

Role of prostaglandin E2 receptor subtypes in ovarian follicle growth in the rat *in vivo*. Correlation with interleukin-8 and neutrophils

N. El-Nefiawy¹, K. Abdel-Hakim², N. Kanayama¹ and T. Terao¹

¹Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine and

²Department of Radiology, Hamamatsu University School of Medicine, Hamamatsu, Japan

Summary. This study was conducted to elucidate the role of three of prostaglandin E2 (PGE2) receptor subtype (EP2, EP3, and EP4) agonists in the process of follicular growth. The influence of these agonists on ovarian expression of intimately related factors to follicle development (neutrophils and interleukin-8 (IL-8)) was also investigated. Immature female Wistar rats were injected once with these agonists and killed 48 hours later. Another group of rats were injected pregnant mare serum gonadotrophin. For evaluation of follicle growth, morphometric assessment of antral and ovulatory follicles was performed in serial ovarian sections. The study demonstrated that, EP2 and EP4 agonists showed the maximum follicle counts and diameters versus the control. EP2 and EP4 agonists mimicked PMSG induced follicle growth. Injection of the three agonists induced neutrophil infiltration into theca layer. EP4 agonist showed the most intense ovarian neutrophil accumulation. In addition, dense ovarian IL-8 expression was observed only after EP4 agonist injection. Conclusions: Our data suggests that: 1) EP2 and EP4 receptors are the key PGE2 receptors engaged in follicle growth. 2) Ovarian IL-8 expression and neutrophil infiltration are chiefly mediated via the EP4 receptor. EP2 and EP4 receptor agonists may be candidates for promising reagents that induce follicle maturation in clinical or agricultural fields. This knowledge could provide numerous targets for manipulation of fertility.

Key words: Rat, ovary, PGE2 receptor agonists, follicle growth, IL-8, Neutrophils

Introduction

Much evidence suggests a crucial role of the inflammatory mediator, prostaglandin E2 (PGE2), in the process of follicular growth (Espey, 1980; Davis et al., 1999). Follicular PGE2 synthesis is dramatically increased in the hours preceding ovulation in several species (Armstrong, 1981; Tanaka et al., 1991; Murdoch et al., 1993; Priddy and Killick, 1993; Mikuni et al., 1998). Recent investigation demonstrated that PGE2 is involved in the final differentiation process of the ovarian follicle (Nuttinck et al., 2002). The authors demonstrated the expression of COX2 and production of PGE2 by the cumulus cells during *in vivo* and *in vitro* maturation. Moreover, PGE2 was found to induce cumulus expansion *in vitro* (Eppig, 1981).

In addition, previous reports suggest a crucial role for PGE2 in cellular mitogenesis and survival, a process that is essential for follicular growth and maturation. In colon epithelial cells, over expression of COX2 and enhanced synthesis of PGE2 have been shown to promote proliferation and survival of cells through inhibition of apoptosis (Tsuji and Dubois, 1995; Sheng et al., 1998). It is reasonable that in the rat ovary, PGE2 may be utilizing similar mechanisms that promote follicular cell proliferation and/or survival.

In the ovary the sources of PGE2 are numerous. PGE2 can be derived from resident macrophage population, follicular granulosa cells (GC), and leukocytes (Takaya et al., 1997). PGE2 exerts its autocrine/paracrine effects on target cells by coupling to four subtypes of receptors, which have been classified as EP1, EP2, EP3, and EP4 (Narumiya et al., 1999; Sugimoto et al., 2000). Reviewing the literature, there has been no study so far evaluating the roles of PGE2 receptor subtypes in process of follicular growth. Therefore, we decided to evaluate which EP receptors are involved in PGE2-induced follicular growth in immature rats by using highly selective EP receptor agonists. In addition, to explore the molecular

mechanism of action of these agonists, ovarian localization of neutrophils and the expression of interleukin 8 (IL-8) was also investigated. Morphologic, morphometric, and immunofluorescent techniques were used in the study.

