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Expression and Localization of Inducible Nitric Oxide Synthase in the Rat Ovary: A Possible Involvement of Nitric Oxide in the Follicular Development

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In the present study, using Northern blot analysis, we demonstrated that the level of iNOS mRNA in the ovary of immature rats decreased after 6 h of pregnant mare serum gonadotropin administration and recovered gradually up to the untreated level 48 h after the administration. Both *in situ* hybridization and immunohistochemistry revealed that iNOS mRNA and protein was predominantly localized in granulosa cells in most of immature follicles, but not in mature follicles with an antrum, which was a consistent finding regardless of gonadotropin treatment. Furthermore, we found that cultured granulosa cells had the ability to express iNOS mRNA in the presence of cytokines such as tumor necrosis factor-α, interleukin-1β and interferon-γ, which are inherently detectable in the ovary. These results raise the possibility that locally produced NO synthesized by iNOS may be involved in the developmental status of ovarian follicles in concert with gonadotropin.

Key Words: **nitric oxide (NO); nitric oxide synthase (NOS); ovarian follicles; granulosa cells.**

Recent studies have revealed that endogenously generated nitric oxide (NO) synthesized from L-arginine by nitric oxide synthase (NOS) is a mediator of a variety of physiological and pathological phenomena (1, 2). NOS isoforms are categorized into three types; i.e. neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS is expressed predominantly in neuronal tissues, whereas eNOS is found in mainly endothelial cells. These two types of NOS are expressed constitutively and their enzymatic activities are regulated by intracellular calcium concentration.

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On the other hand, iNOS is shown to be expressed in a wide array of tissues and organs, and its expression is induced by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-γ (IFN-γ) (1, 2). The expression of iNOS results in sustained synthesis of nitric oxide over long periods. A large amount of NO produced by iNOS is thought to be involved in pleiotropic cytokine-mediated biological functions and manifest cytotoxic or cytoprotective properties. Recent studies have demonstrated that NO arrests neuronal cell growth (3), and acts as an antiproliferative agent during *Drosophila* embryonic development by inhibiting DNA synthesis (4). A recent study from our laboratory demonstrated that NO arrests cell cycle in human hepatocellular carcinoma cells (5).

In the ovary, gonadotropin stimulates the development of a set of follicles, most of which undergo atretic degeneration through apoptosis of granulosa cells as well as oocytes and the remaining follicles which evade atresia are destined to ovulate (6). The development of follicles is considered to be regulated by various factors such as cytokines, growth factors and locally-produced hormones. Among them, NO is of particular interest since it is involved in the regulation of several physiological functions of the ovary such as ovulation and steroidogenesis (7-21). Recently, it has been reported that iNOS mRNA is localized in granulosa cells of immature follicles (16), however, its protein is localized in theca-interstitial cells (20).

In the present study, we investigated the change in iNOS mRNA levels in the rat ovary during the process of follicular development by Northern blot analysis and also cellular localization of iNOS protein as well as its mRNA using immunohistochemistry and *in situ* hybridization, respectively. We further examined whether iNOS could be expressed in rat granulosa cells

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by various cytokines which are inherently present in the ovary.

MATERIALS AND METHODS

Chemicals and biochemicals. Recombinant human TNF-α, IL-1β and IFN-γ were generous gifts from Dainippon Pharmaceutical Co.(Osaka, Japan), Banyu Pharmaceutical Co.(Tokyo, Japan) and Shionogi Pharmaceutical Co.(Osaka, Japan), respectively. All other chemicals were of the highest grade commercially available.

Animal treatment. Twenty-five-day-old immature female Wistar rats were purchased from Charles River Japan, Inc. (Yokohama, Japan) and housed in a temperature-controlled room with a 12-hr light/12-hr dark schedule. Pelleted food and water were provided *ad libitum*. On the 26th day of age, five rats were sacrificed as the control (time 0h), To initiate follicular development, rats were treated with intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG) in 0.2 ml saline and were sacrificed 6, 12, 24 and 48 h later by cervical dislocation. Similarly, rats which were injected with 0.2ml saline alone instead of PMSG were sacrificed 6, 12, 24 and 48 h later. At each time point, five rats were sacrificed. Removed ovaries were immediately cleaned of surrounding connective tissues as described previously (22). Ovaries were snap-frozen in liquid nitrogen and stored at -80 C until used for Northern blot analysis. For histochemical analyses, ovaries were fixed in 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4 C for 3 h and embedded in paraffin by the standard procedures. Three-micron sections were taken on silane-coated glass slides.

