Inhibition of ovulation by a lipoxygenase inhibitor involves reduced cyclooxygenase-2 expression and prostaglandin E₂ production in gonadotropin-primed immature rats

Shiro Kurusu, Masako Jinno, Hirosato Ehara, Tomohiro Yonezawa and Mitsumori Kawaminami

Laboratory of Veterinary Physiology, Kitasato University School of Veterinary Medicine, Towada, Aomori 034-8628, Japan

Correspondence should be addressed to S Kurusu; Email: kurusu@vmas.kitasato-u.ac.jp

Abstract

Potential roles of cyclooxygenase (COX) pathway of arachidonic acid (AA) metabolism are established in a murine model of induced ovulation. Pharmacological inhibition of an alternative lipoxygenase (LOX) pathway has been shown to cause defective ovulation, but the mechanism is still undefined. This study investigated the effects of two LOX inhibitors and their time dependency on ovulation and COX activity in gonadotropins (eCG and human chorionic gonadotropin (hCG))-primed immature rats. Intra-ovarian bursal treatment with a general LOX inhibitor nordihydroguaiaretic acid (NDGA) at 0 h post-hCG (hCG0h) dose dependently inhibited ovulation rate. The drug was still but less effective when treated at hCG6h. A more specific inhibitor, 3,4-dihydroxyphenyl ethanol (DPE) was also inhibitory when treated at hCG0h but not at hCG6h. Interestingly, treatment with DPE at hCG0h resulted in attenuated expression of immunoreactive PTGS2 in granulosa layers and concomitant decrease in ovarian prostaglandin E₂ (PGE₂) content at hCG8h. NDGA treatment reduced immunoreactive PTGS2. Ovulatory impairment by both inhibitors was prevented by systemic administration of PGE₂ at hCG6h. Immunohistochemistry revealed the expression of ALOX5 and ALOX12 in both thecal and granulosa layers of preovulatory follicles and, notably, the augmented immunoreactivities during 8 h after hCG treatment. Our results indicate the probable presence of multiple LOX isoforms and that specific inhibition of LOX at an early stage of hCG-signaling led to reduced PTGS2 activity and thus defective ovulation. They reveal a probable relationship between two pathways of AA metabolism and account at least partly for the mechanism by which the LOX inhibitor causes impaired ovulation.

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Introduction

Ovulation involves LH signaling-initiated early induction of several sets of genes, the gene products-dependent generation of bioactive lipid substances, and further genetical and cytological alterations by local action of these substances (Richards *et al.* 2002, Sirois *et al.* 2004a, Espey & Richards 2006). Eicosanoids are one of these two major lipid substances and are generated through three steps, 1) arachidonic acid (AA) release from membrane phospholipids by phospholipase A₂ (PLA₂), 2) conversion of AA to prostaglandin (PG) H₂ and hydroperoxyeicosatetraenoic acid by cyclooxygenase (COX) and lipoxygenase (LOX) pathways respectively, and 3) formation of terminal products having a variety of biological activities by respective synthetases (Shimizu & Wolfe 1990, Funk 2001). Modes of action of eicosanoids include the specific interaction with cell surface or nuclear receptor(s) mainly in autocrine and paracrine manners and in certain cases in intracrine or endocrine (transition of uterine-derived luteolytic PGF_{2α} to ovarian *corpus luteum*) manners (Funk 2001).

Roles of the COX pathway in ovulation are well established. The inducible type PTGS2 and PGE₂-EP₂ receptor signaling play crucial roles in proper follicle rupture and cumulus expansion in a murine model of induced ovulation (Hizaki *et al.* 1999, Matsumoto *et al.* 2001, Ochsner *et al.* 2003, Sirois *et al.* 2004b). Pharmacological or genetical inhibition of PTGS2 activity results in potential suppression of ovulation (Espey *et al.* 1986, Tanaka *et al.* 1991, Lim *et al.* 1997, Mikuni *et al.* 1998a). On the other hand, the involvement of the alternative LOX pathway in ovulation process was first suggested by Reich *et al.* (1983) who demonstrated that chemical inhibition of LOX blunted ovulatory response in gonadotropins-primed immature rats. Since then, several following studies using a LOX inhibitor (Yoshimura *et al.* 1991, Downey *et al.* 1998, Mikuni *et al.* 1998b) have supported this notion, but others (Carvalho *et al.* 1989, Hellberg *et al.* 1990) did not. These discrepancies appear to have arisen from differences in the dose and the route of drug administration, experimental systems, and animal species

