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Cell-specific expression and immunolocalization of nitric oxide synthase isoforms and the related nitric oxide/cyclic GMP signaling pathway in the ovaries of neonatal and immature rats*

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Abstract: Objective: The present study is designed to investigate the cellular expressions and immunolocalizations of three different nitric oxide synthase (NOS) isoforms and the related nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling pathway in the ovaries of neonatal and immature rats. Methods: The ovaries were obtained from ICR (Institute for Cancer Research) female Sprague-Dawley rats at postnatal days 1, 5, 7, 10, and 19. Then we carried out the histologic examination, immunohistochemistry, measurement of NOS activity, and modifications within the NO/cGMP pathway. Results: During postnatal days 1, 5, 7, 10, and 19, all three isoforms of NOS were mainly localized to the oocytes and expressed as a gradual increase in granulosa cells and theca cells within the growing follicle. The ovarian total NOS activities and NO levels were increased at postnatal days 7 and 10 compared with other days. Conclusions: Our findings suggest that the locally produced NO and the NO/NOS signaling systems are involved in the follicular development to puberty.

Key words: Nitric oxide synthase, Nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway, Ovary, Neonatal rats, Prepuberty
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1 Introduction

Nitric oxide (NO) has been recognized as a gas molecule that regulates significantly the biologic and physiologic processes of the reproductive system (Rosselli *et al.*, 1998). The major regulator of NO production is NO synthase (NOS), which appears in three isoforms: brain NOS (bNOS) or neuronal NOS (nNOS or NOS1), endothelial NOS (eNOS), and inducible NOS (iNOS). Both nNOS and eNOS, also named constitutive NOS (cNOS), are responsible for the continuous basal release of NO and require calcium/calmodulin for activation (Griffith and Stuehr, 1995; Snyder, 1995). The iNOS is expressed in response to inflammatory cytokines and lipopolysaccharides (Nathan and Xie, 1994). It is known that NO is an important modulator of folliculogenesis, atresia, steroidogenesis, prostaglandin biosynthesis, ovulation, luteolysis, oocyte growth, and maturation (Hattori and Tabata, 2006).

Given the important physiologic role of NO, many studies have been carried out to examine the expressions and localization patterns of NOS isoforms in reproductive systems in the mouse, rat, sheep, and pig (Kim *et al.*, 2005). It is reported that eNOS and iNOS isoforms are expressed in the luteinized ovary of the immature female Sprague-Dawley rat after treatment with several hormones (Olson *et al.*, 1996). Only the ovarian iNOS expression level has been found to be changed significantly during the prepubertal period among the three NOS isoforms in

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rats (Srivastava *et al.*, 1997). In addition, iNOS and eNOS are localized in oocytes and theca cells of the immature mouse (Mitchell *et al.*, 2004). However, there are still no data on the expression and localization of NOS isoforms in the neonatal and immature rats.

NO binds the heme group of soluble guanylyl cyclase (sGC) and significantly stimulates the activity of sGC, then subsequently increases cyclic guanosine monophosphate (cGMP) production (Arnold *et al.*, 1977). The cGMP is an important cyclic nucleotide that regulates many ovarian functions (Nakamura *et al.*, 2002; Chen *et al.*, 2003). Therefore, understanding changes in the NO/cGMP pathway in the ovary has become increasingly critical. However, there is a lack of such knowledge in the neonatal and immature rats.

The aim of the present study was to investigate the cell-specific expressions and localizations of the NOS isoforms and the related NO/cGMP pathway in the ovaries of neonatal and immature rats, in order to reveal the mechanisms of the NO/NOS system involved in the follicular development prior to puberty.

2 Materials and methods

2.1 Animals

Fifty immature ICR (Institute for Cancer Research) female Sprague-Dawley rats at postnatal days 1, 5, 7, 10, and 19 (10 rats per time point) were obtained from the Experiment Animal Center of Nanjing Medical University, China. The rats were sacrificed and ovaries were collected under a stereomicroscope. Immediately afterwards, the fresh ovaries were washed thrice with ice-cold Hank's solution. One side's ovary was fixed in 40 g/L paraformaldehyde at room temperature for 24 h and then kept in 70% alcohol for histologic examination and immunohistochemistry; the other side's ovary was frozen and kept in −80 °C freezer for the measurement of NOS activity, NO contents, etc. The experimental protocols involving rats were approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Nanjing Agricultural University, China.