Cell culture and treatment. On the 26th day of age, rats were treated with intraperitoneal injection of 10 IU PMSG. Forty eight hours after PMSG administration, granulosa cells were collected from ovaries as described previously (22). Cells were seeded onto 10 cm plastic culture dishes (Beckton Dickinson Co., Lincoln Park, NJ) at a density of 10^6 cells/dish in the medium and cultured in a humidified atmosphere of 5% CO2 in 95% air at 37C. After 24 h, granulosa cells were treated with various combinations of the following cytokines; TNF-α (500 IU/ml), IL-1β (10 ng/ml) and IFN-γ (100 IU/ml). After an additional 24 h incubation, granulosa cells were harvested for RNA extraction.

RNA isolation and Northern blot analysis. Northern blot analysis of iNOS mRNA expression was carried out using 15 μg total RNA essentially based on the method described previously (23-25). The total RNA was extracted by the guanidine thiocyanate method (23). Fifteen μg of total RNA were separated by electrophoresis on a 1% agarose gel containing 6% formaldehyde and transferred to a Hybond-N membrane (Amersham, Little Chalfont, UK). Prehybridization and hybridization were carried out as described previously (24, 25). A 700 bp fragment of the 5′ portion of cloned rat liver iNOS cDNA was labeled with [α-^{32}P]CTP (3000 Ci mmol^{-1}), using a random prime labeling kit (Amersham). The membranes were washed with 2× standard saline citrate (SSC; 1×SSC=0.15 M NaCl, 0.015 M sodium citrate)/0.1% SDS containing 0.2% sodium pyrophosphate at 58C for 30 min 4 times and exposed to an imaging plate (Fuji Photo Film Co., Japan). The relative intensities of Northern blot hybridization signals were determined using a Bio-imaging analyzer BAS2000 (Fuji Photo Film Co., Japan). To normalize iNOS mRNA levels based on β-actin mRNA level, the filter was rehybridized with a ^{32}P-labeled cDNA probe of cloned rat β-actin (24, 25). iNOS mRNA levels were compared among the treatment groups after normalization by β-actin mRNA content.

Reverse transcription-coupled polymerase chain reaction (RT-PCR). RT-PCR of RNA samples was carried out in essentially the same way as described previously (24, 25). First-strand cDNA was synthesized in a reaction volume of 15 μl containing 5 μg of total RNA and 0.2 μg of random hexamer primers using a commercial kit according to the manufacture's instructions (Pharmacia Biotech, Uppsala, Sweden). For the detection of iNOS and β-actin mRNAs, PCR amplification was performed with the following oligonucleotide primers: i-NOS, 5′-TCCAACCTGCAGGTCTTCGATGC-3′ (sense) and 5′-GGACCAGCCAAATCCAGTCTGC-3′ (antisense); β-actin, 5′-ATCCGCAAAGACCTGTACGC-3′ (sense) and 5′-TGTGTGGACT-TGGGAGAGGA-3′ (antisense). Denaturation, annealing and elongation in the PCR reaction were carried out at 94, 57 and 72C for 30 sec, 1 min and 2 min, respectively, for 40 cycles in the case of iNOS and for 25 cycles in the case of β-actin.

In situ hybridization. Rat iNOS complementary DNA (cDNA; DNase I fragments) was thymine-thymine (T-T) dimerized by UV irradiation (26, 27). The procedure of *in situ* hybridization with double-strand cDNA probes was essentially the same as described previously (28, 29). Briefly, deparaffinized and rehydrated sections were immersed in 0.3% H2O2 in methanol to block endogenous peroxidase activity and then, treated with 0.2N HCl (20 min) and 10 μg/ml proteinase K (37C, 15 min), successively. After postfixation with 4% PFA in PBS, prehybridization was carried out as described previously (28). Hybridization was carried out at 37C for 12 h with 4 μg/ml labeled probe dissolved in hybridization medium. After 5 times-washing in 50% deionized formamide in 0.5× SSC with 0.075% Brij 35 at 37C for 1 h each and at 45C once for 30 min, the sections were reacted with the blocking solution and reacted with anti-T-T mouse IgG (1:100) diluted with the blocking solution for 12 h. Then, the sections were reacted with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:200) diluted with the blocking solution for 1 h and washed four times in PBS with 0.075% Brij 35 for 15 min each. The sites of HRP were visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB) and H2O2 in the presence of nickel and cobalt ions to enhance DAB staining (30). As a negative control, T-T dimerized λDNA was used. Because granulosa cells have very scanty cytoplasm, the hybridization signal was enhanced using an image analyzer (SP 500, Olympus, Tokyo, Japan)

