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Role of nitric oxide in eicosanoid synthesis and uterine motility in estrogen-treated rat uteri

(*in vitro* incubation/[^{14}C]arachidonate/nitroprusside/hemoglobin/N^G-monomethyl-L-arginine)

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ABSTRACT **Cholinergic stimulation of vascular endothelin activates NO synthase (NOS), leading to generation of NO from arginine. This NO diffuses to the overlying vascular smooth muscle and causes vasodilatation. NOS has also been found in the central and peripheral nervous systems and it is clear now that NO plays an important role as a neurotransmitter. Here we investigate the role of NO in controlling contraction of uterine smooth muscle. Our previous work showed that NO activates the cyclooxygenase enzyme in the hypothalamus, leading to production of prostaglandin E_2 (PGE_2). We began by determining whether NO was involved in production of arachidonic acid metabolites in the uterus. Uteri were removed from female rats that had been treated with estrogen (17β-estradiol). Control animals were similarly injected with diluent. Tissues were incubated *in vitro* in the presence of [^{14}C]arachidonic acid for 60 min. Synthesis of PGs and thromboxane B_2 (TXB_2) was markedly stimulated by sodium nitroprusside (NP), the releaser of NO. The effect was greatest on TXB_2; there were no significant differences in increases of different PGs. The response to NP was completely prevented by Hb, a scavenger of NO. The inhibitor of NOS, N^G-monomethyl-L-arginine (NMMA), significantly decreased synthesis of PGE_2 but not the other prostanoids (6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$). Addition of Hb to scavenge the spontaneously released NO inhibited synthesis of 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$, but not TXB_2. There was a much lesser effect on products of lipoxygenase, such that only 5-hydroxy-5,8,11,14-eicosatetraenoic acid (5-HETE) synthesis was increased by NP, an effect that was blocked by Hb; there was no effect of NMMA or Hb on basal production of 5-HETE. Thus, NO stimulates release of the various prostanoids and 5-HETE; blockade of NOS blocked only PGE_2 release, whereas Hb to scavenge the NO released also blocked synthesis of 6-keto-$PFG_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$, indicating that basal NO release is involved in synthesis of all these PGs, especially PGE_2. Presumably, NMMA did not block NOS completely, whereas Hb completely removed released NO. This may explain the different responses of the various prostanoids to NMMA and Hb. To determine the role of these prostanoids and NO in control of spontaneous *in vitro* uterine contractility in the estrogen-treated uterus, the effect of blocking NOS with NMMA and of scavenging NO produced by Hb on the time course of spontaneous uterine contractility was studied. Surprisingly, blockade of NOS or removal of NO by Hb prevented the spontaneous decline in uterine motility that occurs over 40 min of incubation. We interpret this to mean that NO was released in the preparation and activated guanylate cyclase in the smooth muscle, resulting in production of cGMP, which reduces motility and induces relaxation. When the motility had declined to minimal levels, the effect of increased NO provided by NP was evaluated; apparently by stimulating the release of prostanoids, a rapid increase in motility that persisted for 10 min was produced. This effect was completely blocked by Hb. The action of NP was also blocked by indomethacin, indicating that it was acting via release of PGs. Apparently, when motility is low, activation of PG synthesis by NO to activate the cyclooxygenase enzyme causes a rapid induction of contractions, whereas, when motility is declining, NO acts primarily via guanylate cyclase to activate cGMP release; the action of the prostanoids released at this time is in some manner blocked.**

Nitric oxide (NO), previously identified as the main endothelium-derived relaxing factor (1), is now also recognized as a neurotransmitter in the central and peripheral nervous systems (2). It is well known that NO is formed from L-arginine by NO synthase (NOS). In the presence of NADPH and other cofactors, NOS converts L-arginine into citrulline and NO (3). The major mechanism of action of NO in most tissues is activation of soluble guanylate cyclase with subsequent formation of cGMP (4). There is a growing family of guanylate cyclases, but only the soluble heme-containing forms are activated by NO (5). Reduced iron (Fe^{2+}) complexed by anions and especially protoporphyrin to form heme has a high binding affinity and reactivity with NO (6). The binding of NO by heme, which alters its conformation, is the mechanism by which the reactive gas activates guanylate cyclase (6).

