Distinct regulation of nitric oxide and cyclic guanosine monophosphate production by steroid hormones in the rat uterus

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It has previously been reported that uterine nitric oxide (NO) production is enhanced during rat pregnancy compared to non-pregnant, labouring or postpartum states. The present hypothesis is that these changes in uterine NO production during pregnancy are caused by the interplay of oestrogen and progesterone. It is further postulated that changes in cyclic guanosine monophosphate (cGMP) production closely follow the changes in uterine NO synthesis. To test these hypotheses a variety of hormonal regimens (17β-oestradiol, progesterone and combinations) were applied to different rat models (prepubertal, non-pregnant intact and ovariectomized as well as pregnant rats). The production of nitric oxide (NO) as well as basal and in-vitro NO-stimulated cGMP tissue content were measured in parallel. NO production was measured by the accumulation of nitrites and nitrates in a 24 h incubation medium as analysed by Greiss reaction. cGMP content was measured by radioimmunoassay. Diethylenetriamine/NO (DETA/NO) was used as NO donor. NO production in the rat uterus was markedly increased by pregnancy compared to other physiological (prepubertal, or cycling dioestrus) and experimentally induced (OVX) states. In contrast, uterine cGMP was significantly decreased in pregnancy. Pregnancy also inhibited the elevation in uterine cGMP after in-vitro NO challenge. Chronic 17β-oestradiol treatment in prepubertal and/or OVX models increased NO production and also mimicked the effect of pregnancy on cGMP. Administration of progesterone in prepubertal rats induced a parallel decrease in both uterine NO and cGMP. In conclusion, sex steroid hormones distinctly regulate uterine NO and cGMP production depending upon the dose and regimen used, as well as the animal’s reproductive state.

Key words: guanylate cyclase/myometrium/nitric oxide/oestrogen/progesterone

Introduction

Nitric oxide (NO) is synthesized from one of the terminal guanidino nitrogens of L-arginine by the three isoforms of NO synthases (NOS). These NOS isoforms have been described in a variety of tissues including myometrium. Two of the isoforms (endothelial, eNOS and neuronal, nNOS) are constitutive, Ca2+ calmodulin-regulated and the third isoform (inducible, iNOS) is inducible, Ca2+ calmodulin-independent. More importantly, these isoforms differ in the amounts of NO they generate (i.e. picomoles for the constitutive and nanomoles for the inducible NOS) (Berdeaux, 1993).

Initially it was believed that the sole target for NO is the haem moiety of the soluble guanylate cyclease, which contains protoporphyrin IX and iron in the ferrous form (Murad et al., 1990). Indeed, most of the physiological effects of NO can be explained by activation of soluble guanylate cyclease which catalyses the formation of cyclic guanidine monophosphate (cGMP) which in turn stimulates different kinases (Cornwell and Lincoln, 1989), activates K+ channels (Robertson et al., 1993) and lowers cytosolic calcium (Twort and vanBreemen, 1988). More recently, however, it has become increasingly evident that, besides guanylate cyclease, NO has other possible functional targets such as ion channels (Fukami et al., 1998; Li et al., 1998), soluble ADP-ribosylating enzymes (Brune and Lapetina, 1990) and transcription factors through which NO can directly affect gene transcription (Kroncke et al., 1994) or mRNA translation via activation or deactivation (Weiss et al., 1993). On the other hand, the second messenger cGMP may arise from the activity of other enzymes besides the NO-stimulated soluble guanylate cyclease such as hae-moxygenases, which stimulate the soluble guanylate cyclease via carbon monoxide (Acevedo and Ahmed, 1998) or particulate guanylate cyclases, which are activated by peptide ligands (e.g. natriuretic peptides) (Foster et al., 1999).

Although there is strong evidence supporting the role of NO and NO-generated elevations in cGMP as regards smooth muscle (i.e. vascular, airway, gastrointestinal) relaxation (Moncada et al., 1991), there is somewhat conflicting data on
the involvement of the NO and/or cGMP pathway within the myometrium as a regulator of uterine quiescence or contractility (Sladek et al., 1997; Garfield et al., 1998a,b). Several groups reported on the relaxant effects of NO on myometrial contractility (Yallampalli et al., 1994; Buhimschi et al., 1995; Izumi and Garfield, 1995; Norman et al., 1997; Bradley et al., 1998; Kuenzli et al., 1998; Ekerhovd et al., 1999). However, a number of investigators have suggested that myometrial cGMP concentrations may not directly correlate either with NO concentrations or with the degree of smooth muscle relaxation (Nakatsu and Diamond, 1989; Weiner et al., 1994a; Bradley et al., 1998; Kuenzli, 1998; Hennan and Diamond, 1998; Word and Cornwell, 1998).

