The role of nitric oxide in the process of implantation in rats

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Nitric oxide (NO) plays an important role in cell signalling in many physiological systems, including reproduction. During pregnancy, oestrogen modulates uterine NO generation, and NO may play an intermediary role in the oestrogen-mediated effects on the uterus. Since oestrogen is actively involved in inducing endometrial receptivity to support the process of implantation, the role of NO in the process of implantation in rats was investigated. Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase (NOS), was administered in utero with or without sodium nitroprusside (SNP), a generator of NO, on different days during the preimplantation phase of gestation. The status of gestation in respect of implantation failure, endometrial receptivity and embryo development were assessed. L-NAME was administered at various doses (2–5 mg per uterine horn) and on different days of pregnancy (days 2–6 of pregnancy) to optimize the pregnancy terminating dose (absence of implantation site on day 8 of pregnancy) and the effective day of treatment. L-NAME led to failure of implantation when administered at 2.5 mg per uterine horn on day 3 of pregnancy. The characteristic preimplantation permeability changes in the uterus were significantly attenuated and embryo growth was retarded. The L-NAME-mediated effects were significantly reversed when SNP (100 μg) was co-administered with L-NAME. These findings suggest a role for NO in the process of implantation. The possible mechanism by which inhibition of the NO–NOS system may interfere with implantation is discussed.

Introduction

Nitric oxide is now acknowledged as an important multifunctional molecule that mediates a number of diverse physiological functions, including different reproductive processes. NO is synthesized from arginine by NOS. It has been demonstrated that the arginine–NO system is present in the uterus (Yallampalli et al., 1993). Uterine generation of NO is upregulated during pregnancy and downregulated at term (Natuzzi et al., 1993; Sladek et al., 1993; Yallampalli et al., 1994a,b). Therefore, Dong et al. (1996) proposed a possible role of NO in the adaptive vasodilatory changes in maternal circulation and the preservation of uterine quiescence for maintenance of pregnancy and initiation of labour. However, the relative contribution of NO varies depending on the species (Sladek et al., 1997).

Theoretical consideration may also be given to whether NO plays a role in implantation. Pregnancy is associated with high circulating concentrations of oestrogen and progesterone. Preovulatory release of progesterone and release of oestrogen at the preimplantation stage are involved in the process of implantation. Oestrogen acts on the endometrium after preconditioning with progesterone and this is responsible for the characteristic pattern of endometrial sensitivity that is an essential prerequisite to implantation. The earliest endometrial response to oestrogen at implantation includes a localized increase in vascular permeability leading to the development of stromal oedema. There are reports that some of the vasodilatory substances produced and released under the influence of oestrogen, induce or modulate endogenous NO (Moncada et al., 1991; Jovanovic et al., 1994; Shifren et al., 1996). Oestrogen also stimulates uterine generation of NO by inducing available arginine (Van Bruen et al., 1992). The vasoregulatory role of NO is well documented (Molnar et al., 1994). It has been hypothesized that NO is involved as an effector molecule in mediation of the effect of oestrogen on the uterus (Rao et al., 1995). Progesterone also appears to influence uterine NO production because antiprogestins have been reported to reduce the NOS II isozyme, the major isoform of NOS that is responsible for upregulation of NO production during pregnancy (Dong et al., 1996). Therefore, it is plausible that implantation may involve NO. The aim of the present investigation was to study implantation in rats under the influence of altered NO concentrations in the uterus.

Materials and Methods

Test compounds

L-NAME was obtained from Sigma (St Louis, MO) and SNP from E. Merck (Darmstadt). These test compounds were dissolved in sterile 0.9% (w/v) saline. L-NAME was administered alone or in combination with SNP to a final injectable volume of 50 μl.
Animals

Adult Sprague Dawley rats (180–200 g) were maintained under standard husbandry conditions with controlled temperature and light (12 h light: 12 h dark photoperiod). Regularly cyclic females at the pro-oestrous phase of the cycle were caged overnight with males of the same age of proven fertility. Mating was confirmed by the presence of spermatzoa in a vaginal smear on the next morning and this was termed day 1 of pregnancy.

Treatments

After midventral laparotomy under light ether anaesthesia, intraperitoneal drug administration was effected from the cervical end through a 26 gauge needle fitted with a tuberculin syringe. Care was taken to prevent entry of air bubbles or leakage during injection. In each rat, test compounds were injected in one uterine horn, while the contralateral horn received 0.9% (w/v) saline and served as a control. The incision was closed by sutures and the animals were maintained under a care regimen appropriate for the surgical procedures performed.

