

The influence of steroid hormones on *in vitro* NOx production by porcine fetal membranes

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SUMMARY

The aim of the study was to examine: 1/ allantochorial concentrations of nitrate/nitrite (NOx) and 2/ plasma concentration of NOx in pigs on days 25, 35, 40 and 60 of pregnancy as well as 3/ the influence of estradiol-17 β (E₂) and/or progesterone (P₄) on NOx production by porcine fetal membranes on the studied days of pregnancy. Total NOx concentration was determined using a microplate assay method based on the Griess reaction. Fetal membrane NOx content gradually increased from day 25 to day 60 of gestation. Blood plasma NOx concentration decreased from day 25 to 40, and then plasma NOx concentration significantly increased on day 60. In addition, the stimulatory effect of E₂, P₄ and E₂+P₄ on NO *in vitro* production by porcine fetal membranes was demonstrated. The stimulatory effect of steroid hormones on NOx release depended on steroid dose and day of pregnancy. It is possible that the observed differences in the strength of the stimulatory action of E₂, P₄ and E₂+P₄ on fetal membrane NOx production are associated with an activation of

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INTRODUCTION

The increase in conceptus/fetus estrogen production is usually observed after microvillar attachment of the porcine conceptus to the uterine wall, which occurs approximately on 18th day of gestation [15, 16]. From this time, the placenta and uterus undergo a marked growth connected with the formation of new blood vessels (angiogenesis; [20]). In the pig, characterized by a noninvasive diffuse type of epitheliochorial placenta, placental development is maximal by day 60 of gestation [16]. Angiogenesis during early pregnancy is necessary to increase uterine and fetoplacental blood flow and, consequently, the supply of nutrients from maternal to fetal blood [11]. Thus, placental growth is a critical factor for intrauterine fetal development [20]. The allantoic sac was traditionally considered as the reservoir of fetal wastes. Interestingly, there are high concentrations of ornithine and arginine in porcine allantoic fluid on days 35-40 of gestation which decreased during the second half of gestation [28]. Arginine is a precursor of ornithine and nitric oxide (NO). The latter is a free radical with tremendous physiological importance including its role in the regulation of uterine and fetoplacental blood flow during pregnancy [26, 27].

Nitric oxide is synthesized from L-arginine by nitric oxide synthases (NOSs), the family of enzymes with three identified isoforms: neuronal NOS (nNOS) and endothelial NOS (eNOS), responsible for the continuous basal release of NO, and inducible NOS (iNOS), which is expressed in response to many factors including cytokines [17]. Nitric oxide is a highly reactive inorganic free radical that is well recognized as a major mediator of numerous biological processes including vasodilation [14], ovulation

[23], fertilization [13] as well as successful embryonic attachment and development in the uterus [12]. Nitric oxide also plays an active role in angiogenesis [34] and regulating placental function including relaxation of the vascular bed during pregnancy [24]. In our earlier studies, both eNOS and iNOS were identified in porcine cyclic and gravid uteri [1, 3]. The elevated NOS activity in fetal membranes were found in humans [7], rats [19] and sheep [35]. In rats, the production of NO, measured as total nitrite and nitrate concentration (NO_x, stable products of NO oxidation), increases during mid-gestation and markedly decreases during spontaneous delivery and postpartum period [31]. An increase in NOS content in the uterus may be important in the maintenance of pregnancy, and a decrease in NOS activity at term may be involved in initiation of labour.

It has been suggested that ovarian steroids, progesterone (P₄) and estradiol-17β (E₂), are important modulators of NOS activity [4, 9]. Administration of E₂ to ovariectomized pigs increased NADPH-diaphorase (NOS marker) activity in the endothelium of the uterine broad ligament blood and lymph vessels [32, 33]. In the rat uterus, E₂ inhibited iNOS expression but stimulated eNOS [29] and *in vitro* NO production [5]. The effect of P₄ on NOS expression is tissue-dependent. In the rat uterus and placenta, P₄ up-regulates iNOS expression and NO production [5]. Information concerning the steroid influence on NO production by fetal membranes, especially in the pig, are very limited. Therefore, in this study we determined the effect of E₂ and P₄ on NO_x production by the porcine fetal membranes during the first 60 days of pregnancy, a critical period for fetal development.