Materials and methods

Experimental design

Using exogenous injection of three selective PGE2 receptor agonists (EP2A, EP3A, and EP4A), we explored how activation of each of these EP receptors affects:

1- Follicle growth through assessment of follicle number and diameter in comparison with pregnant mare serum gonadotropin (PMSG).

2- Ovarian expression of interleukin-8 (IL-8) and localization of neutrophils.

Animals and drug treatments

Female Wistar-Albino rats around 50 g body weight and at 22 days of life were used in the study. Animals were purchased from Japan SLC Co. (Hamamatsu, Japan). Rats were maintained under routine conditions in the laboratory animal center of our university with free access to food and water. Room condition was; temperature 23±1°C, humidity 55±5%, and 12 h light: 12 h darkness.

Rats were divided into 5 treatment groups, 10 animals/group [control group, single dose of the vehicle used to dissolve the drugs; PMSG group, single dose of 20 IU PMSG (Sigma-Aldrich, St. Louis, Missouri, USA); EP2A group, single injection of 50 µg EP2A (Butaprost, Cayman Chemical Co., Ann Arbor, MI, USA); EP3A group, single injection of 50 µg EP3A (Sulprostone, Cayman Chemical Co., Ann Arbor, MI, USA); EP4A group, single injection of 50 µg EP4A (APS-999 Na, Toray Industries, Inc., Tokyo, Japan)]. We have previously used the same treatment conditions and model to demonstrate the action of PGE2 EP4 receptor agonist on immature rat ovary (El-Nefiawy et al., 2005).

Injections were administered via subcutaneous route. All animals were sacrificed 48 hours after the injection with a lethal dose of ether according to an animal protocol that was approved by the animal care and use committee of Hamamatsu University School of Medicine, Japan. All experiments were performed in triplicate (a total of 150 rats were used).

Morphological characterization of ovarian follicles

Stages of follicular development were classified according to Wulff et al. (2002) as primary follicles (the oocyte is surrounded by a single layer of cuboidal granulosa cells, GCs), secondary follicles (the oocyte is surrounded by multiple layers of cuboidal GCs without an antrum), tertiary follicles (follicles containing an

antrum), and ovulatory follicles (follicles with large continuous antral space). Healthy follicles were recognized by having a normal shaped oocyte surrounded by GCs that were regularly apposed on an intact basement membrane with a normal appearance of GC nuclei without signs of pyknosis.

Morphometric assessment of follicular growth

Many studies have used follicle count and diameter measurement to investigate follicular development (e.g., (Roby 2001; Mazaud et al., 2002)). In the present study we employed the following: 1) morphological assessment of the number of follicles that reached the antral and ovulatory stages. 2) morphological assessment of follicular diameter of antral and ovulatory follicles (mm), the largest diameter of the follicle was measured since some follicles had oblong configurations.

After sacrifice of the rats, ovaries were extracted and immediately fixed in 4% formaline in water, processed and embedded in paraffin. The embedded ovaries were serially sectioned (thickness, 5 mm). The serial sections were placed in order on glass microscope slides and stained with hematoxylin and eosin (H&E) for examination under VANOX AHBS3 (Olympus) light microscope.

Every 10th section was marked for analysis, and the total number of antral and ovulatory follicles in each of the marked sections was calculated. The starting section was selected randomly. To avoid double counting of follicles, only follicles containing an oocyte with a visible nucleus were counted.

To morphologically assess the size of follicles, the diameters of all antral and ovulatory follicles in the marked sections were measured using a calibrated micrometer on a light microscope at x25 magnification. The mean of all follicle diameters was calculated to estimate the total mean diameter of all antral and ovulatory follicles in each ovary. Only follicles containing an oocyte with a visible nucleus were measured to avoid double measurements and to help ensure that a consistent area of each follicle was measured.

To avoid bias, all ovaries were analyzed without knowledge of the group examined. In addition, two investigators independently counted and measured follicles in all sections and compared their results; inconsistencies were resolved before including the data in the analysis. It should be noted that we did not apply any correction factor prior to data presentation as obtaining the absolute total values for follicle numbers or diameters were not the focus of this study.

Antibodies

Polyclonal anti interleukin-8 antibody that was used at a dilution of 1:100 (Sigma Chemical Co., St. Louis, MO, USA).