Pontamine sky blue reaction

On day 5 of pregnancy rats received 0.5 ml of 1% pontamine sky blue (GT Gurr Ltd, London) in 0.9% (w/v) saline i.v. through the tail vein under ether anaesthesia. After 15 min the animals were killed and the spaced blue spots along the length of individual uterine horns were counted and recorded.

Embryo collection

The uterine horns of day 5 pregnant rats were cleared of adhering fat and mesentery and then flushed with PBS from the oviductal end. The flushings were collected in a watch glass. Embryos were then physiologically assessed under the microscope.

Statistical analysis

The results are presented as means ± SEM. Statistical analysis of the results was carried out using Student’s t test and chi-squared test as applicable. Differences were considered significant if \( P < 0.05 \).

Experiment 1

Pregnant rats were randomized and allocated to two major groups each consisting of several subgroups. Group 1 rats, subgrouped on the basis of number of days after mating, received L-NAME in a single intrauterine dose of 5 mg between days 2 and 6 of pregnancy. Group 2 consisted of rats on day 3 of pregnancy, and subgrouping was based on dosage of L-NAME between 2 and 5 mg per uterine horn. All animals were killed on day 8. Gravid uteri were dissected out and inspected for the presence of implantation sites.

Experiment 2

Rats received unilateral intrauterine injection of L-NAME (2.5 mg) or L-NAME (2.5 mg) and SNP (100 \( \mu \)g) on day 3 of pregnancy. On day 5 of pregnancy, a subgroup of rats was subjected to pontamine sky blue reaction or killed to collect uterine embryos for morphological assessment. The remaining subgroup of rats was killed on day 8 of pregnancy and the presence of implantation sites was determined.

Results

Experiment 1

Intrauterine administration of L-NAME at 5 mg dose between days 3 and 5 of pregnancy resulted in termination of pregnancy. Implantation sites were not observed in treated horns on day 8 of pregnancy. Administration of L-NAME on day 2 of pregnancy failed to terminate pregnancy but the number of viable implantation sites significantly decreased compared with the vehicle-treated control horn. L-NAME exerted no adverse effects when administered on day 6 of pregnancy (Table 1). Intrauterine administration of a dose of L-NAME between 2.5 and 5 mg on day 3 of pregnancy led to termination of pregnancy. Implantation sites were not observed in treated horns on day 8 of pregnancy (Table 2). A dose of 2 mg did not lead to termination. Based on these observations, all subsequent investigations were conducted in rats on day 3 of pregnancy using 2.5 mg of L-NAME.

Table 1. Effect of \( N^\text{6-} \text{nitro-L-arginine methyl ester} \) on pregnancy termination in rats

<table>
<thead>
<tr>
<th>Day of treatment after mating</th>
<th>Number of implantation sites per uterine horn (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control horn</td>
<td></td>
</tr>
<tr>
<td>Day 2 (( \mu = 9 ))</td>
<td>5.50 ± 1.15</td>
</tr>
<tr>
<td>Day 3 (( \mu = 10 ))</td>
<td>5.40 ± 0.68</td>
</tr>
<tr>
<td>Day 4 (( \mu = 10 ))</td>
<td>5.30 ± 0.61</td>
</tr>
<tr>
<td>Day 5 (( \mu = 8 ))</td>
<td>5.80 ± 0.44</td>
</tr>
<tr>
<td>Day 6 (( \mu = 7 ))</td>
<td>4.80 ± 0.55</td>
</tr>
<tr>
<td>Treated horn</td>
<td></td>
</tr>
<tr>
<td>Day 2 (( \mu = 9 ))</td>
<td>3.40 ± 0.81</td>
</tr>
<tr>
<td>Day 3 (( \mu = 10 ))</td>
<td>Nil**</td>
</tr>
<tr>
<td>Day 4 (( \mu = 10 ))</td>
<td>Nil**</td>
</tr>
<tr>
<td>Day 5 (( \mu = 8 ))</td>
<td>Nil**</td>
</tr>
<tr>
<td>Day 6 (( \mu = 7 ))</td>
<td>4.85 ± 0.49</td>
</tr>
</tbody>
</table>

\( N^\text{6-} \text{nitro-L-arginine methyl ester} \) (5 mg per uterine horn) was injected in a 50 \( \mu l \) volume.