examined. In all of the studies with a significant impact of LOX inhibition, the action mechanism of the inhibitor on ovulatory process has been virtually obscure. The specificity of the widely used inhibitor nordihydroguaiaretic acid (NDGA) against LOX is somewhat doubted (Van Wauwe & Goossens 1983, Miyazawa *et al.* 1985). The effect of timing of drug administration relative to that of LH/human chorionic gonadotropin (hCG) stimulus has not been taken into consideration. Furthermore, while actual preovulatory changes of the LOX pathway eicosanoids in rat ovarian tissues *in vivo* or isolated perfused system have been intensively studied (Espey *et al.* 1989, 1991, Tanaka *et al.* 1991, Higuchi *et al.* 1995, Mikuni *et al.* 1998b), the information on the identity of LOX enzymes present in the preovulatory follicle (Pridham *et al.* 1990) and their possible regulation by LH/hCG stimulus (Reich *et al.* 1985) is still limited.

To address these issues, this study further defines the effects of LOX pathway inhibition on ovulation by means of two different drugs and implicates LOX activity in the early phase of hCG-initiated follicular signaling in induction of PTGS2 activity. Supplementary to this is the immunohistochemical demonstration of LOX isoforms in rat preovulatory follicles.

Results

Effects of NDGA and 3,4-dihydroxyphenyl ethanol (DPE) on ovulation rate

In the current gonadotropins-priming protocol, normal rats had 14.7 ± 1.5 ($n=6$) ovulations/one ovary. An effect of systemic (i.p.) injection of a great amount (10 mg per rat) of NDGA simultaneously with hCG (at hCG0h) was preliminarily tested. There was no change in ovulation rate (15.4 ± 3.4 , $n=6$). This was consistent with the previous results (Tanaka *et al.* 1991, Gaytan *et al.* 2006) but not with the other one (Mikuni *et al.* 1998b), all of which challenged the nearly doubled dose in an identical experimental model. Instead, abdominal swelling with ascites and intestinal stagnation probably due to the drug's side effect(s) were observed in these rats. Therefore, the LOX inhibitor was administered locally (into ovarian bursa) in all of the following experiments while hCG and/or PGE₂ was injected intraperitoneally.

Effects of two different inhibitors and time dependency of their administration were examined. In the first series of experiment, one side of ovary was treated with vehicle or increasing doses of NDGA and the contralateral intact ovary served as control. Vehicle treatment at hCG0h resulted in a subtle, insignificant decline in ovulation rate compared with that of control group (Fig. 1A). NDGA treatment at hCG0h caused a dose-dependent inhibition of ovulation. The least dose tested (0.01 mg per ovary) was found to be effective ($P<0.01$) and the largest dose (1.0 mg) gave a 95.0% inhibition of that of

control ovaries. When NDGA treatment was delayed as late as hCG6h, it also reduced ovulation rate but the inhibitory effect was modest compared with those of the same doses of administration at hCG0h.

In the second series of experiment, both ovaries of a rat were treated with NDGA or a novel, more selective LOX inhibitor (DPE; Kohyama *et al.* 1997) at hCG0h and in some cases followed by systemic administration of vehicle or 80 µg PGE₂ at hCG6h. Vehicle treatment had no effect (Fig. 1B). Whereas treatment with 0.01 mg DPE per ovary was ineffective, 0.1 mg inhibitor induced a significant decrease in ovulation rate ($P<0.05$, 65.5% of that of vehicle treatment group). However, treatment with the same dose at hCG6h had no significant effect. Treatment with 0.1 mg NDGA resulted in reduction to 39.2% of that of vehicle treatment group, which was very consistent with the above-described result where one side of ovary was treated and another was intact (Fig. 1A).