It is likely that guanylate cyclase is not the only target of NO action. Indeed, NO inhibits several heme-containing enzymes of the mitochondrial electron transport complex and the citric acid cycle enzyme aconitase (7). Cyclooxygenase and lipoxygenase are enzymes that contain iron at the active site of the molecule (8–10). NO, by interacting with iron in the heme group that is needed as a cofactor for cyclooxygenase or the iron at the active site of lipoxygenase, may modify the activity of these enzymes.

Recently, we reported that sodium nitroprusside (NP), a classic donor of NO, increased prostaglandin E_2 (PGE_2) release from hypothalamic fragments. N^G-monomethyl-L-arginine (NMMA), an inhibitor of NOS, blocked the PGE_2 release induced by norepinephrine but had no effect on basal release of PGE_2 (11). We subsequently showed that NO-induced PGE_2 release mediated luteinizing hormone-releasing hormone (12) and corticotropin-releasing hormone (13) release from the hypothalamus.

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Abbreviations: NOS, nitric oxide synthase; NP, nitroprusside; PG, prostaglandin; NMMA, N^G-monomethyl-L-arginine; 5-HETE, 5-hydroxy-5,8,11,14-eicosatetraenoic acid; TXB_2, thromboxane B_2; IDT, isometric developed tension.

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We hypothesized that activation of cyclooxygenase by NO, which we had demonstrated in the hypothalamus (11), may also be operative in the uterus, where uterine cyclooxygenase and lipoxygenase can readily metabolize exogenous arachidonic acid to different prostanoids (14, 15). Therefore, we studied the action of NP, a NO donor; Hb, a NO scavenger; and NMMA, an inhibitor of NOS on the synthesis of prostanoids in uterine tissue of estrogen-treated rats.

To link the stimulation of both enzymes, which was induced by NO, to the physiologic function of these prostanoids in the uterus, we examined the possible effects of blocking NO synthesis with NMMA and introduction of exogenous NO with NP on the spontaneous motility of isolated uterine strips from estrogen-treated rats as well. The results indicate that NO plays an important role in uterine contractility both by inducing uterine relaxation via activation of guanylate cyclase and subsequent cGMP production and by activation of cyclooxygenase, which induces synthesis and release of PGs and contraction of the uterus. Thus, NO has complex opposing actions on uterine contractility. It relaxes uterine smooth muscle by inducing the release of cGMP and contracts it via stimulating synthesis and release of PGs by activation of cyclooxygenase.

MATERIALS AND METHODS

Female rats of the Wistar strain (200–230 g body weight) were used. They were housed in group cages under controlled conditions of light (lights on 500–1700 h) and temperature (23°C–25°C). Rat chow and water were freely available. The rats were treated with estrogen by injecting 1 μg of 17β-estradiol in 0.2 ml of ethanol (30%) subcutaneously. Controls were injected with an equal volume of the solvent solution only. Twenty-four hours later, the animals were stunned by a blow on the neck and their uterine horns were removed.

