Changes in Nitric Oxide Synthase Activity in the Ovary of Gonadotropin Treated Rats: The Role of Nitric Oxide during Ovulation

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Abstract. Immature rats receiving equine chorionic gonadotropin (eCG) and human CG (hCG) were used to study the time course changes in nitric oxide synthase (NOS) activity in the ovary during ovulation. To study the role of NO in ovulation, the effects of intrabursal injection of L-NG-monomethylarginine (L-NMMA, 125 pg/20 μl/bursa), a NOS inhibitor, on the number of ova shed were also examined. Rats were sacrificed at -48, 0, 3, 6, 9, 12, and 24 h after hCG injection, and the ovaries were collected for the NOS activity assay, Western blotting, NADPH-diaphorase histochemistry and immunohistochemistry. Total NOS and constitutive NOS activities in the ovary increased significantly at 9 h after hCG injection and the values remained high thereafter. Inducible NOS (iNOS) activity was detectable as a small peak at 3 and 6 h after hCG injection. Endothelial NOS (eNOS) protein production increased after hCG injection with a peak at 12 h, whereas iNOS protein production decreased at 12 and 24 h after hCG injection. NADPH-diaphorase positive cells increased at the thecae of growing follicles after hCG injection, appeared at mural granulosa cells before ovulation, and were detected in newly formed corpora lutea, which coincided with the results in eNOS positive cells by immunohistochemistry. L-NMMA given to rats at 5 or 7 h after hCG was most effective in reducing the number of ova shed. These results indicate that the NOS activity and NOS positive cells increased after hCG injection, and that eNOS was likely the main NOS increasing in the ovary during ovulation. It is concluded that NO produced between 5 and 9 h after hCG might play a supportive role in ovulation.

Key words: NOS activity, Ovulation, NADPH-diaphorase, Immature rat, L-NMMA

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local inflammatory reaction [11]. NO is well
documented as a local generator in inflammation
[10], and participates in ovulatory processes [7, 8]
including ovarian steroidogenesis [4]. The increase
in intraovarian progesterone concentrations after the
LH surge is an important event in ovulation [12].
In this study, we examined the changes in NOS
activities and localizations in the ovary during ovu-
lation in immature rats receiving equine CG (eCG)
and hCG. In addition, the effects of intrabursal in-
jection of L-N\textsuperscript{\textdegree}O
-monomethylarginine (L-NMMA), a
NOS inhibitor, on the numbers of ova shed and se-
rum progesterone, testosterone and estradiol levels
were examined to study the role of NO in ovulation.

**Materials and Methods**

**Materials**

\[3\text{H}\]-L-arginine (35-70 Ci/mmol) was obtained
from Amersham International plc (Buckingham-
shire, England). L-N\textsuperscript{\textdegree}O
-monomethylarginine (L-
NMMA) was purchased from Wako Pure Chemical
Industries, Ltd. (Osaka, Japan). eCG, hCG,
nitroblue tetrazolium (NBT), \(\beta\)-nicotinamide adenine
dinucleotide phosphate (\(\beta\)-NADPH) and nuclear fast
red were purchased from Sigma Chemical Co. (St.
Louis, MO, USA). Dowex AG 50 WX-8 column
(Na\textsuperscript{+} form), a Western blot detection kit (ECL kit)
and O.C.T. compound were purchased from Bio-
Rad Laboratories (Hercules, CA, USA), Life Science
(Tokyo, Japan) and Miles Inc. (Elkhart, IN, USA),
respectively. Immunohistochemistry kit (HISTO-
FINE SAB-PO kit) was purchased from Nichirei Co.
(Tokyo, Japan), and monoclonal antibody to eNOS
was from Transduction Laboratory (Lexington, KY,
USA). Polyclonal antibody to iNOS was generously
supplied by Dr. Stephen Russell, The University of
Kansas Medical Center, Kansas City, KS, USA.

