Influence of estradiol-17β and progesterone on nitric oxide (NO) production in the porcine endometrium during first half of pregnancy

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SUMMARY

The purpose of the study was to examine: 1/ endometrial concentrations of nitrate/nitrite (NOx) in pregnant pigs, and 2/ the influence of estradiol-17β (E₂) and/or progesterone (P₄) on NOx production by porcine endometrium during the first half of pregnancy. Total NOx concentrations were determined using a microplate assay method based on the Griess reaction. Evident fluctuations of endometrial NOx content were found during the examined time of pregnancy (days 5, 10, 15, 20, 25, 30, 35, 40 and 60 of pregnancy). The NOx concentration was highest on days 10 and 15, and then lowered until day 60 of pregnancy. In addition, we demonstrated the stimulatory effect of E₂ and/or P₄ on NO in vitro production by porcine endometrial slices. The medium content of NOx depended on the steroid type, treatment dose and day of pregnancy. It is possible that the observed differences in the strength of the stimulatory action of E₂ and/or P₄ on

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**INTRODUCTION**

Nitric oxide (NO) is a major mediator of numerous biological processes, including vascular functions [15], neurotransmission [5], hormone secretion [24] and inflammation [21]. Nitric oxide is synthesized from L-arginine by nitric oxide synthases (NOSs), the family of enzymes in which three isoforms have been identified: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS; [18]).

It has been demonstrated that NO generated in the cyclic and gravid uterus [1, 2, 30] plays an important role in maintaining uterine quiescence during pregnancy [9, 12]. Moreover, NO affects the endometrium which the vascular function changes during the estrous cycle and pregnancy [4, 22, 30]. In many species NO generation is up-regulated during pregnancy and down-regulated during delivery [6, 12, 23, 26, 31, 32]. In rats, production of NO, measured as nitrites and nitrates concentrations (NOx, stable products of NO oxidation), increases during mid-gestation and markedly decreases during spontaneous delivery and postpartum period [32]. Others have also reported a decrease in NOS activity in rat [23] and rabbit [26] uterine tissue at term. An increase in NOS content in the uterus may be important in the maintenance of pregnancy, and a decrease in NOS at term may play a role in initiation of labour.

It has been suggested that ovarian steroid hormones, progesterone (P₄) and estradiol-17β (E₂), are important modulators of NOS activity [3, 11]. Administration of E₂ to ovariectomized pigs increased NADPH-diaphorase (marker for NOS) activity in the endothelium of the blood and lymph vessels of the uterine broad ligament [34, 35]. In the rat uterus, E₂ inhibited iNOS expression but stimulated eNOS [33] and in vitro NO production [7]. The effect of P₄ on NOS expression is tissue-dependent. In the rat uterus and placenta, P₄ up-regulates iNOS expression and NO production [7].
Since NO is a molecule with a short half-life and reacts rapidly with free oxygen, oxygen radicals, redox metals, sulfhydryls, disulfides and oxygenated hemoglobin, the measurement of NO production is rather difficult [27]. The measurement of nitrite (\(\text{NO}_2^-\)) and nitrate (\(\text{NO}_3^-\)) concentrations (Griess reaction) allows changes in the concentration of NO release in biological fluids to be monitored [14]. To our knowledge, the effect of \(\text{E}_2\) and \(\text{P}_4\) on NO production in the porcine uterus during pregnancy has not been examined. Therefore, this study was carried out to investigate the influence of the steroids on endometrial NOx production during the first 60 days of pregnancy in gilts.

**MATERIALS AND METHODS**

**Animals and isolation of endometrium**

Forty-five primiparous crossbred (Large White×Landrace) gilts randomly assigned to a pregnant group, after exhibiting two estrous cycle of normal length, were bred at the onset of estrous (day 0), and then 12 h and 24 h later. The animals were housed on a farm. Three days before slaughtering animals were transported to the animal facility and kept in individual stalls under natural light and temperature. They were fed a commercial grain mixture and tap water *ad libitum*. The experimental procedures were approved by the Local Ethics Committee.