Polyclonal anti neutrophil elastase antibody (Santa

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Cruz Biotechnology, Inc.; U.S. 1:100).

Fluorescein-conjugated rabbit antibody was used as the secondary antibody (Dako, Botany, NSW, Australia, Inc; 1:40).

Indirect Immunofluorescence

Ovarian sections were deparaffinized in xylene baths, rehydrated through descending grades of alcohol, and rinsed in phosphate buffered saline (PBS, pH 7.2). Sections were then fixed in cold acetone for 10 min at room temperature. Thereafter, the slides were preincubated with normal rabbit serum in a 1:50 dilution in PBS with 1% bovine serum albumin (BSA, Sigma) for 10 min to block non-specific reactions. Subsequently, the slides were incubated with the primary antibodies prepared in 2% BSA in PBS at their appropriate dilutions for 2 hours at room temperature. After washing in PBS (3x1; 5 min each) slides were incubated with FITC conjugated secondary antibody for 30 min at room temperature. After final wash, glass cover slips were mounted on glass slides with mounting fluid, sealed with clear nail varnish. Slides were examined under a Zeiss Axiophot fluorescence microscope (Germany) equipped with filters for discrimination of fluorescein. Negative control staining was conducted by replacing all the primary antibodies by their non-immune IgG isotypes at the same concentrations.

Image analysis

A computer-assisted image analysis system was used to analyze the stained slides. Light microscopic images were captured then transformed into 32-bit color images with 945x738 resolution. For transformation a digital

camera (FUJIX HC-2000; Fuji Photo Film, Tokyo, Japan) attached to a light microscope (VANOX AHBS3; Olympus, Tokyo, Japan; with x 40 objective) was used together with software (Adobe Photoshop 5.5; Adobe Systems, San Jose, CA) run on a Macintosh computer (Apple Computer, Cupertino, CA). To determine the area of interest, Mac SCOPE Image Analysis was used (Version 2.5.6; Mitani, Maruoka, Japan).

Enumeration of positive stained cells

The number of the immunoreactive cells (for anti-neutrophil elastase or anti-IL-8 antibodies) was calculated in ten randomly chosen fields of each stained section. Counting was performed by two independent observers (blinded to the specimen details), and the mean of the two observations was calculated to reflect the distributional density of positive cells.

Statistical analysis

Data are presented as mean \pm SEM. All statistical analyses were performed on absolute values. Analysis of variance (one-way ANOVA) followed by post-hoc a two-tailed Student's t test was used to compare between groups. Significance was assumed at $P < 0.05$. The tests were performed using SPSS version 6.1 for Macintosh (SPSS, Inc., Chicago, IL). The quoted P values in the results section were obtained by t test without correction for multiple testing.

Results

Effect of PGE2 receptor agonists on follicle development

Follicle count

EP4A demonstrated a highly statistical significant

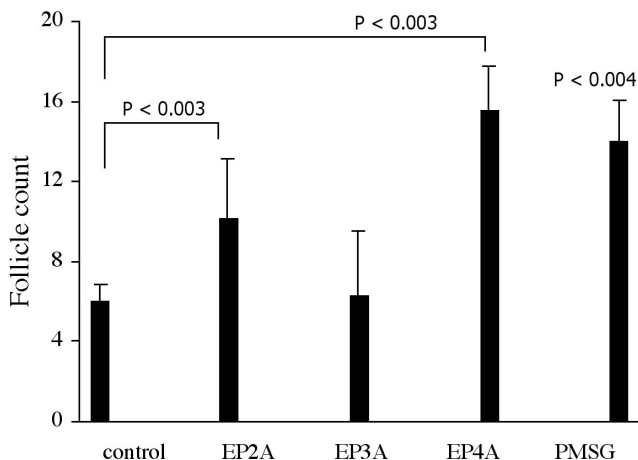


Fig. 1. Estimation of mean follicle (antral and ovulatory) counts in ovaries of the study groups. Statistically significant increase of follicle count was induced by EP4A and EP2A versus the control and in a similar fashion to PMSG.