2.2 Protein homogenate preparation

Protein was obtained from the frozen ovaries in a Dounce homogenizer (Wheaton, Millville, NJ, USA) by disrupting the tissues with radioimmunoprecipitation (RIPA) lysis buffer (50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% (v/v) NP-40, 20% (v/v) glycerol, 25 mmol/L benzamidine, 0.5 mg/ml leupeptin, 0.7 mg/ml pepstatin A, 2 mg/ml aprotinin, and 10 mg/ml trypsin inhibitor). After homogenization, samples were incubated for 30 min on ice and centrifuged at 12000×*g* for 10 min at 4 °C. Total protein concentrations were determined by the Bradford dye-binding assay (Bradford, 1976; Shi *et al.*, 2004) using BSA standards assay. The optical density (OD) value was measured at 595 nm using a Synergy™ 2 Multi-function Microplate Reader (Bio-Tek Instruments Inc., Winooski, Vermont, USA). The remaining supernatant was snap-frozen and stored at −80 °C.

2.3 Histologic examination

To evaluate the follicular development of neonatal rats, ovaries kept in 70% alcohol were dehydrated through increasing alcohol concentrations, embedded in paraffin, and sectioned serially at 5 μm. We then randomly chose 10 slides at every time point, then stained them with hematoxylin and eosin (H&E), and observed any histopathology under a light microscope (Nikon Inc., NY, USA).

2.4 Immunohistochemistry

After fixation, the ovaries from female immature rats at postnatal days 1, 5, 7, 10, and 19 were embedded in paraffin, and then 5-μm sections were cut and mounted on slides. The sections were then processed for immunohistochemical analysis using polyclonal antibodies raised in rabbits against nNOS, iNOS, and eNOS obtained from Boster Biological Technology, Ltd., Wuhan, China, and polyclonal antibodies to sGC α_1 and β_1 subunits (Sigma Chemical Co., St. Louis, MO, USA). The sections were incubated at room temperature overnight with polyclonal rabbit immunoaffinity-purified antisera directed against nNOS (1:100), iNOS (1:100), eNOS (1:100), and sGC α_1 (1:1000) and β_1 (1:1000). The immunoreactivity assay of specific protein was visualized by the Elite ABC kit (BioGenex, San Ramon, CA, USA) and reaction with 0.05% 3,3′-diaminobenzidine tetrachloride (DAB; Sigma Chemical Co.) in 10 mmol/L phosphate-buffered saline (PBS) containing 0.01% H_2O_2 for 3 min. The negative control was examined using

normal rabbit serum instead of primary antibody (Boster Biological Technology). Then the sections were counter-stained by H&E and mounted with coverslips for identifying the structure and types of cells in the rat ovary. Three independent observers were asked to examine the pictures and assessed the intensity of staining using a scale: −, no staining detected; +, weak; ++, moderate; +++, strong staining (Shi *et al.*, 2004; Kim *et al.*, 2005). All observers evaluated all slides, and observations outside the 5%–95% of the remaining observations for the treatment group were considered outlying data and excluded from analysis. Relative levels of immunostaining were evaluated and repeated at least four times. The results represent consistently observed patterns of immunohistochemistry (Zhou *et al.*, 2007).

2.5 Measurement of NOS activity

The total NOS and iNOS activities were measured using a commercial reagent (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, the NOS activity was determined by measuring the release of lactate NO generated via a five-electron oxidation of terminal guanidinium nitrogen on L-arginine by NOS (Palmer and Moncada, 1989). Then, NO bound to the nucleophilic materials and generated a colored compound. After that, the reaction was terminated with citric acid. The optical density (OD) value was measured at 530 nm using a Synergy™ 2 Multi-function Microplate Reader (Bio-Tek Instruments Inc., Winooski, Vermont, USA). In the present study, the concentration of ovarian protein homogenates was 800 μg/ml. However, to further address the eNOS activity, the ovarian protein homogenates were treated with spermidine trihydrochloride ($C_7H_{19}N_3 \cdot 3HCl$), a special inhibitor of nNOS, at an optimal dose of 120 μmol/ml. The procedures were performed strictly according to the manufacturer's protocols.

2.6 Measurement of NO content

The content of NO was measured using a commercial reagent (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, NO gas reacts in vivo and yields the stable end products, nitrite (N_2^-) and nitrate (N_3^-) (Moncada *et al.*, 1991; Lewis and Deen, 1994). Then nitrate reductase reduces N_3^- to N_2^-. The optical density (OD) value was measured at 550 nm using a Synergy™ 2 Multi-function Microplate Reader. In the present study, the concentration of ovarian protein homogenates was 320 μg/ml. The procedures indicated by the kits were performed strictly according to the manufacturer's protocols.