On days 5, 10, 15, 20, 25, 30, 35, 40, 60 of pregnancy (n=5 per day), the gilts were euthanized by electrical shock and exanguined. The uteri were collected and transported (3 min) on ice to the laboratory. Early pregnancy (days: 5, 10, 15) was confirmed by the presence of at least four conceptuses in the uterine horns. Uterine horns were cut, placentas (from day 20 and subsequent days of pregnancy) were carefully isolated from the uterus and pieces of the endometrium were 1/ used immediately for *in vitro* incubation of endometrial slices, and 2/ frozen in liquid nitrogen (-80°C) for NOx measurement. Frozen tissue samples were washed in sterile saline and homogenized with 0.9% NaCl on ice. Homogenized
tissue were centrifuged at 1500 g for 10 min at 4°C. Supernatants were used for NOx determination.

**Incubation of endometrial slices**

Pieces of endometrial tissue (100 mg) were rinsed in minimum essential medium without phenol red. Preincubation and incubation of the tissues were conducted in a shaking water bath (37°C, 5% CO₂/air atmosphere). After 30 min preincubation, the tissue was incubated (triplicates/tissue/hormone) for 24 h in DMEM (without phenol red) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin (2 µg/ml; all reagents from Sigma St. Louis, MO, USA). The culture medium also contained 0, 10, 20 and 40 ng/ml of P₄, 0, 0.05, 0.2, 0.5 ng/ml of E₂, or the combination of 0.05 ng/ml E₂+10 ng/ml P₄, 0.2 ng/ml E₂+20 ng/ml P₄ and 0.5 ng/ml E₂+40 ng/ml P₄ (both steroids from Sigma St. Louis, MO, USA). The concentrations of E₂ and P₄ were chosen on the basis of their concentrations in plasma and uterine lumen [16, 25] and the results of our preliminary study. After 24 h culture period, the conditioned media were collected, clarified by centrifugation (16 000 g, 15 min, 4°C) and stored at -80°C for NOx analysis. Control samples with medium only were included in each experiment (non-tissue controls).

**Measurement of nitrates and nitrites**

Total NOx concentrations were determined using a microplate assay method based on the Griess reaction (according to Sigma protocol). 100 µl of medium or supernatant were incubated with 10 µl of nitrate reductase and 10 µl of NADPH for 20 min at room temperature. Next, 100 µl of Griess reagent was added to all samples and incubated in dark. After 15 min, the absorbance was measured using a 540 nm filter and plate reader (Bio-Rad, Hercules, CA, USA). The assay sensitivity was 0.065 µg/ml and the standard curve was produced for NOx concentrations ranging from 0.05 to 6.9 µg/ml. Results were normalized against the weight of the tissue and expressed as ng/mg of endometrium.
**Statistical analysis**

Experimental data are presented as mean±SEM of each experiment performed in triplicates. The statistical significance of differences in endometrial NOx content among particular days of pregnancy was analyzed by one-way analysis of variance ANOVA followed by Tukey’s multiple comparison test (GraphPad PRISM). The *in vitro* effect of E₂ and/or P₄ on NOx content was calculated by one-way analysis of variance (ANOVA) for repeated measures followed by Dunett’s post-hoc test (GraphPad PRISM). A value of p<0.05 was considered significant.

**RESULTS**

**Concentration of NOx in the endometrium on studied days of pregnancy**

Dramatic changes in endometrial NOx concentrations were observed during the first 60 days of pregnancy in gilts (fig. 1). The highest levels of

