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INCREASED EXPRESSION OF ENDOTHELIAL CONSTITUTIVE NITRIC OXIDE SYNTHASE IN RAT AORTA DURING PREGNANCY

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SUMMARY. The mechanisms underlying enhanced vascular reactivity in pregnancy are not yet defined. In this study we have investigated the potential role of endothelium-derived vasodilator nitric oxide (EDNO). EDNO-mediated dilatory responses *in vitro* were markedly increased in aorta of pregnant as compared with nonpregnant rats. This increase in EDNO-releasability was accompanied by a two-fold increase in mRNA of endothelial constitutive nitric oxide synthase (NOS-III). Chronically substituted estrogen, but neither progesterone nor testosterone induced an upregulation of NOS-III mRNA in aorta of gonadectomized rats which amounted to about half that induced in aorta of pregnant rats. Thus, increased EDNO-releasability and increased NOS-III mRNA contribute to enhanced vascular reactivity in pregnancy.

Previous *in vivo* and *in vitro* studies have demonstrated a blunted vascular responsiveness to vasoconstrictor autacoids in pregnancy, which is endothelium-dependent and is abolished in almost all vascular beds by inhibition of nitric oxide synthase and/or cyclooxygenase (1-4). Furthermore, vascular sensitivity and responses to the endothelium-dependent vasodilator acetylcholine are enhanced in pregnancy (5, 6). These findings have implicated endothelium-derived nitric oxide (EDNO) as having an importance in mediating alterations of vascular reactivity during pregnancy.

The present study was designed in order to investigate, a) whether increased EDNO-releasability accounts for altered reactivity of rat aorta during pregnancy, b) whether changes in EDNO-releasability are accompanied by altered endothelial constitutive nitric oxide synthase (NOS-III) gene expression, and c) whether NOS-III gene expression is regulated by changes in circulating levels of sex steroid hormones.

ABBREVIATIONS

EDNO, endothelium-derived nitric oxide; NOS-III, endothelial constitutive nitric oxide synthase; ACh, acetylcholine; SNP, sodium nitroprusside; RT, reverse transcription; PCR, polymerase chain reaction; cDNA, complementary DNA.

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MATERIALS AND METHODS

Animals. Eight-week-old female Sprague-Dawley rats were purchased from the Versuchstieranstalt Hannover, FRG. Rats were nonpregnant (n=16), pregnant or gonadectomized, and pregnant animals were of 7 days (early; n=5), 16 days (middle; n=15) or 21 days (late; n=5) gestation (full gestation period 21-22 days). Gonadectomized animals received sex steroid hormone substitution for two weeks and were randomly assigned to one of the following groups: group E (n=6) 17β-estradiol, 1.0 mg/kg/day s.c., group P (n=6) progesterone, 0.4 mg/kg/day s.c., group T (n=6) testosterone, 1.0 mg/kg/day s.c., and sham (n=5) benzylbenzoate-castor oil (3 volumes : 10 volumes) s.c.. At the time of sacrifice, a 1-ml blood sample was withdrawn from ether-anesthetized animals for determination of serum sex steroid hormone levels. The thoracic section of aorta was carefully excised, and either placed immediately into ice-cold Tyrode's solution for functional analysis by perfusion *in vitro* or frozen in liquid nitrogen and stored at -80°C until use for mRNA quantification.

Determination of serum sex steroid hormone levels. 17β-estradiol serum concentrations were determined using the Delfia-test (Pharmacia Ltd., Freiburg, FRG) with a sensitivity of 13.6 pg/ml, progesterone serum concentrations using the Amerlite-test (Kodak Diagnostik Ltd., Braunschweig, FRG) with a sensitivity of 0.11 ng/ml and testosterone serum concentrations using the Testosteron-RIA COAT-A-COUNT (Biermann Diagnostica Ltd., Bad Nauheim, FRG) with a sensitivity of 0.04 ng/ml.