**Animals and Treatments**

The experimental protocols were reviewed by the
Committee for the Ethics on Animal Experimenta-
tion at Yamaguchi University School of Medicine
and carried out under the Guidelines for Animal Ex-
periments at Yamaguchi University School of Medi-
cine under Law (No. 105) and Notification (No. 6) of
the Government.

Twenty-one day old Sprague-Dawley rats (Japan
SLC Inc., Hamamatsu, Japan) were housed in rooms
with lights on from 0500 to 1900 h and free access to
standard rat chow and water. All rats received a
subcutaneous injection of 15 IU eCG and 48 h later
an injection of 15 IU hCG; those rats ovulated
around 12 h after hCG injection. Rats were killed by
decapitation under ether anesthesia before eCG in-
jection (−48 h), immediately before hCG injection
(0 h), or at 3, 6, 9, 12 or 24 h after hCG injection.
Ovaries were quickly removed, frozen in liquid N\textsubscript{2}
and stored at −70°C for NOS activity assays and
Western blot analysis. For the NADPH-diaphorase
histochemical study, rats were anesthetized with
ether, their blood drawn and infused with saline fol-
lowed by 20 ml of freshly prepared 4% paraformal-
dehyde in 0.1 M sodium phosphate buffer (PBS, 4°C,
ph 7.4) at each time described above. Ovaries were
removed, immersed in 4% paraformaldehyde in 0.1
M PBS (4°C, ph 7.4) for 1 h, placed in 15% sucrose
in PBS at 4°C for 24 h, and then embedded in
O.C.T. compound. For immunohistochemistry,
ovaries were fixed in Bouin's solution and then em-
bedded in paraffin.

Immature rats given eCG and hCG were divided
into ten groups: ovarian intrabursal injection of L-
NMMA (125 µg/20 µl/bursa, the L-NMMA group)
or the same volume of saline (control group) at 0, 3,
5, 7 or 9 h after hCG injection. All rats were
sacrificed 24 h after hCG injection, the number of
ova shed was counted by irrigating the fallopian
tubes with saline, and the serum was saved for
progesterone determinations by radioimmunoassay
(RIA). Additional rats receiving the intrabursal
injection of L-NMMA 5 h after hCG were also
sacrificed at 10 h after hCG injection when serum
progesterone levels were at their peak [12, 13], and
trunk blood was collected. Serum progesterone,
testosterone and estradiol concentrations were deter-
mined by RIA.

**NOS Activity Assay**

Frozen ovaries were homogenized at 0°C in 25% of
homogenizing buffer which consisted of 50 mM
Tris-HCl, 250 mM sucrose, 1 mM dithiothreitol
(DTT), 0.1 mM EDTA, 0.1 mM EGTA, 1 µM
leupeptin, 1 µM pepstatin A and 0.1 mM 4-
amidinophenylmethanesulfonyl fluoride. The homo-

genates were centrifuged at 15,000 × g for 30 min

at 0°C, and the supernatant was saved. NOS activity

in the ovaries was measured using [3H]-L-arginine

which is converted to [3H]-L-citrulline by NOS [14].

Briefly, each sample was incubated with [3H]-L-argi-

nine in 100 μl assay solution (50 mM Tris-HCl, pH

7.4, 1 mM DTT, 2 mM CaCl₂, 1 mM β-NADPH,

1 μg calmodulin, 10 μM tetrahydrobiopterin, 5 μM

flavin adenine dinucleotide) for 30 min at 37°C, and

the reaction was terminated by addition of 200 μl of

ice cold HEPES buffer (20 mM, pH 5.5) containing

2 mM EDTA and 0.2 mM EGTA. The incubated

sample was then applied onto a Dowex AG 50 WX-8

column (Nat form), and the eluted [3H]-L-citrulline

was measured by liquid scintillation counting. The

specific NOS activity was determined as the difference

between total counts and the nonspecific counts de-

termined by the reaction in the presence of 1 mM

EGTA and 1 mM L-NMMA. The specific iNOS ac-

tivity was determined as the difference between the

counts in the absence of Ca-Calmodulin with 1 mM

EGTA and the nonspecific counts described above.