Observations were made on day 8 of pregnancy.

\( ^a \)Numbers of animals shown in parentheses.

\( \text{**}P < 0.001 \) versus control.
Table 2. Effect of different doses of N\textsuperscript{\textalpha}-nitro-L-arginine methyl ester on pregnancy termination in rats at day 3 of pregnancy

<table>
<thead>
<tr>
<th>Dose of N\textsuperscript{\textalpha}-nitro-L-arginine methyl ester (mg)</th>
<th>Number of sites per uterine horn (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control horn</td>
<td>Treated horn</td>
</tr>
<tr>
<td>5 (n = 10)\textsuperscript{a}</td>
<td>5.4 ± 0.68</td>
</tr>
<tr>
<td>4 (n = 8)</td>
<td>6.0 ± 0.53</td>
</tr>
<tr>
<td>3 (n = 8)</td>
<td>6.8 ± 0.48</td>
</tr>
<tr>
<td>2.5 (n = 10)</td>
<td>6.8 ± 0.48</td>
</tr>
<tr>
<td>2 (n = 10)</td>
<td>6.2 ± 0.24</td>
</tr>
</tbody>
</table>

N\textsuperscript{\textalpha}-nitro-L-arginine methyl ester was injected in a 50 µl volume. Observations were made on day 8 of pregnancy. \textsuperscript{a}Numbers of animals shown in parentheses. **P < 0.001 versus control.

Experiment 2

Co-administration of L-NAME and SNP failed to terminate pregnancy. The number of implanted sites on day 8 in the L-NAME–SNP treated horns was not significantly different compared with the vehicle-treated controls (Table 3).

Pontamine sky blue reaction

There were significantly fewer blue spots in L-NAME-treated horns compared with control horns (P < 0.001). However, co-administration of L-NAME with SNP resulted in a pontamine reaction in the L-NAME-treated uterine horns that was not significantly different from that of the control horns (Table 3).

Morphological assessment of embryos

Embryonic growth was retarded in L-NAME-treated uterine horns. Embryos collected from L-NAME-treated horns were at the morula stage with an intact zona pellucida (100%), while those recovered from vehicle-treated horns were at the blastocyst stage (93%) without a zona pellucida. The majority of embryos (90.5%) from the L-NAME–SNP-treated horns were at the blastocyst stage with or without a zona pellucida, while the embryos in the vehicle-treated horns in this experiment were at the blastocyst stage without a zona pellucida (100%) (Table 4).

Discussion

Hormone-dependent changes in cell proliferation and differentiation in all parts of the endometrium are essential prerequisites in the implantation process. Oestrogen acting on the endometrium after preconditioning with progesterone for at least 2 days is responsible for the pattern of sensitivity and subsequent refractory characteristics of the preimplantation period. Oestrogen, and possibly also progesterone, modulate uterine generation of NO (Van Bruen et al., 1992; Dong et al., 1996) and NO may play an intermediary role in the oestrogen-mediated effects on the uterus (Rao et al., 1995).

L-NAME, an inhibitor of NOS that converts L-arginine to NO, significantly inhibited implantation. L-NAME was administered in association with SNP, a spontaneous generator of NO, to ensure that failure of implantation was a consequence of NO deficiency. The results showed that SNP reversed the anti-implantation effect of L-NAME. L-NAME was effective in preventing implantation only when administered on days 3, 4 or 5 of pregnancy. It is critical that fertilized eggs reach the uterus at an appropriate pregestational stage after the endometrium has undergone hormone-dependent changes that lead to receptivity for implantation to occur. The preparatory uterine changes associated with endometrial receptivity were investigated, as well as the effect of L-NAME on the presence of embryos.