It has previously been reported that uterine NO production is enhanced during rat pregnancy compared to non-pregnant, labouring or postpartum states (Yallampalli et al., 1994; Buhimschi et al., 1996). Furthermore, there was an intriguing evolution in uterine NO production throughout gestation, with tissues collected on days 20 and 21 showing a further increase in NO compared to earlier in pregnancy (Buhimschi et al., 1996). Uteri during labour showed a much lower NO synthetic activity compared to any time during pregnancy (Ali et al., 1996). It is well established that in the rat during the days before parturition there are critical alterations in hormone concentrations which enhance uterine contractility and initiate labour (Garfield et al., 1998a). The fall in progesterone (progesterone withdrawal) which occurs after day 18 of gestation in rats is complemented by the rise in oestrogen, which starts on day 20. Oestrogen concentrations remain highly elevated before and during parturition on day 22 (Puri and Garfield, 1982).

A link between steroid hormones (namely oestrogen and progesterone) and the NO–cGMP pathway exists in several tissues. During pregnancy myometrial quiescence in the rat coincides with elevated progesterone concentrations. Prevention of progesterone withdrawal in rats prevents the fall in uterine NO production at term, whereas administration of antiprogesterone before term induces a preterm decrease in myometrial NO (Buhimschi et al., 1996; Sladek and Roberts, 1996). Earlier studies have also shown that while N·NAME (Nω-nitro-L-arginine methyl ester), an inhibitor of NO synthesis, does not induce preterm labour by itself, it does significantly potentiate the effect of antiprogestins in inducing preterm parturition in rats (Yallampalli et al., 1996). These studies suggest that during rat pregnancy myometrial NO production is highly controlled by progesterone. In the non-pregnant state, however, oestrogen seems to be the steroid hormone that increases Ca2+-dependent NOS activity in a number of tissues from female and male guinea-pigs (Weiner et al., 1994b). However, depending on the animal model used, uterine NOS activity (mainly Ca2+-dependent) was either unaffected (Weiner et al., 1994b), decreased (Batra et al., 1998) or increased (Figueroa and Massmann, 1995) by oestrogen. In the myometrium, at least, it is still not clear how sex steroid hormones modulate NOS activity and whether cGMP exhibits concomitant changes. More importantly, the effects of alterations in oestrogen to progesterone ratio, a better determinant of reproductive function than changes in individual hormones, remain unclear.

The present hypothesis is that oestrogen and progesterone are major modulators of uterine NO production in both the pregnant and non-pregnant states. It is further postulated that cGMP will follow closely the changes in uterine NO synthesis. To test these hypotheses the concentration-dependent effects of oestrogen and progesterone were investigated, individually and in combination, on NO production and cGMP content in the rat uterus using different animal models (prepubertal, non-pregnant intact and ovariectomized as well as pregnant rats).

Materials and methods

Animals and in-vivo treatments

Animals were received in the animal care unit from Harlan Sprague Dawley (Houston, TX, USA) and were allowed free access to food and water. They were housed in separate cages and maintained on a 12:12 h light:dark cycle. All procedures were approved by the Animal Care and Use Committee of the University of Texas Medical Branch.

Timed pregnant rats

Female nulliparous rats were received in the animal care unit on day 14 of gestation (day 0 = sperm plug observed). For experiments evaluating nitrate production or cGMP content during gestation, groups of animals were killed daily on day 18, 19, 20, 21, on the morning of day 22 (the expected day of delivery), during active labour (1–3 pups expelled) and on the morning of the first postpartum day. Uterine tissue was collected and processed for nitrite production and cGMP content as described below. For experiments where the effect of 17β-oestradiol on nitrite production or cGMP content was evaluated, pregnant rats on day 17 of gestation were injected with one dose of either 20 or 100 µg/kg 17β-oestradiol. Six hours later animals were killed and uterine tissues collected. The control group was injected with sesame oil.