Metabolism of [^{14}C]Arachidonic Acid. Once the uterine horns were obtained, each horn was opened, trimmed of visible fat, and placed in a Petri dish containing a modified Krebs–Ringer bicarbonate (KRB) solution with glucose (11 mM) for substrate as described elsewhere (12). The metabolism of exogenous arachidonic acid by rat uterine tissue was determined by incubating the tissue for 60 min in KRB medium containing 0.25 μCi of [^{14}C]arachidonic acid (52.9 Ci/mol; 1 Ci = 37 GBq) (New England Nuclear) in an atmosphere of 95% O_2/5% CO_2 with constant shaking at 60 cycles per min at 37°C. For each determination, ≈200 mg of uterine tissue was used. The uterine strips were randomly treated with sodium NP (100 μM), Hb (2 μg/ml), or NMMA (300 μM). These reagents were added to the incubation medium alone or in combination as described in *Results*. The above-mentioned drugs were obtained from Sigma. The controls were incubated in medium alone. At the end of the incubation period, tissues were removed and lipoxygenase products were extracted (three times) with 2 ml of ethyl acetate. Pooled ethyl acetate extracts were dried under nitrogen. The residue was suspended in 0.2 ml of chloroform/ethanol (2:1; vol/vol) and applied to a silica gel TLC plate. Authentic 5-hydroxy-5,8,11,14-eicosatetraenoic acid (5-HETE) and 12-HETE (Paesel, Frankfurt) were applied to other plates, which were run in parallel. The plates were developed in the solvent system ethyl acetate/isooctane/acetic acid/water (76:67:20:100; vol/vol). The spots were visualized in iodine vapor and scraped off the plates. Average R_f values for arachidonic acid, 5-HETE, and 12-HETE were 0.85, 0.70, and 0.92, respectively. Results for each compound were expressed as percentage total radioactivity of the plate determined by liquid scintillation counting.

After extraction of lipoxygenase products, the remaining incubation medium was acidified to pH 3.0 with 1.0 M HCl in 1 vol of ethyl acetate and extracted twice for PGs. Pooled ethyl acetate extracts were dried under nitrogen. The residues were suspended in chloroform/methanol and applied to silica gel TLC plates. The plates were developed in a solvent system of benzene/dioxane/glacial acetic acid (60:30:3; vol/vol). The position of the authentic eicosanoids was visualized by spraying the dried plates with 10% phosphomolybdic acid in ethanol followed by heating at 110°C for 10 min. Average R_f values were 0.30 for 6-keto-$PGF_{1\alpha}$, 0.35 for $PGF_{2\alpha}$, 0.47 for PGE_2, 0.57 for thromboxane B_2 (TXB_2), and 0.80 for arachidonic acid. Radioactivity from TLC zones for arachidonic acid and for different prostanoids, was measured by liquid scintillation counting. The area of each of the radioactive peaks corresponding to authentic prostanoids was calculated and expressed as percentage of the total radioactivity of the plates.

Spontaneous Motility Studies. The uterine horns were obtained as described above. The tissue was removed and each horn was divided by a transverse cut into two equal-length segments. The segments were placed in Petri dishes containing KRB at room temperature and constantly gassed with 95% O_2/5% CO_2. Each segment was immediately opened by a cut along the mesosalpinx insertion; one end was attached to a glass holder and immersed in a tissue chamber filled with 20 ml of KRB (pH 7.4; 37°C) and continuously gassed. The other end of the tissue was attached to a strain gauge coupled to an amplifier driving a direct writing oscillograph. After a resting tension of 1 g was applied to each strip by means of a micrometric device, isometric developed tension (IDT) was measured. The mean IDT values (expressed in mg) were obtained by measuring the mean amplitude of all the contractions recorded over a 10-min period. The constancy of IDT for 40 min after mounting was expressed as percentage change from control value at the end of the first 10 min (time 0 in the figures).

All uterine strips were preincubated in the chambers without tension, in the presence of NMMA (300 μM), Hb (2 μg/ml), indomethacin (1 μM), or an equal volume of medium alone for 20 min, after which motility was recorded for 40 min with these compounds in the medium.

Each time one strip from one half horn was used as control and the other half was submitted to experimental treatment. The pieces were chosen at random.

Statistics. Data were analyzed by one-way analysis of variance and the Student–Newman–Keuls multiple comparison test for unequal replicates. Differences with $P < 0.05$ were considered significant.

RESULTS

Effect of NO on Synthesis of PGs. The synthesis of PGs was markedly stimulated by the releaser of NO, NP. The effect was greatest with TXB_2; however, there were no significant differences in the increases with the different PGs evaluated, which included 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$. The response of NP was completely prevented by the scavenger of NO, Hb (Fig. 1).

