BIOLOGY OF REPRODUCTION 58, 875–879 (1998)

Nitric Oxide as a Regulator of Embryonic Development

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ABSTRACT

The role of nitric oxide (NO) in activation of cGMP is well established. It has been proposed that the ratio of cAMP to cGMP may be important in the regulation of preimplantation embryonic growth and differentiation. Therefore, we determined the ability of murine preimplantation embryos to produce NO. In addition, NO as an endogenous smooth muscle relaxant and vasodilator is a candidate for involvement in embryo implantation because this process requires increased vascular permeability and uterine quiescence at the sites of blastocyst apposition. Nitrite assays, an indirect measure of NO production, indicate that preimplantation murine embryos produce NO. This production was reversibly inhibited by culture of embryos in medium containing a nonspecific NO synthase (NOS) inhibitor (N^G-nitro-L-arginine). Additionally, inhibition of normal development was observed in embryos cultured with NOS inhibitor. NO levels increased in culture medium when ovariectomized progesterone-treated animals were exposed to estrogen for 1 h in utero. Such hormonal treatment induces implantation. These data indicate that NO levels are regulated by estrogen and may be important in regulation of implantation. In addition, these data demonstrate for the first time that NO production appears to be required for normal embryonic development.

INTRODUCTION

Nitric oxide (NO) is a free radical molecule that has been demonstrated to be an intracellular messenger (reviewed in [1, 2]). It is produced in various tissue and cell types, and its functions include smooth muscle relaxation, vasodilation, neuronal signaling, and stimulation of immune responses (reviewed in [2–5]). NO is produced when the enzyme NO synthase (NOS) catalyzes the oxidation of L-arginine to L-citrulline. Various isoforms of NOS have been isolated and include neuronal NOS (nNOS), inflammatory NOS or macrophage NOS (iNOS), and endothelial NOS (eNOS) (reviewed in [5]). These isoforms are expressed in a variety of cell types, and several isoforms been shown to be constitutive producers that are also capable of induced production, i.e., nNOS and eNOS. The third isoform, iNOS, is produced only in response to a stimulus.

Mitotic division of mouse zygotes begins following fertilization, although embryonic transcription does not occur until the 2-cell stage (reviewed in [6]). All proteins produced in the 1-cell embryo therefore are derived from maternal mRNA. Culture of preimplantation embryos in a simple defined medium [7, 8] results in the progression of development to the blastocyst stage, suggesting that embryonic cellular division does not require reproductive tract-specific components.

It has been previously proposed that the ratio of cAMP to cGMP may be important in the regulation of preimplantation embryonic growth and differentiation [9]. Because NO mediates many of its effects via activation of soluble guanylate cyclase resulting in elevated cGMP, we have investigated the ability of preimplantation embryos to produce NO and its possible role in embryo development. In addition, we have determined the effect of estrogen on the production of NO from blastocyst-stage embryos. This was investigated because initiation of the implantation process is regulated by estrogen and one of the requirements for successful implantation is the progression of embryo development to the blastocyst stage.

MATERIALS AND METHODS

Animals

The animal experiments reported in this study were performed in adherence to the guidelines established in the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. All animals used were humanely cared for in accordance with guidelines from PHS, USDA, and the University of North Carolina at Charlotte Institutional Animal Care and Use Committee.

CD-1 mice (Charles River Laboratories, Raleigh, NC) were kept on a 10L:14D cycle and allowed free access to drinking water and Purina Laboratory Chow (Ralston-Purina, St. Louis, MO).

Virgin females (20–25 g) were housed overnight with virile males. The following morning, females were checked for the presence of a vaginal sperm plug. The day of vaginal plug was designated Day 1 of pregnancy. Delayed implantation was induced by bilateral ovariectomy before 1100 h on Day 4 of pregnancy. Animals were maintained on progesterone (P_4, 2 mg/0.1 ml corn oil, s.c.; Sigma Chemical Co., St. Louis, MO) for 4 days. On the fourth day of P_4 administration, the animals were given a single injection of estradiol-17β (E_2, 20 ng/0.1 ml corn oil, s.c.; Sigma).

Embryo Culture

Embryos were recovered from oviducts on Days 1 through 3 and from the uterus on Day 4 (4–6 mice per day) and in delayed-implanting mice (10–20 mice per group). After recovery, embryos were rinsed several times with Whitten's medium [7, 10] and then cultured in groups of 7–15 for 4 h in 100 μl of Whitten's medium at 37°C in a humidified CO_2 chamber. Culture of all groups was performed in either duplicate or triplicate. Medium for individual groups was collected and concentrated to a pellet that was resuspended in 200 μl sterile water.