Prepubertal rats

Prepubertal rats were received on day 17 of life weighing 50–70 g and were weaned on day 19 of life. Beginning with day 21 of life, the following three regimens of injections were employed.

Protocol 1. 17β-oestradiol, progesterone, 17β-oestradiol + progesterone, or vehicle were administered daily for 3 consecutive days. 17β-Oestradiol was administered in doses of 1, 5, 20, 100 or 500 µg/kg/day. Progesterone was administered in doses of 10, 20 or 40 mg/kg/day. For the combination, 17β-oestradiol (100 µg/kg/day) with progesterone (20 mg/kg/day) were injected simultaneously. The daily dose was diluted in sesame oil and split into two injections, of 0.1ml each. All animals were killed on the morning of the fourth day by CO2 inhalation immediately before uterine tissues were collected.

Protocol 2. Prepubertal animals were first primed with 20 µg/kg 17β-oestradiol and 18 h later injected with a single dose of either 10, 20 or 40 mg/kg progesterone diluted in 0.1 ml sesame oil. Control animals received two injections of vehicle 18 h apart. Tissues were collected after 24 h from the progesterone injection.

Protocol 3. Prepubertal rats were acutely injected with 20, 100 or 500 µg/kg 17β-oestradiol diluted in 0.2 ml sesame oil. Control animals were injected with 0.2 ml oil only. Animals were killed after 2, 6 and 24 h from the oestradiol injection and uterine tissue collected.

Non-pregnant intact rats

Virgin rats weighing 150–170 g were separated by vaginal smear into the three phases of oestrous cycle (dioestrus, pro-oestrus and oestrus) and uterine tissue was collected.
Virgin rats weighing 150–170 g were ovariectomized 14 days prior to drug administration. 17β-oestradiol was injected in doses of 10 or 100 μg/kg/day for 3 days. The control group was injected with 0.2 ml sesame oil for 3 days.

**Non-pregnant ovariectomized rats**

**Tissue incubation and nitrite (NO2⁻) assay**

Pieces of uterine tissue (weighing 100–120 mg from mature animals and 60–80 mg from prepubertal rats) were minced thoroughly in minimum essential medium without phenol red (MEM; Gibco, Grand Island, NY, USA), and further cut with a scalpel blade in a relatively consistent number of full-thickness segments. In some of the treatment groups (e.g. control or progesterone-treated animals) it was necessary to pool tissue from two different animals to accumulate the necessary mass of tissue. *n* values represent the number of incubations. The tissue was further incubated in MEM with 1% antibiotics (penicillin-streptomycin, Gibco) in a CO₂ incubator with a humidified chamber at 37°C for 24 h. Following incubation, the medium was collected, clarified by centrifugation at 16 000 g for 15 min at 4°C and stored at −70°C for a maximum of 2 weeks. In each 24-well incubation plate, at least two wells with medium only were incubated and labelled as non-tissue controls. The 100–120 mg tissue from mature rats was incubated in 0.5 ml medium and the 60–80 mg tissue from prepubertal rats was incubated in 0.4 ml medium. After incubation the tissues were weighed before (wet weight) and after overnight drying at 60°C (dry weight).

Nitrates in the medium were reduced to nitrites by the acid-treated cadmium method (Davison and Woof, 1978) using 300 mg Cd for 0.7 ml medium diluted 1:3.5. Total nitrites (endogenous nitrites in the medium plus nitrites obtained after reduction of nitrates) were measured using the Greiss reaction (Green et al., 1982). Briefly, 50 μl of Greiss reagent was added to 50 μl aliquots of reduced medium and optical densities were measured at 550 nm in a microtitre plate using NaNO₂ as standards. The values obtained from the wells in which medium alone had been incubated without tissue (non-tissue controls representing background non-specific optical density measurements) were averaged for each incubation plate and the average subtracted from the values of each well containing tissue sample. The concentration of total nitrites in each well was normalized against the wet weight of the tissue. It had previously been observed that the major determinant of reproducibility is the reduction step. For this reason NaNO₃ aliquots at the same dilution as those used for the standard curve were also run through the reduction process as an indication of efficacy. In addition, all samples from each individual experiment (the values presented in each figure panel) were reduced simultaneously and the results expressed as percentage of the values from each control group. The control group comprised the animals treated with vehicle or, in the case of the non-pregnant cycling...
animals, the dioestrous group. A slight but not statistically significant increase in the ratio dry/wet weight in the oestradiol-treated prepubertal animals from a mean ± SEM of 12.8 ± 0.7% in the control group to 13.0 ± 0.7% following 3 day treatment with 1 µg/kg 17ß-oestradiol and up to 13.8 ± 0.2% following 3 day treatment with 100 µg/kg 17ß-oestradiol was observed. It was concluded therefore that normalization of NO production against wet weight was appropriate. The potential error due to a differential water content between the various regimens of steroids was assumed to be insignificant.