Ovulatory impairment by DPE (0.1 mg) treatment at hCG0h was significantly ($P<0.01$) reversed by systemic administration of PGE₂ at hCG6h (Fig. 1B). NDGA-treated ovary was also rescued by this PG supplementation. The counts of proper ovum release into the oviduct ampulla in both groups were comparable with those of vehicle-treated and intact groups.

Effects of LOX inhibitors on ovarian PTGS2 expression, PGE₂ content, and progesterone secretion

Based on the established role of PGE₂ (Davis *et al.* 1999, Matsumoto *et al.* 2001, Ochsner *et al.* 2003) and the current data of a possible rescue effect of PGE₂ on the LOX inhibitors-treated ovaries, the authors analyzed whether tissue PGE₂ synthetic response was impaired in the drug-treated ovaries. PGE₂ content in vehicle-treated ovaries showed a 7.1-fold increase ($P<0.01$) at hCG8h (Fig. 2). NDGA (0.1 mg)-treated ovaries also showed a significant ($P<0.05$, versus hCG0h value) increase, but the PGE₂ synthetic response was modest ($P=0.10$, versus vehicle-treated ovaries). In DPE (0.1 mg)-treated ovaries, no significant increase was elicited following hCG and there was a significant ($P<0.05$) reduction in tissue PG level compared with that of temporarily matched control.

The study then pursued to ask whether hCG induction of PTGS2 was altered in NDGA- or DPE-treated ovaries. Immunoreactive PTGS2 was absent in the ovary at hCG0h (Fig. 3A) and highly induced throughout the granulosa layer of most mature follicles at hCG8h (Fig. 3B). This result was consistent with the previous studies on PTGS2 expression in the time course of an identical experimental model (Sirois *et al.* 2004b). Neither NDGA nor DPE treatment altered general histology of the granulosa layer of ovulatory follicles at hCG8h (Fig. 3C and D). But importantly, NDGA- and DPE-treated ovaries both showed attenuated expression

Figure 1 Effects of local administration with LOX inhibitors on ovulation rate. Rats were induced to ovulate by treatment with eCG followed by hCG 48 h later. Ovulation rates (= number of eggs released per ovulation) were counted at hCG24h. (A) Vehicle or different doses of NDGA (0.01, 0.1, and 1.0 mg) was administered into the left ovarian bursa at hCG0h or hCG6h. Contralateral (right) intact ovaries served as control in each individual. Ovulation rates were transformed as percentage of that of the control ovary and were expressed as mean $\pm$ S.E.M. Numbers in parenthesis indicate numbers of rats. Difference between treated and control ovaries were evaluated by paired *t*-test. ****P* < 0.001 compared with those of control. NS, not significant. (B) Intact rats and rats treated on both sides of ovary with vehicle, DPE (0.02 or 0.1 mg), or NDGA (0.1 mg) at hCG0h and/or hCG6h were examined for ovulation at hCG24h. Some animals that received local DPE or NDGA (both 0.1 mg) at hCG0h further received a single dose (80 μ g, sc) of PGE₂ or vehicle at hCG6h. Ovulation rates were expressed as mean $\pm$ S.E.M. whose ovarian sample numbers are shown in each parenthesis, and statistically evaluated by Tukey–Kramer’s multiple comparison test. **P* < 0.05; ****P* < 0.001 versus vehicle-treated group. #*P* < 0.05; ###*P* < 0.001, between indicated groups.

of granulosa PTGS2 (Fig. 3C and D). The uniformity of positive immunoreaction throughout the mural granulosa layer was lost and there was a tendency for granulosa cells near the side of the thecal layer to become negative. PTGS2 immunoreactivity in mature follicles, whose largest diameters were over 500 μ m on the slide section, was scored and semi-quantitatively analyzed among vehicle-, NDGA-, or DPE-treated groups (Table 1). The score of PTGS2 reaction was