The protein content in the supernatant was measured

by the Lowry method [15] and the NOS activity was

expressed as pmol [3H]-L-citrulline/min/mg protein.

The cNOS activity was calculated by subtracting

iNOS activity from total NOS activity. All assays

were performed in duplicate.

Western Blot Analysis

Forty μg protein for eNOS or 60 μg protein for

iNOS of the supernatant in the NOS activity assay

was separated by SDS-PAGE in 7.5% gels under

reducing conditions. The proteins on the gel were
electrophoretically transferred to nitrocellulose mem-

branes and reacted with either eNOS monoclonal

antibody (1 : 500) or iNOS polyclonal antibody (1 :

20). The membranes were then immersed in the
reaction buffer containing peroxidase-conjugated
rabbit anti-mouse IgG for eNOS or peroxidase-conjugated
swine anti-rabbit IgG for iNOS, respec-
tively. The reacted band was developed on a film

with an ECL kit. Reacted bands of NOS were

scanned and their relative optical densities were

measured by NIH Image. The same experiments

were repeated three times.

NADPH-Diaphorase Histochemistry

O.C.T. compound embedded ovaries were sec-
tioned at 10 μm, and the sections were mounted on

clean slides in a humidified box. To demonstrate the

NADPH-diaphorase positive cells which indicate the

NOS positive cells [16], sections were incubated in

0.1 M PBS (pH 8.0) containing 0.3% Triton X-100,

0.1 mg/ml NBT and 1.0 mg/ml β-NADPH at room

temperature for 30 min. The reaction was terminat-
ed by rinsing the sections with cold PBS. After

several rinsings, the slides were air-dried and stained

with nuclear fast red.

Immunohistochemistry

Paraffin embedded ovaries were sectioned at 4 μm.
The sections were mounted on egg-white coated

slides, and immunohistochemistry was made with a

HISTOFINE SAB-PO kit as follows. The sections

were deparaffinized and treated with 0.3% H₂O₂ in

methanol for 30 min to block endogenous peroxidase

activity. The slides were then washed with PBS, and

treated with 10% normal rabbit serum to block non-
specific binding. The serum was decanted, and the

slides were incubated overnight at 4°C with the

monoclonal antibody (diluted 1 : 250 with 1% BSA in

PBS). After washing with PBS, the slides were in-
cubated with biotinylated rabbit anti-mouse immu-

noglobulin (10 μg/ml) for 10 min at room tem-

perature. After washing with PBS, the slides were

incubated with peroxidase conjugated streptavidin

(100 μg/ml) for 5 min at the room temperature,
washed again in PBS, and developed with 0.2 mg

DAB/ml of 0.05% H₂O₂ in Tris-buffered saline

(TBS) (pH 7.4, 0.05 M) for 2 min. The sections were

stained with hematoxylin solution.

Steroid Assays

Serum progesterone, testosterone and estradiol
concentrations were determined by RIA as reported

previously [17]. The specific antisera to progester-
one (GDN 337), testosterone (GDN 250) and es-
tradiol (GDN 244) were kindly provided by Dr.
Gordon D. Niswender, Colorado State University,
Fort Collins, CO, USA. The lower limits of these

assay sensitivities were 5.4 pg/tube for progesterone

and...
and 2.7 pg/tube for testosterone and estradiol, respectively.

**Statistical Analysis**

Data were analyzed by ANOVA and Duncan's new multiple range test; where appropriate, Student's t-test was used. Differences were considered significant if $P<0.05$.

**Results**

**Changes in NOS Activities during Ovulation**

Total NOS and cNOS activities significantly ($P<0.01$) increased at 9 h after hCG injection, and it remained at the same levels until 24 h after hCG injection. On the other hand, iNOS activity declined after eCG injection and slightly increased at 3 and 6 h after hCG injection; but those changes were not significant (Fig. 1).

