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European Journal of Pharmacology 516 (2005) 158 – 164

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Stimulatory effects of eicosanoids on ovarian angiogenesis in early luteal phase in cyclooxygenase-2 inhibitor-treated rats

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Received 21 October 2004; received in revised form 4 February 2005; accepted 2 March 2005
Available online 25 May 2005

Abstract

Our previous study demonstrated that impaired ovarian vasculature is responsible for the decrease in serum progesterone observed in cyclooxygenase (COX)-2 inhibitor-treated rats. To explore the role of arachidonic acid metabolites in the formation of the corpus luteum, we determined in the present study the effects of prostaglandin (PG) and thromboxane (TX) receptor agonists together with vascular endothelial growth factor (VEGF) on progesterone secretion and angiogenesis in the newly formed corpus luteum in NS-398 (a selective inhibitor of COX-2)-treated rats. Uterine injection of PGE_2 or U-46619 (TXA_2 receptor agonist) prevented decreased levels of serum progesterone and ovarian hemoglobin, an indicator of amounts of vasculature in NS-398-treated rats. Luteal capillary vessel establishment was inhibited by NS-398, as determined by histological examination of ovarian vascular plexuses, while administration of PGE_2 reversed the effect. VEGF enhanced the levels of serum progesterone and ovarian hemoglobin, and increased the density of ovarian capillaries. However, VEGF-induced angiogenesis was inhibited by NS-398 treatment. These results suggest that PGE_2 and TXA_2 stimulate angiogenesis in the newly formed corpus luteum and that there is a possibility that these eicosanoids are involved in VEGF-induced progesterone production and the increase in luteal blood flow.

Keywords: Angiogenesis; Eicosanoid; Ovary

1. Introduction

The ovarian corpus luteum formed from ovulated follicles plays a critical role in female reproduction. Corpus luteum development is dependent on angiogenesis (Tamura and Greenwald, 1987; Smith et al., 1994; Reynolds et al., 2000). Angiogenesis, the formation of new blood vessels via endothelial replication, occurs actively during the early stage of the corpus luteum lifespan. The newly formed corpus luteum is rapidly invaded by blood vessels, and the vessels in thecal cells outside the corpus luteum are interspersed toward the center through the space among the granulosa-derived luteal cells. The primary secretory product of the corpus luteum is progesterone, which is essential for the establishment and maintenance of pregnancy. Luteal angiogenesis is needed for the supply of large amounts of cholesterol required for progesterone synthesis as well as for the delivery of progesterone to the circulation during pregnancy.

Vascular endothelial growth factor (VEGF) is critical for angiogenesis in the corpus luteum (Ferrara et al., 1998). VEGF induces endothelial proliferation, migration, and tube formation to regulate angiogenesis (Ferrara and Davis-Smyth, 1997). Luteal angiogenesis is suppressed by neutralization of VEGF in marmoset monkeys (Fraser et al., 2000). In most species, VEGF mRNA is detected in the granulosa-derived luteal cells of the newly formed corpus luteum, and VEGF protein is localized in steroidogenic cells of the corpus luteum (Shweiki et al., 1993; Geva and Jaffe, 2000).

Autocrine or paracrine effects of luteal prostaglandins may be involved in the control of the corpus luteum lifespan and functions (Arosh et al., 2004). Our previous study

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doi:10.1016/j.ejphar.2005.03.003

demonstrated that cyclooxygenase (COX)-2 activity may be important for angiogenesis in the early developing corpus luteum in rats (Sakurai et al., 2003). When gonadotropin-primed rats were injected with a COX-2 inhibitor, NS-398, for 2 days after ovulation, serum progesterone levels decreased together with vasculature impairment in the developing corpus luteum. VEGF stimulates the expressions of COX-2 and prostaglandin (PG) E synthase mRNAs in rat luteal cells (Sakurai et al., 2004). Thus, COX-2 may be involved in the physiological angiogenesis of the corpus luteum that takes place during the early luteal phase in rats. To address the role of eicosanoids in luteal angiogenesis in the present study, we examined the effects of exogenous PGE_2, Ciprostene (PGI_2 analogue), and U-46619 (TXA_2 receptor agonist) on progesterone secretion and angiogenesis using the gonadotropin-induced ovulation model in rats. We also examined the possible role of eicosanoids in VEGF-induced ovarian angiogenesis.