*Figure. 1. Endometrial content of total NOx (means±SEM) in pregnant pigs (days 5, 10, 15, 20, 25, 30, 35, 40, 60, n=5 per group). Means were compared by one-way analysis of variance followed by Tukey’s multiple comparison test. Means with different letters are significantly different (p<0.01)*
Figure 2. The effect of E₂ and/or P₄ on in vitro NOx production (means±SEM) by endometrial slices on days 5, 10, 15, 20, 25, 30, 35, 40 and 60 of pregnancy (n=5 per group). Tissues (100 mg) were incubated for 24 hours in DMEM (without phenol red) supplemented with 10% of calf serum. Means were compared by one-way analysis of variance for repeated measures followed by Dunett’s post-hoc test. Asterisks indicate means different from controls, * p<0.05, ** p<0.01, *** p<0.001
NOx (p<0.001) were found on days 10 and 15 of pregnancy. On subsequent days (days 20 and 25; p<0.001), NOx concentration in endometrium decreased. An increase in NOx content, compared to days 25 (p<0.001), 35 (p<0.001) and 40 (p<0.01), was observed on day 30. The next significant increase was seen on day 60 of pregnancy (p<0.01). The lowest level of NOx endometrial content was found on days 25, 35 and 40 of pregnancy.

**The influence of E\textsubscript{2} and P\textsubscript{4} on *in vitro* NOx production**

The stimulatory effect of E\textsubscript{2} and/or P\textsubscript{4} on *in vitro* production of NOx by endometrial tissues is depicted in Figure 2. Two lower doses of E\textsubscript{2} (p<0.01) as well as the lowest dose of the steroid (p<0.001) increased NOx production by porcine endometrium collected on days 5 or 15 of pregnancy, respectively. Endometrium from day 20 released more NOx in response to the intermediate dose of E\textsubscript{2} (p<0.05). On the remaining days of pregnancy, an increase in endometrial release of NOx was caused by either the highest dose (days 10, 25, 35; p<0.01) or by two higher doses (days 30, 40, 60; p<0.01) of estrogen.

The intermediate dose of P\textsubscript{4} increased (p<0.001) NOx production by endometrium collected on days 5 and 15 of pregnancy. When day 10 endometrium was incubated, the level of NOx rose in response to all three doses of P\textsubscript{4} (p<0.01). The two lowest P\textsubscript{4} doses affected endometrial NOx production on days 20, 25 and 30 of pregnancy. The two highest doses of P\textsubscript{4} increased (p<0.01) NOx secretion on day 35 of pregnancy. Production of NO was not affected by P\textsubscript{4} on days 40 and 60.

The combination of the lowest as well as the highest doses of both hormones increased (p<0.001) NOx production by endometrium collected on days 5, 40 and 60. Two higher doses of combined steroids enhanced (p<0.001) NOx medium content during the incubation of porcine endometrium collected on day 15 of pregnancy. Only the highest doses of combined E\textsubscript{2} and P\textsubscript{4} increased (p<0.01) endometrial NOx secretion on days 20 and 30 of pregnancy. All doses of both hormones affected (p<0.001) NOx production by endometrium on day 25 of pregnancy. No effect of combined steroid treatment was found on days 10 and 35 of pregnancy.
DISCUSSION

This is the first study demonstrating the influence of $E_2$ and $P_4$ on NO production in the endometrium during the first 60 days of pregnancy in pigs. Evident fluctuations of endometrial NOx content were found during the examined time of pregnancy. The NOx concentration was highest on days 10 and 15, and then decreased until day 60 of pregnancy. Since steroid hormones are among other regulatory agents affecting secretion of NO, the increased NOx concentration on days 10 and 15 may be linked to estrogens produced by porcine blastocysts (days 10-12; [29]). On the other hand, NO is known to increase vascular permeability and blood flow as well as the secretion of histotrophe from luminal endothelial cells, and thus the high NO production on days 10-15 may be associated with the preparation of the implantation window and implantation itself [4].

Our previous study [1] demonstrated increased NADPH-diaphorase activity in the luminal epithelial cells on days 5-12 and its gradual increase in the glandular epithelium on days 10-17 of pregnancy in pigs. Because pigs possess a true epitheliochorial type of placenta, a high NO concentration may be toxic for trophoblast and endometrial luminal cells. In turn, the augmented NO expression in the endometrial glands is probably involved in increased vascular permeability and nutritional function that occur in the endometrium before trophoblast attachment is accomplished [4].