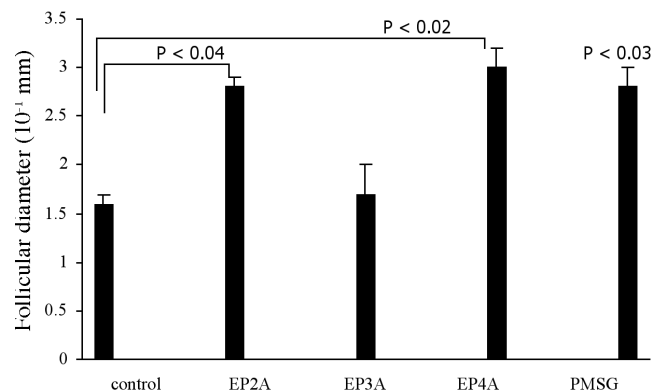


Fig. 2. Mean follicle (antral and ovulatory) diameters in ovaries of the study groups. EP4A and EP2A induced pronounced increase of follicle diameters compared with the control and in a comparable degree to PMSG.

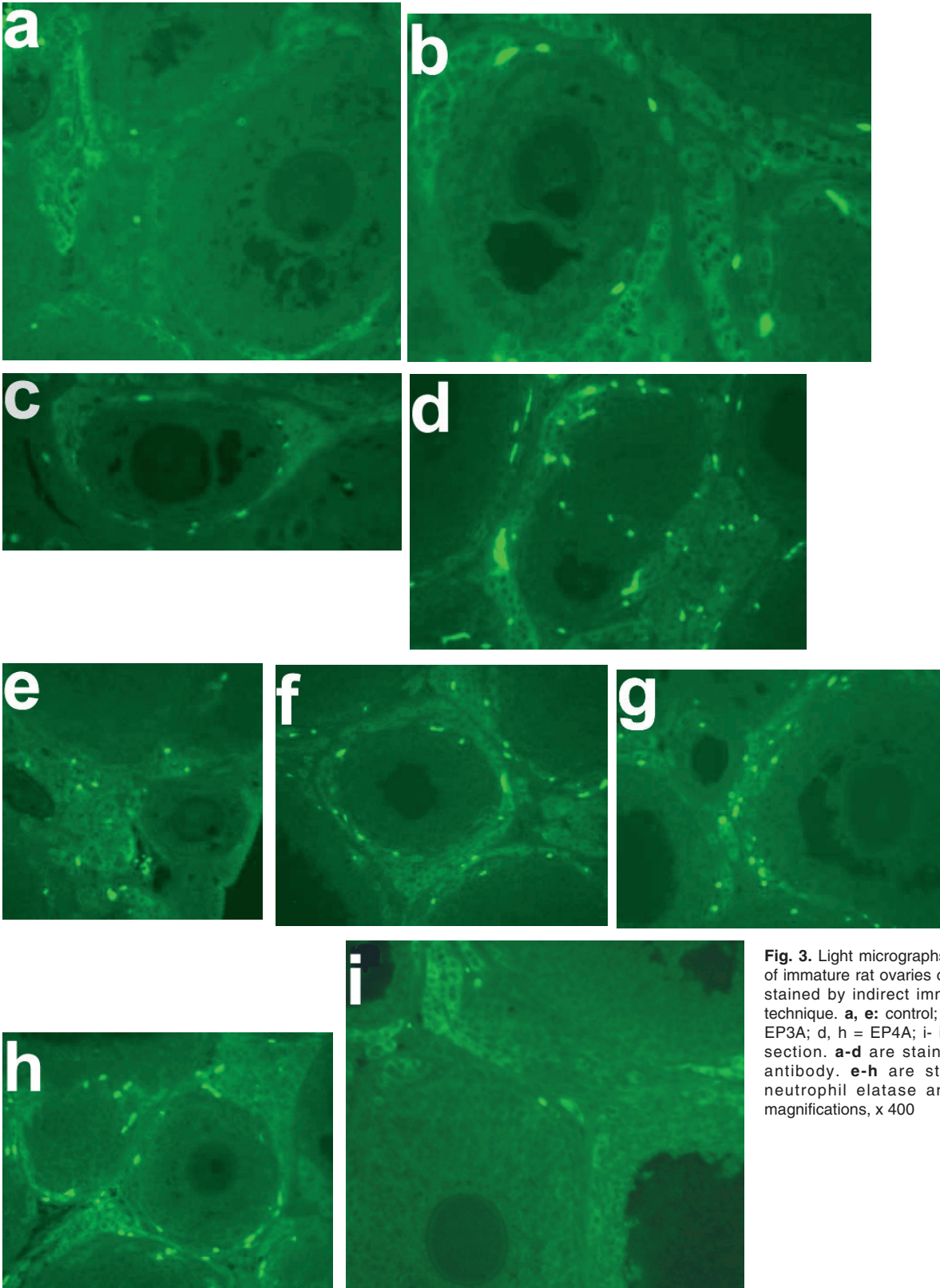


Fig. 3. Light micrographs showing sections of immature rat ovaries of the study groups stained by indirect immunofluorescence technique. **a, e:** control; **b, f:** EP2A; **c, g:** EP3A; **d, h =** EP4A; **i-** is negative control section. **a-d** are stained with anti-IL-8 antibody. **e-h** are stained with anti-neutrophil elatase antibody. Original magnifications, x 400

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increase in follicle (antral & ovulatory) count (15.5 ± 2.2 , $P = 0.003$) compared with the control (6 ± 0.8). Similarly, EP2A revealed a marked increase of follicle count (10.1 ± 2.9 , $P < 0.005$). On the other hand, EP3A did not contribute to follicle growth as revealed from low follicle count 6.2 ± 1.2 . PMSG group revealed mean follicle count of 14 ± 2 ($P < 0.004$). Statistical comparison between PMSG, EP2A and EP4A groups did not reveal significant differences. Figure 1 demonstrates follicle counts for all groups.

Follicle diameter

Measurement of follicle (antral & ovulatory) diameters coincided with results of follicle count. A highly significant statistical increase of follicle diameter was obtained in EP4A (3.0 ± 0.2 , $P < 0.02$) as well as EP2A (2.8 ± 0.1 , $P < 0.04$) groups versus the control group (1.6 ± 0.1). EP3A did not induce an increase in follicle diameter (1.7 ± 0.3). PMSG group revealed mean diameter of 2.8 ± 0.2 ($P < 0.03$). EP4A and EP2A resulted in an increase of follicle diameter in a comparable fashion to PMSG with no statistical difference. Figure 2 illustrates follicle diameter measurements.