MATERIALS AND METHODS

Animals and isolation of fetal membranes

Twenty primiparous crossbred (Large White×Landrace) gilts randomly assigned to a pregnant group, after exhibiting two estrous cycles of normal length, were bred at the onset of estrus (day 0), and then 12 h and 24 h later.

The animals were housed on a farm, and three days before slaughtering animals were transported to the local animal house 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 in Olsztyn.

On days 25, 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. Uterine horns were cut, placentas were carefully isolated from the uterus and slices of fetal membranes were 1/ used immediately for *in vitro* incubation, and 2/ frozen in liquid nitrogen (-80°C) for NO_x measurement. Frozen tissues were washed in sterile saline and homogenized with 0.9% NaCl on ice. Homogenized tissues were centrifuged at 1500×g (10 min, 4°C). Supernatants were used for NO_x determination. Additionally, the blood samples were collected on studied days of pregnancy to determine plasma NO_x concentration. The blood plasma was separated by centrifugation (2000×g, 10 min, 4°C) and stored at -20°C until NO_x determinations were completed.

Incubation of fetal membrane slices

Slices of fetal membranes (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 [22] and the results of our earlier study [2]. After 24 h culture period, the

conditioned media were collected, clarified by centrifugation (1×600 g, 15 min, 4°C) and stored at -80°C for NO_x analysis. Control samples with medium only were included in each experiment (non-tissue controls).

Measurement of NO_x concentration

Total NO_x concentrations were determined using a microplate assay method based on the Griess reaction [2]. 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 NO_x concentrations ranging from 0.05 to 6.9 µg/ml. Results were normalized against the weight of the tissue and expressed as µg/mg of placenta and in the blood plasma as µg/ml.

Statistical analysis

Experimental data are presented as mean±SEM of each experiment (n=5 pigs per each day) performed in triplicates. The statistical significance of differences in NO_x content in fetal membranes and blood plasma among particular days of pregnancy was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (GraphPad PRISM; GraphPAD Software, Inc., San Diego, CA, USA). The *in vitro* effect of E₂, P₄ and E₂+P₄ on NO_x content was calculated by one-way analysis of variance (ANOVA) for repeated measures followed by Dunett's post-hoc test (GraphPad PRISM; USA). A value of p<0.05 was considered as significant.

RESULTS

Fetal membrane and plasma concentration of NO_x

Along with the development of fetal membranes, the gradual increase in fetal membrane NO_x content was observed on days 25 (34.28 µg/mg), 35

(45.78 $\mu\text{g}/\text{mg}$), 40 (67.84 $\mu\text{g}/\text{mg}$) and 60 (77.92 $\mu\text{g}/\text{mg}$) of pregnancy (fig. 1). The significant increase ($p < 0.001$) in NOx content, in comparison to day 25 of pregnancy, was observed on days 40 and 60 of pregnancy. Compared to day 35, a higher level ($p < 0.001$) of NOx in the fetal membranes was found on days 40 and 60 of pregnancy.

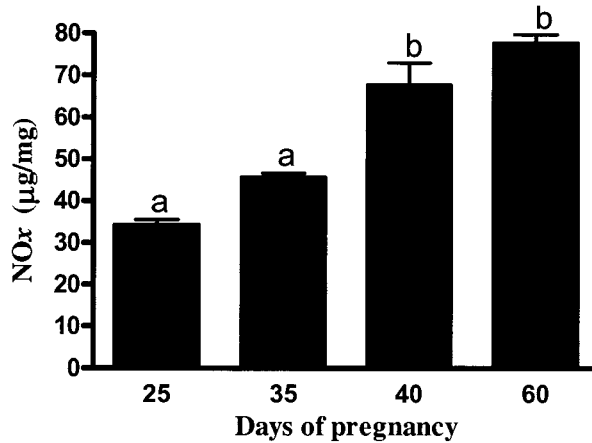


Figure 1. Concentration of total NOx (means \pm SEM) in porcine fetal membranes collected on days 25, 35, 40 and 60 of pregnancy (n=5 per group). Means with different letters are significantly different ($p < 0.01$)