Accepted November 6, 1997.
Received March 27, 1997.
[1]This research was supported by the Charlotte Mecklenburg Hospital Authority Health Services Foundation grant #012-892.
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Nitrite Assays

NO concentrations in the conditioned culture medium from murine embryos were determined indirectly by measurement of nitrites (NO_2). Nitrites are stable degradation products of NO and are considered to be a reliable indicator of NO production in cell cultures. NO secretion was detected by a microtiter plate Greiss assay [11]. Nitrite assays were performed with equal amounts of Greiss reagent (0.5% sulfanilamide, 0.05% naphthalene diamine dihydrochloride in 2.5% orthophosphoric acid), noncultured medium, and the concentrated cultured medium. All samples were run in duplicate. Assays were performed at room temperature for 10 min. Microtiter plates were read at 550-nm absorbance. Linear regression was used to determine NO concentration from the standard curve of $NaNO_2$.

Inhibition of NO Production

N^G-nitro-L-arginine (L-NA, 500 μM), an analogue of arginine that cannot be converted to NO by any of the NOS isoforms, was used to test whether embryonic production of NO could be inhibited. Embryos were recovered on various days of preimplantation pregnancy and cultured for 1 h in groups of 5–16 as described above (see section on embryo culture). Embryos were then placed in new microdrops for 24-h or 32-h culture at 37°C. As controls for toxicity, embryos were cultured in media supplemented with L-arginine (500 μM) or L-NA for 7 h and then transferred to L-arginine-containing medium for 24 h or 72 h. A dose response was determined using Day 2 embryos cultured in various L-NA and L-arginine concentrations (0.5 mM, 0.25 mM, and 0.05 mM). Embryonic development was observed at 24 h.

Immunocytochemistry

Delayed embryos recovered after 1-h exposure to E_2-treatment in utero were stained for the presence of the endothelial and induced isoforms of NOS by immunocytochemistry. Embryos were cytocentrifuged (3000 × g) onto poly-L-lysine (Sigma)-coated slides and fixed with formalin (10%) for 10 min at room temperature. Slides were treated with 0.2% Triton X-100 (Sigma) and 0.1 M Tris solution for 10 min to permeabilize embryos. A Histostain-SP Kit (Zymed Laboratories, San Francisco, CA) was used to perform the immunocytochemistry. Blastocysts were incubated with a 1:250 dilution of either the primary eNOS or iNOS antibody (ABR Inc., Golden, CO). The rabbit polyclonal eNOS antibodies were raised against the following peptide sequence: P_{599}YNSSPRPEQHKSYK_{613}-C. The rabbit polyclonal iNOS antibodies were raised against the following peptide sequence: CK_{1131}KGSALEEPKATRL_{1144}. Incubation with the primary antibody was performed at room temperature for 4 h in a humidified chamber. After immunocytochemical staining, embryos were counterstained with hematoxylin for 1 min. Negative controls were processed in the same manner but were not incubated with the primary antibody solution. All antibodies have been previously characterized through immunocytochemistry and western blot analysis as specific for either eNOS or iNOS (ABR, technical notes).

Statistical Analysis

The results of the experiments were analyzed using various statistical methods. A one-way ANOVA was used to test for significant difference in the mean concentration of NO production by preimplantation mouse embryos on different days of pregnancy as well as by delayed embryos collected from control mice and those treated with E_2. Chi-square analysis (from 2 × 2 contingency tables) was used to test for difference in proportions of embryos at the appropriate stage of development in the control embryos versus those cultured in NOS inhibitor. Chi-square analysis was also used to test for differences in proportions of embryos at the appropriate stage of development in embryos cultured in different concentrations of NOS inhibitor.