Immunohistochemistry. iNOS was localized immunohistochemically using the polyclonal antibody described previously (31, 32). Deparaffinized and rehydrated sections were immersed in 0.01 M citrate buffer (pH 6.0) and then autoclaved at 120C for 10 min as described previously (33). Then, the sections were immersed in 0.3% H2O2 in methanol to block endogenous peroxidase activity and were preincubated with 500 μg/ml goat IgG and 1% BSA in PBS for 1 h at room temperature. The sections were reacted with the primary antibody (1:800) diluted with 1% BSA in PBS 12 h at room temperature in a humidified atmosphere. After washing in PBS with 0.075% Brij 35, the sections were incubated with goat anti-rabbit IgG (1:400) diluted with 1% BSA in PBS for 1 h at room temperature, and washed as described above. The HRP sites were visualized as described for *in situ* hybridization. As a control, serial sections were reacted with normal rabbit serum in place of the specific antibody.

Statistical analysis. All experiments were repeated at least three times. Data were given as the mean ± SEM from three independent experiments. The statistical analysis was performed by Student's *t*-test.

RESULTS

Expression of iNOS mRNA in the Whole Ovary

Northern blot analysis was used to detect changes in the level of iNOS mRNA in the whole ovaries after gonadotropin stimulation. Figure 1 illustrates a representative result of Northern blot analysis, demonstrating that iNOS mRNA was constitutively expressed in the ovary from immature rats. Six hours after PMSG administration, iNOS mRNA decreased transiently to an undetectable level, and thereafter returned gradu-

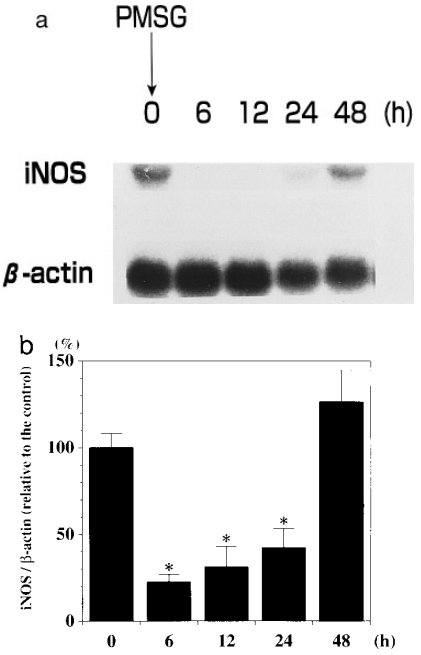

FIG. 1. Effect of gonadotropin administration on iNOS expression in the ovary from immature rats. a, Northern blot analysis of iNOS mRNA expression in the immature rat ovary. Expression of iNOS mRNA was analyzed by Northen blotting using 15 μg of total RNA isolated from ovaries at various time points after PMSG administration. The amount of total RNA analyzed was evaluated by hybridization with rat β-actin probe. The result was representative of five independent experiments. b, Quantitative analysis of iNOS expression. Densitometry was used to quantify relative signal densities. The ratio of the density of iNOS mRNA to that of β-actin mRNA was calculated and the result was expressed as percentage of that of the control (time 0 h). Each point represents mean ± SEM (n= at least 5). *, $P < 0.05$ *vs.* the control (time 0 h) by Student's *t* test.

ally to the untreated level 48 h after PMSG administration. In the untreated control group, the level of iNOS mRNA remained unchanged throughout the experimental period (data not shown).

Histochemical Analyses of iNOS Expression in the Rat Ovary

Localization of iNOS mRNA and protein was histochemically analyzed in the ovary from untreated and PMSG-treated immature rats. iNOS mRNA was detected in the cytoplasm of granulosa cells in most of immature follicles both 0 h and 48 h after PMSG administration, while the intensity of iNOS mRNA signal was considerably fluctuated among granulosa cells (Figure 2). On the contrary, granulosa cells in mature follicles with an antrum were essentially negative for iNOS mRNA. Oocytes and theca-interstitial cells were also negative for iNOS mRNA, irrespective of follicular developmental stages.