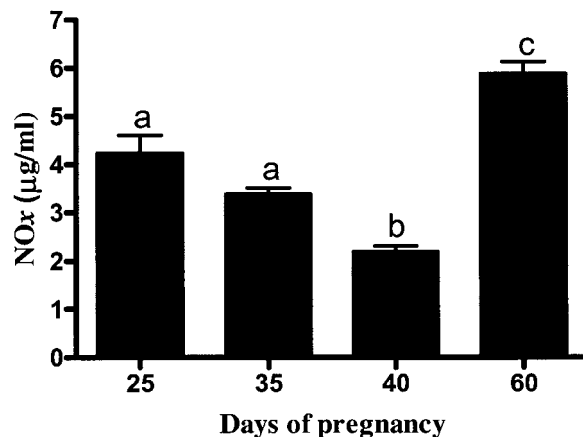


Figure 2. Plasma concentration of total NOx (means \pm SEM) in pigs on days 25, 35, 40 and 60 of pregnancy (n=5 per group). Means with different letters are significantly different ($p < 0.01$)

Plasma concentrations of NO_x on particular days of porcine pregnancy is presented in Figure 2. A gradual decrease in plasma NO_x concentration was observed from day 25 to day 40. A significant increase ($p < 0.001$) in NO_x concentration was noted on day 60 of pregnancy.

The influence of E₂ and P₄ on *in vitro* NO_x production

Two higher doses of E₂ ($p < 0.001$) increased NO_x production by porcine fetal membranes collected on day 25 of pregnancy. The tissues from day 35 released more of NO_x in response to E₂ at a dose of 0.2 ng/ml ($p < 0.05$; fig. 3). The enhancement of medium NO_x content was also noted on day 40 after fetal membrane treatment with E₂ at dose of 0.2 ng/ml ($p < 0.01$; fig. 4). All doses of E₂ stimulated ($p < 0.05$) NO_x production by fetal membranes on day 60 of pregnancy (fig. 4).

Fetal membranes from day 25 increased NO_x production in response to the two lowest doses of P₄ ($p < 0.001$ and $p < 0.01$, respectively). The tissue collected on day 35 of pregnancy released more NO_x after treatment with the highest dose of P₄ ($p < 0.001$; fig. 3). When tissues from day 40 were incubated, the level of NO_x rose after the addition of P₄ at the lowest dose ($p < 0.01$). The two highest doses of P₄ increased ($p < 0.05$) NO_x secretion on day 60 of pregnancy (fig. 4).

The combination of the two highest doses of E₂+P₄ increased ($p < 0.001$ and $p < 0.05$, respectively) NO_x medium content during incubation of porcine fetal membranes collected on day 25 of pregnancy. No effect of combined steroid treatment was found on day 35 of pregnancy (fig. 3). The two lowest doses of both hormones augmented ($p < 0.01$ and $p < 0.05$, respectively) fetal membrane NO_x secretion on day 40 of pregnancy. The combined two highest doses of E₂ and P₄ enhanced ($p < 0.01$) NO_x production by fetal membranes on day 60 of pregnancy (fig. 4).

