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# IL-1$\alpha$ Induces Inducible Nitric Oxide Synthase in Uterine Smooth Muscle

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**In a previous report, we have identified interleukin-1$\alpha$ (IL-1$\alpha$) in amniotic fluid of women with premature rupture of the amniotic membrane (PROM), and suggested that this cytokine may play a role in the development of the fetus. To clarify the functional role of amniotic IL-1$\alpha$, we studied its effect on the induction of nitric oxide (NO) synthesis in the uterus. IL-1$\alpha$ administered intraperitoneally to rats induced an increase in the amount of mRNA encoding the macrophage-type iNOS which was determined by reverse transcription-polymerase chain reaction. NO measurement by NO selective electrode revealed that NO production in the rat and human uterus treated with IL-1$\alpha$ was promoted by the addition of 1 mM L-arginine to the culture medium. These findings suggest that in women with PROM, amniotic IL-1$\alpha$ may reduce uterine contractions, in part, by inducing of iNOS.** © 1997 Academic Press

Interleukin-1 (IL-1) is a cytokine, involved in homeostatic processes, such as the immune system and in inflammatory reactions (1). It is produced not only by immunologically competent cells but also by a wide variety of epithelial cells, and is involved in the growth and differentiation of various cells through a paracrine or autocrine mechanism.

Human amniotic fluid (AF) is produced during early embryonic stage and believed to be important for the growth and protection of the fetus. It contains various cytokines, such as tumor necrosis factor and IL-1 (2-6). Romero et al. (6) demonstrated elevated IL-1, especially IL-1$\beta$, in women during preterm labor and clinical chorioamnionitis and during spontaneous parturition at term with or without infection. In most women without labor pain or infection, amniotic IL-1 was at undetectable levels, i.e., below the sensitivity of the assay used. Thus, amniotic IL-1$\beta$ may trigger the onset of labor by inducing the synthesis of prostaglandin in the amniotic membrane or decidua, and promote subsequently an immune reaction in the event of a concomitant intrauterine infection.

A sensitive enzyme-linked immunosorbent assay using monoclonal antibodies was recently developed, whereby IL-1 in amniotic fluid can be measured in subjects with premature rupture of the membrane (PROM), without labor pain or infection (7). A noteworthy finding was that IL-1$\alpha$ levels were significantly elevated in women with PROM. Also IL-1$\alpha$ but not IL-1$\beta$ was localized in chorionic plate of the trophoblast and in the epithelial cells of the umbilical cord by an immunohistochemical method (7). Thus, the production of IL-1$\alpha$ by the fetus is probably a response to the stress of PROM, and that it might have some beneficial effects on the fetus. Relative to this contention is our previous findings, suggesting that IL-1$\alpha$ may promote maturation of the proliferating mucosal epithelium to act as a barrier to protect the fetus against the hostile extrauterine environment (8).

Relaxation of the uterus may be another important mechanism to protect the viability of the fetus in the event of PROM. Indeed, we found that the inducible type of nitric oxide synthase (iNOS) was expressed in the lipopolysaccharide (LPS) stimulated myometrium of rat uterus and that NO production from cultured human uterine smooth muscle cells (9). NO is known to be an endothelium-derived relaxing factor and is involved in the uterine quiescence state during pregnancy (10-12). The present paper is the first report describing the physiological significance of elevated IL-1$\alpha$ as a regulator of uterine contractility during PROM by the upregulation of the iNOS system of the myometrium. That this metabolic pathway acts as a physiological protective mechanism for the developing embryo in the event of PROM is discussed.

## MATERIALS AND METHODS

*In vivo stimulation by IL-1$\alpha$.* 10 to 12-week-old female Wistar rats were used in the *in vivo* study to clarify the action of IL-1$\alpha$.

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Rats were injected intraperitoneally with IL-1$\alpha$ (15 $\mu$g/kg) (recombinant IL-1$\alpha$, Otsuka, Tokushima, Japan) or vehicle as control. To examine the effect of glucocorticoid on NO production by IL-1$\alpha$, dexamethasone (3 mg/kg, i.p., Sigma, St. Louis, MO) was injected 40 min prior to the administration of IL-1$\alpha$. The animals were killed four hours after the injection. Uteri were immediately removed and the total mRNA was isolated and used to RT-PCR.

