Nitric oxide synthase inhibition and the uterotrophic response to oestrogen in immature rats

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The role of nitric oxide in the uterotrophic action of oestradiol after 6 h or 72 h was studied in immature (19–21 days old) female Wistar rats by use of L-arginine, the amino acid from which nitric oxide is synthesized, and N^ω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase. Oestradiol at single s.c. doses of 2.5, 5.0 and 10.0 µg per rat induced dose-dependent uterine oedema in 6 h. L-NAME (10 and 20 mg kg⁻¹, i.p.) administered 30 min before oestradiol (10 µg per rat) injection suppressed the formation of uterine oedema in a dose-related manner. This action of L-NAME on oestradiol-induced uterine oedema was effectively blocked by pretreatment of rats with L-arginine (600 mg kg⁻¹, s.c.), a precursor of nitric oxide, but not by L-lysine, an amino acid not involved in the generation of nitric oxide. In addition, L-NAME at similar doses significantly prevented oestradiol-induced (3 µg per rat, s.c. on three successive days) increases in uterine growth after 72 h; however, this effect was mitigated by L-arginine (600 mg kg⁻¹). These results suggest the involvement of an L-arginine–nitric oxide system in the oestradiol-induced uterotrophic effect in immature rats.

Introduction

Oestrogen induces a sustained increase in uterine blood flow, stromal oedema and epithelial proliferation (Makowski, 1977; Phaily and Senior, 1978; Magness and Rosenfeld, 1989). These effects arise from the interactions of oestrogen with a specific receptor protein in the uterine artery and in endometrial stromal cells (Tamaya *et al.*, 1979; Cunha *et al.*, 1983). However, the precise mechanism by which oestrogen brings about these changes in the uterus is not completely understood. Stromal oedema is probably dependent on a synergism between mediators that increase vascular permeability and mediators that enhance blood flow, whereas cellular hypertrophy is probably dependent on the synthesis and regulation of polypeptide growth factors. In this regard, numerous vasoactive substances, like adenosine, acetylcholine, bradykinin, histamine and prostaglandins (Resnick *et al.*, 1976; Still and Greiss, 1978; Clark *et al.*, 1981), and peptide growth factors, such as transforming growth factor- α /epidermal growth factor (TGF- α /EGF) and insulin-like growth factor I (IGF-I) (Murphy *et al.*, 1987; Gardner *et al.*, 1989; Nelson *et al.*, 1992), have been implicated as the potential mediators because of their vasodilator or growth-promoting actions, but to date no specific antagonist has been able to inhibit or antagonize totally the uterotrophic action of oestrogen.

Yallampalli *et al.* (1994) hypothesized that an L-arginine–nitric oxide (NO)–cyclic guanosine monophosphate (cGMP)

system is present in the uterus and modulates uterine contractility and Van Buren *et al.* (1992) suggested that oestrogen could induce the available arginine to produce NO. Nitric oxide apparently stimulates soluble guanylate cyclase, increases the cellular concentration of cGMP and relaxes vascular smooth muscle (Ignarro *et al.*, 1986). In addition, it appears to contribute to maintenance of basal vascular tone and to attenuate the actions of vasoconstrictors in fetal–placental circulation and regulate blood pressure during pregnancy (Myatt *et al.*, 1992). Chronic inhibition of NO synthesis in pregnant rats can produce a pre-eclampsia-like syndrome in which there is sustained hypertension and intrauterine growth retardation (Molnar *et al.*, 1994). These studies strongly suggest a vasoregulatory role for NO. Oestrogen is also vasoactive and is, therefore, available in high concentration during the follicular phase of the ovarian cycle and in pregnancy. Some of the vasodilatory substances produced and released under the influence of oestrogen, such as acetylcholine, histamine, angiotensin and bradykinin, were shown to induce or modulate endogenous NO (Moncada *et al.*, 1991; Jovanovic *et al.*, 1994). Therefore, it is plausible that the uterotrophic effect of oestrogen involves NO.

Most of the biological assays for oestrogen are based upon the increased wet mass in immature or spayed rats (Calhoun *et al.*, 1971). In the present study, we used this bioassay to investigate the possible involvement of NO in the uterotrophic action of oestradiol, using L-nitroarginine methyl ester (L-NAME) as an inhibitor of NO synthase (Rees *et al.*, 1990) and immature rats as a model system (Ramalay, 1979).