On the other hand, when the inhibitor of NOS, NMMA, was incubated with the tissue, the resting synthesis of PGE_2 was significantly decreased but the resting synthesis of the other prostanoids was not significantly affected (Fig. 2). When Hb was added to scavenge the NO that was being spontaneously released in the tissue, the synthesis of 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$ was inhibited but there was no effect on the synthesis of TXB_2 (Fig. 2).

Effect of NO on the Products of Lipoxygenase. When NP, the releaser of NO, was incubated with the uteri, there was an increased synthesis of 5-HETE; however, the relative magnitude of this increase was much less than that previously found with PGs (Fig. 3). There was no significant effect of NP on the synthesis of 12-HETE. In the case of 5-HETE, Hb

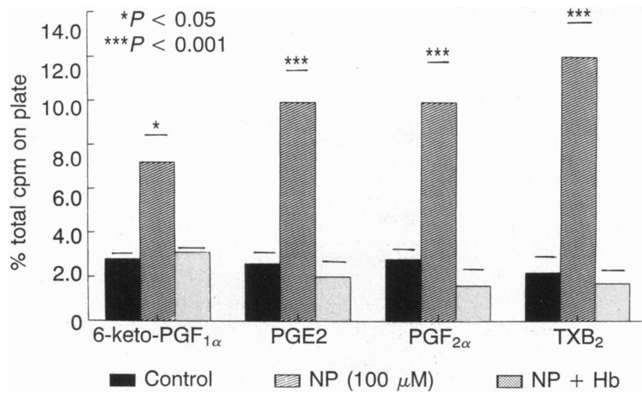

FIG. 1. Effect of NP (100 μM) and Hb (2 μg/ml) on basal metabolism of [^{14}C]arachidonic acid to various cyclooxygenase metabolites: 6-keto-$PGF_{1\alpha}$, $PGE_{2\alpha}$, and TXB_2. Each column represents mean + SEM of five preparations from different animals. In this and subsequent figures, P values are versus control.

completely prevented the increased synthesis induced by NP.

There was no significant effect of the inhibitor of NOS, NMMA, on the basal synthesis of either 5-HETE or 12-HETE; similarly, Hb to scavenge the NO produced had no significant effect on basal synthesis (Fig. 3).

Effect of NO on Spontaneous Uterine Contractility. When the estrogen-treated uterus was incubated *in vitro*, the motility declined over time, reaching quite low levels by 40 min of incubation (Fig. 4). When the NO being produced in the tissue was scavenged by Hb, this reduction of motility was almost completely blocked (Fig. 4).

Furthermore, the inhibitor of NOS, NMMA, very similarly blocked the decrease in motility (Fig. 4). This blockade of the decrease of motility was associated with more frequent contractions than those of the controls, which also tended to be of higher magnitude than those of the controls. Individual examples of the effect of Hb (Fig. 5) and NMMA (Fig. 6) are illustrated.

Effect of NO on the Quiescent Uterus. In contrast to the apparent inhibitory effect of NO on motility shown in the preceding experiments, when the uterine motility had been nearly completely reduced by incubation for 40 min *in vitro*, the results were completely different. For example, when NP was added to the bath, it induced a rapid onset of uterine contractions without change in base line tension, which lasted ≈10 min and then dissipated (Fig. 7). These results were similar to these previously reported for PGs (11). The effect of NP was completely blocked by addition of Hb to

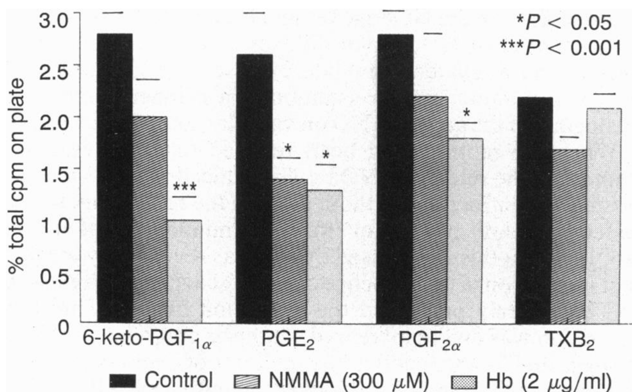

FIG. 2. Effect of NMMA (300 μM) and Hb (2 μg/ml) on basal metabolism of [^{14}C]arachidonic acid to various cyclooxygenase metabolites. Each column represents mean of five preparations from different animals.