Dilatory reactivity of aorta *in vitro* . Segments of thoracic aorta (10-15 mm) were mounted in an organ bath (for details of the experimental setup, see ref.7) containing oxygenated (95% O_2/ 5% CO_2, pH 7.25) Tyrode's solution at 37°C (composition, in mM: Na^+ 144.0, K^+ 4.0, Ca^{2+} 1.6, Mg^{2+} 1.0, Cl^- 140.0, HCO_3^- 11.9, $H_2PO_4^-$ 0.36, glucose 11.2 and calcium disodium EDTA 0.025). After a 60-minute equilibration period, intraluminal perfusion of the aortic segments was commenced with Tyrode's solution, gassed with 75% N_2/ 20% O_2/ 5% CO_2, pH 7.38, in addition to extraluminal perfusion. All drugs were applied intraluminally, and vasomotions were recorded continuously as changes of external vessel diameter by means of a photoelectric device (8). After precontraction with phenylephrine to 90% of starting diameter, vasodilation was studied in response to acetylcholine, Ca^{2+}-ionophore A23187 and sodium nitroprusside. A washout period for return to initial precontraction level was allowed between each agonist. Dose-response-curve to sodium nitroprusside was performed during inhibition of endogenous nitric oxide formation by Nitro-L-arginine (0.03 mM). Four aortic segments in each group were exposed to indomethacin (0.03 mM) intra- and extraluminally throughout the perfusion period. ED_{50} values of the vasodilators were calculated from mean dose-response-curve as dose required for 50% dilation of precontracted vessel segment.

Quantification of NOS-III mRNA in aorta. NOS-III mRNA was quantified by competitive RNA-polymerase chain reaction as described by Studer et al. (9).

a) primer oligonucleotides for PCR. Rat aortic cDNA was amplified by PCR with primer oligonucleotides selected on the basis of human and bovine NOS-III cDNA sequences (10, 11). PCR-products of expected size were isolated and subcloned between the SmaI / BAP sites of pUC 18. Southern blot-positive inserts of expected size were then subjected to sequence analysis. On the basis of these sequence data the oligonucleotides 5'-CTGCGCTGGTATGCCCTCC-3', corresponding to 1017-1035 of bovine NOS-III cDNA (16), and 5'-AAGAGCCTCCCCAGCTGCTG-3', corresponding to 1041-1660 of bovine NOS-III cDNA, were selected as sense and antisense primer, respectively.

b) construction of competitor RNA for PCR. A 125 bp fragment was released from the bovine NOS-III cDNA clone (10) by digestion with the restriction enzymes PflMI and BglII. The shortened NOS-III cDNA clone was isolated, blunted and religated. After linearization by digestion with the restriction enzyme Eco81I, the shortened NOS-III cDNA clone was subjected to PCR using the primer pair designed on the basis of the sequenced rat NOS-III related cDNA fragments (see section a)). The amplified cDNA fragment (518 bp) was blunted, phosphorylated and ligated into a blunt-ended,

dephosphorylated Bluescript SK(-) vector. After linearization by digestion with the restriction enzyme XhoI, the 518 bp NOS-III cDNA clone was subjected to *in vitro* transcription into RNA. The purified NOS-III-construct RNA was quantified spectrometrically.

c) isolation of RNA. Total cellular RNA was isolated from aortic tissue by acid guanidinium thiocyanate-phenol-chloroform extraction (12). RNA was quantified spectrometrically and run on ethidium bromide stained agarose gels in order to check for its integrity.

d) competitive RNA-PCR. Reverse transcription (RT) of RNA into first-strand cDNA was performed with constant amounts of total aortic RNA (312.5 ng) together with different amounts of competitor NOS-III-construct RNA (500 to 6.25 x 10^5 molecules). RT reaction mixture (25 µl) contained aortic and competitor RNA, 50 mM TRIS-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 250 pmol of random hexanucleotide primers, 1.25 mM of each dNTP, 25 mM of dithiothreitol, 5 units of RNase inhibitor (Life Technologies Ltd., Eggenstein, FRG) and 25 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies Ltd., Eggenstein, FRG). The RT reaction was performed by incubation of the mixture at 42°C for 60 minutes. Amplification of cDNA by PCR was performed in duplicate with 10 µl of RT reaction mixture added to 40 µl of PCR reaction mixture comprising 20 mM TRIS-HCl pH 8.3, 50 mM KCl, 75 mM $MgCl_2$, 0.5 pmol of each primer and 2.5 units of Thermus aquaticus DNA polymerase (Amersham Buchler Ltd., Braunschweig, FRG).The reaction mixture was overlaid with mineral oil and was then subjected to 30 PCR-cycles with a temperature profile of 94°C for 1 min, 65°C for 2 min and 72°C for 3 min. The PCR-products were separated by gel electrophoresis, stained with ethidium bromide, visualized by UV illumination and photographed. The amounts of target and competitor NOS-III PCR-products were quantified by densitometric scanning of the film negative. To correct for differences in size of target (643 bp) and competitor (518 bp) NOS-III PCR-product, the amounts of competitor PCR-product were multiplied by 1.24 (643 : 518). The ratio of competitor to target NOS-III PCR-product was plotted against the number of competitor NOS-III-construct RNA molecules on a log-log scale (Fig. 1). At the point, where equal molar amounts of target and competitor NOS-III product were yielded by PCR (i.e. competition ratio = 1), the original number of target NOS-III transcripts in the aortic sample was defined by the known input of competitor NOS-III-construct RNA molecules.