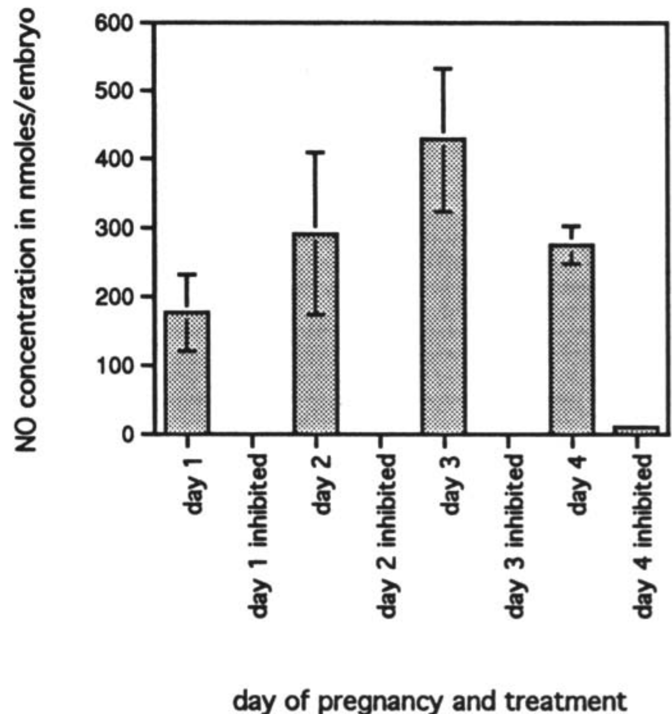

FIG. 1. NO production by preimplantation mouse embryos. Embryos from each day of pregnancy were collected from oviducts and cultured, in groups of 10–20, in L-arginine (500 μM) or L-NA (500 μM) for 24 h. Assay for NO production was performed on the culture medium. NO concentration is given in nmol/embryo, and each day represents mean ± SEM of 20 embryos. ANOVA was performed on the data, and no statistical difference was found in NO production between days of control groups. * Statistically significant differences were found when analysis was performed between the inhibited and control groups on each day of pregnancy, $p < 0.05$.

RESULTS

NO_2 was present in the conditioned medium from embryos collected on all 4 days of pregnancy (Fig. 1). All embryos cultured with inhibitor had negligible concentrations of NO in the conditioned medium, suggesting that the use of a NOS blocker can inhibit NO secretion by preimplantation embryos.

In addition, embryos cultured with L-NA were assessed to determine whether they made a successful transition from one developmental stage to the next (Fig. 2). Development of Day 1 embryos from the 1-cell stage to the 2-cell stage was not affected by L-NA. While 97% of the control embryos collected on Day 2 of pregnancy developed to the 4-cell stage or beyond, only 17% of those cultured with L-NA developed past the 2-cell stage. Morulae cultured for 32 h in medium with L-NA compared to control morulae did not progress to the blastocyst stage (25.8% vs. 53%). All Day 4 blastocysts cultured with inhibitor died (as detected by loss of cell structure), whereas only 14% of the

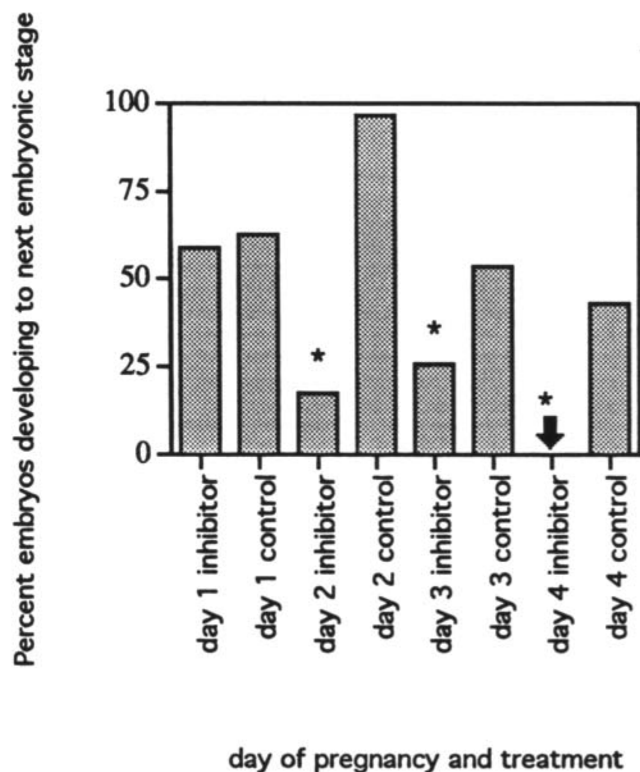

FIG. 2. Preimplantation development of embryos cultured with or without NO inhibitor. Day 1 embryos inhibited, n = 17; arginine, n = 16. Day 2 embryos inhibited, n = 23; arginine, n = 29. Day 3 embryos inhibited, n = 31; arginine, n = 30. Day 4 inhibited, n = 14; arginine, n = 14. * Chi-square statistical analysis was performed between the inhibited and control groups on each day of pregnancy, and all were significantly different with the exception of Day 1 embryos, $p < 0.05$.