2. Materials and methods

2.1. Animals

Immature (21 days old) Wistar–Imamichi rats (Imamichi Institute for Animal Reproduction, Ibaraki, Japan) were maintained in an air conditioned room (temperature: 23±1°C; humidity: 55±5%) under controlled lighting (12 h light/day schedule) with free access to food and water. All experimental protocols with rats used in this study were reviewed and approved by the Institutional Animal Care Committees at the Tokyo University of Pharmacy and Life Science, in compliance with institutional guidelines for experimental animal care. Pseudopregnancy was induced by high dose gonadotropin, equine chorionic gonadotropin (eCG; Teikoku Hormone MFG Co., Tokyo, 50 IU on day 23), and human CG (hCG; Teikoku Hormone, 25 IU on day 25) administered 54 h after eCG treatment (Sakurai et al., 2003).

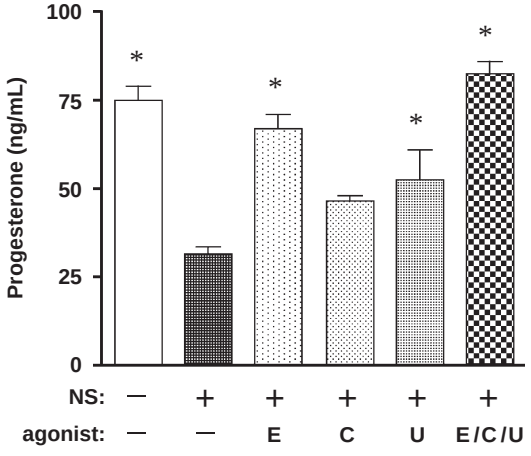

Fig. 1. Effects of eicosanoids on serum levels of progesterone in NS-398-treated rats. Immature rats were treated with 50 IU of eCG and 25 IU of hCG on days 24 and 26, respectively. NS-398 (5 mg/kg, s.c.) was administered at 0900 h on days 27 (the day of ovulation) and 28. At 1200 h (3 h after NS-398 injection) on day 27, prostaglandin (PG) E_2, Ciprostene (stable PGI_2 derivatives), and U-46619 (TXA_2 receptor agonist) (0.3 μg/10 μl each) were infused into the uterus after the removal of the ovary on the other side. Two days later (0900 h on day 29), blood samples were collected. Each bar is the mean±S.E.M. of 6–16 rats from three experiments. *$P<0.01$ vs. NS (NS-398). E: PGE_2; C: Ciprostene; U: U-46619; E/C/U: the combination of E, C, and U.

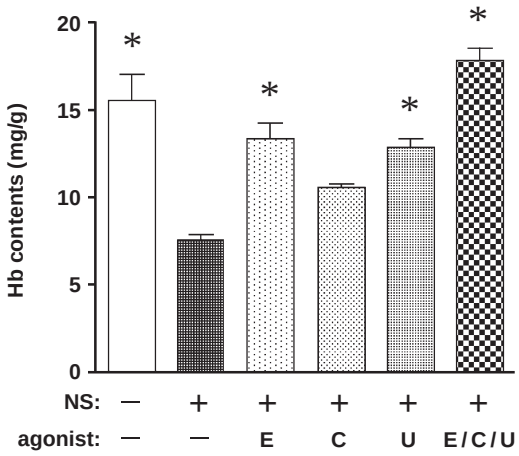

Fig. 2. Effects of eicosanoids on ovarian hemoglobin content in NS-398-treated rats. The same treatments as described in Fig. 1 were administered. Ovaries were collected at 0900 h on day 29 before blood collection and hemoglobin (Hb) levels in the ovary were measured. Each value shows the mean±S.E.M. of 6–16 rats. *$P<0.01$ vs. NS (NS-398). E: prostaglandin E_2; C: Ciprostene; U: U-46619; E/C/U: the mixture of E, C, and U.