In accord with the result of *in situ* hybridization, immunohistochemistry revealed that iNOS protein was localized in granulosa cells in immature follicles and signal intensity was variable among the cells. The granulosa cells in mature follicles with an antrum and oocytes were again negative. Theca-interstitial cells were negative or faintly stained (Figure 3).

Expression of iNOS mRNA in Granulosa Cells Cultured with Cytokines

RT-PCR and Northern blot analysis were used to examine whether iNOS mRNA was induced by cytokines in cultured granulosa cells. RT-PCR demonstrated that expected 700-bp DNA fragment from granulosa cells cultured without cytokines was amplified and that the same level of PCR product was observed in the cells cultured with a single cytokine (Figure 4). Interestingly, any combination of two or more cyto-

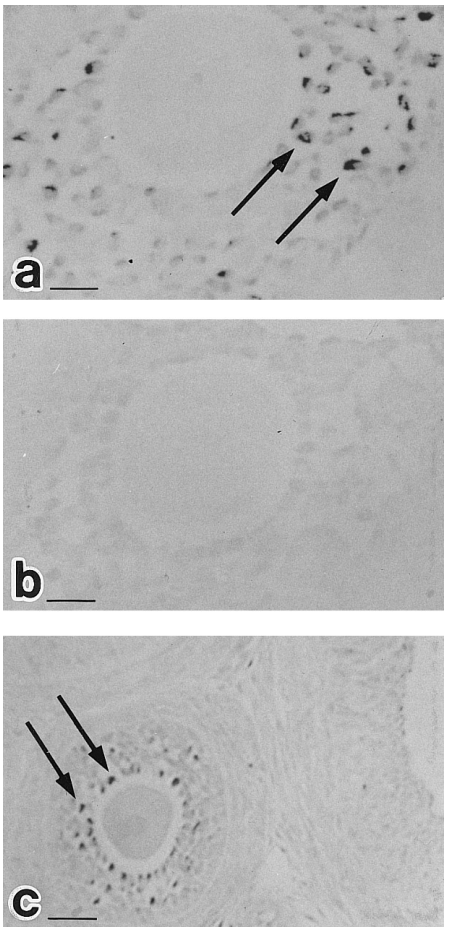

FIG. 2. *In situ* hybridization of iNOS mRNA in serial sections of the immature rat ovary 48 h after PMSG administration. a, An immature follicle hybridized with iNOS cDNA probe. iNOS mRNA was expressed in the cytoplasm of granulosa cells in an immature follicle (*arrow*) (magnification, ×400; bar= 25 μm). b, Control section hybridized with λDNA probe (magnification, ×400; bar= 25 μm). c, iNOS mRNA was expressed in granulosa cells in an immature follicle (*arrow*), but not in theca-interstitial cells (magnification, ×200; bar= 50 μm).