cGMP measurements

Uterine strips (~20 mg) were incubated for 60 min in 10 ml Krebs solution maintained at 37°C and continuously bubbled with 95% O₂ and 5% CO₂. In some of the experiments, the strips were exposed to the NO donor diethylenetriamine/NO (DETA/NO: 1–100 µmol/l) during the last min of incubation (1, 3, 10 min). No phosphodiesterase inhibitor was added because we did not wish to change artificially the cGMP content in a direction that may not occur in vivo.

At the end of the incubation period, tissue samples were immediately transferred into liquid nitrogen and homogenized in ice-cold 10% trichloroacetic acid. The homogenate was centrifuged at 10 000g for 15 min at 4°C. The pH of the supernatant was neutralized by the addition of excess CaCO₃. The sample was then clarified by low speed centrifugation (1000 g) and aliquots were assayed for cGMP in duplicate by radioimmunoassay using a cGMP-[¹²⁵] assay system with Amerlex-M™ magnetic separation (Amersham, Arlington Heights, IL, USA). In some of the experiments we also measured cGMP concentration in the 10 ml incubation medium which was close to zero.

Chemicals used

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless specified. DETA/NO was a kind gift from Dr L. Keifer (National Cancer Institute, Frederick, MD, USA). The Krebs solution was of the following composition (in mmol/l): 116 sodium chloride, 5.4 potassium chloride, 2.5 calcium chloride, 12 monosodium phosphate, 11.2 d-glucose, 22.0 sodium bicarbonate and had a pH of 7.4.

Statistical analysis

Results are expressed as mean ± SEM. After subjecting all data sets to a test of normality (Kolmogorov–Smirnov), statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) (parametric) or Kruskall–Wallis ANOVA on ranks (non-parametric) followed by Dunnett’s or Dunn’s post-hoc tests (versus control groups), or Student–Newman–Keuls (among multiple groups) as appropriate. A two-tailed P < 0.05 was used to denote statistical significance.

Results

Uterine NO and cGMP production during gestation

The production of total NO₂⁻ in vitro by the rat uterus was estimated at 186.24 ± 16.5 nmol/g on day 19 of gestation (n = 14) (Figure 1A). Uteri obtained from animals on day 20 and 21 of gestation exhibited a statistically significant increase in NO₂⁻ accumulation in the incubation medium compared to animals on day 19 (P < 0.05). Uteri during labour and postpartum produced significantly less NO than uteri on day 19. When cGMP tissue content was evaluated, a completely different trend was seen, with highest uterine cGMP observed in labouring tissues. However, statistical analysis did not show any differences throughout gestation when day 19 uterine cGMP tissue content (2.98 ± 0.1 nmol/g, n = 6) was taken as reference. To investigate whether the uterine tissue was able or not to respond to NO by increasing cGMP tissue content, uterine strips were incubated on either day 19 of gestation or during labour with the NO donor DETA/NO (100 µmol/l) and cGMP was measured after 10 min of exposure (Figure 1C). The results of this experiment showed that DETA/NO was unable to induce a statistically significant increase in cGMP in both tissues studied.

These findings suggest that the NO production by rat uterus is maximal on days 20 and 21 of gestation followed by a marked decrease during labour and postpartum. However, this high NO output is not paralleled by an increase in cGMP tissue content, rather the state of pregnancy induces a refractoriness of the myometrium to the cGMP-generating ability of NO.

