

# Vascular endothelial growth factor increases messenger RNAs encoding cyclooxygenase-II and membrane-associated prostaglandin E synthase in rat luteal cells

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## Abstract

Vascular endothelial growth factor (VEGF) is known to be necessary for the vascularization of the developing corpus luteum. Our recent data suggested that cyclooxygenase-II (COX-II) may play a role in the formation of vascular plexuses in developing corpora lutea of the rat. Here we examined the relationship between VEGF and the expression of prostaglandin (PG)-metabolizing enzymes in rat ovarian luteal cells. VEGF treatment caused a dose-dependent increase in the expression of COX-II and membrane-associated PGE synthase (mPGES) mRNA in cultured rat luteal cells. However, pretreatment of the luteal cells with a selective COX-II inhibitor, NS-398,

abolished the VEGF-enhanced mPGES mRNA expression. VEGF also increased PGE<sub>2</sub> secretion. Conversely, PGE<sub>2</sub> dose-dependently stimulated VEGF mRNA expression. Furthermore, VEGF induced VEGF mRNA expression, but this effect was abolished by NS-398 pretreatment. These findings suggest that VEGF enhances PGE<sub>2</sub> production by stimulating COX-II and mPGES expression in rat corpus luteum and that the effect of VEGF on luteal cells may be partially mediated by this stimulation of PGE<sub>2</sub> production.

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## Introduction

The corpus luteum is formed from the cellular components of the follicle after ovulation and it plays a critical role in the secretion of progesterone (P4), which maintains early pregnancy. Angiogenesis is dramatically induced in the corpus luteum as it grows and matures. During the establishment of pregnancy, the corpus luteum rapidly increases its size due to the augmented numbers of endothelial cells and increased luteal blood flow (Bruce *et al.* 1984). The acquirement of corpus luteum function is known to be dependent on the growth of new capillary vessels (Tamura & Greenwald 1987, Smith *et al.* 1994, Ferrara *et al.* 1998, Reynolds *et al.* 2000) and it appears that this prominent vascularization of the corpus luteum may provide the luteal cells with the large amounts of cholesterol that are needed for P4 synthesis as well as aiding the delivery of P4 into the circulation.

Prostaglandins (PGs) are believed to modulate vascular permeability and angiogenesis (Ziche *et al.* 1982, Form & Auerbach 1983). When the activity of cyclooxygenase (COX)-II – a rate-limiting enzyme for producing PGs – is inhibited, angiogenesis in colon cancer and tumor growth were suppressed (Tsujii *et al.* 1998). In addition, PGE<sub>2</sub> reverses the inhibition of *in vitro* angiogenesis of rat aortic

endothelial cells that is caused by the COX-II inhibitor NS-398 (Jones *et al.* 1999). We recently found that the activity of COX-II may be related to the formation of functional corpora lutea because it stimulates angiogenesis in immature rats (Sakurai *et al.* 2003). Briefly, we found that if gonadotropin-primed rats were injected with NS-398 for 2 days after ovulation, serum P4 levels decreased, and this effect may be due to the NS-398-induced change in the vasculature of the developing corpus luteum. COX-II may be involved in the physiological angiogenesis of the corpus luteum that takes place during the early luteal phase in rats.

Vascular endothelial growth factor (VEGF) elicits angiogenesis by inducing endothelial proliferation, migration and tube formation, and plays a critical role in the regulation of vascular permeability and angiogenesis. This process involves the activation of multiple genes; candidate genes may be COX (Bryant *et al.* 1998, Gallo *et al.* 2001, Hernandez *et al.* 2001) and their products, eicosanoids (Tsujii *et al.* 1998, Amano *et al.* 2001). Furthermore, COX-II activity has been suggested to play a significant role in angiogenesis in carrageenin-induced granulation tissue (Ghosh *et al.* 2000) through its ability to stimulate VEGF production. Alternative splicing of the single VEGF gene results in several VEGF isoforms comprised of 121,

145, 165, 189 and 206 amino acids (VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub> and VEGF<sub>206</sub> respectively) (Neufeld *et al.* 1999). VEGF<sub>165</sub> and VEGF<sub>121</sub> are secreted and act mitogenically on endothelial cells. VEGF is highly expressed in the luteinizing ovary (Ferrara *et al.* 1998). VEGF protein expression increases in luteinizing granulosa cells of the ovulatory follicle as well as in the developing corpus luteum in primates (Smith *et al.* 1994, Reynolds *et al.* 2000). Treatment in the preovulatory stage with an anti-VEGF receptor 2 antibody inhibits luteal angiogenesis in mice (Zimmermann *et al.* 2001). Thus, VEGF may be essential for the follicular angiogenesis that takes place during the early stage of luteinization in various species.

In the present study, we examined whether VEGF affects COX-II and membrane-associated prostaglandin E synthase (mPGES) mRNA expression in rat luteal cells. Moreover, we assessed whether PGE<sub>2</sub> in turn influences VEGF mRNA expression.