Expression of IL-8 after injection of PGE2 receptor agonists

In the control group, positive cells were predominantly observed inside ovarian blood vessels located mainly in the medulla rather than in the cortex of the ovary. No positive staining was observed in the follicles.

In sections of PGE2 agonists, IL-8 positive cells were observed inside the theca layer of secondary and preovulatory follicles but not primary follicles. Also, positive cells were noticed inside ovarian vasculature as

in the control group. Figure 3 a-d illustrates micrographs of IL-8 staining for all groups.

Enumeration of IL-8 positive cells revealed the highest statistical significant increase of cell count for the EP4A group versus all study groups. Noteworthy, in EP4A group markedly dilated blood vessels were distended with a tremendous number of positive stained cells. EP2 and EP3 agonists resulted in mild insignificant increase of IL-8 positive cells.

Mean number of positive cells was 221.6 ± 33.9 ($P < 0.005$) for EP4A group, 50.3 ± 12.6 ($P < 0.3$) for EP2A, 52.5 ± 8.6 ($P < 0.1$) for EP3A, and 38.7 ± 13.1 for the control group (Fig. 4).

Localization of neutrophils after injection of PGE2 receptor agonists

In the control group, neutrophils were seen inside ovarian blood vessels evenly distributed all over the ovary. EP2 and EP3 agonists induced neutrophils infiltration of the theca lutein cell layer of growing secondary and preovulatory follicles but not primary follicles. In the EP4A group, the markedly dilated ovarian blood vessels revealed a huge number of neutrophils while follicular infiltration with neutrophils was less. Figure 3 e-h illustrates micrographs for neutrophil immune staining (Fig 3i is for negative control staining). The immune localization of neutrophils was comparable to that for IL-8 protein previously mentioned suggesting that neutrophils were the source of IL-8.