It was further postulated that the above findings may be due to the interplay of steroid hormones (oestrogen and progesterone) during rat pregnancy. To test this hypothesis,
Figure 3. (A) Total nitrites (nmol/g nitrites + reduced nitrates; as % of dioestrus mean) produced by uterine tissues from animals at different stages of the oestrous cycle (dioestrus: n = 7; pro-oestrus: n = 8; oestrus: n = 7 incubations representing seven, eight and seven different animals in each group, respectively). Statistical analysis: one-way analysis of variance (ANOVA) (P = 0.047). The mean with asterisk is statistically different from the dioestrus mean at the level of P < 0.05 (Dunnett’s test). (B) Tissue cGMP content (as %, mean ± SEM, from dioestrus concentrations) of full-thickness strips of uteri from animals at different stages of the oestrous cycle (dioestrus: n = 7; pro-oestrus: n = 8; oestrus: n = 7 animals). Statistical analysis: one-way ANOVA (P = 0.52). (C) cGMP content (nmol/g) in uterine tissues of animals during pro-oestrus (n = 4) and oestrus (n = 5) in the absence (control: CRL) or presence of the NO donor diethylenetriamine/NO (DETA/NO; 0.1 mmol/l for 10 min). Statistical analysis: Student’s t-tests between control and DETA/NO-treated: P = 0.047 (pro-oestrus); and Mann–Witney rank sum test: P = 0.016 (oestrus).

Uterine NO and cGMP production during different physiological states

In order to examine the effect of hormonal manipulations on different animal models it was necessary to compare baseline concentrations (both for NO and cGMP) between these models. NO production by the rat uterus was markedly increased by the state of pregnancy when compared to other physiological (prepubertal: 52.71 ± 9.7 nmol/g, n = 18; cycling dioestrus: 27.14 ± 5.1 nmol/g, n = 7) and experimentally induced (49.8 ± 5.1 nmol/g, n = 5) states (Figure 2). In contrast, uterine cGMP content was significantly decreased by the state of pregnancy compared to immature (32.59 ± 4.6 nmol/g, n = 7) or cycling dioestrus animals (27.14 ± 5.1 nmol/g, n = 7) (P = 0.03).

Uterine NO and cGMP production in the uterus of non-pregnant rats during oestrous cycle

To examine how changes in endogenous steroids may impact on uterine NO and cGMP, the production of total NO and cGMP content were measured during the stages of oestrous cycle and it was observed that the rat uterus during pro-oestrus produced significantly more NO compared to during dioestrus and oestrus (Figure 3A). In contrast, cGMP content did not differ significantly between the three phases of the oestrus cycle (Figure 3B). Figure 3C illustrates the uterine cGMP content after 10 min exposure to 100 μmol/l DETA/NO, and

experiments were designed using hormonal manipulations in animals during different physiological states and NO and cGMP were analysed in parallel. The results of these experiments will be described below.
it suggests that in the non-pregnant state exogenous NO is able to induce a significant increase in myometrial cGMP. This increase was of the same magnitude in both pro-oestrous and oestrous animals.

**Effects of hormonal manipulation in ovariectomized rats**

17β-oestradiol injection in ovariectomized animals resulted in a dose-dependent rise in NO production (Figure 4). Whereas 10 µg/kg/day 17β-oestradiol for 3 days produced only a small increase, the administration of 100 µg/kg/day 17β-oestradiol was followed by a sustained and statistically significant (P = 0.025) increase in the nitrite production.