2.7 Radioimmunoassay (RIA) of cGMP

The level of cGMP was determined by competition binding with [^{125}I]-succinyl guanosine 3′,5′-cyclic monophosphate tyrosyl methyl ester (ScGMP-TME) (Theilig *et al.*, 2001). The amount of bound radioactivity was determined in a γ-counter. The cGMP level of each sample was measured using commercial RIA kits (Shanghai University of Traditional Chinese Medicine, China). The minimal detection limit for cGMP was 0.1 pmol/ml for nonacetylated samples; cross-reaction rate with cyclic adenosine monophosphate (cAMP) was less than 0.001%. The intra-coefficients of variations for cGMP were less than 6%.

2.8 cGMP-phosphodiesterase (PDE) activity assay

The cGMP-PDE activity was measured using 0.1 mmol/L cGMP as substrate, according to the method detailed previously (Sette and Conti, 1996; Nichols and Morimoto, 2000). Samples were assayed in a total volume of 200 ml of reaction mixture including 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.1 mmol/L cGMP. After incubation for 30 min at 37 °C, the reaction was terminated by adding an equal volume of 40 mmol/L Tris-HCl (pH 7.5), which contained 10 mmol/L ethylenediamine tetraacetic acid (EDTA), then heat-denaturizing for exactly 1 min at 100 °C. In each reaction tube, the cGMP level was measured using RIA. Briefly, PDE activity is reported as the amount of cGMP hydrolyzed by PDEs in the assay samples. Blank (background) control reactions were conducted using tissue samples that had been incubated in a boiling water bath for 5 min. The cGMP-PDE relative activity was the value of the PDE activity per unit of total protein concentration.

2.9 Statistical analysis

Continuous variables were expressed as the mean±standard error of the mean (SEM). Statistical analysis was performed using a commercially available program, Statistical Package for the Social Sciences (SPSS Version 13.0; Chicago, IL, USA).

Comparisons among groups were performed using one-way analysis of variance (ANOVA) with Scheffe's post-hoc test and univariate correlation analysis, as appropriate. $P<0.05$ was considered to be statistically significant.

3 Results

3.1 Histologic examination of follicular development in neonatal rats

Follicular development in neonatal and immature rats was evaluated in ovaries at postnatal days 1, 5, 7, 10, and 19. At day 1, germ cell nests broke down shortly after birth and then the primordial follicles were formed. The vast majority of follicles were primordial follicles, and the largest follicles observed contained a single layer of GC in neonatal rats at postnatal day 1. In addition, by day 5, the oocytes of the largest follicles were surrounded by cuboidal and squamous granulosa cells, and some had grown, as evidenced by the appearance of primary follicles surrounded by a single layer of cuboidal granulosa cells. At day 7, secondary follicles with two or more layers of squamous granulosa cells appeared. At day 10, the oocytes in the largest early tertiary follicles with antra were surrounded by more than three layers of squamous granulosa cells. At day 19, we observed several significant tertiary follicles.

3.2 Immunohistochemical analysis of NOS isoforms and sGC subunits

In order to investigate the localizations of the different NOS isoforms in the ovary during follicular development in neonatal and immature rats, immunohistochemical staining of three NOS isoforms was performed in the rat ovaries at postnatal days 1, 5, 7, 10, and 19. Our results showed that eNOS was markedly immunolocalized in the oocytes of primordial, primary, secondary, and tertiary follicles (Table 1 and Figs. 1a–1e), and strong staining was also evident in the oocytes of other staged follicles at different postnatal days (Table 1 and Fig. 1i). In addition, immunostaining of eNOS was slight in the granulosa cells and theca cells of secondary and tertiary follicles at postnatal days 7, 10, and 19 (Table 1 and Figs. 1c–1e). Immunostaining patterns of nNOS were completely different from those of eNOS, because nNOS was not detected in the theca cells of multilaminar follicles and only slightly stained oocytes were observed at postnatal days 1, 5, 7, 10, and 19, compared with eNOS and iNOS (Table 1 and Figs. 1g–1k). Furthermore, positive staining of iNOS was detected in the oocytes and granulosa cells of primordial, primary, secondary, and tertiary follicles at postnatal days 1, 5, 7, 10, and 19 (Table 1 and Figs. 1m–1q), and strong staining was also evident in the oocytes of other staged follicles at different postnatal days (Table 1 and Figs. 1p–1q). At day 19, iNOS was also found in the theca cells of tertiary follicles (Table 1 and Fig. 1q).