**Changes in NADPH-Diaphorase Activity Localizations in the Ovary during Ovulation**

NADPH-diaphorase positive cells were present scarcely at interstitium and only moderately at the small follicles before eCG injection (Fig. 2, A), and increased at the thecal layer of the growing follicles 48 h after eCG injection (Fig. 2, B). The positive cells were mainly observed at the thecal layer at 3 and 6 h after hCG injection (Fig. 2, C, D) and then appeared also at the mural granulosa cell layer at 9 h after hCG injection (Fig. 2, E, H). At 12 h after hCG injection, the positive cells increased at the granulosa cell layer and decreased at the thecal layer (Fig. 2, F, I). At 24 h after hCG injection, positive cells were present inside of the corpus luteum and in the thecal layer of small follicles (Fig. 2, G).

**Changes in eNOS and iNOS Protein and their Localization in the Ovary during Ovulation**

Western blot analysis indicated that eNOS protein increased after hCG injection with a peak at 12 h, whereas iNOS proteins slightly increased at 3 h and subsequently decreased at 12 and 24 h after hCG injection (Fig. 3). The eNOS positive cells were present at the thecal and interstitial layers at 3 h (Fig. 4, A), and appeared also at the mural granulosa cell layer at 9 h after hCG injection (Fig. 4, C). At 24 h after hCG injection, the eNOS immunostains could be detected in newly formed corpora lutea (Fig. 4, E). The iNOS stain, however, was indefinite.

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Fig. 1. Changes in intraovarian total NOS (solid line), cNOS (dot line) or iNOS (solid thin line) activities during follicular development and ovulation in eCG and hCG treated immature rats. The vertical lines are SEM of 6–9 rats. Each value was normalized to the mean value of total NOS activity before eCG injection (-48 h). Total NOS and cNOS activities at 9 h and after of post hCG increased significantly compared with the value at -48 or 0 h. Data were analyzed by Duncan’s new multiple range test. a, d: $P<0.01$ compared with the value at -48 h. b: $P<0.05$ compared with the value at -48 h. c, f: $P<0.05$ compared with the value at 0 h. e: $P<0.01$ compared with the value at 0 h.
Fig. 2. Time-related changes of histochemical localization of NADPH-diaphorase positive cells in the ovary at -48 (A), 0 (B), 3 (C), 6 (D), 9 (E, H), 12 (F, I) and 24 (G) h after hCG injection. Blue stains indicate the presence of NADPH-diaphorase activity. (f) = follicle, (cl) = corpus luteum, (t) = thecal layer, (g) = granulosa cell layer. Original magnification: A–G, × 20; H, I, × 50. Scale bars = 100 μm in A–G. Scale bars = 40 μm in H and I.
Fig. 3. Western blot analysis of intraovarian eNOS and iNOS protein expressions during follicular development and ovulation in eCG and hCG treated immature rats. The representative blotting bands are shown in the left panel and the changes of mean values with three repeated experiments are shown in the right panel. Solid line shows eNOS values, and dotted line shows iNOS values. Values are normalized by the value at -48 h as 100%.

Fig. 4. Immunohistochemical localization of eNOS in the ovary at 3 (A, B), 9 (C, D) and 24 (E, F) h after hCG injection. eNOS stains are present at the thecal (t) and interstitial (i) layers (arrows in A) at 3 h, increase at theca, and are present at the mural granulosa (g) cells (arrows in C) at 9 h, and decrease but are present in the newly formed corpora lutea (cl) (arrows in E). There were no positive stains in the controls (B, D, F). Original magnification, ×100. Scale bars=20 μm.
Effects of Intrabursal L-NMMA Injection on the Number of Ova Shed and Serum Steroid Levels

L-NMMA given at 5 or 7 h after hCG injection reduced the number of ova shed (P<0.05) and slightly but not significantly reduced serum progesterone levels by 24 h after hCG when compared with the control group (Table 1). L-NMMA given at 0, 3 or 9 h after hCG injection had no effect on the number of ova shed or serum progesterone concentrations (Table 1). Serum estradiol levels at 10 h after hCG injection were significantly higher in the group given L-NMMA at 5 h after hCG injection than in the control group, but serum progesterone and testosterone levels were not different between the two groups (Table 2).