**Effects of hormonal manipulation in prepubertal rats**

Administration of 17β-oestradiol (1, 5 and 20 µg/kg/day) for 3 days (Protocol 1) produced a dose-dependent increase in NO production in the rat uterus (Figure 5A). A decrease towards values not different from control was recorded with the highest dose (100 µg/kg). Interestingly, at a lower dose of oestrogen prepubertal animals were much more responsive on a percentage change basis. Unlike NO, cGMP content did not change significantly across the different oestradiol concentrations (Figure 5B). When uterine strips were challenged in vitro with DETA/NO (Figure 5C and D), cGMP increased only in control uteri for exposure to 0 dose (P < 0.01). The strips of uterine tissues were incubated in vitro for 50 min. in oxygenated Krebs solution and for an additional 10 min in the absence (0 dose: open symbols) or presence of the NO donor diethylenetriamine/nitric oxide (DETA/NO: closed symbols: 1, 10 or 100 µmol/l). The time of exposure to DETA/NO was varied (1, 3 or 10 min). n = 5, representing five different animals at each time point. Statistical analysis: two-way ANOVA followed by Dunnett’s tests: P < 0.001 both for oestrogen and DETA/NO variables. The mean with asterisk is significantly different from 0 dose values (P < 0.01). (D) The strips of uterine tissues were incubated in vitro for 50 min. in oxygenated Krebs solution and for an additional 10 min in the absence (0 dose: open symbols) or presence of 100 µmol/l DETA/NO. The time of exposure to DETA/NO was varied (1, 3 or 10 min). n = 5, representing five different animals at each time point. Data are presented as mean ± SEM. Statistical analysis: two-way ANOVA followed by Dunnett’s tests: P = 0.001 for oestrogen dose and P = 0.002 for the time of exposure variable. The means marked are different from time 0 values (P < 0.01). E2 = 17β-oestradiol.
cGMP content was consistently decreased in animals treated with progesterone for 3 days compared to control animals (Figure 6B). Since the effects of progesterone in an animal model without a prior exposure to oestrogen may seem controversial due to the lack of progesterone receptors, an experiment was also designed where the animals were primed with a single dose of oestradiol 18 h prior to the administration of progesterone (Protocol 2). An increase in NO production was only observed when the lowest dose of progesterone (10 mg/kg) was used (Figure 7A). Myometrial cGMP content was again significantly decreased \((P = 0.037)\) by the two higher doses of progesterone (20 and 40 mg/kg) (Figure 7B).

These results suggest that in prepubertal rats chronic oestrogen treatment increases uterine NO output while progesterone opposes this effect. The increase in NO production is not paralleled by an increase in cGMP. Moreover, chronic oestrogen treatment induces a decrease in cGMP generation in response to NO \textit{in vitro}. Progesterone lowers uterine NO production and is also able to decrease uterine cGMP both in presence and absence of oestrogen priming.

To identify the time course of 17\(\beta\)-oestradiol on the stimulatory effect of NO production and inhibitory effect on cGMP, an experiment was designed where prepubertal animals were injected acutely with 17\(\beta\)-oestradiol (three doses) and NO and cGMP were measured at 2, 6 or 24 h from the oestrogen injection (Protocol 3). It was possible to determine that at 6 h (Figure 8A) with all doses used (20, 100 and 500 \(\mu\)g/kg) uterine NO production was significantly elevated \((P < 0.01)\). The concentrations returned to normal after 24 h in the groups injected with 20 and 100 \(\mu\)g/kg. The highest dose produced a sustained increase in NO production, which remained statistically significant after 24 h \((279.9 \pm 62.6\% , P < 0.01)\). Myometrial cGMP content in these animals (Figure 8B) increased significantly \((P < 0.01)\) only in the group injected with the lowest dose of 17\(\beta\)-oestradiol (20 \(\mu\)g/kg) compared to the group of animals killed at time 0 (before injection).
Regulation of NO and cGMP by steroid hormones in rat uterus

Figure 8. Nitric oxide (NO) production (A) and cGMP content (B) in uterine tissues of prepubertal rats injected acutely with 17β-oestradiol 20 µg/kg (circles), 100 µg/kg (squares) or 500 µg/kg (triangles). Uterine tissues were collected before (time 0) and at 2, 6 or 24 h after the acute administration of 17β-oestradiol. For NO production (A) n = 12, representing 12 different animals. For cGMP content (B) n = 6, representing six different animals. Data are presented as mean ± SEM. Statistical analysis: two-way analysis of variance followed by Dunnett’s tests: \( P < 0.001 \). The means marked with asterisk are significantly different from the NO or cGMP values before injection, respectively (\( P < 0.01 \)). \( E_2 = 17β\)-oestradiol.

This increase occurred as early as 2 h after injection. At 6 h (when these tissues have the maximal NO-generating ability) cGMP content decreased significantly compared to concentrations recorded at the previous time point (2 h) both in the groups injected with 20 and 100 µg/kg oestradiol. In animals injected with the highest dose of oestradiol, cGMP content showed a sustained trend towards a decrease despite the sustained NO release by the myometrium.