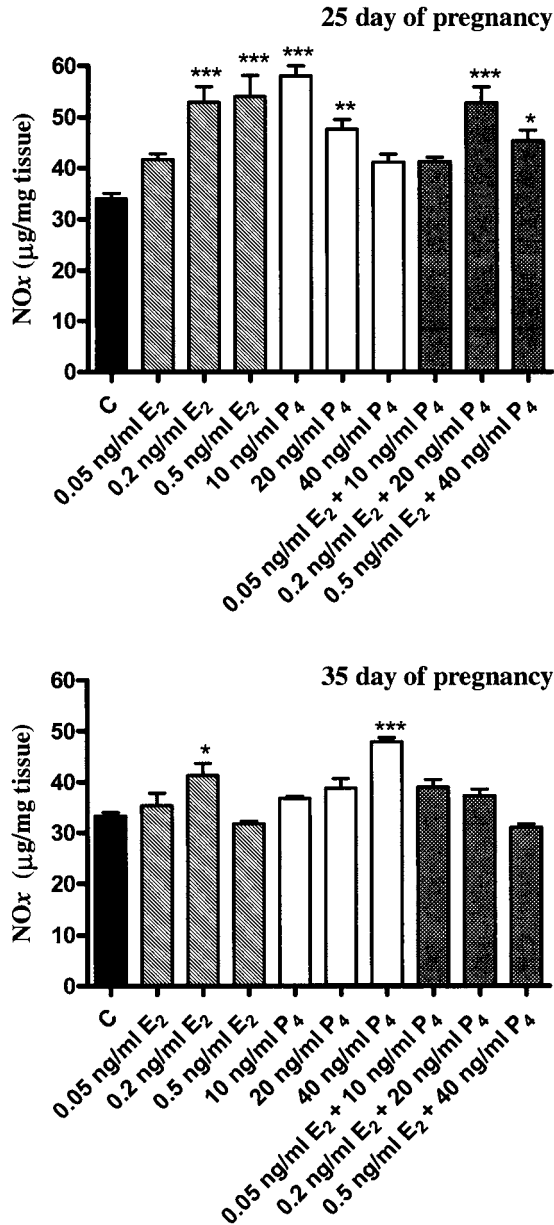


Figure 3. The effect of E₂ and/or P₄ on in vitro NO_x production (means±SEM) by porcine fetal membranes on days 25 and 35 of pregnancy (n=5 per group). Tissues (100 mg) were incubated for 24 hours in DMEM (without phenol red) supplemented with 10% of calf serum. Asterisks indicate means different from controls (C), *p<0.05, **p<0.01, ***p<0.001

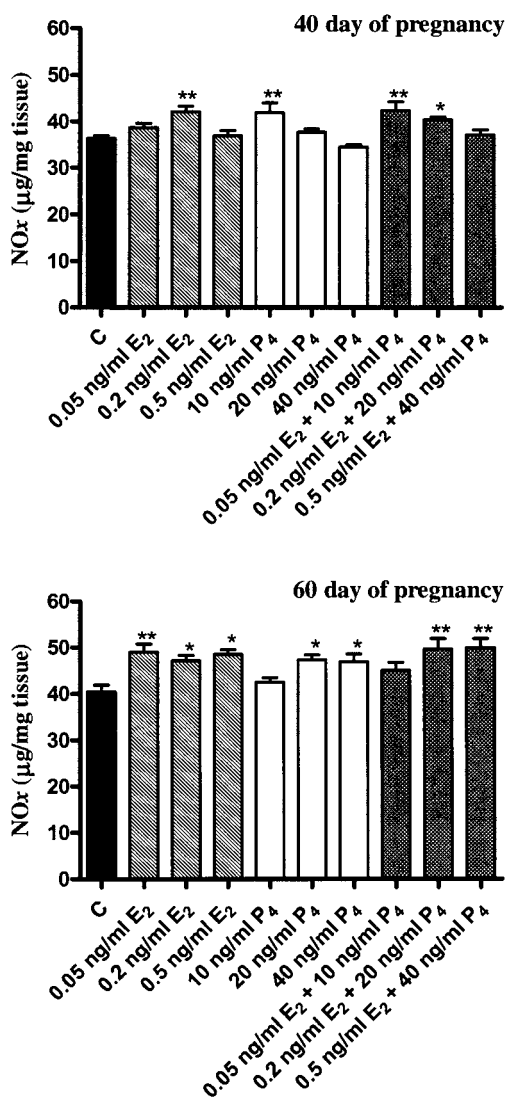


Figure 4. The effect of E₂ and/or P₄ on in vitro NOx production (means±SEM) by porcine fetal membranes on days 40 and 60 of pregnancy (n=5 per group). Tissues (100 mg) were incubated for 24 hours in DMEM (without phenol red) supplemented with 10% of calf serum. Asterisks indicate means different from controls (C), *p<0.05, **p<0.01, ***p<0.001

DISCUSSION

In this study we reported for the first time porcine fetal membrane concentration of NO_x and we demonstrated the effect of E₂ and P₄ on NO production by the membranes during the first half of pregnancy. The allantochorial NO_x concentration gradually increased from day 20 to day 60 of pregnancy. The formation of the amnion by folding is completed on day 18. The allantois appears approximately on day 14 of gestation and grows rapidly. On day 19, the mesodermal covering of allantois makes contact with a small area of the chorion. Allantoic vessels proceed into the chorion and by day 30 of pregnancy, the chorion is extensively vascularized by allantoic blood vessels. The rapid increase in allantochorial fluid volume, connected with increased permeability of placental cells to water is observed from day 20 to day 30 of gestation [16] and from day 50 to term [21, 22]. The elevated level of E₂ and P₄ 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 [21, 25]. Additionally, the rapid increase in allantoic fluid volume around day 30 of pregnancy is correlated with the initial expansion of chorioallantoic membranes and establishment of intimate contact between placenta and endometrial surfaces. Estrogens and NO are known to increase the permeability of cells to water. Our previous study revealed fluctuations in NO_x production in the porcine endometrium with peaks on days 10-15, 30 and 60 of pregnancy [2]. Thus, increase in fetal membrane NO_x concentration observed in this study may be connected with changes in the accumulation of allantoic fluid and placental length [16].