Oestrogen that is released at about the time of ovulation initiates the first wave of epithelial proliferation, and a decline in its concentration is responsible for a wave of activity in the glands on day 3 of pregnancy. Progesterone from the corpus luteum conditions the stroma, and oestrogen released on day 4 of pregnancy induces proliferation of the

Table 3. Effect of sodium nitroprusside on N\textsuperscript{\textalpha}-nitro-L-arginine methyl ester-induced termination of pregnancy and pontamine blue reaction in rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Number of sites per uterine horn\textsuperscript{a} (mean ± SEM)</th>
<th>Number of spots per uterine horn\textsuperscript{a} (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control horn</td>
<td>Treated horn</td>
</tr>
<tr>
<td>L-NAME (n = 10)</td>
<td>5.30 ± 0.68</td>
<td>Nil***</td>
</tr>
<tr>
<td>L-NAME–SNP (n = 6)</td>
<td>5.30 ± 0.61</td>
<td>4.80 ± 1.07</td>
</tr>
</tbody>
</table>

L-NAME, N\textsuperscript{\textalpha}-nitro-L-arginine methyl ester; SNP, sodium nitroprusside. \textsuperscript{a}Observations were made on day 8 of pregnancy. \textsuperscript{b}Observations were made on day 5 of pregnancy. ***P < 0.001 versus control and L-NAME–SNP-treated group.
stroma on days 4 and 5 in preparation for implantation (Weitlauf, 1994). Oestrogen-mediated stromal oedema involves increased local vascular permeability and enhanced blood flow (Magness and Rosenfield, 1989), which are the earliest known responses of the sensitive endometrium. Endometrial capillary permeability was investigated using the pontamine sky blue reaction. Increased endometrial permeability causes macromolecular colourants like pontamine blue to leave the circulation at precisely the sites where the blastocysts are in contact with the luminal epithelium, and produces spaced blue spots along the length of the uterine horn (Psychoyos, 1973). The results showed that oestrogen-induced permeability changes were significantly reduced in the L-NAME-treated horns.

A number of vasodilatory substances that are produced and released under the influence of oestrogen, such as acetylcholine, angiotensin, histamine, bradykinine and vascular endothelial growth factor, have been implicated as the potential mediators of oestrogen-induced changes in capillary permeability (Resnick et al., 1976; Still and Griess 1978; Clark et al., 1981; Cullinan-Bove and Koos, 1993). These substances have been reported to induce or modulate endogenous NO (Moncada et al., 1991; Jovanovic et al., 1994), an effecter molecule that is well known for its vasoregulatory role (Molnar et al., 1994). It has been proposed that the effect of oestrogen on the uterus involves NO (Van Bruen et al., 1992). Recent reports suggest that vascular endothelial growth factor, an oestrogen-responsive endothelial-specific mitogen with potent angiogenic activity, plays an important intermediary role in the oestrogen-mediated hyperpermeability and proliferation of uterine blood vessels (Sifren et al., 1996). Vascular endothelial growth factor regulates the baseline synthesis and release of endothelial NO (van der Zee et al., 1997) and modulates the microvascular permeability via a signalling cascade involving NO synthesis (Wu et al., 1996). It has been demonstrated that gestation increases NO-mediated vasodilation in rat uterine arteries (Ni et al., 1997), and the uterotrophic effect of oestrogens potentially can be prevented by inhibiting NOS activity (Rao et al., 1995). Therefore it is likely that in the present study oestrogen-mediated endometrial bed preparation was inhibited due to a block in endogenous NO generation. This proposal is supported by the fact that in rats, an oestrogen surge occurred on the evening of day 4 of pregnancy, and L-NAME only prevented implantation when administered around day 4 (days 3–5). Reversal of the L-NAME-mediated effect by coadministration of SNP also supports this proposal.

In addition to endometrial receptivity, the presence of embryos in the uterus at the appropriate stage of maturity is essential for implantation to occur. On the morning of day 5 of pregnancy, all pre-embryos recovered from L-NAME-treated uterine horns were at the morula stage with an intact zona pellucida. In the control horns the embryos were at the blastocyst stage. Attenuated growth of pre-embryos may be a secondary consequence of NO deficiency, because in L-NAME–SNP-treated uterine horns 90.5% of the embryos recovered on day 5 of pregnancy had reached the blastocyst stage. However, whether uterine NO influences embryonic growth remains to be investigated.

Previous studies have reported that human preimplantation embryos produce NO, which may have a role in early embryonic development and mediate implantation (Fukuda et al., 1996). Therefore, it is possible that in the present study the embryonic NO–NOS system was adversely affected by L-NAME leading to retardation of embryonic growth and consequent implantation failure. In conclusion, this study suggests a role for NO in implantation. However, since selective inhibition of uterine and embryonic NO–NOS systems was not investigated the relative importance of these two sources with regard to implantation remains unknown.

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