2.2. Experimental schedule

Gonadotropin-primed rats were injected with NS-398 (5 mg/kg; Cayman Chemical, Ann Arbor, MI, USA), a selective COX-2 inhibitor, at 0900 h on the day of ovulation (day 26) and the following day (day 27). The dose of NS-398 sufficient to inhibit COX activity was determined in our previous study (Sakurai et al., 2003). At 1200 h on day 26, rats were anesthetized with ether, the uterus was exposed, the middle of the uterine horn was ligated, and the ovary on the side opposite of where the injection would occur was removed. PGE_2 (0.3 μg/10 μl; Cayman), Ciprostene (a stable analog of PGI_2, 0.3 μg/10 μl; Cayman), or U-46619 (a TXA_2 receptor agonist, 0.3 μg/10 μl; Cayman) was injected into the ligated uterine lumen near the ovary. The doses of eicosanoids used were determined based upon the physiological contents in the preovulatory ovary reported by Brown and Poyser (1984). The mixture of these three eicosanoids (0.3 μg each/10 μl) was also administered as a treatment. Animals in the control group were injected with the same amount of PBS containing 0.15% gelatin as a vehicle. After the injections, animals were sutured with silk thread. Two days later (day 28), animals were anesthetized with ether and decapitated, and their ovaries were immediately removed to measure hemoglobin content. Blood collected from the abdominal aorta was centrifuged. The serum was separated by centrifugation and stored at −80 °C until assayed for progesterone. Some animals were infused with heparin–saline solution followed by a Mercox solution to visualize ovarian vascular vessels (see below).

In another experiment, animals were treated with gonadotropin and NS-398, as described above, and injected with VEGF (recombinant human VEGF, 10 ng/10 μl; R&D Systems, Inc., Minneapolis, MN, USA) into the ovarian bursa at 1200 h on day 26. To determine the dose of VEGF administration, we referred to

the data showing the concentration of VEGF in the corpus luteum (Kashida et al., 2001). Some rats were injected with PGE_2 (0.3 μg/10 μl) into the lumen of the uterine horn at the same time. The serum levels of progesterone, ovarian hemoglobin contents, and histological analysis of capillary vessels using corrosion casting were determined.

2.3. Progesterone assay by radioimmunoassay (RIA)

The concentration of progesterone in the serum or culture media was measured by RIA, as described previously (Tamura et al., 1991).

2.4. Hemoglobin assay

The ovarian hemoglobin content was determined with an assay kit employing the SLS hemoglobin method (Hemoglobin B test; Wako Pure Chemical Industries Ltd., Osaka, Japan), as described previously (Sakurai et al., 2003).

2.5. Histological examination of ovarian vascular plexuses

The procedure was basically performed according to our previous report (Sakurai et al., 2003). Under deep anesthesia with ether, the ovary was exposed, and a small hole was made in the right atrium of the heart for drainage of fluid. Mercox (Okenshoji, Tokyo, Japan) was injected via the ventricle and abdominal aorta using a syringe with a 22-gauge needle. Animals were kept at 55°C for 30 min to allow for complete polymerization, and the ovaries were removed and placed in warm (60 °C) water for 5 h followed by digestion in 10% NaOH. Samples were examined microscopically and photographed (Leica DC 300 F; Leica Microsystems, Tokyo, Japan).

2.6. Statistical analysis

All experiments were conducted with at least five animals and values are given as mean ± S.E.M. All experiments were independently replicated at least twice. The statistical significance of the