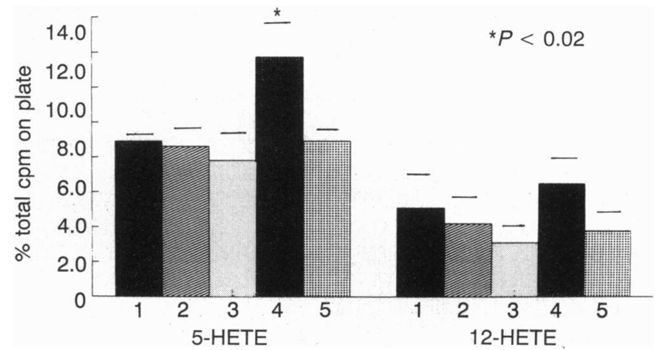

FIG. 3. Effect of NP, NMMA, and Hb on basal metabolism of [^{14}C]arachidonic acid to lipoxygenase products (5-HETE and 12-HETE). Each column represents mean of five preparations from different animals. Columns: 1, control; 2, NMMA (300 μM); 3, Hb (2 μg/ml); 4, NP (100 μM); 5, NP + Hb.

scavenge the NO released. That this effect was due to the stimulatory effect of NP on the cyclooxygenase enzyme and the synthesis and release of newly formed PGs was shown by the ability of indomethacin, an inhibitor of cyclooxygenase, to block completely the stimulation of motility induced by NP (Fig. 7).

DISCUSSION

The main purpose of the present study was to investigate whether endogenous or exogenous NO is able to modify the metabolism of labeled arachidonic acid in the uterus. Recently, we demonstrated that NP, a classic NO donor, increased PGE_2 release from rat hypothalamic fragments (11). In the present study, the action of NO on cyclooxygenase activity was confirmed. Indeed, we have discovered that NO released by NP stimulated significantly the synthesis and release of 6-keto-$PGF_{1\alpha}$, PGE_2, $PGF_{2\alpha}$, and TXB_2. The increment observed was 2- to 4-fold, being the highest with TXB_2. On the other hand, the NO scavenger Hb, when added simultaneously with NP, inhibited completely the stimulatory action of NO. The action of NP was also demonstrated on the lipoxygenase pathway. The synthesis of 5-HETE, a metabolic product of 5-lipoxygenase was stimulated significantly by NP, and this action was inhibited by Hb. The relative enhancement of 5-HETE synthesis induced by NP was much less than that occurring with the PGs and the synthesis of the related compound 12-HETE was unaffected by NP.

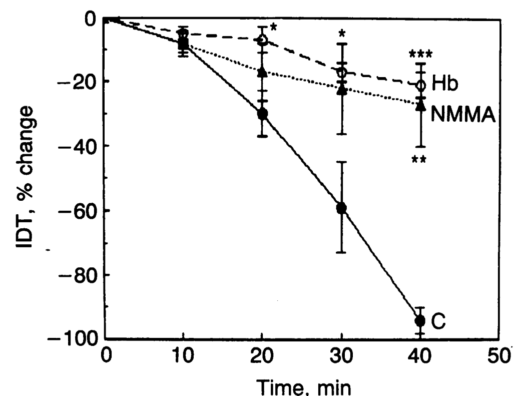

FIG. 4. Effect of NMMA (300 μM) and Hb (2 μg/ml) on spontaneous IDT of uterine strips isolated from estrogen-treated rats. C, control. Points are means of five uterine strips obtained from different animals and bars represent SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