*Culture of rat uterine tissues.* Rat uterine tissues were also used to measure NO production by *in vitro* IL-1$\alpha$ stimulation. Namely they were cut into 3 mm cubes, and placed into each well of a 24-well culture plate with Hank's balanced salt solution (Sigma, St. Louis, MO) with or without IL-1$\alpha$ (5 ng/ml). The plate was incubated in the humidified incubator with 5% carbon dioxide in air at 37 °C for 24 hrs. The effect of glucocorticoid was also examined by addition of hydrocortisone (10 $\mu$M, Sigma, St. Louis, MO) in culture medium.

*Culture of human uterine smooth muscle cells.* Informed consent was obtained from each patient before surgery. Smooth muscle cells were isolated from uterine tissues obtained from the upper incisional edge of the lower uterine segment of pregnant women at the time of cesarean section and cultured. Single smooth muscle cells were obtained by enzymatic dissociation according to the method of Young and Herndon-Smith (13). The dissociated cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 U/ml, Sigma, St. Louis, MO), and streptomycin (100 g/ml, Sigma, St. Louis, MO), at a density of $10^5$-$10^6$ cells/ml. Aliquots of the suspended cells were placed into each well of a 24-well culture plate. The plates were incubated in a humidified incubator in 5% carbon dioxide in air at 37°C. The smooth muscle cells were used after 5 to 10 days of culture. Contamination with fibroblasts was excluded by detecting $\alpha$-smooth muscle actin using an immunofluorescent staining technique (14). The cells were stimulated as described above and used to measure NO production by *in vitro* stimulation of IL-1$\alpha$.

*Reverse transcription-polymerase chain reaction (RT-PCR).* Total RNA was prepared from *in vivo* stimulated rat uterus by the acid-guanidium isothiocyanate phenol chloroform (AGPC) method (15). RT-PCR was performed by a modification of the method described by Liu et al (16). Briefly, 10 $\mu$g of total RNA from each tissue was reverse transcribed into cDNA using an oligo-(dT) 15 primer and MMLV reverse transcriptase (USB, Cleveland, OH). RT-generated cDNAs encoding macrophage iNOS and glyceraldehyde-3- phosphate dehydrogenase (G3PDH) were both used as positive controls and as internal standards. Genes were amplified by PCR using oligonucleotide primers for iNOS (17) and G3PDH (18) that corresponded to rat macrophage iNOS and rat G3PDH cDNA. PCR amplification was performed by standard methods using Taq polymerase (Perkin Elmer, Branchburg, NJ) according to the following conditions, utilizing a programmed temperature control system (PC700, ASTEC, Tokyo, Japan): 1) an initial denaturation for 4 min at 94°C, 2) 30 amplification cycles (96°C for 1 min, 55°C for 1 min, and 72°C for 2 min), 3) a 10 min extension at 72°C. PCR products were electrophoresed in 2% agarose gel containing 0.2 $\mu$g/ml ethidium bromide. The gel was then photographed under ultraviolet transillumination. For semi-quantification, PCR bands on the gel photographs were scanned by a densitometer linked to a computer analysis system (Densitograph, ATTO, Tokyo, Japan) and densitometric data were expressed as arbitrary density units. We normalized the iNOS signal to the corresponding G3PDH signal from the same RNA sample. Data are expressed as an iNOS/G3PDH ratio.

*Measurement of NO.* To investigate the production of NO from confluent human uterine smooth muscle cells or the uterine tissue excised from rats (net weight 50 mg/ml solution), 1 mM L-arginine was added to the culture medium and the produced NO was measured using a NO selective electrode (Model NO 501, Intermedical, Nagoya, Japan), fitted with a porphyrinic-based microsensor (19). The probe measures the concentration of NO gas in aqueous solutions. The NO concentration is displayed as a redox current. In this system, NO that diffuses through the membrane is oxidized at the working electrode, resulting in an electrical current. The current flow is proportional to the rate of diffusion through the membrane's outer surface. Each electrode has a differing sensitivity, that required daily calibration of the electrodes prior to the performance of the using S- nitroso-N- acetyl DL-penicillamine. Measurements were performed at 32-35 °C.

Furthermore, we confirmed that production of NO after addition of 1 mM L-arginine was failed in each experiment in which 1 mM L-NAME, a specific blocker of L-arginine/NO pathway, was added simultaneously.

*Solutions and chemicals.* Normal Tyrode's solution contained 140 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 10 mM Na-[3{N-morpholino}-propanesulfonic acid (MOPS)], and 5 mM glucose. The solutions described above were adjusted to pH 7.2 with NaOH. L-arginine, NG-L-nitroarginine (L-NAME) were purchased from Wako Chemicals (Tokyo, Japan).

*Statistical analysis.* Data were expressed as mean ± SEM. Statistical analysis was performed by an one way analysis of variance followed by Student's t test. A level of $p<0.05$ was considered to be statistically significant.