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Materials and Methods

Animals and treatments

Immature female Wistar rats (19–21 days old) 25–38 g, were used. All animals were allowed free access to food and water and were maintained on a 12 h light:12 h dark cycle. For experimentation, they were randomly assigned to groups.

Drugs

The following drugs from Sigma Chemical Co., (St Louis, MO) were used: oestradiol, L-N^ω-nitroarginine methyl ester (L-NAME), L-arginine and L-lysine. Oestradiol was dissolved in corn oil and all other drugs were dissolved in sterile 0.9% (w/v) saline. The test drugs used did not have any effect on uterine mass.

Bioassay of oestrogen

The technique described by Calhoun *et al.* (1971) to measure oestrogen was used. Groups of 6–10 immature female rats were administered oestradiol in a single s.c. dose of 2.5, 5 or 10 µg per rat to study the uterine oedema response after 6 h or in repeated doses of 3 µg per rat per day for three consecutive days to study the uterine growth response after 72 h. At the end of the 6 h or 72 h period, the animals were killed by an excess of ether and the wet and dry uterine masses were obtained and expressed in mg per 40 g body mass. While assessing the influence of test drugs, the increase in uterine wet mass after 6 h and the increase in uterine dry weight after 72 h were taken as the parameters of oestradiol-induced uterine oedema and growth effects, respectively.

The test drugs, L-NAME (5–20 mg kg⁻¹, i.p.), L-arginine and L-lysine (600 mg kg⁻¹, s.c.) or their combinations were injected 30 min before to study their influence on the uterotrophic actions of oestradiol. In the study of uterine growth response to oestradiol, the test drugs were administered twice a day. Control animals received corn oil (0.1 ml per rat, s.c.).

Statistical analyses

The results are reported as means ± SEM. Statistical analysis of differences between groups was performed by ANOVA and the Newman-Keuls' or Dunnett's test, with significance set at $P < 0.05$.

Results

Uterine oedema response to oestradiol

At 6 h after oestradiol (2.5, 5 and 10 µg per rat) administration, a dose-related increase ($P < 0.05$) was observed in uterine wet mass (Fig. 1) but not in dry mass, indicating that oestradiol induces uterine oedema. Systemic administration of L-NAME (10 and 20 mg kg⁻¹), the NO synthase inhibitor, significantly decreased the uterine wet mass increase induced by 10 µg oestradiol (Fig. 2). The L-NAME (20 mg kg⁻¹)-induced

Fig. 1. The uterotrophic response to oestradiol administration after 6 h in immature rats. Corn oil (control) or oestradiol was given as a single s.c. injection in a volume of 0.1 ml. The animals were killed 6 h after oestradiol injection and the uterine masses were obtained. Values are means ± SEM ($n = 6$). * $P < 0.05$ (versus control; Dunnett's test).

attenuation of uterine mass gain provoked by oestradiol was inhibited significantly by 600 mg L-arginine kg⁻¹ but not by L-lysine at a similar dose (Fig. 2).

Uterine growth response to oestradiol

In rats treated with oestradiol (3 µg per rat) for 72 h, the uterine wet and dry masses were significantly increased, indicating oedema inducing as well as growth promoting properties of oestradiol. These increases were significantly attenuated in animals pretreated with L-NAME (10 and 20 mg kg⁻¹). The effect of L-NAME (20 mg kg⁻¹) was almost completely reversed by L-arginine.

Discussion

Immature rats were used to examine whether NO plays a role in the uterotrophic effects of oestradiol because, at this age, serum concentrations of oestrogen and progesterone are very low, whereas the appropriate receptors are present at adult concentrations (Ramalay, 1979; Rendt *et al.*, 1992). Six hours after single s.c. injections of oestradiol, the oedema-inducing effect was apparent, as evidenced by a significant dose-dependent enhancement in uterine wet mass but not in dry mass. The wet mass increase after oestradiol administration occurs because oestrogen enhances uterine blood flow, capillary permeability and causes oedema of the endometrial stroma in rats (Psychoyos, 1966; Phaily and Senior, 1978; Marshall and Senior, 1986).