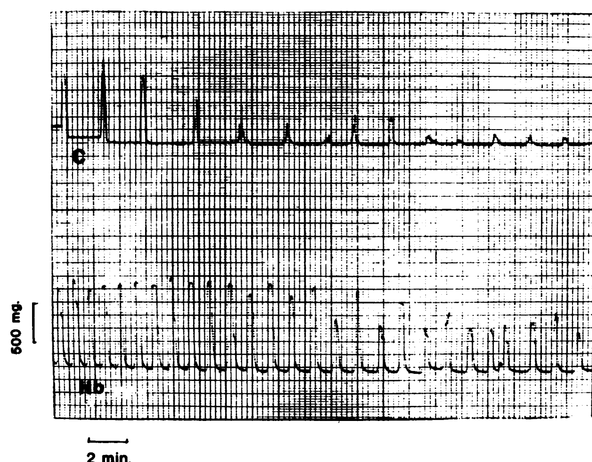

FIG. 5. Typical individual recording of the effect of Hb (2 μg/ml) on spontaneous motility of uterine strips from an estrogen-treated animal compared to the control (C) strip in the absence of Hb. Resting applied tension was 500 mg.

In rat hypothalamic fragments, we found no participation of endogenous NO in the "basal" synthesis of PGE_2 (11). On the contrary, in the estrogen-treated rat uterine tissue, NMMA, an inhibitor of NO synthase, decreased significantly the basal production of PGE_2 without altering the synthesis of other eicosanoids. When Hb was used as a scavenger of the NO released, not only the synthesis of PGE_2 but also that of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were decreased. On the other hand TXB_2 synthesis was not modified by either NMMA or Hb. Presumably, NMMA did not block NOS completely, whereas Hb completely removed released NO. This may explain the different responses of the various prostanoids to NMMA and Hb.

Because of these dramatic effects on PG synthesis and with the knowledge that PGs have powerful stimulatory effects on contraction of uterine muscle, it appeared of interest to examine the effect of NO on uterine motility in estrogen-treated rat uteri. To our surprise, instead of inhibiting uterine activity via blockade of PG synthesis and release, we found that the inhibitor of NOS, NMMA, or the scavenger of NO, Hb, almost completely prevented the gradual reduction in uterine motility that occurred during the first 40 min of incubation. Previously, we have shown that *in vitro* preparations of uteri from proestrous or estrous rats contract significantly less than strips from metestrous or diestrous rats (16). Moreover, uterine preparations isolated from ovariectomized rats treated with 17β-estradiol exhibited less spontaneous motility than those from untreated animals (17). We postulate that the decrement of spontaneous motility, which occurred during the first hour of incubation of uterine strips from estrous rats, could be due to the release of some endogenous compound with negative inotropic action. Indeed, we have demonstrated that estrogen treatment inhibited uterine motility *in vivo* and depressed *in vitro* contractions. These actions were presumably due to the release of histamine since they were antagonized by cimetidine, the H2 receptor blocker (18–20).

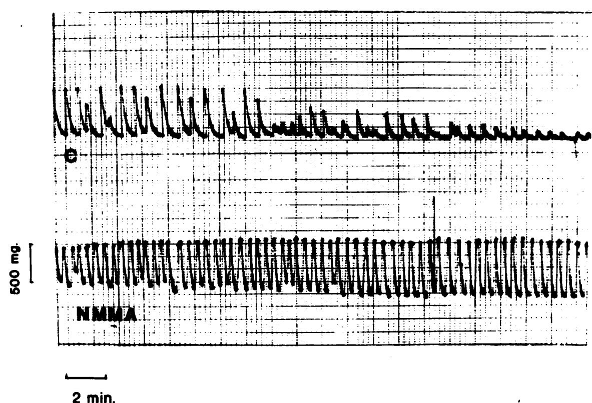

FIG. 6. Typical individual recording of the effect of NMMA (300 μM) on spontaneous motility of uterine strips from an estrogen-treated animal compared to control (C).