EP4A revealed the highest number of neutrophils versus all groups (208.7 ± 34 , $P < 0.008$). Although, EP2A (59.2 ± 10.8 , $P < 0.01$) and EP3A (49.7 ± 12.8 , $P < 0.05$) showed a statistically significant increase of neutrophil count compared with the control (30.5 ± 6.8) (Fig. 5).

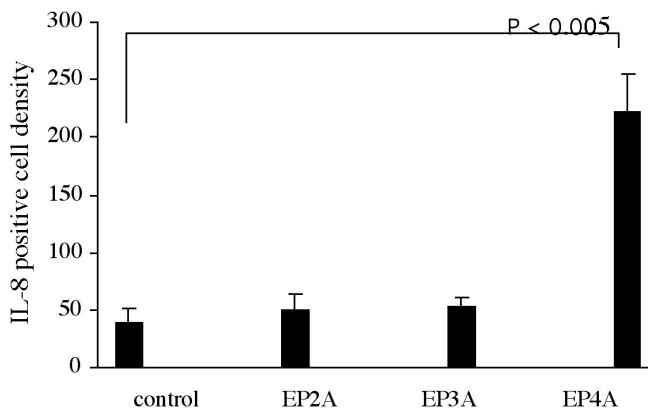


Fig. 4. Ovarian IL-8 expression after injection of PGE2 receptor agonists. EP4A resulted in a remarkable increase in the number of IL-8 positive cells compared with the control. EP2 and EP3 agonists revealed insignificant increases of IL-8 positive cells.

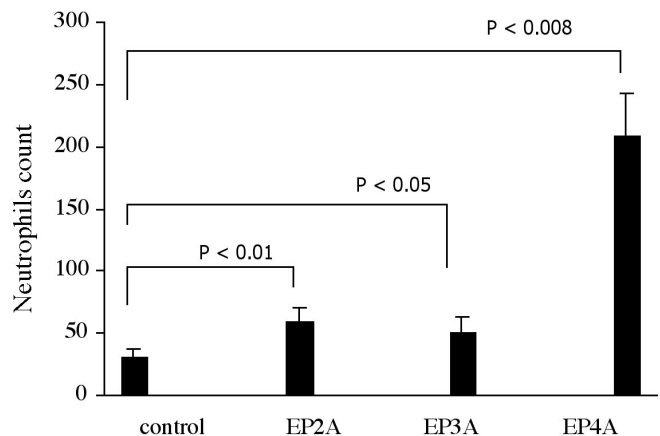


Fig. 5. Density of ovarian neutrophils after treatment with PGE2 receptor agonists. EP4A induced the highest number of neutrophils versus all groups. EP2 and EP3 agonists revealed a significant increase of neutrophil counts versus control.

Discussion

The present study demonstrated for the first time the effects of exogenous injection of three of PGE2 receptor agonists (EP2, EP3, EP4) on the processes of follicular growth in the immature rat *in vivo*. Also, the study investigated the impact of these receptor agonists on ovarian neutrophil localization and IL-8 expression in an attempt to role out their underlying molecular mechanism of action.

Role of EP agonists in follicular growth

The specific receptor subtype (s) that mediates PGE2 action in follicular growth is still not determined and is becoming a focus of intense research. Chinese hamster ovary cells were shown to stably express the four PGE2 receptors (Kiriya et al., 1997). Narko et al. (2001) detected transcripts for EP2 and EP4 receptors in freshly isolated human ovarian GCs. Another study also revealed that human GCs express functional EP1 and EP2 receptors (Harris et al., 2001). Stimulation of the two receptors with ligands that can bind to both resulted in cAMP formation and progesterone synthesis. The investigators revealed that GCs expressed mRNA transcripts encoding both EP1 and EP2 receptors using Reverse-transcription PCR. In mouse periovulatory follicles, expression of mRNA for EP2 and EP4 receptors has been recently detected (Segi et al., 2003). The authors also revealed that hCG administration induced expression of EP4 mRNA in GCs and cumulus cells all through till ovulation. In the previous studies, the expression pattern for the EP1, EP2, and EP4 receptors being localized in GCs suggests a possible role in follicular growth. Here, we detected follicle growth after exogenous injection of EP2 and EP4 agonists but not EP3A. Our data suggests that EP2 and EP4 receptors are the key receptors engaged in follicle growth and goes in line with previous studies.