The increase in dry mass of uteri in rats treated with oestradiol for 72 h represents the true growth response and is associated with cellular hypertrophy and hyperplasia of the uterus. This growth response may arise from interactions with soluble receptor proteins in the uterine tissue and subsequent stimulation of gene transcription (Clark and Peck, 1979; Parker, 1986; Halachmi *et al.*, 1994) involving polypeptide growth factors such as IGF-I, EGF and TGF-α (Mukku and Stancel, 1985; Nelson *et al.*, 1992; Sahlin *et al.*, 1994).

Fig. 2. The inhibitory effect of N^G -nitro-L-arginine methyl ester (L-NAME) on uterotrophic response induced by 10 μ g oestradiol after 6 h in immature rats and its reversal by L-arginine. Oestradiol was given s.c. and L-NAME i.p., 30 min before oestradiol. Controls received 0.1 ml of corn oil. ^a $P < 0.05$ (versus control; ANOVA and Newman-Keuls' test). ^b $P < 0.05$ (versus oestradiol alone; ANOVA and Newman-Keuls' test).

Table 1. The inhibitory effect of L-NAME on the uterotrophic response to oestradiol after 72 h in immature rats and its reversal by L-arginine

Treatment	Uterine mass (mg per 40 g)	
	Wet	Dry
Control	50.6 $\pm$ 4.9	21.3 $\pm$ 1.8
Oestradiol 3 μ g per rat, s.c. on 3 successive days	197.8 $\pm$ 15.7 ^a	48.6 $\pm$ 2.8 ^a
L-NAME + oestradiol 5 mg kg ⁻¹ , i.p. twice a day for 3 days	170.2 $\pm$ 14.1 ^a	41.2 $\pm$ 3.1 ^a
L-NAME + oestradiol 10 mg kg ⁻¹ , i.p. twice a day for 3 days	152.6 $\pm$ 5.8 ^{ab}	38.8 $\pm$ 3.7 ^{ab}
L-NAME + oestradiol 20 mg kg ⁻¹ , i.p. twice a day for 3 days	139.3 $\pm$ 6.6 ^{ab}	34.0 $\pm$ 2.5 ^{ab}
L-NAME + L-arginine + oestradiol (20 mg kg ⁻¹ , i.p.) (600 mg kg ⁻¹ , s.c.) twice a day for 3 days	206.0 $\pm$ 16.6 ^a	46.6 $\pm$ 5.4 ^a

Values are means $\pm$ SEM from six to ten animals. Controls received 0.1 ml of corn oil s.c. on three successive days. The differences in uterine wet and dry masses between the control (vehicle) and drug-treated rats are considered indicative of uterine oedema and growth responses, respectively. L-NAME (N^G -nitro-L-arginine methyl ester) and L-arginine were administered 30 min before oestradiol. ^a $P < 0.05$ (versus control, ANOVA and Newman-Keuls' test).

^b $P < 0.05$ (versus oestradiol alone, ANOVA and Newman-Keuls' test).

The oestradiol-induced uterine oedema and proliferative responses were significantly attenuated in a dose-related manner by pretreatment of rats with L-NAME, an inhibitor of NO synthase that converts L-arginine to NO. It is likely that these effects of L-NAME are due to dose-related decreases in blood flow to the uterine muscle. To ensure that the effect of L-NAME was mediated only by NO, we tested L-NAME (20 mg kg⁻¹) in association with an excess of L-arginine (600 mg kg⁻¹), a precursor of NO, or with an excess of L-lysine (600 mg kg⁻¹), an amino acid that is not involved in NO biosynthesis. The results evidenced that an excess of L-arginine could reverse the attenuating effect of L-NAME on oestradiol-induced uterine oedema and growth. It indicates the

presence of an arginine-NO system in the uterus that is stimulated by oestrogen, and that the enzyme involved is a constitutive isoform of NO synthase. The source of the NO is not clear from this study. Oestrogen may induce endothelium-dependent, as well as independent, NO through activation of NO synthase in the uterine artery endothelial cells or in the vasodilator neurones supplying the uterine artery (Van Buren *et al.*, 1992; Morris, 1993; Shew *et al.*, 1993; Weiner *et al.*, 1994). In conclusion, our study suggests a role for NO in the uterotrophic action of oestrogen in immature rats.

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