We further addressed the changes in sGC proteins, which bind to NO and then produce cGMP in the ovary, during the different postnatal days 1, 5, 7, 10, and 19. Immunohistochemical staining of sGC α_1 and β_1 subunits was also examined in the same samples. Our results indicated that both sGC α_1 and β_1 subunits are mainly localized in the granulosa cells of primordial, primary, tertiary, and small developing follicles, and the staining intensity decreased commensurately with follicle development.

3.3 Measurement of NOS activity

Measurement of NOS activity showed that the total NOS activities did not change at postnatal days 1 and 5, but strongly increased at days 7 ($P<0.05$) and 10 ($P<0.01$), decreasing subsequently at day 19 (Fig. 2a). In addition, as demonstrated in Fig. 2b, the eNOS activity was significantly higher at postnatal days 7 ($P<0.05$) and 10 ($P<0.01$), compared with other days. Similarly, the iNOS activity was also significantly higher at postnatal days 7 ($P<0.05$) and 10 ($P<0.01$), compared with other days (Fig. 2c). However, the nNOS activity was not changed throughout the experimental (Fig. 2d). Notably, the value for iNOS activity was the highest among the three NOS isoforms. Then univariate correlation analysis (Table 2) revealed that the activity of total NOS was significantly correlated with those of eNOS and iNOS, but not with nNOS.

3.4 Measurement of ovarian NO contents at postnatal days 1, 5, 7, 10, and 19

As shown in Fig. 3, the ovarian concentrations of NO did not change at postnatal days 1 and 5, but strongly increased at days 7 ($P<0.05$) and 10 ($P<0.01$), and then decreased at day 19.