Drugs. Progynon-Depot-10 (estradiol valerate, 10mg/ml), Proluton-Depot (hydroxyprogesterone caproate, 250 mg/ml) and Testoviron-Depot-50 (testosterone propionate, 20mg/ml, + testosterone enantate, 55mg/ml) were obtained from Schering (Berlin, FRG). Phenylephrine hydrochloride, acetylcholine hydrochloride, Ca^{2+}-ionophore A23187, sodium nitroprusside, Nitro-L-arginine and indomethacin were purchased from Sigma Chemicals (Deisenhofen, FRG). Phenylephrine hydrochloride, acetylcholine hydrochloride and Nitro-L-arginine were dissolved in Tyrode's solution; A23187 was dissolved in dimethylsulfoxide, sodium nitroprusside in 1.0 mM sodium acetate, indomethacin in ethanol-$NaHCO_3$ 0.1 M (1 volume-3 volumes) and diluted with Tyrode's solution.

Statistical analysis. All data are presented as mean ± SEM.
Student's t-test for unpaired data was used to compare mean values between the pregnant and nonpregnant group. One way analysis of variance followed by the Bonferroni t-test was performed for comparison of mean values between the different groups of gonadectomized rats. Differences were considered statistically significant at a p-value less than 0.05.

RESULTS AND DISCUSSION

Dilatory reactivity of isolated perfused aortic segments to the endothelium-dependent agonist acetylcholine (ACh) were markedly enhanced in thoracic aorta of pregnant as compared with nonpregnant rats ($p<0.05$; Fig. 2). The ED_{50} of ACh was decreased by