**NO and cGMP production in the pregnant uterus after administration of a single dose of oestrogen**

To investigate whether the same doses of 17β-oestradiol that were able to elevate NO production (at 6 h) in the prepubertal model would have a similar effect on the pregnant rat uterus, pregnant animals were injected (on day 17 of gestation) with 20 and 100 µg/kg 17β-oestradiol and the uteri sampled at 6 h. It was observed that oestrogen was not able to affect NO production if the animals were pregnant (Figure 9A). cGMP was also unaltered by this hormonal manipulation in pregnant animals (Figure 9B).

**Discussion**

The results of the present study demonstrate that (i) NO production by the rat uterus was markedly increased by pregnancy compared to other physiological (prepubertal, or cycling dioestrus) and experimentally induced (ovariectomized) states. In contrast, uterine cGMP content was significantly decreased by pregnancy. Pregnancy also inhibited the elevation in uterine cGMP after in-vitro NO challenge. (ii) Chronic oestradiol treatment in non-pregnant models mimics the effects of pregnancy on NO and cGMP: NO production is generally increased by oestrogen with a dissociation in myometrial NO and cGMP production. In addition, oestrogen treatment decreased the ability of NO to elevate cGMP in the rat uterus. (iii) Progesterone decreases NO and cGMP in parallel in the rat uterus and antagonizes the stimulatory effect of oestrogen on NO production. These studies suggest that the increase in NO and decrease in cGMP during pregnancy in the uterus may be a consequence of elevated oestradiol concentrations during pregnancy. Moreover, steroid hormones may alter NO and cGMP production by a complex and intricate mechanism, and a change in NO may not necessarily result in parallel changes in cGMP. The effect of sex steroid hormones on uterine NO and cGMP production is complex and depends upon the animal model, dose and regimen used.

Previous studies have shown that NO inhibits uterine contractility both in isolated strips of pregnant myometrium as well as in vivo. Authentic NO gas, L-arginine (the substrate for NO) (Izumi *et al.*, 1993; Yallampalli *et al.*, 1994) and NO donors (Buhimschi *et al.*, 1996; Sladek and Roberts, 1996; Ali *et al.*, 1997) are all capable of inhibiting myometrial...
contractility. Previous studies have also shown that there is an absolute elevation in the NO and cGMP production in the pregnant rat uterus (Yallampalli et al., 1994; Buhimschi et al., 1996) which declines at term. However, the evidence for an interaction between NO and cGMP systems within the myometrial smooth muscle cell remains circumstantial. The action of NO on the myometrium may be through an effect independent of soluble guanylate cyclase and/or the increase in myometrial cGMP content may be the result of activation of particulate guanylate cyclases or haemoxigenases, both of which are expressed in the rat uterus (Acevedo and Ahmed, 1998; Vaillancourt et al., 1998). The relationship between NO and cGMP can be even more intricate as several feedback loops between NO and cGMP (Inoue et al., 1995; Miller et al., 1996) or between intracellular Ca$^{2+}$ and NO-induced cGMP synthesis (Xu et al., 1994) have been described at least for vascular smooth muscle cells. The present finding, that changes in cGMP in response to physiological states, sex steroid hormones or during the ovulatory cycle do not necessarily mirror changes in NO, is in support of separate mechanisms controlling NO and cGMP production in the uterus: NOS enzymes, guanylate cyclases and phosphodiesterases (the enzymes that degrade cGMP) may all be distinct targets of hormonal modulation.

NOS enzymes, in particular the constitutive endothelial isoform, are up-regulated both by the state of pregnancy and oestriadiol treatment (Weiner et al., 1994b; Magness et al., 1997; Vagoni et al., 1998). Khorram et al. (1999) has recently shown that both endogenous ovarian steroids and exogenous sex hormones markedly influence uterine NOS expression in women with differential spatial responses in endometrium versus the myometrium.

The present data in non-pregnant and prepubertal animals suggest that oestrogen up-regulates NO production in the myometrium whereas progesterone opposes this effect. However, when administered during pregnancy, the same dose of oestrogen does not have any consequences (Figure 9). One possible explanation is that pregnant animals have a much higher baseline NO production compared to prepubertal or ovariectomized animals (Figure 2) (probably due to the very high endogenous oestrogens associated with pregnancy) and the lack of response to exogenous oestrogens may mean that the tissue has already reached its maximal NO output that can be afforded by oestrogen. Interestingly, there may also be some degree of inhibition due to progesterone action, which may explain why, after progesterone withdrawal occurs (on 19 of gestation in rats), NO production increases further (on days 20 and 21 of gestation: Figure 1). However, the marked decrease in NO during parturition has to be induced by other mechanisms that override the stimulatory effect of oestradiol on NO since it occurs in the presence of highly elevated oestrogen concentrations associated with the parturition process (Puri and Garfield, 1982).