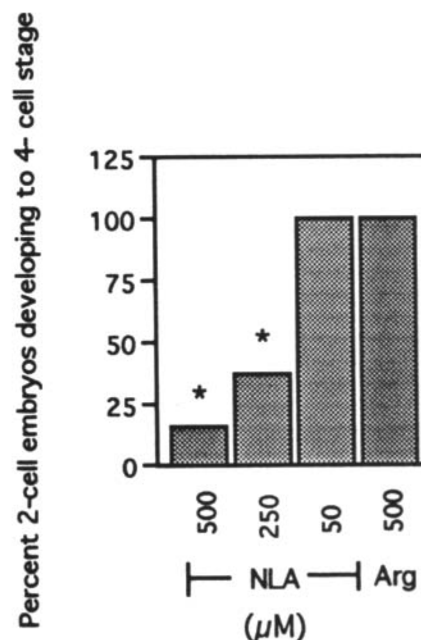

FIG. 3. Dose-response curve of L-NA on embryonic development. * Chi-square analysis indicated significant difference between the lowest groups and the two higher L-NA concentrations, $p < 0.05$.

controls died and 42.9% hatched in culture. The percentage of Day 2 embryos, cultured in varying concentrations of L-NA (5–500 μM), that developed past the 4-cell stage was found to be inversely related to concentration of L-NA (Fig. 3). As a further control for toxicity of L-NA, embryos were cultured for 7 h in medium with inhibitor (500 μM) and then transferred to medium with L-arginine. The control group was cultured in L-arginine-containing medium only. There was no difference in development between the two groups either at 24 h (Fig. 4) or 72 h (data not shown), indicating that the L-NA was not directly toxic to the embryos.

Delayed blastocysts recovered 1 h after exposure to E_2 produced the highest levels of NO (Fig. 5). These embryos were therefore examined by immunocytochemistry for the presence of eNOS and iNOS. The endothelial isoform of NOS is a constitutively produced calcium-calmodulin-dependent enzyme and was chosen because embryos from each day of early pregnancy produced NO. In addition, other factors such as the requirement of calmodulin for compaction of morula, and increased capillary permeability at the site of implantation, indicated that this may be one form of NOS present in embryos [12]. The inducible isoform of NOS was chosen because its production in macrophages and other cell types is induced, and we determined that after exposure to E_2 the production of NO increased. Trophoblast cells of delayed embryos after 1 h of E_2 exposure stained positively with both eNOS and iNOS primary antibodies (Fig. 6, A and B). Trophoblast cells adhere to and penetrate the uterine endometrium; thus they would be likely candidates in the elaboration of signals for communication with uterine epithelial cells that line the lumen and stromal cells underlying the point of embryonic adherence. The cellular distribution of eNOS in the embryo is thus consistent with a role in regulating vascular events at the site of implantation.

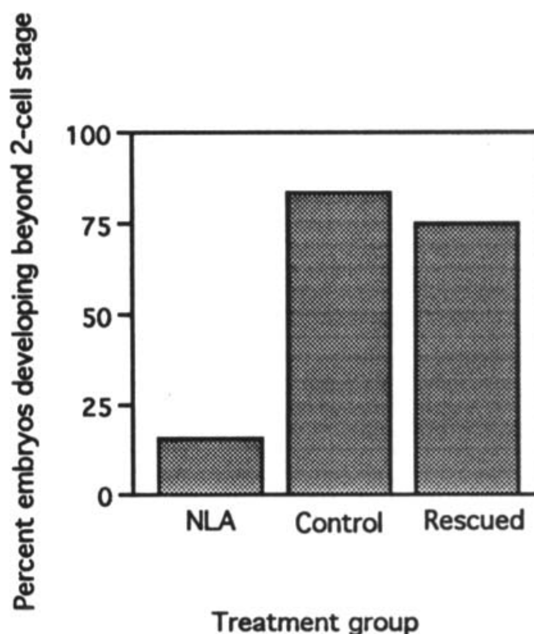

FIG. 4. Toxicity of NO inhibitor. Embryos collected on Day 2 of pregnancy were cultured in L-NA (NLA, 500 μM)-containing medium for 7 h and then transferred to medium with L-arginine (rescued, 500 μM) or NLA for 17 h, or in L-arginine-containing medium for 24 h (control), to determine whether embryos could be rescued from developmental delay. Chi-square analysis indicated statistical significance between treatment groups, $p < 0.05$. NLA, n = 25; control, n = 28; rescued, n = 22.