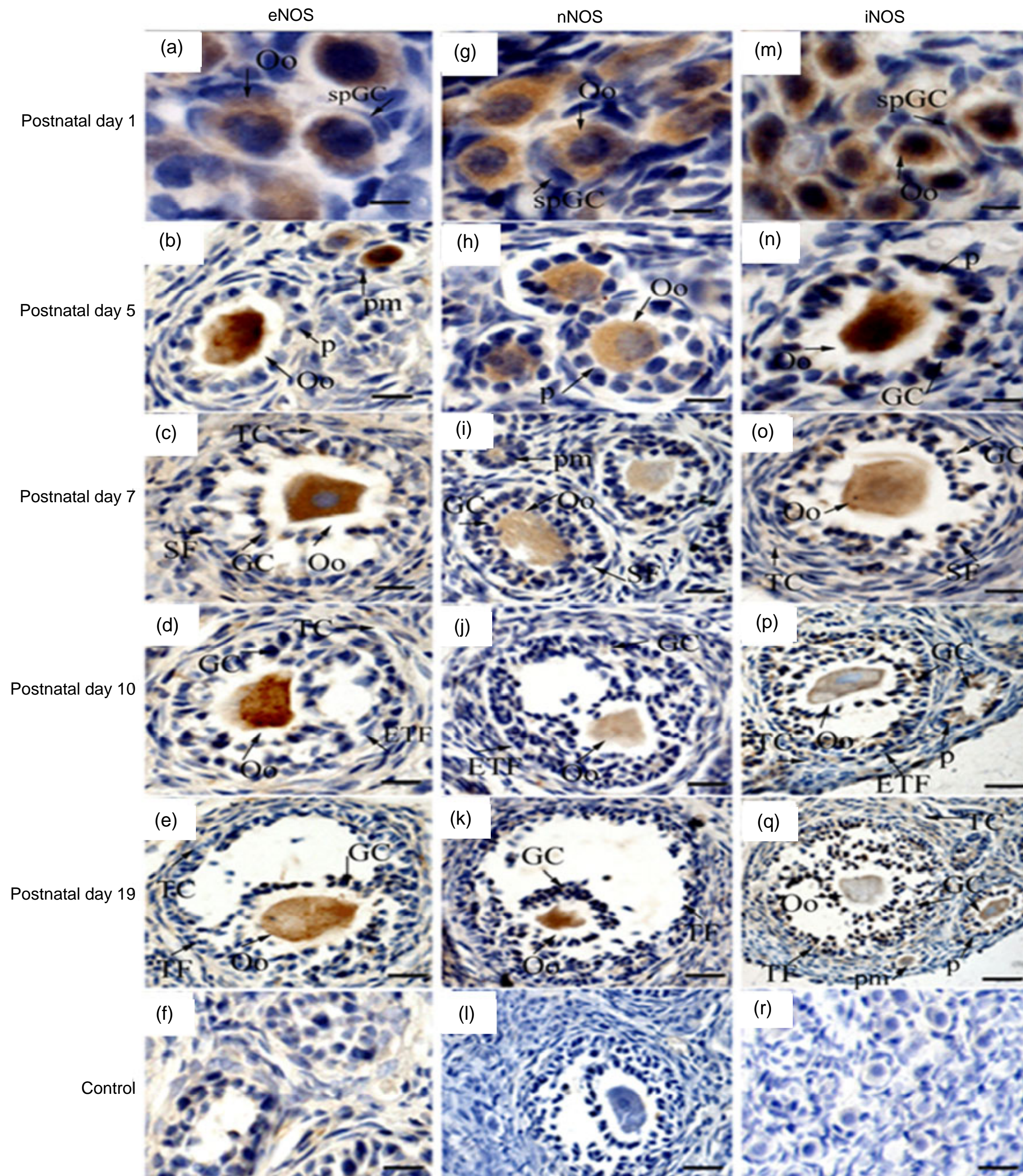

Fig. 1 Cellular localizations of eNOS, nNOS, and iNOS proteins during follicular development at postnatal days 1, 5, 7, 10, and 19

Ovarian sections were immunostained for eNOS, nNOS, and iNOS and counterstained with H&E. The immunohistochemical signals appear brown and the counterstaining background appears blue in color. At postnatal day 1, eNOS (a) and iNOS (m) immunoreactivities were markedly localized to the oocytes (arrows), but nNOS (g) immunoreactivity was weak. At postnatal day 5, eNOS (b) and nNOS (h) immunoreactivities were localized to the oocytes of primordial follicles (arrows). However, positive staining of iNOS (n) was detected in the oocytes and granulosa cells of primary follicles. At postnatal day 7, eNOS (c) and iNOS (o) immunoreactivities were markedly localized to the oocytes and granulosa cells of primary and secondary follicles (arrows), but nNOS (i) immunoreactivity was also weak and was not detected in granulosa cells. At postnatal days 10 and 19, eNOS (d, e) and iNOS (p, q) immunoreactivities were markedly localized to the oocytes, granulosa cells, and theca cells of secondary and early tertiary follicles (arrows), but nNOS (j, k) immunoreactivity was weak and only present in the oocytes. Negative controls lacking primary antibody remained unstained (f, l, r). pm: primordial follicle; p: primary follicle; SF: secondary follicle; ETF: early tertiary follicle; TF: tertiary follicles; spGC: squamous pre-granulosa cell; GC: granulosa cell; Oo: oocyte; TC: theca cell. Scale bars=50 μm

Table 1 Relative abundances of eNOS, nNOS, and iNOS in the rat ovary during follicular development before prepuberty

Follicular development	Staining intensity														
	PD1			PD5			PD7			PD10			PD19		
	eNOS	nNOS	iNOS	eNOS	nNOS	iNOS	eNOS	nNOS	iNOS	eNOS	nNOS	iNOS	eNOS	nNOS	iNOS
Oocyte															
Primordial	++	+	++	++	+	++	++	+	++	++	+	++	++	+	++
Primary	NA	NA	NA	+++	+	+++	+++	+	+++	+++	+	+++	+++	+	+++
Secondary	NA	NA	NA	NA	NA	NA	+++	+	+++	+++	+	+++	+++	+	+++
Early tertiary	NA	NA	NA	NA	NA	NA	NA	NA	NA	+++	+	+++	+++	+	+++
Tertiary	NA	NA	NA	NA	NA	NA	NA	NA	NA	+++	+	+++	+++	++	+++
Atretic	+	+	+	+	+	+	+	+	+	++	+	++	++	+	++
Granulosa cells															
Primordial	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Primary	NA	NA	NA	−	−	++	+	−	++	+	−	++	+	−	++
Secondary	NA	NA	NA	NA	NA	NA	++	+	++	++	−	++	++	−	++
Early tertiary	NA	NA	NA	NA	NA	NA	NA	NA	NA	+++	−	+++	+++	−	+++
Tertiary	NA	NA	NA	NA	NA	NA	NA	NA	NA	+++	−	+++	+++	−	+++
Atretic	NA	NA	NA	−	−	+	+	+	+	+	−	+	+	−	+
Theca cells															
Secondary	NA	NA	NA	NA	NA	NA	+	−	+	+	−	+	+	−	+
Early tertiary	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	−	+	+	−	+
Tertiary	NA	NA	NA	NA	NA	NA	NA	NA	NA	+	−	+	+	−	+
Atretic	NA	NA	NA	NA	NA	NA	+	−	+	+	−	+	+	−	+