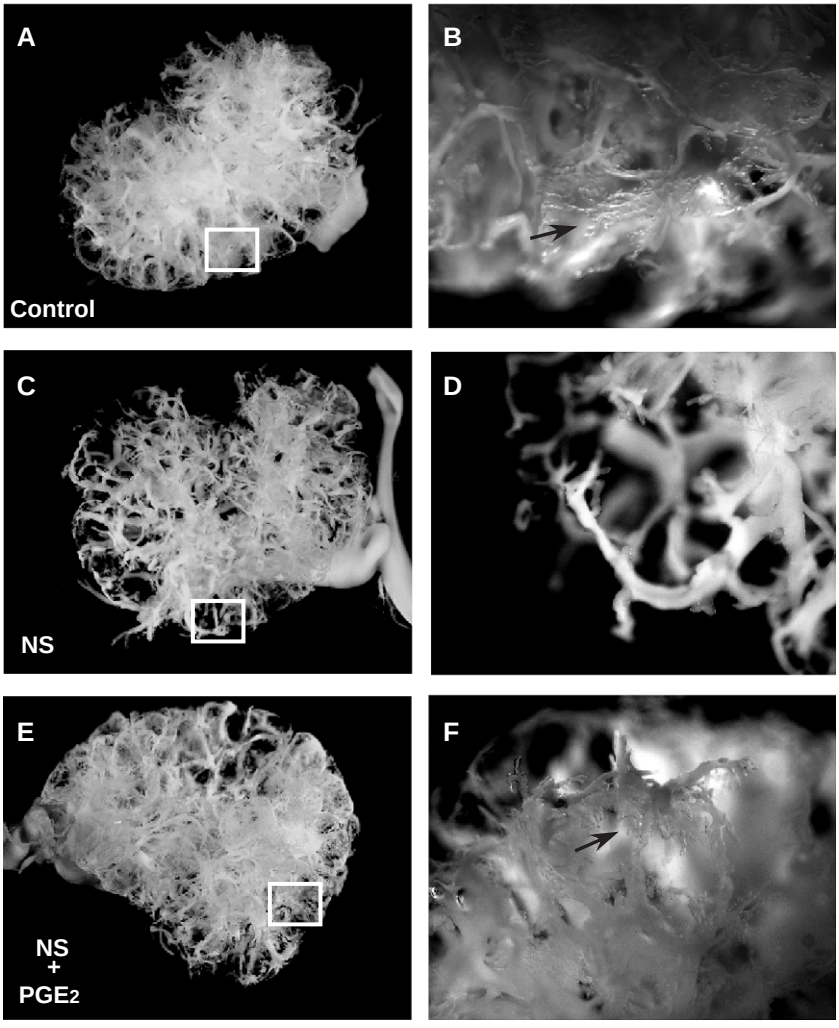

Fig. 3. Effects of prostaglandin E_2 on ovarian vascular plexuses in NS-398 (NS)-treated rats. The same treatments as described in Fig. 1 were administered. Mercox was infused into the ventricle and abdominal aorta on day 29. The pictures on the right (original magnification: B, D, and F × 40) are enlargements of the boxed area of pictures on the left (original magnification: A, C, and E × 10). The arrow indicates capillary vessels in the corpus luteum. (A and B) Control; (C and D) NS-398-treated; (E and F) NS-398 and PGE_2-treated.

results was tested by Dunnett's test for multiple comparisons. Differences with P values <0.05 were considered significant.

3. Results

3.1. Effects of eicosanoids on the levels of serum progesterone and ovarian hemoglobin and the formation of vascular plexuses in rats treated with a COX-2 inhibitor

We examined whether the selective COX-2 inhibitor-induced reduction of serum progesterone levels is restored by treatment with PGE_2, Ciprostene (PGI_2 analogue), or U-46619 (TXA_2 receptor agonist). As shown in Fig. 1, PGE_2 and U-46619 treatment significantly increased progesterone levels, compared with the group treated only with the COX-2 inhibitor, NS-398. Ciprostene tended to increase the levels. Moreover, treatment with the mixture of these three eicosanoids completely enhanced the serum levels of progesterone up to control levels. Hemoglobin content in the ovary was elevated basically in parallel with changes in progesterone levels (Fig. 2). PGE_2 or U-46619, as well as the combined mixture of eicosanoids, increased ovarian hemoglobin content. Fig. 3 shows the histological examination of vascular plexuses using the corrosion casting method. Capillary development observed in highly luteinized ovaries was absent in ovaries exposed to NS-398 (Fig. 3C and D). PGE_2 treatment of NS-398-treated rats inhibited the loss of vascular capillaries, so that the capillary density was comparable to control levels (Fig. 3E and F).