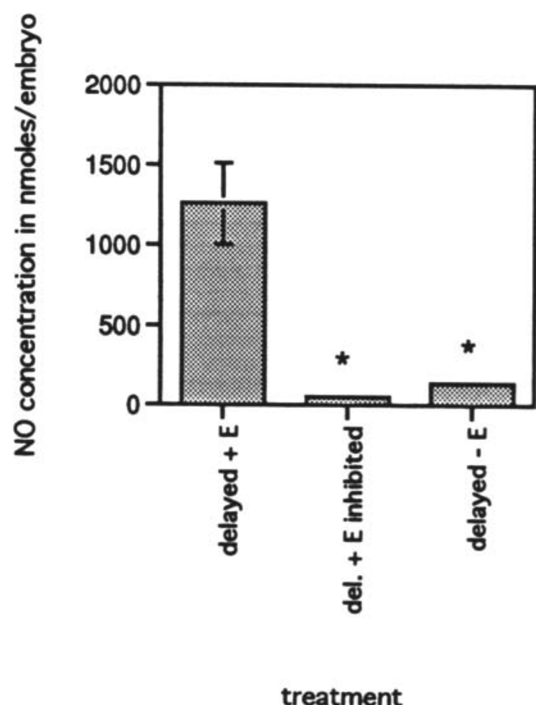

FIG. 5. NO production by delayed embryos. Embryos were flushed from delayed animals 1 h and 0 h after exposure to E_2. After 24-h culture, medium was collected and assayed for NO. NO concentration is given in nmol/embryo, and each group represents mean ± SEM of 20 embryos. * ANOVA was performed on the data, and significant differences were found between groups, $p < 0.01$.

DISCUSSION

These data indicate that embryos cultured in medium containing inhibitor, from the 2-cell to the blastocyst stage, were developmentally delayed or became nonviable as compared to embryos cultured in L-arginine, the substrate for NOS enzyme (Figs. 2 and 3). We believe that the lack of inhibition of 1-cell embryos to the 2-cell stage is due to the presence of maternal factors regulating mitosis. It is not until the 2-cell stage that activation of embryonic transcription occurs; therefore it is not surprising that the 1-cell embryos did not respond in the same manner as the later-stage embryos.

At the blastocyst stage of development, the embryo adheres to and penetrates the uterine endometrium. The complex process of implantation requires both P_4 and E_2. In the mouse, implantation occurs on the morning of Day 4 of pregnancy. At this time an endogenous ovarian E_2 surge occurs to induce a receptive state for implantation in the P_4-dominant uterus [13–15]. If the source of the E_2 is removed, the uterus can be maintained in a neutral state and implantation can be delayed. A single injection of E_2 will initiate a state of uterine receptivity and activate the blastocyst.

Increased capillary permeability at the site of blastocyst attachment has been shown to be a requisite for implantation in all species [13]. We suggest that NO, which is known to increase blood flow and affect capillary permeability at higher concentrations, is an important embryonic factor involved in the initiation of the implantation process. Nitrites were measured in conditioned culture medium from delayed implanting embryos recovered at 0 and 1 h after exposure to estrogen. This assay indicated significant dif-