Figure 2 Effects of local administration with LOX inhibitors on ovarian PGE₂ level. Rats were treated locally with vehicle, NDGA (0.1 mg), or DPE (0.1 mg) at hCG0h. PGE₂ levels in intact ovaries at hCG0h and treated ovaries sampled at hCG8h were determined by EIA. Data are mean $\pm$ S.E.M. of whose ovarian sample numbers are indicated in each parenthesis, and statistically evaluated by Tukey–Kramer’s multiple comparison test. **P* < 0.05; ***P* < 0.01 versus hCG0h group.

significantly attenuated in follicles pretreated with NDGA (*P* < 0.002, by Mann–Whitney *U* test) and DPE (*P* < 0.02).

Plasma progesterone levels in intact rats were 56.9 ± 8.4 and 17.2 ± 2.9 ng/ml at hCG8h and hCG24h respectively. There were no significant changes in the circulating steroid level between vehicle- and LOX inhibitor-treated rats at these time points (data not shown).

Distribution of immunoreactive LOX isoforms in the preovulatory follicle

The final experiment of this study examined cellular distribution of immunoreactivity of LOX isoforms in preovulatory follicles. Preliminary experiments checked the cross-reactivity of the used antibodies against respective LOX antigens with lung (ALOX5 and -12) and spleen (ALOX12) positive controls. As was consistent with the previously reported results (Maruyama *et al.* 1989, Covin *et al.* 1998, Kawajiri *et al.* 2000), alveolar epithelial cells in the lung and white pulp in the spleen of adult rats showed positive immunoreaction for respective antigen (data not shown).

Moderately positive immunoreactivity of ALOX5 was present in both the thecal and granulosa layers of only equine CG (eCG)-treated mature follicles (at hCG0h; Fig. 4A). Upon subsequent hCG stimulation, the thecal layer showed augmented immunoreactivity at hCG8h (Fig. 4B). Granulosa cells also exhibited increased positive reactions but with less intensity than that of the thecal layer. Immunoreactivity of ALOX12 was also present in preovulatory ovaries prior to hCG stimulation (Fig. 4C). At hCG8h, thecal layer showed increased

Figure 3 Effects of the LOX pathway inhibitor on immunoreactive PTGS2 induction. Effects of pre-treatment with LOX inhibitors on hCG-induced PTGS2 expression were evaluated by immunohistochemistry. Local injection of vehicle, NDGA (0.1 mg), or DPE (0.1 mg) was performed at hCG0h and ovaries sampled at hCG8h were processed for histology. PTGS2 immunostaining was revealed by DAB (brown color) with hematoxylin (blue) counterstaining. (A) The ovary at hCG0h was entirely negative for PTGS2 immunoreactivity. (B) The immunoreaction was clearly induced in mural granulosa cells of preovulatory follicles in vehicle-treated ovaries at hCG8h. Its immunoreactivity was attenuated by (C) NDGA- and (D) DPE- pretreatment. A scale bar represents 200 μ m throughout.

ALOX12 immunoreaction and scattered signals appeared in some, but not all, of the mature follicles (Fig. 4D). Replacement of the primary antibody with non-immune IgG produced no immunoreaction in the ovary at hCG0h (Fig. 4E).

Discussion

Major findings of this study are summarized as follows: 1) demonstration of immunoreactive ALOX5 and ALOX12 in the thecal and granulosa layers of preovulatory follicles, which are sensitive to hCG stimulus, 2) acute inhibition of LOX pathway inhibited hCG-induced ovulation in a dose- and time-dependent manner, 3) the decreased ovum release was preceded by reductions in hCG-stimulated PTGS2 protein expression and PGE₂ production, and 4) the ovulatory inhibition by the LOX inhibitor could be prevented by PGE₂ supplementation. Collectively, these data suggest that pre-existing LOX activity enhances hCG induction of PTGS2 and may give a novel insight into an auto-regulatory mechanism

for AA metabolic cascade system, an essential part of LH-evoked ovulatory cascade.

