO-Fe-DTCS complex in fresh blood. B: EPR spectrum with Fe-DTCS trapping of the fetal placenta after administration of L-NAME (100 mg/kg-body wt) on day 15.5 of gestation. C: EPR spectrum of an NO-Fe-DTCS complex detected in the fetal placenta on day 15.5 of gestation. The heights of the NO-Fe-DTCS (a) and MnO (b) signals were measured simultaneously. The ratio (a/b) of the signal heights was used to quantify the levels of NO production.

The change in the ratio of the heights of the NO-Fe-DTCS and MnO signals was determined for the fetal placenta of rats at day 13.5 to day 21.5 of gestation. As revealed by EPR analysis, NO was detected in the placenta throughout gestation. NO production was higher in the 15.5-day placenta than in the 13.5-day or 17.5-day placenta (Fig. 2). The EPR spectra observed were in accordance with those observed in previous studies [22, 27], and, as expected, the EPR spectra derived from NO-Fe-DTCS almost disappeared after administration of L-NAME. These findings confirm that the EPR spectra obtained in the present study were derived from endogenous NO in the placenta. In addition, the data show that NO production reached a peak at day 15.5 of gestation and declined during the last few days of gestation, as NO production in the 15.5-day placenta was 2.6-fold greater than that of the term placenta. Although several studies have demonstrated the presence of NOS in the placenta [5, 13, 14, 23], to the best of our knowledge, the present study is the first report that shows the

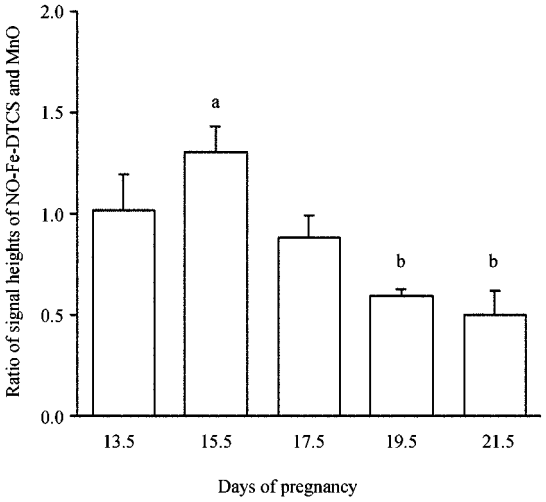

Fig. 2. Developmental changes in the ratio of the heights of the NO-Fe-DTCS and MnO signals in the placenta. EPR spectra were recorded in the fetal placentas on days 13.5 to 21.5 of gestation. Data are expressed as the means ± SEM of five individual experiments. Bars with different letters at the top differ significantly ($p<0.05$).

developmental time-course of NO production in the rat placenta during pregnancy.

To examine the expression of NOS isoforms responsible for NO production in the fetal placenta of rats, quantitative RT-PCR analysis was performed on total RNA extracted from those placentas. The NOS II mRNA expression on day 15.5 of gestation was significantly higher than on day 13.5, and NOS II mRNA levels were significantly lower in the 19.5-day placenta (Fig. 3A). In contrast, placental NOS III mRNA levels did not show marked changes over the course of gestation (Fig. 3C).

Furthermore, we examined the expression of NOS isoforms in the placenta to determine which NOS isoform is responsible for NO production. NOS II mRNA expression on day 15.5 of gestation was significantly higher than at other stages; by contrast, NOS III mRNA was detected at similar levels during pregnancy. Expression of the NOS II isoform was in good agreement with NO levels as revealed by EPR spectroscopy. Together with the fact that NOS II has high NO production capability, while NOS III has low NO production capability [11], the present findings suggest that NO production in the fetal placenta of rats, especially the peak of NO production observed on day 15.5 of gestation, is catalyzed predominantly by NOS II. Placental blood

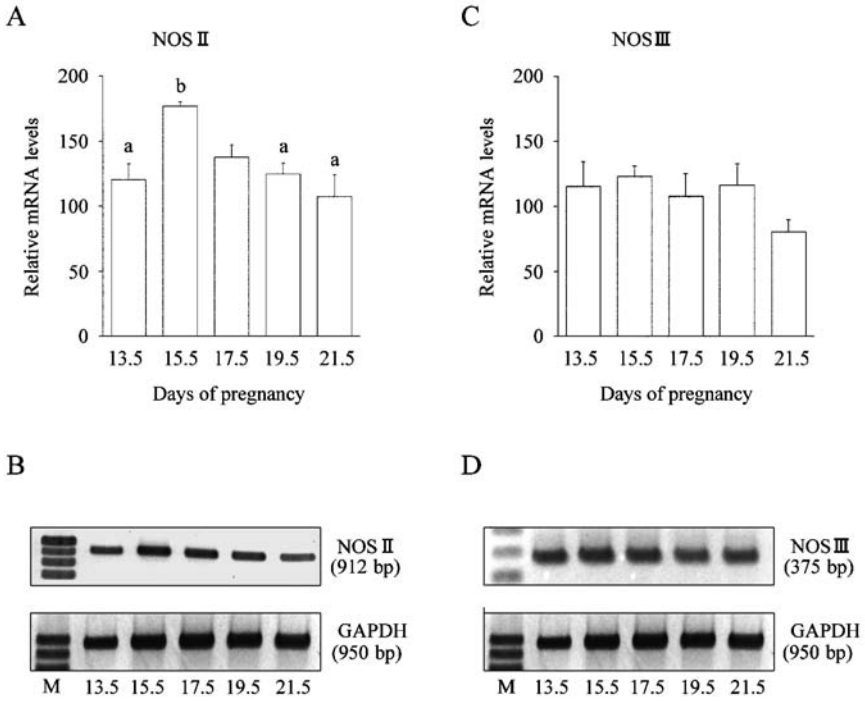

Fig. 3. Changes in NOS II and NOS III mRNA levels in the placenta over time. A, C: Relative levels of NOS II (A) and NOS III (C) were calculated as a percentage relative to levels of an appropriate control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are expressed as the means ± SEM of five individual experiments. Bars with different letters at the top differ significantly ($p<0.05$). B, D: A representative separation by 1.5% agarose gel electrophoresis of the RT-PCR products amplified from total RNA extracted from the fetal placentas on days 13.5 to 21.5 of gestation. The expected product sizes were 950 bp for GAPDH, 912 bp for NOS II (B), and 373 bp for NOS III (D). M: DNA size markers.

flow is not regulated by the nervous system, because the placenta is a non-innervated organ [18]. It has been reported that the blood flow of the placenta is regulated by the direct action of the NO on placental blood vessels [17]. The embryo and the fetal placenta develop rapidly in midgestation in rats, and, in fact, we confirmed that the weight of the fetal placenta increased dramatically from day 13.5 to day 15.5 of gestation (data not shown). Therefore, the peak of NO production on day 15.5 of gestation may be related to placental growth.

The present data also provide a baseline for further research to evaluate the regulatory mechanisms for the expression of specific NOS isoforms and NO functions in the placenta. Although the fetal placenta of rats can be distinguished from the decidua basalis, previous studies that measured NOSs in the placenta did not clearly distinguish between the fetal placenta and the decidua basalis. Thus, levels of NOSs in the fetal placenta could not be distinguished from overall levels in the fetal placenta plus the decidua basalis. Moreover, we regard the measurement of NO and NOS levels over time in the placenta as providing important information that is not available from read-outs of baseline levels from mixed tissues.

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