Another aspect that deserves comment, and may explain the differences in NO production among the physiological states studied, is the relative contribution of the three NOS isoforms to NO production in the uterus between the pregnant and non-pregnant states. Outside of pregnancy, the constitutive isoforms (NOS I and NOS III) are the primary isoforms responsible for NO production in the uterus (Buhimschi et al., 1996; Dong et al., 1996, 1998; Zang et al., 1999). During pregnancy, at least in the rat uterus, a marked elevation in iNOS occurs (Buhimschi et al., 1996; Dong et al., 1996; Ali et al., 1997). The uterine iNOS mRNA expression during pregnancy can be further up-regulated by anti-oestrogens (Dong et al., 1998). Therefore, it is possible that steroid hormones regulate the various NOS isoforms differently. In the sheep uterus, where the NOS activity is mainly calcium-dependent both in the pregnant and non-pregnant states (Massmann et al., 1999), oestrogens modulate the calcium-dependent NOS isoforms (NOS I and NOS III) distinctively (Zhang et al., 1999).

Furthermore, in this study full-thickness uterine tissues were used, which are comprised of many cell types including vasculature. Studies have been performed on the effect of pregnancy and steroid hormones on uterine vasculature and have shown that the intense uterine vasodilation of pregnancy can be mimicked to some extent by oestrogen administration both in the non-pregnant sheep (cycling and steroid-treated) (Magness et al., 1997, 1998) and postmenopausal women treated with steroid replacement therapy (Khorram et al., 1999). Therefore spatial changes in the relative expression of NOS isoforms between the myometrium, endometrium and vasculature may explain the differences in NO production between physiological states as previously suggested (Magness et al., 1997, 1998; Khorram et al., 1999).

The discrepancy between the changes in NO and uterine cGMP may also lie in the direct modulation of guanylate cyclases both by pregnancy and sex steroids. Weiner et al. suggested that, in guinea-pigs, pregnancy induces a down-regulation of soluble guanylate cyclase (the preferential source of NO-stimulated cGMP) while increasing particulate guanylate cyclase activity (San Martin Clark et al., 1995). Although not examined directly by the present study, a direct down-regulation of soluble guanylate cyclase by pregnancy is also suggested by the current data [i.e. very low myometrial cGMP content during pregnancy compared to the other states analysed (Figure 2B) and lack of cGMP elevation of pregnant uterus after exposure to DETA/NO in vitro (Figure 1C)]. This effect was entirely mimicked by oestrogen administration in the prepubertal model (Figure 5C and D), suggesting that oestrogen may induce a selective down-regulation of the soluble guanylate cyclase in the rat uterus. Furthermore an altered cyclic nucleotide metabolism during pregnancy or steroid treatment by direct modulation of phosphodiesterase activity may add to the discrepancies between NO and cGMP as the cGMP content measurements were performed in the absence phosphodiesterase inhibitors.

In conclusion it was found that NO production by the rat uterus was markedly increased by pregnancy compared to other physiological (prepubertal, or cycling dioestrus) and/or experimentally induced (ovariectomized) states. In contrast, uterine cGMP content was significantly decreased by pregnancy. Pregnancy also inhibited the elevation in uterine cGMP after in-vitro NO challenge. Chronic oestradiol treatment in non-pregnant models generally mimics the effects of pregnancy on uterine NO and cGMP. Oestrogen up-regulates NO inde-
pendently of cGMP in the uterus of non-pregnant and prepuberal rats. Progesterone opposes the effects of oestrogen and down-regulates NO and cGMP simultaneously. It should be emphasized that the effects of sex steroid hormones on uterine NO and cGMP production are complex and depend upon the animal model, hormonal state, dose and regimen used. Extrapolation of results between NO and cGMP and between animal models should therefore be made with caution. Measurement of cGMP as an indicator of NO activity in the uterus and vice versa may thus result in erroneous conclusions.

References


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