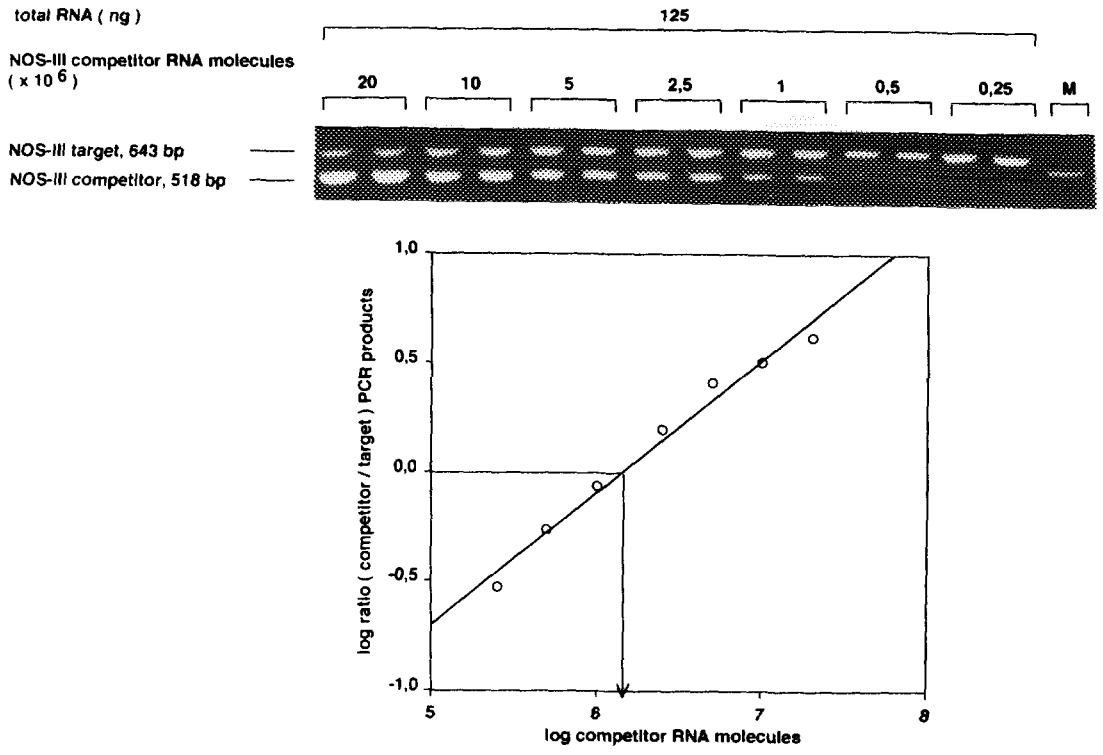

Fig. 1. Quantitative analysis of PCR-products. As indicated at the top of the gel, a constant amount of total RNA was used for competitive RNA-PCR with different numbers of NOS-III-construct RNA molecules as competitor. The original number of NOS-III transcripts in the aortic sample was calculated by plotting the ratio of competitor to target NOS-III PCR-product against the number of competitor NOS-III-construct RNA molecules on a log-log scale. At the point where equal amounts of competitor and target NOS-III PCR-product were yielded, i.e. where the logarithm of the competition ratio (NOS-III competitor : NOS-III target) is zero, the original number of NOS-III transcripts in the sample was defined by the known number of competitor NOS-III RNA molecules. M, DNA size marker (603 bp fragment).

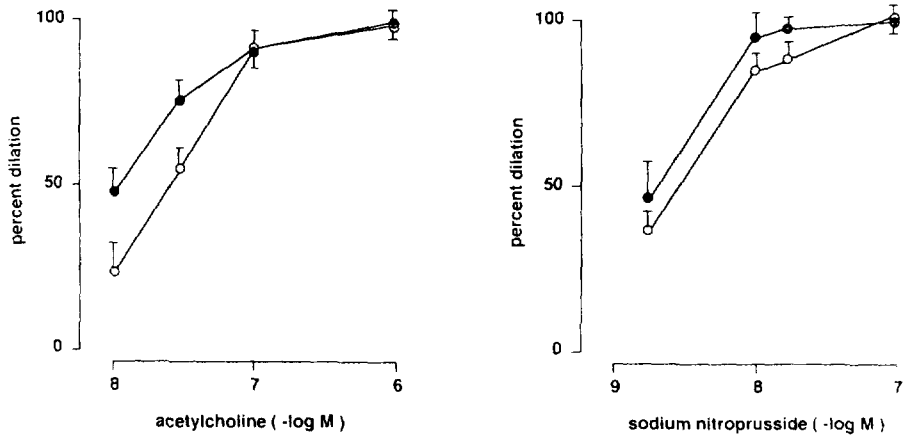

Fig. 2. Dose-response curves of aortic segments from pregnant (filled symbols) and nonpregnant rats (open symbols) to acetylcholine and sodium nitroprusside. Dilatory responses are expressed as percent of maximum phenylephrine precontraction steady state level.

about two-fold, while the maximal dilator response to ACh was unaltered in pregnant as compared with nonpregnant rats (ED_{50}; 11.8 vs 24.0 nmol). There was no difference in acetylcholine responses between aortic segments taken from early, middle and late pregnant animals (ED_{50}; 12.3 vs 13.1 vs 10.1 nmol). Similar results were observed with Ca^{2+}-ionophore A23187, an endothelium-dependent, but receptor-independent acting vasodilator (ED_{50}; 11.0 vs 19.5 nmol). Inhibition of cyclooxygenase with indomethacin did not affect the ACh and A23187 responses. Inhibition of nitric oxide synthase by Nitro-L-arginine, however, completely suppressed ACh- and A23187-induced dilations of rat aorta, implying that they are mediated by EDNO. In contrast to endothelium-dependent dilatory reactivity, the responses to the endothelium-independent vasodilator sodium nitroprusside were only marginally potentiated in aortic segments of pregnant as compared with nonpregnant rats (ED_{50}; 2.0 vs 2.7 nmol; Fig. 2).