3.2. Effects of VEGF on progesterone levels, ovarian hemoglobin content, and vascular plexuses in COX-2 inhibitor-treated rats

As shown in Fig. 4, VEGF treatment tended to stimulate progesterone levels in control animals. However, the inhibition of progesterone levels was not significantly increased by VEGF treatment in NS-398-treated rats. Additional PGE_2 treatment in VEGF-treated animals increased progesterone concentrations up to control levels. Ovarian hemoglobin content also tended to be elevated after VEGF treatment in the NS-398-treated group compared with the group treated only with NS-398, but not significantly (Fig. 5). As observed with progesterone levels, combined treatment with VEGF and PGE_2 significantly increased the hemoglobin content up to intact levels. Comparison of vascular capillary appearance in the ovaries is shown in Fig. 6. The density of the vascular cast in the ovarian cortex in VEGF-treated rats was visually concentrated when compared with that in control rats (Fig. 6D vs. A). NS-398 treatment caused the loss of capillaries (Fig. 6B), as depicted previously in Fig. 2. VEGF administration to NS-398-treated animals partially restored the development of capillaries (Fig. 6C), and the combined treatment of VEGF and PGE_2 promoted the formation of the vascular plexuses to the same levels as the control group, as determined by visual appearance (Fig. 6E).

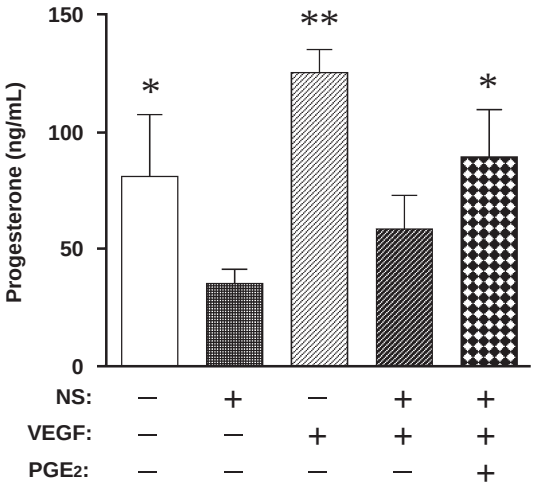

Fig. 4. Effects of vascular endothelial growth factor (VEGF) on the serum levels of progesterone in NS-398-treated rats. Gonadotropin and NS-398 treatments were administered as indicated in Fig. 1. At 1200 h (3 h after NS-398 injection) on day 27, VEGF (10 ng) was injected into the ovarian bursa after removal of the ovary on the other side. Some animals were also treated with PGE_2 injection into the uterus. Blood samples were collected at 0900 h on day 29. Each bar is the mean±S.E.M. of 10–13 rats from three experiments. *$P<0.01$ vs. NS (NS-398) (−)/VEGF (−)/PGE_2 (−).

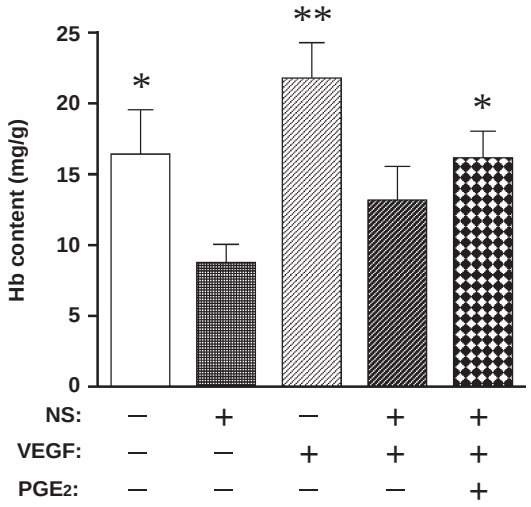

Fig. 5. Effects of vascular endothelial growth factor (VEGF) on ovarian hemoglobin content in NS-398-treated rats. Treatments were administered as indicated in Fig. 4. Ovaries were collected at 0900 h on day 29 before blood collection and hemoglobin (Hb) levels were measured. Each bar is the mean±S.E.M. of 10–13 rats. *$P<0.01$ vs. NS (NS-398) (−)/VEGF (−)/PGE_2 (−).

4. Discussion

Our previous study suggested that the decrease in serum progesterone levels induced by NS-398 treatment may be caused by inhibition of angiogenesis during luteinization in rats (Sakurai et al., 2003). The up-regulation of various ovarian eicosanoids including PGE_2, PGI_2 (Brown and Poyser, 1984), and TXA_2 (Wilken et al., 1990), which are mainly induced by increased expression and activity of COX in the ovary around the time of ovulation after the LH surge, might be involved in the control of angiogenesis. Some prostaglandins stimulate vascular permeability and angiogenesis (Ziche et al., 1982; Form and Auerbach, 1983).