The current use of a novel specific inhibitor DPE has further established a definitive role of the LOX pathway in ovulation, at least in rats. Comparing its effects and those of the classical inhibitor NDGA has revealed common and somewhat different features. As inhibition of ovulation as well as PTGS2 induction were commonly seen following both inhibitor treatments at the early phase, it is very likely that those alterations occurred as results of specific inhibition of LOX activity. It is also notable that the effect on ovulation of acute and probably transient LOX inhibition tends to differ between the early (hCG0h ~) and the late (hCG6h ~) phases of hCG signaling. More marked inhibition of ovulation by NDGA might be ascribed to two additional effects of this inhibitor. One is an antioxidant property (Carlson *et al.* 1995, Takami *et al.* 1999) since oxidative stress contributes to the ovulation process (Riley & Behrman 1991), and another is some cross-reactivity with COX that becomes expressed at the high dose (Van Wauwe & Goossens 1983, Miyazawa *et al.* 1985).

Table 1 PTGS2 immunoreactivity in preovulatory follicles.

Time	Group	Number of ovaries	Score				Difference versus Veh
			0	1	2	3	
hCG0h	Intact	3	20	0	0	0	
hCG8h	Veh	4	3	2	2	23	
hCG8h	NDGA	4	9	4	12	9	$P < 0.002$
hCG8h	DPE	3	7	3	8	13	$P < 0.02$

Preovulatory follicles that looked healthy and were with the largest diameter over 500 μ m were examined for indicated numbers of ovaries in each group. Staining intensity, as shown in Fig. 3, was evaluated semi-quantitatively and scores with a scale grading from negative (0), faintly positive (1), moderately positive (2), to strongly positive (3) were statistically compared between vehicle (Veh) and the inhibitor-treated group by Mann-Whitney *U* test.

Figure 4 Localization of immunoreactive LOXs in preovulatory follicles. Normal ovaries at (A, C, and E) hCG0h and (B and D) hCG8h were immunostained for ALOX5 and ALOX12. LOX immunostaining was revealed by DAB (brown color) with hematoxylin (blue) counterstaining. ALOX5 signals were moderate but evident in both thecal and granulosa layers prior to hCG stimulus (A) and greatly induced in thecal layers after hCG (B). ALOX12 immunoreactivity was also present in both thecal and granulosa layers and stroma (C) and was induced in the theca cells similarly to that of ALOX5 (D). Scattered, positive signals (D, arrows) were noted in the thecal layer. (E) Non-immune IgG application served as negative control. A scale bar represents 100 μ m throughout.

The primary result of this study is to link the early phase LOX activity to following induction of PTGS2 activity. Pretreatment with NDGA or DPE tended to attenuate or did prevent PGE₂ synthetic response to hCG respectively. This is consistent with a previously reported result that NDGA inhibition of ovulation was accompanied with reduced PGE₂ and PGF_{2 α} production (Mikuni *et al.* 1998b). Importantly, the present finding of attenuated PTGS2 expression after inhibitors treatment has implicated the reduced PTGS2 activity in decreased PGE₂ response. As DPE does not inhibit, and may actually enhance, COX activity (Kohyama *et al.* 1997), attenuated PGE₂ response by this drug must have occurred as a result of the specific direct inhibition of LOX and subsequent indirect inhibition of COX. Furthermore, the inhibitors-induced impaired follicular rupture would have been mediated, at least partly, by the reduction in PGE₂ synthesis, as their anti-ovulatory effects were significantly restored by PGE₂ supplementation. Taken together, it is very likely that PTGS2/PGE₂ pathway functions downstream of the LOX pathway in the ovulatory cascade. There appear to be some little inconsistencies among alterations in PTGS2

immunoreactivity, PGE₂ level, and ovulation rate in NDGA versus DPE treatment groups. There are several possibilities for these causes that 1) PGE₂ content in whole ovarian tissues was determined, 2) AA availability and/or PGE₂ synthase activity might be altered, 3) granulosa PTGS2 immunoreactivity and ovarian PGE₂ level were assayed at only one time point, and 4) factor(s) other than PTGS2/PGE₂ pathway might also be responsible for determining ovulatory outcome.