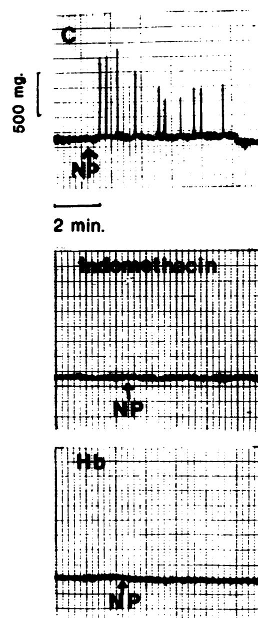

FIG. 7. Typical individual recording of the effect of NP (100 μM) on spontaneous motility of uterine strips from estrogen-treated animals in control medium and in the presence of indomethacin (1 μM) or Hb (2 μg/ml).

On the other hand, NMMA and Hb, by decreasing NO concentrations in the tissue, should have reduced the synthesis and release of PGs, which have a stimulatory action on uterine motility. We hypothesize that the NO released endogenously during this period was not only stimulating PG synthesis by activating cyclooxygenase but was also activating guanylate cyclase, leading to the production of cGMP, which caused decreased uterine motility as has been described by others (21). Presumably, the released cGMP overcame the stimulatory effects of the PGs.

Since histamine mediates via H2 receptors the decreased motility in the estrogen-primed uterus, we hypothesize that H2 receptors on the NOergic terminals activate NOS, leading to generation of NO, which diffuses to the uterine smooth muscle and activates guanylate cyclase with generation of cGMP and induction of relaxation in a manner completely analogous to the action of NO on vascular smooth muscle (4).

When the motility had been reduced to low levels, we found that the release of NO by NP induced a rapid increase in uterine contractions without altering the basal tone, which lasted for nearly 10 min, an effect that mimicked that of PGs (16, 17). That this was caused by NO was revealed by the fact that incubation in the presence of Hb to scavenge the released NO completely prevented the activation by NP. That this activation was due to increased synthesis and release of PGs is suggested by the fact that the inhibitor of cyclooxygenase, indomethacin, completely blocked the effect of NP.

Therefore, it appears that NO has two distinct effects on metabolism in uterine muscle—namely, (*i*) to activate guanylate cyclase, which generates cGMP, which inhibits motility, and (*ii*) to stimulate synthesis and release of PGs, which

enhance motility, but only from the relaxed uterus. Obviously, more work needs to be done *in vivo* and *in vitro* in different conditions of uterine motility to determine clearly the relative roles of cGMP and prostanoids in the control of uterine motility. Also, it would be important to study the system under different hormonal conditions—for example, in the untreated ovariectomized rat, and in the estrogen-primed animal treated with progesterone.

The importance of these findings is amplified by the recent description by Shew *et al.* (22) of the presence of NADPH diaphorase-positive neuronal axons in the rat uterus. These fibers are embedded in the uterine wall, including vasculature, endometrium, and myometrium. Since NOS and NADPH diaphorase are identical, the existence of NOergic neuronal terminals in the rat uterus was postulated (23). Indeed, we have now demonstrated histochemically the presence of a large number of NOergic fibers in the rat uterine wall by using a rabbit antiserum against the synthetic peptide included in the sequence of neuronal NOS.¶ Therefore, it is quite clear that NO is indeed produced in the uterus and probably is in large part responsible for regulation of uterine motility.

In fact, it appears that NO is involved in reproduction at all levels of the organism from the stimulation of leuteinizing hormone-releasing hormone release, which induces mating behavior and leuteinizing hormone release (12), to the induction of penile erection in males (5) and uterine motility in the female.

¶Gimeno, M., Franchi, A. M., Rettori, V., Chaud, M., Polack, J. M. & Suburo, A. M., 3rd European Congress on Prostaglandins in Reproduction, Aug. 25–27, 1993, Edinburgh, Scotland (abstr.).

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