−: no staining detected; +: weak; ++: moderate; +++: strong; NA: not available; PD: postnatal day

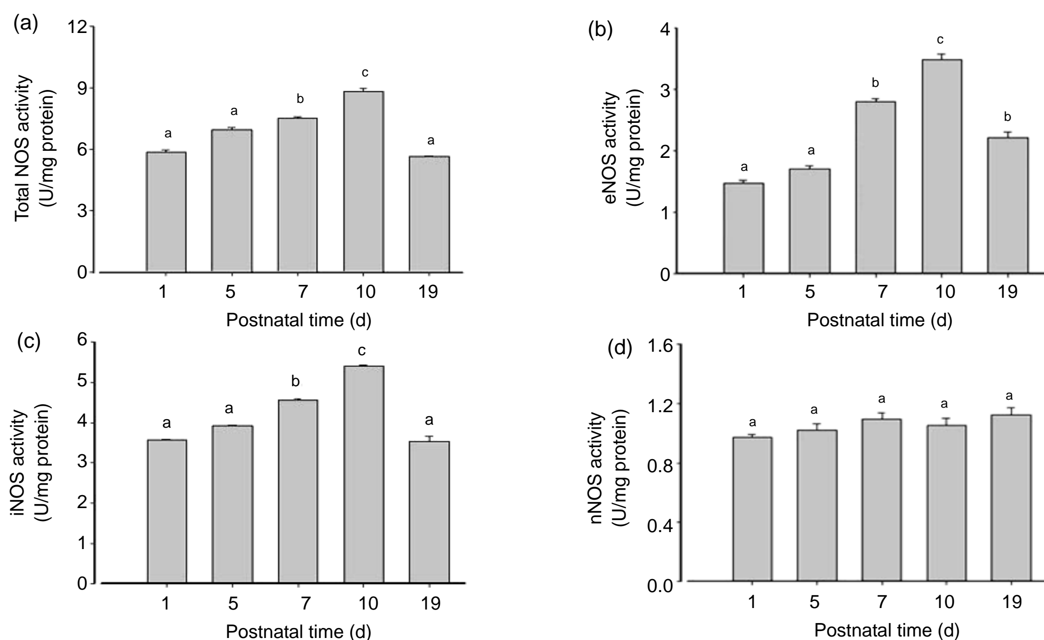

Fig. 2 Nitric oxide synthase (NOS) activities of ovaries during follicular development at postnatal days 1, 5, 7, 10, and 19
(a) Changes in total NOS activity; (b) Changes in eNOS activity; (c) Changes in iNOS activity; (d) Changes in nNOS activity. Data are expressed as mean±SEM for three repeats. One-way ANOVA followed by Scheffe's post-hoc test was used. Different letters above the bars indicate a statistically significant difference among the days: $^{b}P<0.05$, $^{c}P<0.01$

Table 2 Univariate analysis between the activities of three NOS isoforms

NOS isoform	*R*	*P*
eNOS	0.9854	0.002
iNOS	0.9562	0.011
nNOS	0.3871	0.520

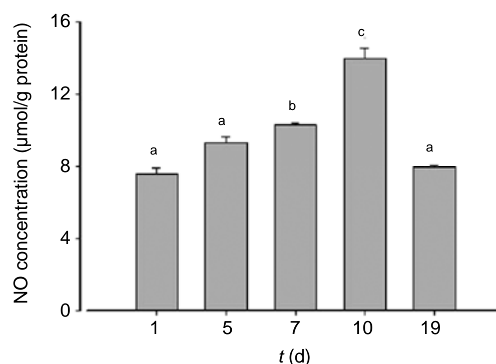

Fig. 3 Nitric-oxide (NO) concentrations of ovaries during development of follicle at postnatal days 1, 5, 7, 10, and 19
Data are expressed as mean±SEM for three repeats. One-way ANOVA followed by Scheffe's post-hoc test was used. Different letters above the bars indicate a statistically significant difference among the days: $^{b}P<0.05$, $^{c}P<0.01$