## RESULTS

### *Detection of iNOS and G3PDH mRNA by RT-PCR in Rat Uterus*

The PCR products amplified using the iNOS- and G3PDH- specific primers showed clear bands at the predicted sizes of 444 and 309 bp, respectively (Fig.1). The nucleotide sequences of the PCR-amplified iNOS cDNA from uterus were matched with the cloned rat vascular smooth muscle iNOS cDNA sequence.

A summary of the ratios of the iNOS/G3PDH for uterine tissues are given in Table 1. A weak band of iNOS mRNA was detected in the control uterus of all 5 rats. A strong iNOS signal was observed in all uteri from animals treated with IL-1$\alpha$ (15 $\mu$g/kg) ($p<O.O5$). These signals were significantly suppressed in all tissues by pretreatment with dexamethasone (3 mg/kg)

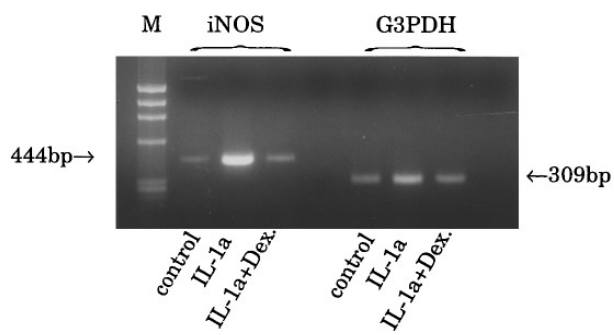


**FIG. 1.** *In vivo* effect of interleukin-1$\alpha$ (15 $\mu$g/kg) (IL-1$\alpha$) treatment on inducible nitric oxide synthase (iNOS) mRNA expression in rat uterus. Gel photograph shows PCR-amplified iNOS and glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) cDNA derived from iNOS and G3PDH mRNA. In control uterus, small signal was observed. IL-1$\alpha$ treatment induced marked expression of iNOS mRNA. Dexamethasone pretreatment (3mg/kg) (Dex.) inhibited the IL-1$\alpha$-induced expression of iNOS mRNA. M: size marker.

**TABLE 1**

Signal for iNOS mRNA in Rat Uterus after *in Vivo* IL-1$\alpha$ Stimulation

| | Control (n = 5) | IL-1$\alpha$ (n = 5) | IL-1$\alpha$+ dexamethasone (n = 5) |
|---|---|---|---|
| iNOS | 88.8 ± 73.0[a] | 351 ± 130 | 98.4 ± 55.2 |
| G3PDH | 984 ± 277[a] | 1015 ± 344 | 977 ± 274 |
| iNOS/G3PDH | 0.131 ± 0.082[b] | 0.514 ± 0.167* | 0.158 ± 0.090 |

[a] Density units of PCR band (mean ± SEM).
[b] Ratio of mRNA signal for iNOS to G3PDH (mean ± SEM).
* $p < 0.05$ compared to control.

($p<0.05$). Neither IL-1$\alpha$ nor dexamethasone treatment had any significant effect on G3PDH signals.

### *Production of NO by Cultured Rat Uterus*

Fig. 2 shows the *in vitro* stimulatory effect of IL-1$\alpha$ on the production of NO in cultured rat uterus, measured with an NO selective electrode. In unstimulated uterus (control), the addition of 1 mM of L-arginine did not induce any release of NO. Since we detected weak signals for iNOS mRNA in the control rat uterus, the production of NO induced with L-arginine in freshly isolated unstimulated tissue was measured. Very small NO signal was observed in 3 experiments. The amount of NO production was 83.7 ± 33.4 nM (Fig. 3), which is at the lower limits of NO detection assay.

Incubation with IL-1$\alpha$ (5 ng/ml) for 24 hrs markedly increased the production of NO on the addition of 1 mM L-arginine. This production of NO lasted more than 20 min and reached a peak of 367.2 ± 39.4 nM. Pretreatment with 10 $\mu$M hydrocortisone abolished the production of NO induced by IL-1$\alpha$ (Fig.2-3).