These data demonstrate an enhanced dilatory reactivity of rat aorta in pregnancy which is due to an increased EDNO-releasability rather than an increase in smooth muscle responsiveness to nitric oxide. They do not, however, identify whether or not basal as well as agonist-stimulated EDNO-release is enhanced during pregnancy. Previous studies have demonstrated a blunted endothelium-dependent contractile reactivity in different vascular beds in pregnancy which is almost completely reversed by suppression of endogenous nitric oxide formation. These studies have therefore provided substantial evidence of an enhanced basal EDNO release in pregnancy (3, 4).

To determine whether the increased EDNO releasability is due to altered NOS-III gene expression, NOS-III mRNA was quantified in thoracic aorta from another group of pregnant (16 days of gestation) and nonpregnant rats. As shown in figure 3, NOS-III mRNA levels were increased more than two-fold in aorta of pregnant as compared with nonpregnant animals ($2.79 \pm 0.25 \times 10^6$ vs $1.25 \pm 0.17 \times 10^6$ NOS-III transcripts per 125 ng of total RNA, $p < 0.01$). Thus, the enhanced EDNO releasability in rat aorta during pregnancy is paralleled by an increase in NOS-III gene expression.

To estimate potential hormonal influences on the upregulation of NOS-III gene expression in pregnancy, aortic NOS-III gene expression was analyzed in separate groups of gonadectomized rats given sex steroid hormone substitution for two weeks. As illustrated in figure 3, 17β-estradiol substitution (plasma level; 8584.2 ± 165.7 vs 35.0 ± 12.8 pg/ml) led to an approximate 70% increase in aortic NOS-III mRNA level. In contrast, neither testosterone substitution (plasma level; 11401.0 ± 99.4 vs < 40.0 pg/ml) nor progesterone substitution (plasma level; 92.0 ± 3 vs 3.15 ± 0.9 ng/ml) had any effect on NOS-III gene expression in gonadectomized rats. This finding is consistent with the results of previous *in vitro* and *in vivo* studies (13, 14).

The upregulation of NOS-III gene expression induced by 17β-estradiol, however, amounted to only about half that induced by pregnancy. This implies the influence of additional factors other than estrogen in accounting for the upregulation of NOS-III gene expression observed during pregnancy.

In summary, the present study demonstrates increased EDNO-releasability concomitant with an upregulation of NOS-III gene expression in pregnancy. These findings are likely

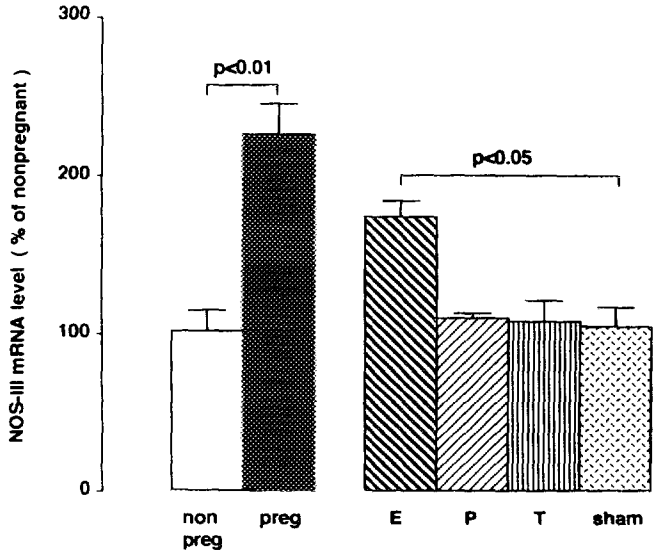

Fig. 3. NOS-III mRNA level in thoracic aorta of nonpregnant (nonpreg), pregnant (preg) as well as gonadectomized rats with sex steroid hormone substitution (17β-estradiol, E; progesterone, P; testosterone, T vs sham). NOS-III mRNA levels are expressed as percent of NOS-III mRNA amount in thoracic aorta of nonpregnant rats.

to account for the enhanced dilatory reactivity of rat aorta during pregnancy, which in part may be mediated by an estrogen-dependent mechanism. Impairment or lack of this adaptive mechanism may be important in the pathophysiology of pregnancy related cardiovascular disease.

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