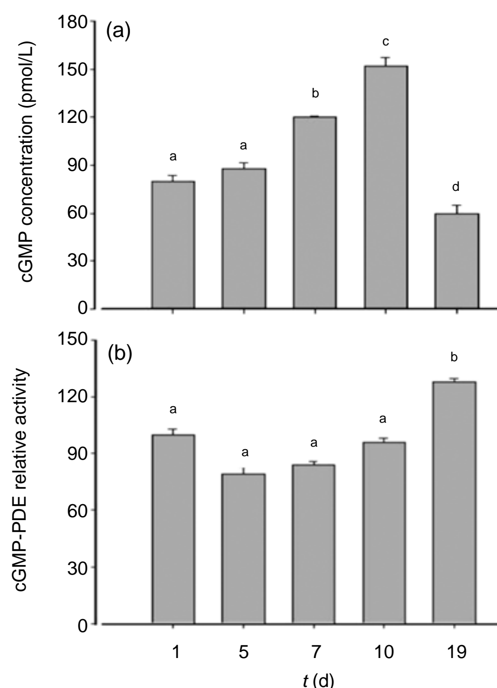

Fig. 4 Ovarian concentrations of cGMP and relative activity of cGMP-PDE in the ovarian homogenates of the rats at postnatal days 1, 5, 7, 10, and 19
(a) Ovarian cGMP levels; (b) cGMP-PDE activity. Values represent mean±SEM for three repeats. One-way ANOVA followed by Scheffe's post-hoc test was used. Different letters above the bars indicate a statistically significant difference among the days: $^{b}P<0.05$, $^{c}P<0.01$

3.5 Ovarian cGMP level and cGMP-PDE activity in postnatal rats

In postnatal rats, ovarian cGMP levels increased at days 1 and 5, and reached their highest levels at days 7 and 10, but decreased dramatically at day 19 (Fig. 4a). Meanwhile, the cGMP-PDE activity was not changed significantly throughout days 1 to 10, but increased significantly at day 19 ($P<0.05$), compared with the other postnatal days (Fig. 4b).

4 Discussion

NO has been identified as a major intracellular as well as intercellular signaling molecule, and the NO/cGMP pathway is involved in diverse physiologic processes throughout the ovary (Tamanini *et al.*, 2003). To our knowledge, this is the first report to demonstrate cellular expressions and immunolocalizations of three different NOS isoforms (eNOS, nNOS, and iNOS) in the rat ovary prior to puberty. Our results clearly show cell-specific expressions of these three NOS isoforms. In addition, ovarian concentrations of NO and activities of total NOS are contributed by eNOS and iNOS, but not by nNOS.

It is interesting that three NOS isoforms are localized mainly in the oocyte. The NOS was gradually expressed and localized in the granulosa cells and theca cells during follicular development at postnatal days 1, 5, 7, 10, and 19. Our immunostaining pattern of iNOS was largely similar to the previous study of Mitchell *et al.* (2004) in the mouse. From the time of follicular organization and continuing throughout ovulation, the oocyte regulates the follicular development by controlling granulosa cell, theca cell proliferation and differentiation by producing adequate amounts of gonadotropins. In turn, the granulosa cells are indispensable for oocyte growth and differentiation, nuclear meiotic status, cytoplasmic maturation, and genomic transcriptional activity (Matsumi *et al.*, 1998, van den Hurk and Zhao, 2005). This knowledge, together with the findings that the expressions and

localizations of different NOS isoforms (eNOS, nNOS, iNOS) in the rat ovary before puberty, suggests a connection between granulosa cells and oocytes via the NO/NOS pathway.