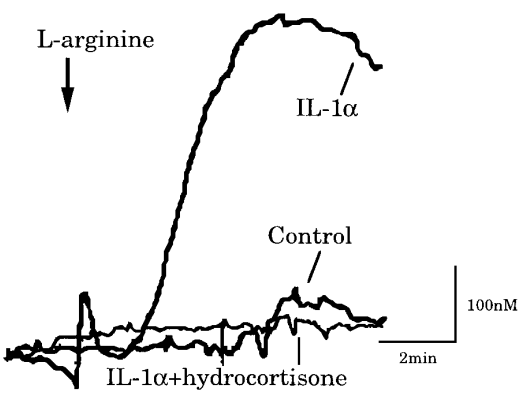


**FIG. 2.** The representative profile of *in vitro* production of nitric oxide (NO) in rat uterus stimulated by IL-1$\alpha$. Addition of 1 mM L-arginine induced NO production in the 5 ng/ml IL-1$\alpha$-treated uterus. In control uterus, NO production was very small. 10 $\mu$M hydrocortisone (HC) blocked IL-1$\alpha$-induced NO production.

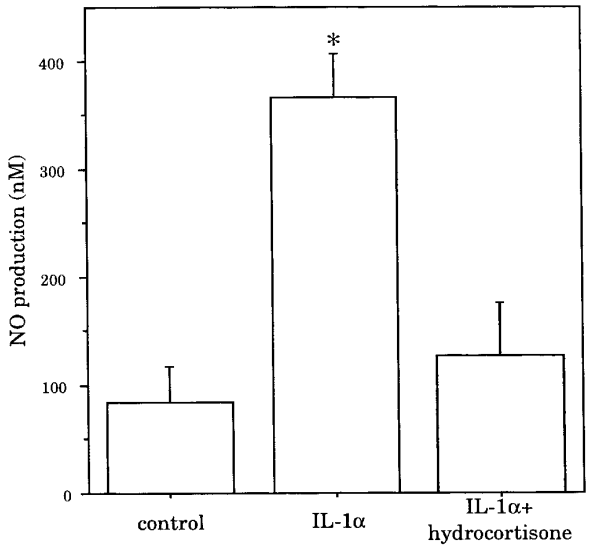


**FIG. 3.** Rat uterine NO production induced with IL-1$\alpha$. Bars represent mean ± SEM of 4 specimens. Note the NO production was very small in the control uteri and hydrocortisone blocked IL-1$\alpha$ induction. *$p<0.05$.

### *Production of NO by Human Uterine Smooth Muscle Cells*

The production of NO by addition of L-arginine in human uterine smooth muscle cells stimulated with IL-1$\alpha$ for 24 hours similar to that obtained with cultured rat uterus (Fig.2 vs. 4). The peak level of NO produced with 1 mM L-arginine was 824 ± 185 nM (n=4). In the control uteri, NO production was nil to barely detectable. The effect of IL-1$\alpha$ was blocked by 10 $\mu$M hydrocortisone (Fig.4).

## DISCUSSION

The present study clearly demonstrated the upregulation of iNOS mRNA by RT-PCR in the rat myometrium following treatment with IL-1$\alpha$. Furthermore, the thesis that the mRNA is translated to produce the enzyme was validated by the measurement of NO production in the presence of L-arginine in the cultured rat uterine tissue and in cultured human smooth muscle cells treated *in vitro* with IL-1$\alpha$. These data are completely concordant with our previous results that LPS induced iNOS in rat and human uterus (9). In the previous study, we demonstrated that NO production in the uterus after LPS stimulation is due to the expression of an inducible type of NOS while the constitutive type NOS (cNOS) was not. This conclusion is based on the characteristics of the band pattern of the PCR product, suppression by dexamethasone, and lack of $Ca^{2+}$ requirement (9). Also, the electrophoretic pattern of the PCR product and suppressive effect of dexamethasone show that IL-1$\alpha$ induces iNOS in the uterus. Similar to the previous finding, a weak signal of iNOS

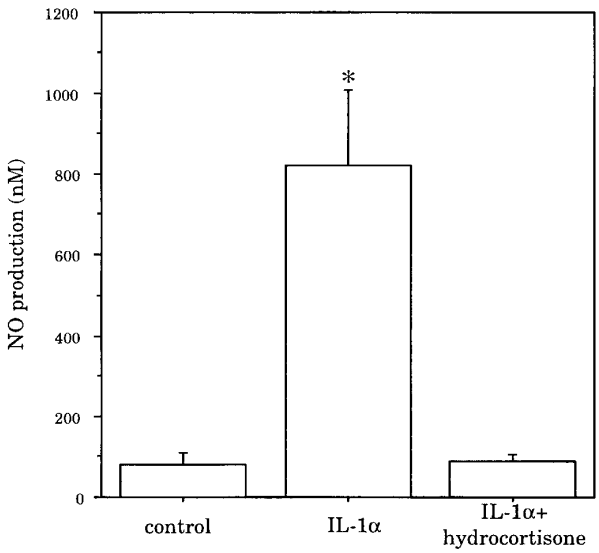


**FIG. 4.** IL-1$\alpha$ stimulation of nitric oxide (NO) production in cultured human uterine smooth muscle cells. The addition of 1 mM L-arginine induced NO production in 5 ng/ml IL-1$\alpha$-treated uterine cells. In control uterine cells, NO production was very small. 10 $\mu$M hydrocortisone (HC) blocked IL-1$\alpha$-induced NO production. Bars represent mean ± SEM of 4 specimens. *$p<0.005$.