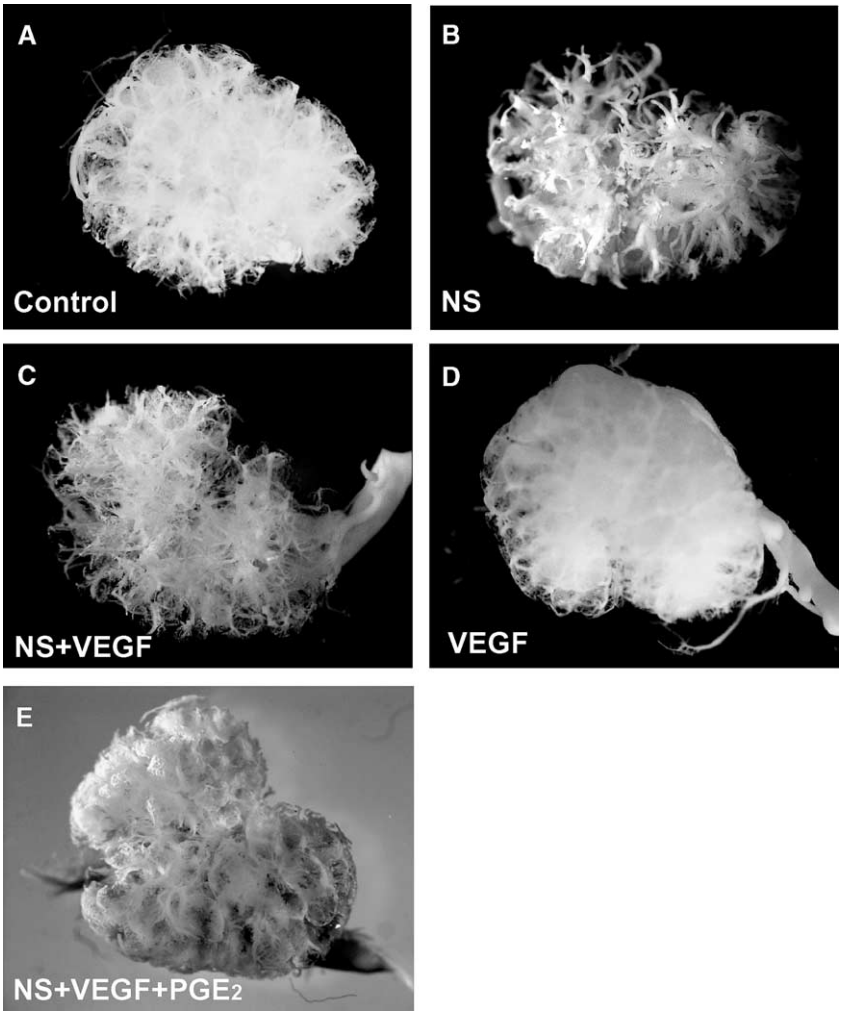

Fig. 6. Effects of vascular endothelial growth factor (VEGF) on ovarian vascular plexuses in NS-398-treated rats. Treatments were administered as indicated in Fig. 1. Mercox was infused into the ventricle and abdominal aorta on day 29. (A) Control; (B) NS-398; (C) NS-398+VEGF; (D) VEGF; (E) NS-398+VEGF+PGE_2. Original magnification: A, B, C, D, and E ×10.

Development of the vasculature in the luteinized ovary is responsible for the increase in serum progesterone (Wulff et al., 2001). We, therefore, examined whether the selective COX-2 inhibitor-induced inhibition of ovarian angiogenesis is restored by treatment with PGE_2, Ciprostene, or U-46619. At the end of the luteal phase in pseudopregnant rodents, $PGF_{2\alpha}$ produced in the uterus is transferred to the ovary via the uterine–ovarian arteriovenous system, and then luteolysis is induced (Pharriss and Wyngarden, 1969; Goldberg and Ramwell, 1975; Horton and Poyser, 1976). Indomethacin administered into the uterine lumen of rats on diestrus I increased the content of progesterone in the ovary 24 h later (Takahashi et al., 1979). Therefore, we injected eicosanoids into the uterus lumen in the present study. Injection of PGE_2 or U-46619 increased the serum levels of progesterone. PGE_2 treatment increased the hemoglobin content and the density of the vascular capillary in the cortex of the ovary. TXA_2, in addition to PGE_2, counteracted the reduced ovarian progesterone secretion and hemoglobin level induced by the COX-2 inhibitor. These data indicate that both eicosanoids stimulate ovarian angiogenesis and restore NS-398-reduced development of corpus luteum.