Discussion

Ovarian NOS activity, mainly cNOS, increased at 9 h after hCG injection in the thecal and interstitial layer. NADPH-diaphorase positive cells after the fixation and incubation made in this study are equal to NOS positive cells [16] and the time course changes of their localizations after hCG injection coincided with the changes of eNOS localization. bNOS protein could not be detected in the ovary by Western blot analysis or immunohistochemistry (unpublished data; Nakamura et al.) as reported for bNOS mRNA expression [1]. Therefore, the main isoform increasing during ovulation would seem to be eNOS. These results agree well with previous reports that eNOS increases after hCG injection and is localized to blood vessels in hilum and stroma [1] or thecal layer and mural granulosa cells [2]. It is well known that the LH surge induces ovarian hyperemia and vasodilation [18], which may be mediated by eNOS [19], a potent vasodilator in arteries [20]. Since immature rats receiving eCG and hCG injections ovulate at 12 h after hCG injection, the increase of eNOS activity at 9 h after hCG indicates an important role for eNOS in local vasodilation during ovulation.

Table 1. Effects of intrabursal injection of L-NMMA given at 0, 3, 5, 7 or 9 h after hCG injection on the number of ova shed and serum progesterone levels at 24 h after hCG injection

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Number of ova shed (/rat)</th>
<th>Serum progesterone levels (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 h)</td>
<td>6</td>
<td>41±2</td>
<td>16.4±1.4</td>
</tr>
<tr>
<td>L-NMMA (0 h)</td>
<td>6</td>
<td>43±4</td>
<td>19.8±3.0</td>
</tr>
<tr>
<td>Control (3 h)</td>
<td>3</td>
<td>42±4</td>
<td>18.0±5.8</td>
</tr>
<tr>
<td>L-NMMA (3 h)</td>
<td>3</td>
<td>47±6</td>
<td>17.0±6.2</td>
</tr>
<tr>
<td>Control (5 h)</td>
<td>6</td>
<td>43±3</td>
<td>22.1±1.9</td>
</tr>
<tr>
<td>L-NMMA (5 h)</td>
<td>6</td>
<td>33±2*</td>
<td>18.8±2.6</td>
</tr>
<tr>
<td>Control (7 h)</td>
<td>6</td>
<td>50±7</td>
<td>22.2±2.0</td>
</tr>
<tr>
<td>L-NMMA (7 h)</td>
<td>6</td>
<td>31±4*</td>
<td>18.5±3.0</td>
</tr>
<tr>
<td>Control (9 h)</td>
<td>6</td>
<td>37±8</td>
<td>34.2±5.8</td>
</tr>
<tr>
<td>L-NMMA (9 h)</td>
<td>6</td>
<td>35±4</td>
<td>22.2±1.8</td>
</tr>
</tbody>
</table>

Data were means±SEM of 3 or 6 rats.

*P<0.05 compared with control group at each time by Student’s t-test.

Table 2. Serum progesterone, testosterone and estradiol levels at 10 h after hCG injection in the groups receiving L-NMMA or saline at 5 h after hCG injection

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum progesterone levels (ng/ml)</th>
<th>Serum testosterone levels (pg/ml)</th>
<th>Serum estradiol levels (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80.5±4.9</td>
<td>927 ± 62</td>
<td>115±33</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>86.1±9.9</td>
<td>882±105</td>
<td>171±31*</td>
</tr>
</tbody>
</table>

Data were means±SEM of 5 rats.