Clearly, many factors contribute to follicular development: gonadotrophic hormones as well as inter- and intra follicular signaling molecules. Recent investigations have demonstrated that gonadotrophic hormone administration induced the expression of COX2 and the production of PGE2 by the follicular cells (Watson et al., 1991; Narko et al., 2001). This implies that gonadotrophic hormone induces follicle growth partially through PGE2. In the present study, relying upon the morphologic and morphometric parameters, EP2 and EP4 agonists mimicked PMSG induced follicle growth. EP2 and EP4 receptor agonists may be candidates for promising reagents that induce follicle maturation in clinical or agricultural fields.

Effect of EP agonists on neutrophils and IL-8

It is known that ovulation has many characteristics of an inflammatory-like reaction (Brännström and Janason, 1991). Leukocytes, especially neutrophils,

dramatically increase in number in response to gonadotrophin, in the theca of preovulatory follicles (Bonello et al., 2004). Previous investigation demonstrated that neutrophils have an essential role in follicle growth (Chang et al., 1998). The authors detected that in the human ovary, neutrophils are restricted to antral rather than preantral follicles. Despite a cardinal role for both PGE2 and neutrophils in the process of follicle growth, no study before has explored the impact of PGE2 treatment on the accumulation of neutrophils in the ovary during follicle growth. In the present study, exogenous injection of the three PGE2 receptor agonists resulted in neutrophil infiltration of the theca cell layer of secondary and ovulatory follicles. EP4A revealed the most significant increase in neutrophil count. These results signify that EP4 receptor is the primary receptor by which ovarian neutrophil infiltration occurs.

IL-8 is a mediator of angiogenesis, mitogenesis of vascular smooth muscle cells, and chemotaxis and activation of neutrophils (Koch et al., 1992; Yue et al., 1994). In addition, previous studies demonstrated that exogenous IL-8 induced ovarian follicle growth (Goto et al., 1997; Chang et al., 1998; Runesson et al., 2000). Investigations dealing with PGE2 mode of action, have demonstrated that PGE2 mediates its action through up-regulation of IL-8 production. In human colonic epithelial cells, exogenous PGE2 was shown to up-regulate IL-8 gene expression and protein production through EP4 receptor (Yu and Chadee, 1998). *In vitro* study on peripheral blood, neutrophils and monocytes derived IL-8 was found to be regulated by PGE2 (Wertheim et al., 1993). In the present study, exogenous EP4A but not other PGE2 receptor agonists resulted in highly significant IL-8 protein expression. This result declares that ovarian IL-8 expression is chiefly mediated via the EP4 receptor.

It is known that neutrophils constitute a rich source for the cytokine IL-8 (Wertheim et al., 1993). Previously, Chang et al., (1998) declared that, neutrophils and IL-8 are associated with follicle development in the normal human ovary. They found expression of IL-8 whenever follicular neutrophils increase in number. We observed the localization pattern of IL-8 expression was comparable to that for neutrophils using immune staining. Hence, it could be assumed that EP4A up-regulated IL-8 expression via intense neutrophil accumulation in the ovary.

In summary, the data of this study suggests that different mechanisms are involved in follicular growth, accumulation of neutrophils, and expression of IL-8 in the immature rat ovary. Follicle growth was detected after stimulation of EP2 and EP4 receptor with their selective agonists. Stimulation of the EP4 receptor resulted in marked neutrophil accumulation and IL-8 expression. The current data provides additional knowledge that enhances our understanding of the biology of follicle growth as well as the mechanism of action of PGE2. Also it may provide new approaches to

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regulation of reproductive function and treatment of pathological conditions of the female reproductive tract.

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