Eicosanoids appear to be associated with angiogenesis in the corpus luteum. At least two possible mechanisms have been postulated. First, eicosanoids may stimulate the formation of vascular plexuses in the corpus luteum via VEGF expression. VEGF plays a critical role in the formation of the corpus luteum by stimulating physiological angiogenesis. Our recent data showed that PGE_2 treatment enhanced VEGF mRNA expression in rat luteal cells (Sakurai et al., 2004). In addition, VEGF mRNA was induced by PGE_2 in osteoblasts (Harada et al., 1994), in rat gastric microvascular endothelial cells (Pai et al., 2001), and in human granulosa luteal cells (Laitinen et al., 1997). Alternatively, eicosanoids may directly stimulate angiogenesis, although the effect of eicosanoids alone in the absence of endogenous VEGF expression or under the blockade of VEGF signaling has not been demonstrated.

The enhanced effect of angiogenesis is clearly seen in E-type prostaglandins (Ziche et al., 1982; Form and Auerbach, 1983; Kanayasu et al., 1989; Salcedo et al., 2003). Jones et al. (1999) indicated that the nonsteroidal anti-inflammatory drug (NSAID)-induced inhibition of in vitro angiogenesis of human microvascular endothelial cells is partially reversed by the addition of PGE_2. Daniel et al. (1999) showed that a TXA_2 receptor agonist reconstitutes both migration and angiogenesis under COX-2-inhibited conditions. Furthermore, a TXA_2 receptor antagonist inhibited basic fibroblast growth factor (bFGF)- and VEGF-stimulated endothelial cell migration in human umbilical vein endothelial cells (Nie et al., 2000). The molecular mechanisms underlying angiogenic actions of PGE_2 and TXA_2 have not been fully elucidated. But Kuwano et al. (2004) have reported that the stimulation of inflammatory cytokine-elicited angiogenesis is induced by TXA_2 and PGE_2 through the TP receptor and EP2/4 receptor, respectively.

In this experiment, VEGF had no significant effect on several parameters examined, possibly because of maximum stimulation in angiogenesis by endogenous VEGF induced by hCG. Further, VEGF did not result in complete recovery of the levels up to control levels in NS-398-treated animals. Interestingly, additional treatment with PGE_2 enhanced progesterone and hemoglobin levels up to levels that were significantly higher than those in the NS-398-treated group. The effect of VEGF appears to be partly mediated by PGE_2 in the developing corpus luteum. VEGF treatment increased COX-2 expression and PGE_2 production in cultured rat luteal cells (Sakurai et al., 2004). In the series of the present study, VEGF injected into the ovary increased COX-2 mRNA expression (data not shown). However, the lack of effect of VEGF in overcoming the inhibition of progesterone and hemoglobin levels might be due to the inhibition of COX-2-independent pathway induced by NS-398. There are increasing evidences showing the inhibitory action of COX-2 inhibitors on multisteps of the signaling pathway for angiogenesis. For example, NS-398 decreases the phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK) in human lung cell line (Liu et al., 2003) and celecoxib suppresses TNF-induced p38 MAPK and extracellular regulated kinase (ERK) activation as well as NF-κB activation (Shishodia et al., 2004). VEGF receptor tyrosine kinases activate phospholipase C (PLC)-γ and induce activation of the Raf–MEK–MAPK pathway to proliferate endothelial cells (Takahashi et al., 2001). Thus, NS-398 might inhibit VEGF signaling for angiogenesis partially via MAPK pathways in addition to the inhibition of COX activity. This may be a reason for no significant effect of VEGF on the inhibition of progesterone and hemoglobin in NS-398-treated animals.

In conclusion, our results indicated that PGE_2 and TXA_2 overcome the inhibition of progesterone release and angiogenesis by COX-2 inhibitor in the newly formed corpus luteum, and that stimulatory effects of VEGF on ovarian angiogenesis become weak in COX-2 inhibitor-treated rats.

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