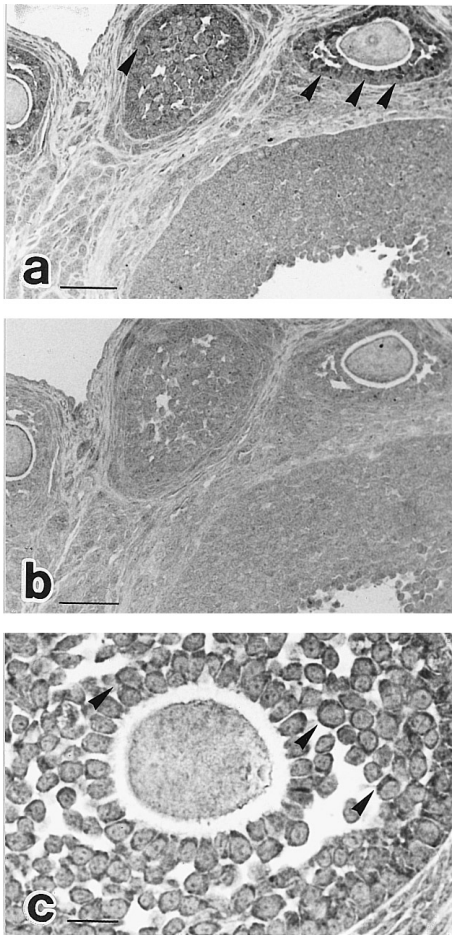

FIG. 3. Immunohistochemical detection of iNOS in serial sections of the immature rat ovary 48 h after PMSG administration. a, Immunohistochemistry with anti-iNOS antibody; iNOS was expressed in granulosa cells in immature follicles (*arrowhead*), but not in mature follicle with an antrum. Theca-interstitial cells were faintly stained. (magnification, ×200; bar= 50 μm). b, A section was reacted with normal rabbit serum as negative control (magnification, ×200; bar= 50 μm). c, iNOS was expressed in the cytoplasm of granulosa cells in an immature follicle (*arrowhead*) (magnification, ×400; bar= 25 μm).

kines increased the level of iNOS mRNA synergistically. Each PCR product was sequenced, and confirmed to be identical to the sequence of iNOS obtained from rat liver (34). As shown in Figure 5, Northern blot analysis failed to detect iNOS mRNA in granulosa cells cultured in the absence of cytokines or in the presence of a single cytokine (Figure 5a). However, the addition of any two cytokines increased the amount of iNOS mRNA up to 2 to 5-fold over the untreated control. The combination of three cytokines enhanced iNOS mRNA expression up to about 100-fold over the untreated control (Figure 5b).

DISCUSSION

In the present study, we examined iNOS mRNA expression in the rat ovary and its regulation by gonadotropin using Northern blot analysis. In the immature rat ovary, iNOS mRNA level was decreased transiently in response to PMSG administration and it returned gradually to the untreated level 48 h after PMSG administration. Using the similar experimental model, thus far, several authors have reported on the expression of iNOS in the rat ovary (16, 18, 20). Jablonka-Shariff et al. demonstrated that during PMSG-induced follicular development, iNOS protein levels remained relatively constant (20). According to the observation by Van Voorhis et al., iNOS mRNA level was maximum in unstimulated immature rat ovaries and reduced 48 h after PMSG administration (16). They determined the level of iNOS mRNA only at time 0 h (control) and 48 h after PMSG administration. In the present study, however, we have clearly demonstrated that the decrease in iNOS mRNA level after PMSG administration was a transient phenomenon by observing its level at shorter intervals.

In situ hybridization revealed that iNOS mRNA was localized in granulosa cells in most of immature follicles but not in mature follicles with an antrum. These findings are in good agreement with that of the previous report (16), where they detected the localization of iNOS mRNA using radioactive RNA probes. Moreover, our nonradioactive method with an excellent resolution enabled us to conclude that the expression of iNOS mRNA in granulosa cells was not homogenous. It is also worth emphasizing that iNOS mRNA was expressed only in the immature follicles regardless of gonadotropin stimulation. Immunohistochemical study as well demonstrated that the expression of iNOS protein was heterogenous among granulosa cells of immature follicles. Theca-interstitial cells were faintly stained as reported previously (20). The likely explanation of this discrepancy between mRNA and protein is that *in situ* hybridization might fail to detect the faint

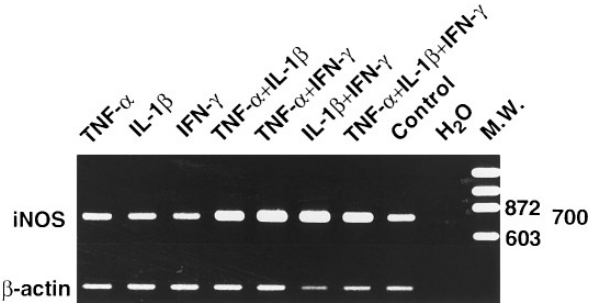

FIG. 4. Effects of TNF-α (500 IU/ml), IL-1β (10 ng/ml) and IFN-γ (100 IU/ml) on the expression of iNOS mRNA in rat cultured granulosa cells. RNA from granulosa cells after incubation for 24 h with cytokines was amplified by RT-PCR. A 700-basepair PCR product was the size predicted based on the cDNA sequence of iNOS. The amplified DNA fragments were separated on a 3% agarose gel and were visualized by ethidium bromide staining. The result was representative of three independent experiments.