mRNA was detected in uterus of control rats. We previously pointed out the possible physiological role of NO to act as a protective factor against infection of the uterus exposed to the external environment (9).

Although whole rat uterus was used in these studies, the site of IL-1$\alpha$ induction of iNOS takes place most likely in the myometrium. since the present in vitro study revealed that cultured human smooth muscle cells are responsive to IL-1$\alpha$ stimulation. The isolated uterine cells were identified definitively as smooth muscle cells immunocytochemically using antibody against $\alpha$-smooth muscle actin, a specific marker for smooth muscle. Furthermore, it is known the IL-1$\beta$ does not induce iNOS in fibroblasts (20).

As described in introduction, IL-1$\alpha$ is predominantly produced from fetus into amniotic fluid in PROM cases and seems to be one of the main biological and pathological factors of maternal and fetal reaction during PROM (7,8). Thus, to examine the physiological role of IL-1 in PROM we used IL-1$\alpha$ as the stimulator to myometrium in all the experiments in this study, although it seems likely that IL-1$\beta$ stimulates NO production in myometrium as well as IL-1$\alpha$ as shown in our preliminary study (9).

NO is known to relax vascular and uterine smooth muscles by increasing guanosine 3, 5-cyclic monophosphate (cGMP) (10-12). The success for prevention of premature labor by the clinical use of NO donor further support the hypothesis that NO has a relaxing effect on the myometrium of pregnant women in vivo. Indeed, NO activates guanylate cyclase and increases the synthesis of cGMP in smooth muscle. By modulating phosphorylation of myosin light chain, cGMP regulates the contractile state. In vitro study using cultured human smooth muscle cells obtained from pregnant uterus, we have also shown that NO activates $Ca^{2+}$-dependent $K^+$ channel ($K_{Ca}$) (Shimano et al., to be published). Opening of the $K_{Ca}$ channels results in the hyperpolarization of the cell membrane and subsequently closing of Ca channels, which reduces entrance of calcium ion into the cells, resulting in the relaxation of the smooth muscle cells. Based on these data, we hypothesize that in PROM case the fetus produces IL-1$\alpha$, which induces iNOS in the myometrium and NO as the metabolic product from circulating L-arginine inhibits uterine contraction by activation of cGMP in a paracrine or autocrine manner.

Recent reports show that NO also activates cyclooxygenase, which mediates the synthesis of prostaglandins (PGs) in murine macrophage cell line, human fetal fibroblast and rat uterus (20-22). Thus, an increase in NO production may not always cause relaxation of smooth muscle cells (21). However, PGs do not always lead to contraction of smooth muscle cells and may occasionally cause relaxation. $PGI_2$, a well-known vasodilator, relaxes non-pregnant human myometrium (24), and $PGE_2$, a vasodilator of uterine artery (25), may have dual effects on human uterus: promotes contraction in low concentration and relaxation in high concentration (26). Whether the uterus contracts or relaxes when it exposed to NO or PGs would depend on the state of the uterus, such as pregnancy, stage of the pregnancy, the presence or absence of infection, or the concentration and the types of PGs produced. Izumi et al. (10) reported that the sensitivity of the myometrium to cGMP differs during the various stages of pregnancy. It is highly sensitive during the mid-gestation stage and becoming less sensitive toward term. Thus, it is likely that, in PROM, especially at mid-gestation, NO might cause relaxation of the uterine myometrium to overcome the contractile effects of PGs by the activation of guanylate cyclase and by the high sensitivity of the myometrium to cGMP.

Considering the fact that fetus produces a large amount of IL-1$\alpha$ during PROM, this factor should be beneficially to the fetus facing a hostile environment. Our previous findings suggesting that IL-1$\alpha$ may induce maturation of the fetal mucosal epithelium are supportive of the above hypothesis. In addition, the present data showing that IL-1$\alpha$ may inhibit uterine contraction by inducing iNOS in the myometrium supports strongly the important role that IL-1$\alpha$ may play to protect the fetus in the event of PROM through various physiological mechanisms.

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