Thecal and granulosa layers of preovulatory follicles are identified as the probable sites of ALOX5 and ALOX12 expression that is sensitive to hCG stimulus. The localization of LOXs is temporarily and spatially associated with that of group IVA PLA₂ (Kurusu *et al.* 1998; this study). This AA-selective PLA₂ isoform and ALOX5 are subject to common post-translational regulations, which are phosphorylation by MAP kinase and the rise in cytosolic Ca²⁺ (Radmark *et al.* 2007). Another line of evidence has shown that the basal activity of group IVA PLA₂ is important for determining the level of PTGS2 induction in a murine model (S Kurusu, A Sapirstein, E O'Leary, X Sun, M Kawaminami and J V Bonventre, unpublished observation). Combined

findings of these histological, biochemical, and functional studies suggest that group IVA PLA₂-ALOX5 pathway regulates the tone of granulosa cell *Ptgs2* gene expression in the early phase of LH-initiated signaling.

The role of the late phase LOX pathway is still undefined. Rat preovulatory follicles responded to hCG stimulus *in vivo* with a fivefold increase in ALOX5 enzymatic activity (Reich *et al.* 1985) and the increased immunoreactivity of ALOX5 and ALOX12 (this study) during 6–8 h. In fact, ovarian contents of 12- and 15-HETEs and LTC₄S/CYSLTR1/E₄ change in a temporal pattern similar to those of COX metabolites PGE₂ and PGF_{2α}, starting to increase around 6 h after hCG and remaining elevated until 12–14 h when follicular rupture occurs (Espey *et al.* 1989, 1991, Higuchi *et al.* 1995). Impacts on ovulation of a bolus LOX inhibitor treatment at hCG6h were reduced or absent. It is also notable that the LOX inhibitor is not so potential as the COX inhibitor indomethacin when the drug is administered to block activated production of eicosanoids during the latter preovulatory phase (Espey *et al.* 1986, 1989, Tanaka *et al.* 1991). These facts suggest that the role of the LOX pathway in ovulatory process is minor than that of the COX pathway and relatively dispensable. They, however, do not exclude out the possibility that the LOX metabolites are with some effects during the period. In the circumstance of ovulatory cascade, which is considered analogous to inflammatory response (Espey 1980, Richards *et al.* 2002), several pathways would function in concerted, overlapped, or redundant manners. It is possible that the drastically induced PTGS2 activity is workable to compensate for the loss of LOX activity.

Preferential localization of ALOX5 and ALOX12 immunoreactivity in thecal layers at hCG8h suggests that this pathway is affected by follicular microvasculature and vice versa. Evident appearance of intense immunoreactivities for ALOX5 and/or ALOX12 is likely elicited by mast or basophilic cells that are shown to accumulate in the preovulatory follicle wall (Richards *et al.* 2002, Espey & Richards 2006). The induced LOX may be involved in follicular microcirculation and hemodynamics such as chemotaxis, vasodilation, and extravasation. To date, only leukotriene B₄ is shown to be a putative effector of the LOX pathway eicosanoid because anti-ovulatory effect of NDGA could be reversed by this eicosanoid (Yoshimura *et al.* 1991, Mikuni *et al.* 1998b) and was mimicked by its receptor antagonist (Matousek *et al.* 2001). Many important issues, however, remain to be answered such as the identification of the effector metabolite(s), the spatial and temporal pattern of their synthesis, and action mechanism for the proper induction of PTGS2 protein and other possible activities including tissue degradation/remodeling processes.