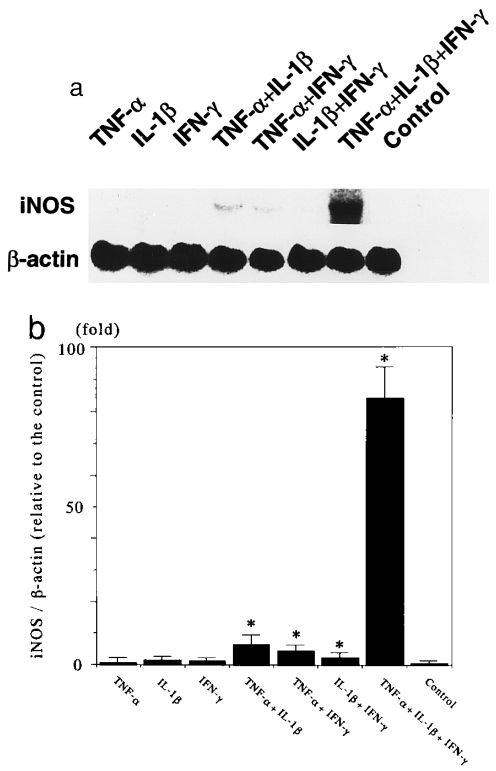

FIG. 5. Effects of TNF-α (500 IU/ml), IL-1β (10 ng/ml) and IFN-γ (100 IU/ml) on the expression of iNOS mRNA in rat cultured granulosa cells. a, Expression of iNOS mRNA in 15 μg of total RNA from granulosa cells after incubation for 24h with cytokines was analyzed by Northern blot analysis. The amount of total RNA analyzed was evaluated by hybridization with rat β-actin probe. The result was representative of three independent experiments. b, Quantitative analysis of iNOS expression. Densitometry was used to quantify relative signal densities. The ratio of the density of iNOS mRNA to that of β-actin mRNA was calculated. Each point represents mean ± SEM (n= 3). *, $P < 0.05$ *vs.* the control by Student's *t* test.

level of iNOS mRNA. An alternative explanation is that theca-interstitial cells are apt to be stained nonspecifically with antibodies as reported previously (22).

Recent studies have demonstrated that NO arrests neuronal cell division required for terminal differentiation (3), and acts as an antiproliferative agent during *Drosophila* development by inhibiting DNA synthesis (4). It has been also shown that NO inhibits mitosis of cultured vascular smooth muscle cells (35), blocks the cell cycle at G2+M phase in mouse macrophage-like cells (36). Similar cell cycle arrest at G1 by NO was observed in a human hepatoma cell line, HepG2 with concomitant p21/WAF1 induction (5). Overall, these findings lead us to suggest that NO may be a cytostatic factor for granulosa cells in immature follicles and PMSG-induced reduction in iNOS mRNA level may be a possible trigger of follicular development.

Using Northern blot analysis, we demonstrated a considerable amount of iNOS mRNA was expressed in granulosa cells cultured in the presence of any combination of proinflammatory cytokines such as TNF-α, IL-1β and IFN-γ. Especially, the combination of all of these cytokines induced a maximal amount of iNOS mRNA in a synergistic way. However, in the absence of these cytokines, the expression of iNOS mRNA was not detected. Interestingly, we confirmed that a considerable amount of iNOS mRNA was detectable in untreated whole ovary. Recent studies have shown that TNF-α, IL-1β and IFN-γ could be derived from infiltrating cells, such as leukocytes and T lymphocytes, inherently localized in the theca layer and stroma (37, 38), and directly modulate the actions of gonadotropin on granulosa cell functions (39-41). Thus, it is possible that cytokines from these cells can be accessible to granulosa cells in an *in vivo* situation, which may be an explanation for the presence of iNOS mRNA in untreated ovaries.

Recent studies demonstrated an inhibitory effect of NO on steroidogenesis in granulosa cells (12-14). NO is postulated to be an antiestrogenic agent through a mechanism of directly inhibiting aromatase activity (12, 14). Considering our histochemical analyses, it is intriguing to speculate that NO might inhibit steroidogenesis by rendering granulosa cells quiescent and thereby preventing their differentiation.

In conclusion, the present study raises the possibility that locally produced NO via iNOS in granulosa cells may be involved in the developmental status of ovarian follicles in concert with gonadotropin. However, the molecular mechanisms by which NO modulates developmental status of follicles await further study.

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