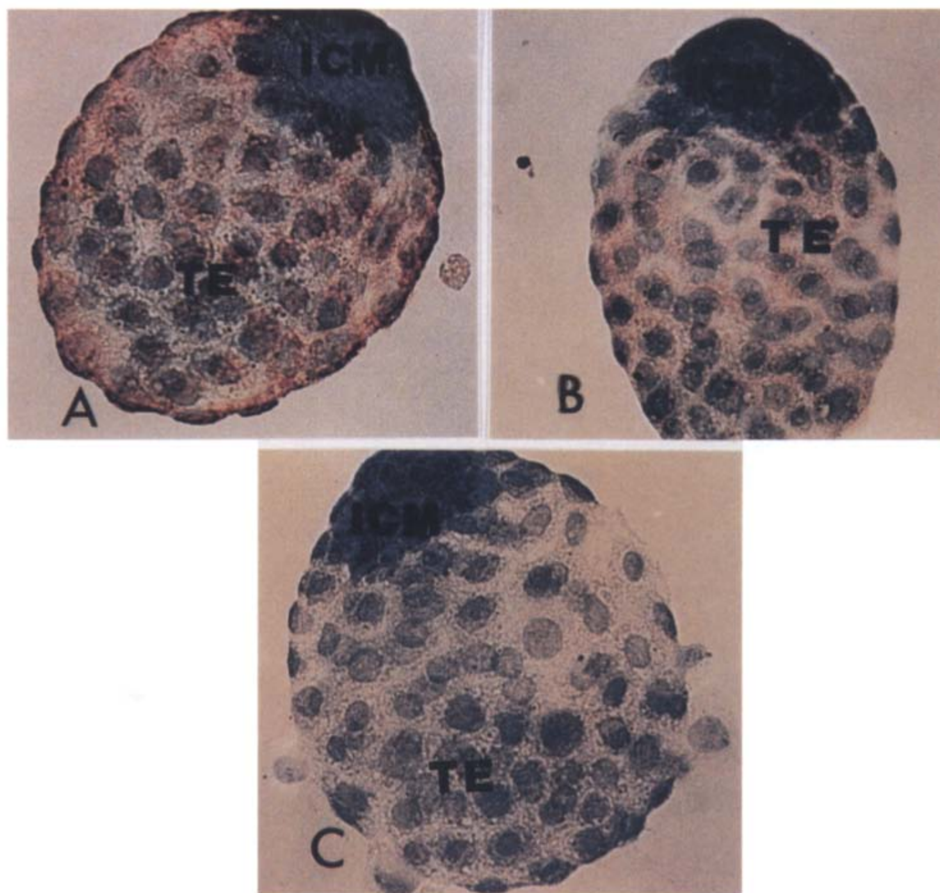

FIG. 6. Immunocytochemical localization of NOS on delayed implanting embryos 1 h after E_2 treatment. Formalin-fixed (10%) whole-mount embryos were stained with the following polyclonal antibodies: **A**) eNOS, **B**) iNOS, **C**) negative control. TE, trophectoderm; the dark, uppermost region is the inner cell mass. ×400 (reproduced at 95%).

ference in NO secretion in embryos between the 1-h E_2 treatment (1255 nmol/embryo) and the 0-h E_2 control group (130 nmol/embryo). The production of NO was inhibited by the addition of L-NA (500 μM) to the culture medium. Further studies need to be done to determine whether estrogens, both those that induce and those that do not induce implantation, have a direct effect on NO production by preimplantation embryos.

We have demonstrated that NO is produced in preimplantation embryos and that its production is required for normal embryonic development. In addition, we have shown that E_2 increases NO production in delayed blastocysts and that eNOS and iNOS are present in the embryonic cells that first interact with the uterus. NO has been shown to have many different functions, but this is the first time a possible role for NO in mitotic division of preimplantation embryos has been shown. While it is true that mice that are deficient in one isoform of NOS [16–18] are viable, we demonstrate that normal embryonic development requires NO production by the embryo. An important finding is that murine preimplantation embryos can produce both iNOS and eNOS. It is possible that several different isoforms of NOS are present in murine embryos and can therefore compensate for the lack of only one isoform during development. Preimplantation embryonic development is inhibited when histamine production is impaired [19]. Histamine increases cAMP levels in preimplantation rabbit blastocysts, whereas NO has been shown, in other tissues, to mediate its responses via activation of cGMP. The inhibition of either histamine or NO alters cyclic nucleotide production and therefore the ratio of cAMP to cGMP; this in turn may affect the transcription of regulators of mitosis. This suggests that the inhibition of NO alters cyclic nucleotide production, which is detrimental to normal embryonic development.

Estrogen has an established role in the induction of implantation, and it appears that, in vivo, it increases NO production in embryos at the time they become activated to implant. In addition to the probable role of NO in regulating embryonic development, the embryo may produce NO as one of many signals to the uterus to stimulate local vasodilation and the increased capillary permeability required for successful implantation.

ACKNOWLEDGMENTS

We would like to thank Dr. Larry Leamy for his statistical assistance and support. Thanks to Dr. Carol Sloan and Dr. Margrett Henault for their support and guidance.

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