It has been reported that different isoforms of NOS play critical roles in the different biologic and physiologic processes of the reproductive system. For example, eNOS-knockout (eNOS−/−) females mice showed a significantly longer estrous cycle, a reduction in ovulatory efficiency, a lower number of embryos, and reduced numbers of ovulated oocytes after superovulation (Drazen *et al.*, 1999; Jablonka-Shariff *et al.*, 1999; Pallares *et al.*, 2008). nNOS knockout (nNOS−/−) female mice exhibited normal numbers of rupture sites, but reduced numbers of oocytes recovered following systemic injections of gonadotropins (Drazen *et al.*, 1999). Moreover, locally produced NO via iNOS in granulosa cells may be involved in the developmental status of ovarian follicles in concert with gonadotropins (Matsumi *et al.*, 1998). There were no data published, however, regarding NOS location and function in the rat ovary before puberty. In our present study, we observed that nNOS immunoreactivity was slightly elevated compared with those of eNOS and iNOS. Then the activity of nNOS was also lowest, but the value of iNOS activity was the highest among the three NOS isoforms in the rat ovary during neonatal and prepubertal stages. Next, univariate analysis (Table 2) revealed that the activity of total NOS was significantly correlated with that of eNOS and iNOS, but not with nNOS. On the other hand, histological examination showed that follicules developed in neonatal and immature rat ovaries at postnatal days 1, 5, 7, 10, and 19. The results are similar to our previous study (Wang *et al.*, 2007). Collectively, according to the descriptions above, iNOS and eNOS were the main NOS isoforms involved in follicular development during postnatal and prepubertal stages.

Moreover, we detected a progressive increase in NO content and NOS activity of the rat ovary during follicular development from prepuberty to postnatal days 1, 5, 7, 10, and 19. The activities of eNOS and iNOS and the related NO/cGMP pathway were not significantly changed at postnatal days 1 and 5, whereas they were markedly higher at postnatal days 7 and 10. Meanwhile, human chorionic gonadotropin (hCG) receptors in the rat ovary were first detected between postnatal days 6 and 8, which coincides with the onset of luteinizing hormone (LH) or hCG sensitivity of the postnatal ovary of rat (Lamprecht *et al.*, 1973). Follicle stimulating hormone (FSH) receptor content per ovary increased ten-fold from days 1 to 7, and LH receptor content per ovary increased 27-fold (Sokka and Huhtaniemi, 1990). On the other hand, during ovarian follicular development of immature female Sprague-Dawley rats (24 d old) treated with pregnant mare serum gonadotropin (PMSG) and hCG, NOS responded to gonadotropin stimulation with distinct cell-specific patterns of expression (Jablonka-Shariff and Olson, 1997). Given that the follicles of the rat ovary developed from secondary to early tertiary follicles at postnatal days 7 and 10, it appears that postnatal days 7 and 10 are very important time points for rat follicle development. Furthermore, eNOS levels can be altered by various stimuli and some studies have reported the expression of iNOS in response to signals that are non-inflammatory or non-immunologic (Huang *et al.*, 1995). Thus, our findings strongly indicate that iNOS and eNOS regulate follicular development via gonadotropins in the rat ovary before puberty, especially at postnatal days 7 and 10.

Furthermore, previous studies have indicated that granulosa cells of preantral follicles will undergo little apoptosis, but the cultured preantral follicles treated with a cGMP analog can inhibit programmed cell death (McGee *et al.*, 1997). We detected changes in the NO/cGMP pathway at postnatal days 1, 5, 7, 10, and 19 and found that cGMP levels were increased from days 1 to 5, with the highest levels observed at days 7 and 10, and a subsequent dramatic decrease at day 19. The decrease of cGMP at day 19 is clearly due to a significant increase in the activity of cGMP-PDE.

In the current study, we observed that three isoforms of NOS were mainly expressed in oocytes and slightly expressed in theca cells at postnatal days 1, 5, 7, 10, and 19, whereas sGC α_1 and β_1 subunits were mainly expressed in the granulosa cells and theca cells, and only slightly in oocytes, in accordance with our previous study (Shi *et al.*, 2004). In biologic systems, as a gas molecule, NO is a lipophilic, highly diffusible, and short-lived physiologic messenger and is thought to diffuse over a wide area (100 nm), moving freely through membranes of neighboring cells. Thus, in agreement with its function as a

paracrine mediator, NO can travel significant distances to reach target cells near the NO-generating cells with little consumption or direct reaction (Lancaster, 1997; Kelm, 1999). These findings suggest that NO may be mainly expressed in the oocytes and transported into the surrounding granulosa cells via gas diffusion.

In conclusion, our overall results for the expressions and localizations of three different NOS isoforms (eNOS, nNOS, iNOS) and the changing ovarian concentrations of the NO-related NO/cGMP pathway suggest that locally produced NO may modulate follicular development, and play an important role in follicular atresia via regulation of granulosa cell apoptosis during postnatal and prepubertal developments. Currently, the precise regulation of endogenous NO and the related NO/cGMP pathway during postnatal and prepubertal developments remain to be explored.

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