We found that the pattern of plasma NO_x concentration differs from that observed in porcine fetal membranes. A progressive decrease in plasma NO_x from day 25 to day 40 of gestation was followed by an increase on day 60 of gestation. The profile of plasma NO_x level is parallel to that observed in endometrial tissue during pregnancy in the pig [2]. These findings suggest that the plasma NO_x concentration may reflect maternal endometrial NO production.

We noticed the stimulatory effect of E₂ and/or P₄ on NO *in vitro* production by porcine fetal membranes. The medium content of NO_x depended on the

steroid type, treatment dose and day of pregnancy. E_2 or P_4 administered alone caused an increase in NO production on all studied days of pregnancy. No effect of combined steroid treatment was found on day 35 of pregnancy. The high sensitivity of fetal membranes to E_2 on day 25 of gestation may be associated with a progressive increase in placental estrogen production from day 20 to 30 of porcine gestation. Our earlier studies revealed a dose-dependent stimulation of *in vitro* endometrial NOx production after E_2 treatment. The most significant increase in NOx concentration was observed on days 30, 40 and 60 of gestation [2]. The rapid increase in allantoic fluid volume on days 20-30 was described by Knight et al. [16]. In addition, the authors reported a decrease in allantoic E_2 concentration and allantoic fluid volume between day 30 and 40. The final period of allantoic fluid accumulation occurred between day 40 and 60 of pregnancy. Because the estrogen as well as NO are known to increase the permeability of placental cell to water, our *in vitro* data may suggest NO participation in the regulation of the allantoic fluid volume and placental length.

It is possible that the observed in this study differences in the strength of the stimulatory action of E_2 and/or P_4 on fetal membrane NOx production are associated with the activation of different isoforms of NOS. In sheep, expression and activity of eNOS increased after E_2 treatment as well as during advancement of pregnancy in the uterus and several other tissues [9, 18]. In contrast, studies by Yallampalli and Dong [29] revealed that E_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 activity. Thus, the exact effect of E_2 on NOS activity may be species-dependent.

Similarly to E_2 , two/three peaks of P_4 plasma level were demonstrated during pregnancy in the pig [16]. Our results showed that P_4 enhanced fetal membrane NOx production in all studied days of gestation in pigs. The previous studies demonstrated that in porcine endometrium, P_4 enhanced NOx concentration, but not later than day 35 of gestation [2]. Although P_4 is well recognized as a major gestational hormone regulating uterine contractility, the precise events involved in the actions of this hormone during pregnancy are not well understood. Yallampalli et al. [30] implied

that P_4 up-regulates NO synthesis in the rat uterus. This suggestion was also confirmed by Dong et al. [8] who showed that P_4 is required for maintaining iNOS expression in the rat uterus during pregnancy. In addition, NO production and/or NOS expression may be affected by interaction between P_4 and E_2 . In rats, E_2 inhibited NO production *via* reducing iNOS expression only in the absence of P_4 [29]. It is possible that progesterone up-regulates a specific isoform of NOS in the gravid uterus. Pregnancy-associated increases in NO synthesis might also be related to cytokine action since cytokines modulate NO production in a variety of tissues and their uterine levels vary during pregnancy [6, 10]. Moreover, E_2 is known to activate progesterone receptors and may be necessary for efficient P_4 action. The combination of E_2 and P_4 , in the current study, was sometimes more effective in stimulation of NO production than application of individual hormones.

In summary, we demonstrated that fetal membrane NOx content increased with the advancement of the first half of porcine pregnancy. Moreover, E_2 and P_4 stimulated *in vitro* production of NO by porcine fetal membranes in a dose- and day-dependent manner. The differences in NOx production observed during pregnancy in pigs may result from the activation of different isoforms of NOS.

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