In conclusion, this study demonstrated the expression of ALOX5 and ALOX12 in the preovulatory follicle and that inhibiting the early phase LOX activity led to

reduction in preovulatory PTGS2 induction and PGE₂ production and thus defective ovulation. These findings may help to understand the mechanism for the involvement of the LOX pathway in LH-triggered ovulatory response, at least the induction of PTGS2 activity, in rats.

Materials and Methods

Chemicals and antibodies

Equine CG (eCG) and hCG were purchased from Shionogi (Osaka, Japan) and Sankyo (Tokyo, Japan) respectively. DPE, a specific inhibitor against both ALOX5 and ALOX12 but not for COX (Kohyama *et al.* 1997), PGE₂, and an enzyme immunoassay (EIA) kit for PGE₂ were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Antibodies against human/rat ALOX5 and against murine leukocyte ALOX12 were also purchased from Cayman. NDGA was obtained from Sigma Chemical Co. All other reagents including 3,3'-diaminobenzidine tetrahydrochloride (DAB) were of analytical grade.

Animals and ovulation induction

Rats of Wistar–Imamichi strain were used in the present study. They were originally introduced from Imamichi Institute of Animal Reproduction (Saitama, Japan) and had been bred in our laboratory. They were housed in light (light on 0500–1900)- and temperature (23 ± 3 °C)-controlled rooms and water and food pellets were always available. Female rats of 25–27 day old and weighing 50–70 g were treated with a single dose of eCG (i.p., 0.2 iu/g of body weight) and followed 48 h later by hCG (5 iu/rat) to induce ovulation. Ovulation rate (= the number of released eggs) was determined at 24 h after hCG (depicted as hCG24h in this paper) as reported previously (Kurusu *et al.* 1998). Briefly, rats were killed by cervical dislocation under light diethyl ether anesthesia. The ovaries and oviducts were sampled. The eggs present in the ampulla of the oviduct were counted under a light microscope. For immunohistochemical study, three adult rats were also killed and lung and spleen were sampled.

All procedures employed in this study were carried out following the guidelines of the Animal Care and Use Committee of Kitasato University.

Experiments

In a pilot experiment, 10 mg NDGA dissolved in 0.1 ml dimethyl sulfoxide (DMSO) was injected intraperitoneally with an ovulatory hCG. As this systemic treatment had no significant effect on ovulation but side effects on gastrointestinal system (described in the Result section), the local (intra-ovarian bursa) treatment was adopted to achieve the localized and more effective actions of the drug in all of the following experiments. As rats in the similar gonadotropins-priming protocol started to ovulate at about 12 h after hCG administration (Espey *et al.* 1989, 1991), the preovulatory period after hCG administration was divided into the early (the first half, hCG0h to hCG6h) and the latter (the second half, after hCG6h) phases and a bolus of

the drug was treated at hCG0h or hCG6h to examine the time dependency of the effect.

Experiment 1

To examine the effects of NDGA, the drug (0.01, 0.1, or 1.0 mg) dissolved in 50 µl of 20% (v/v) DMSO in physiological saline was administered into the left ovarian bursa at hCG0h or hCG6h. The contralateral intact ovary served as each control group. In a separate group the vehicle was challenged. In this series of experiments, the value of ovulation rate was transformed to a percentage of that of the contralateral ovary. As shown in Fig. 1A, 6–10 rats were used for each experimental group.

Experiment 2

To examine the effects of DPE, the drug (0.02 or 0.1 mg) dissolved in the vehicle described above was administered into both ovarian bursa of the same animal at hCG0h or hCG6h. The group of NDGA (0.1 mg) treatment at hCG0h was prepared as well. Vehicle treatment group served as control groups. To test the possible compensatory effect of PGE₂, some rats that had received local DPE or NDGA (both, 0.1 mg) at hCG0h subsequently received systemic challenge with 80 µg PGE₂ at hCG6h.