A triphasic pattern of NOx production was found in the current study with peaks on days 10-15, 30 and 60 of pregnancy. Pig blastocysts start to produce estrogens at the 10 mm spherical stage [13]. Estrogen secretion by pig conceptus/fetus exhibits three peaks: on days 10 to 12 [29], 16 to 30 [25, 29] and 60 to term [17, 25]. In addition, the two/three peaks of $P_4$ plasma level were demonstrated during pregnancy in this species [25, 28]. The rapid enhancement of $E_2$ and $P_4$ concentrations observed in plasma and allantoic fluid around day 30 and day 60 appears to be associated with an increase in placental size and allantoic fluid volume. Additionally, the rapid increase in allantoic fluid volume around day 30 of pregnancy is correlated with initial expansion of chorioallantoic membranes and establishment of intimate contact between placenta and endometrial surface.
Since estrogens as well as NO are known to increase the cell permeability to water, the increase in endometrial NOx concentration demonstrated on day 60 of pregnancy may be related to the final period of accumulation of allantoic fluid and placental length [17]. Similar timing of estrogens and NO actions during early pregnancy as well as their involvement in the regulation of cell permeability suggest that NO production is under hormonal regulation.

Generally, in the present paper the effect of E\textsubscript{2} and/or P\textsubscript{4} on NO in vitro production by porcine endometrial slices was stimulatory. The medium content of NOx depended on the steroid type, treatment dose and day of pregnancy. Estradiol administered alone caused a dose dependent increase in NO production on all studied days of pregnancy, while P\textsubscript{4} increased the concentration of NOx only until day 35 of pregnancy. For all combined doses of E\textsubscript{2}+P\textsubscript{4} the most significant increase in medium NOx content was demonstrated on day 25. These in vitro data are consistent with our in vivo results concerning both the relationship between the increase in permeability and consequent increase in allantoic fluid volume and placental length as well as changes in plasma concentrations of steroid hormones and NO production.

Three known isoforms of NOS may be involved in NO synthesis. The expression of the isoforms is regulated in different manners and depends on the examined tissue and hormonal status of the animal. It is possible that the observed differences in the strength of the stimulatory action of E\textsubscript{2} and/or P\textsubscript{4} on endometrial NOx production are associated with activation of different isoforms of NOS. In sheep, expression and activity of eNOS increased after E\textsubscript{2} administration as well as during pregnancy in the uterus and several other tissues [11, 19]. In contrast, studies by Yallampalli and Dong [33] revealed that E\textsubscript{2} administered to pregnant rats on day 18 of gestation inhibited uterine NO production by suppressing iNOS expression. Surprisingly, this inhibition was accompanied by an increase in eNOS. In ovariectomized rats, E\textsubscript{2} also inhibited iNOS and up-regulated eNOS expression in the uterus [9, 32, 33]. Thus, the exact effect of E\textsubscript{2} on NOS activity may depend on the examined species.

It is of interest that P\textsubscript{4} is required for maintaining iNOS expression in the
rat uterus during pregnancy [10, 32]. Our results showed that P₄ enhanced endometrial NOₓ production on days 5 to 35 of pregnancy in pigs. In addition, NO production and/or NOS expression may be affected by interaction between P₄ and E₂. In rats, E₂ inhibited NO production via reducing iNOS expression only in the absence of P₄ [33]. Also in the current study the combination of E₂ and P₄ was sometimes more effective in the stimulation of NO production than the application of individual hormones.

In summary, in the current study we demonstrated that endometrial NOₓ concentrations changed dramatically during the first 60 days of pregnancy in pigs. Moreover, E₂ and P₄ stimulated NO in vitro production by porcine endometrial slices. The stimulatory effect of steroids depended on the dose of hormones and the day of pregnancy. The differences in NOₓ production observed during pregnancy in pigs may result from the activation of different NOS isoforms.

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