*P<0.05 compared with the control group by Student’s t-test.
The dilated arteries permit important ovulation-related factors to flow into the Graffian follicles, and accelerate further growth and ovulation related processes. The appearance of eNOS positive cells in the granulosa cell layers agreed with the previous report of angiogenesis during ovulation and luteal formation [21]. However, the mechanism by which eNOS is induced in the ovary by hCG is still unclear. Although an addition of hCG increases estradiol production by follicles [22] and estradiol increases eNOS activity [3], the eNOS content in the ovary did not increase by in vitro ovary incubation with hCG (unpublished data; Nakamura et al.). Continuous LH infusion induces superovulation in cyclic hamster, in which it is thought that LH induces vasodilation and thus more FSH can reach growing follicles [23]. Mast cells existing in the ovary release histamine for vasodilation in response to LH [24], but LH could not directly release histamine (unpublished data; Nakamura and Terranova). Substance P, which exists in the ovary [25], has a histamine releasing effect [26], and is also known to induce NO production in the arteries [27]. NO produced by eNOS might be a key factor in vasodilation during ovulation and neuronal-peptidergic network might cause vasodilation during the preovulatory period.

As shown in the present results with iNOS Western blot analysis, Van Voohris et al. have shown that iNOS mRNA levels are highest in the unstimulated ovary and decline with follicle growth, and also that iNOS mRNA is localized to the granulosa cells of secondary follicles and small antral follicles [1]. On the other hand, Jablonka-Shariff and Olson have reported that iNOS protein increases after hCG injection and is localized to the thecal layer and stroma [2]. The present results with iNOS activity assay showed that it declined after eCG injection and increased slightly at 3 and 6 h after hCG injection. There are reports suggesting iNOS involvement in the ovulatory process [7, 8]. Current reports indicate that IL-1, which plays an important role in ovarian function [22, 28, 29], utilizes NO to influence the ovarian cells [30-32]. The IL-1β mRNA expression in the ovary shows a narrow peak at 6 h after hCG injection [33]. In pancreatic β-cells, approximately 3 to 8 h is required for iNOS induction by IL-1 [34, 35]. If this time lag is required for iNOS expression after IL-1 stimulation, it would seem that IL-1 is not an inducer of iNOS in the ovary. We could not detect

iNOS immunostaining in the ovary. Therefore, the mechanism for stimulation of iNOS and its role in the ovary remain unclear. Matsumi et al. recently reported iNOS mRNA localization in immature follicles but not in mature follicles, and speculated its role in follicular development [36].

The number of ova shed was reduced by the intrabursal L-NMMA injection at 5 or 7 h after hCG, but not at 0, 3 or 9 h. L-NMMA is a competitive inhibitor of NOS synthase [37], and its inhibitory effect on NO release of endothelium by acetylcholine is reversed over a period of 90 min [38]. The present results indicate that NO produced between 5 and 9 h after hCG plays an essential role in follicular rupture (ovulation), but NO at other periods is not important for ovulation. The blood volume per ovary doubles its value before hCG injection at 4 h and increases 7-fold at 10 h after hCG injection [39]. NO produced by increasing NOS after hCG injection may contribute to this change of local circulation. Numerous factors are involved in inducing follicular rupture [40]. For example, progesterone, which increases as a result of the LH surge and peaks at 10 h after hCG injection [13], is one of the important factors in ovulation [12]; in fact, progesterone receptor knockout mice fail to ovulate [41]. NO, however, did not affect this progesterone increase at 10 h in rats whose ovulation was disturbed by L-NMMA injection. Moreover, the indispensable role of progesterone is in the first 4 h of the ovulatory process [12]. Therefore, the role of NO in ovulation may not be through progesterone production. The bursal treatment at 9 h after hCG injection seemed to be harmful because saline injection altered the number of ova shed and serum progesterone levels. Interestingly, serum estradiol levels were higher in the L-NMMA injected rats, which agreed with the previous in vitro experiments that NO suppresses progesterone and estradiol production, and that NOS inhibitors increase estradiol but not progesterone production [4, 6]. NO inhibits estradiol production via the suppression of aromatase activity and the reduction of aromatase mRNA expression [42].

In conclusion, the present findings show that NOS activity increases in response to hCG during the ovulatory process, with its main isoform being eNOS, and that NO produced between 5 and 9 h after hCG injection plays an important role in ovulation.
Acknowledgements

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References


22. Nakamura Y, Kato H, Terranova PF (1990) Interleukin-1α increases thecal progesterone production of...