For evaluation of ovulation rates, rats were killed by cervical dislocation after blood sampling by heart puncture and ovaries sampled at hCG24h. Ovarian PTGS2 activity and steroid synthesis at the preovulatory period was evaluated at hCG8h. For their biochemical analyses, rats that were treated locally with vehicle, NDGA (0.1 mg), or DPE (0.1 mg) at hCG0h were killed and ovaries sampled at hCG8h. Intact ovaries at hCG0h were also sampled. Aliquots of ovaries were stored frozen (−80 °C) until the analysis of tissue PGE₂ level, and the other ones were instantly processed for tissue fixation for PTGS2 and LOXs immunohistochemistry. For LOX immunohistochemistry, lungs and spleen were sampled from adult rats. Blood plasma samples were stored frozen (−20 °C) until the steroid assay.

Assay of PGE₂ and progesterone

Ovarian PGE₂ was determined according to a previously published method (Higuchi *et al.* 1995, Kurusu *et al.* 1998) using a commercial EIA kit. Briefly, the ovary was homogenized in 0.05 M Tris–HCl (pH 7.4) containing 0.9% NaCl, 0.01% Triton X100, and 0.0057% thimerosal and then centrifuged at 10 000 g for 20 min at 4 °C. The supernatant was assayed directly without any extraction or separation from other eicosanoids and lipids. PGE₂ values were standardized by protein amount that was determined using a protein assay kit (Bio Rad).

Blood samples in experimental rats collected at hCG8h and at hCG24h were assayed for plasma progesterone levels. Progesterone was extracted by *n*-hexane and assayed by RIA as reported previously (Kurusu *et al.* 2007).

Immunohistochemistry

Preovulatory follicular expression of PTGS2 and LOX isoforms (ALOX5 and ALOX12) was analyzed by immunohistochemistry as reported previously (Kurusu *et al.* 1998). Ovarian tissues obtained at hCG0h, hCG3h, and hCG8h were fixed in Bouin's

fixative, dehydrated, and embedded in paraffin. Samples of three to four individual rats in each group were collected and examined. Tissues were serially sectioned (6 µm in thickness), deparaffinized, and examined immunohistochemically. Endogenous peroxidase was blocked by pretreatment with 0.3% H₂O₂ in methanol for 30 min. Tissue sections were incubated with anti-PTGS2 (used at 1:500), anti-ALOX5 (used at 1:200), or anti-ALOX12 (used at 1:200) at 4 °C overnight. Antigen/antibody complexes were visualized with the Vectastain Elite ABC staining kit (Vector Laboratories, Burlingame, CA, USA) and DAB as peroxidase substrate. Controls were performed with normal (non-immunized) mouse IgG. All slides were then counterstained with hematoxylin.

The first immunohistochemical experiment was undertaken to evaluate PTGS2 expression in hCG8h ovaries that had been treated with vehicle, NDGA or DPE at hCG0h. Ovarian sample at hCG0h served as negative control for PTGS2 immunohistochemistry. More than 30 mature follicles whose largest diameter was over 500 µm from three to four different ovarian specimens in each experimental group at hCG8h were evaluated for comparison of expression of immunoreactive PTGS2. The relative intensity of the immunoreactivity in granulosa tissue was evaluated on a scale grading from negative (0), faintly positive (1), moderately positive (2), to strongly positive (3) and statistically analyzed. The second experiment was to examine the presence of immunoreactivity of ALOX5 and ALOX12 in the preovulatory ovaries just before (at hCG0h) and 8 h after hCG treatment (hCG8h). This experiment was also carried out for three different samples at each time point. Sections of lung and spleen of adult rats served as positive controls for ALOX5 (lung) and ALOX12 (lung and spleen) immunohistochemistry (Maruyama *et al.* 1989, Covin *et al.* 1998, Kawajiri *et al.* 2000).

Statistical analysis

Data were presented as mean with S.E.M. The means among different groups were analyzed by ANOVA followed by Student's paired *t*-test or Tukey–Kramer's multiple comparison test, or Mann–Whitney *U* test as appropriate. A *P* value less than 0.05 was considered to be significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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