## Materials and Methods

### Luteal cell preparation and culture

To obtain luteal cells, female rats were treated subcutaneously with 50 IU equine chorionic gonadotropin (eCG; Teikoku Hormone MFG Co., Tokyo, Japan) and intraperitoneally with 25 IU human chorionic gonadotropin (hCG; Teikoku Hormone MFG Co.) at 23 and 25 days of age respectively; their ovaries were isolated on day 26 and digested with collagenase (Type I; Sigma) and DNase (Sigma). Highly purified luteal cells were collected from the collagenase-digested suspension by Percoll gradient centrifugation as described previously (Sakurai *et al.* 2003). The cells were then cultured at  $1 \times 10^6$  cells/ml in 12-well plates for 24 h in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin-streptomycin and 100 µg/ml gentamicin for 24 h at 37 °C in a 95% air–5% CO<sub>2</sub> humidified environment. The cells were subsequently incubated for 24 h in serum-free DMEM and then treated for 6–30 h with 0.1–30 ng/ml recombinant human VEGF<sub>165</sub> (R&D Systems, Inc., Minneapolis, MN, USA). Alternatively, they were treated for 2 h with 0.003–3 µM PGE<sub>2</sub> or with 10 µM of the selective COX-II inhibitor NS-398 (Cayman Chemical, Ann Arbor, MI, USA). NS-398 was added 1 h before VEGF treatment. The culture media were collected for the P4 assay and the poly (A)<sup>+</sup> RNAs of the cultured cells were isolated for Northern blotting. The P4 that was produced when the cells were exposed to 100 ng/ml ovine luteinizing hormone (LH) (NIDDK, oLH26 (AFP-5551B); obtained from Dr A F Parlow of the National Hormone and Pituitary Program, Harbor/UCLA Med Center, Torrance, CA, USA) was examined to confirm that the cells were steroidogenically responsive.

### RNA extraction and Northern blotting

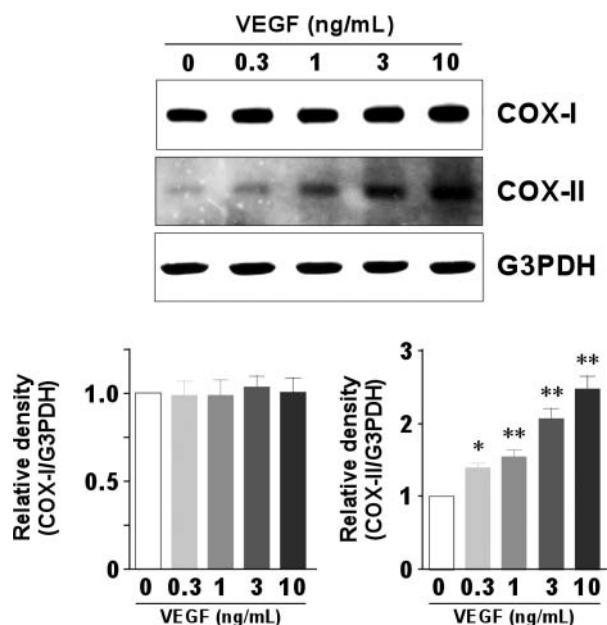
Poly (A)<sup>+</sup> RNA was extracted from the cultured cells by using the QuickPrep *micro* mRNA Purification Kit (Amersham) according to the manufacturer's instructions and quantitated by absorbance at 260 nm. To prepare the cRNA probes, partial cDNAs encoding rat VEGF<sub>164</sub>/VEGF<sub>188</sub> (117 bp, 506–622; kindly provided by Dr M Shibuya, The Institute of Medical Science, University of Tokyo) and rat mPGES (710 bp, 1–709, kindly provided by Dr H Naraba, National Cardiovascular Center Research Institute, Osaka, Japan) were subcloned into the pCR II vector and the pGEM-T easy vector respectively. Moreover, partial cDNAs encoding rat COX-I (319 bp, 717–1036) and COX-II (212 bp, 945–1157) were subcloned into the pGEM-T easy vector. After linearization of each plasmid, the digoxigenin (DIG)-labeled antisense cRNA probes were synthesized by using an *in vitro* transcription kit (Toyobo, Tokyo) (Tamura *et al.* 1998). Northern blotting was performed using the DIG system as described previously (Tamura *et al.* 2003). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression was used as an internal control. The bands on the Kodak scientific imaging film (X-OMAT XB-1; Eastman Kodak) were analyzed by using NIH image (developed at the US National Institutes of Health and available on the Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195 GEI) and each value was normalized against that of the G3 PDH band in the corresponding lane.

### Measurement of PGE<sub>2</sub>

Cultured cells were treated with 10 ng/ml VEGF in the presence or absence of 10 µM NS-398 which was added 1 h before VEGF treatment. The cells were then incubated for 24 h. The levels of PGE<sub>2</sub> in the culture media were determined by using the Prostaglandin E<sub>2</sub> Enzyme Immunoassay Kit (Assay Designs, Inc., Ann Arbor, MI, USA) according to the manufacturer's instructions.

### Statistical analysis

The densitometry values and PGE<sub>2</sub> levels in the culture media were measured. All experiments were repeated three times or more with triplicate wells in each experiment. All the densitometric analyses and the enzyme immunoassay data (the means of the values in each experiment) were used to obtain the mean ± S.E.M. The statistical significance of the results was analyzed by applying Dunnett's test for multiple comparisons. A *P* value of <0.05 was considered to be statistically significant.



**Figure 1** Effect of VEGF on COX-I and COX-II mRNA expression in cultured luteal cells. Luteal cells were collected from eCG-primed immature rats 24 h after hCG treatment and cultured for 6 h in the presence of various doses of VEGF (0.3–10 ng/ml). Poly (A)<sup>+</sup> RNA (0.5 µg RNA) was then extracted for Northern blot analysis. Top panel, representative blots showing COX-I or COX-II mRNA expression. G3PDH mRNA was measured as a loading control. Bottom panel, the COX-I and COX-II mRNA levels in four independent experiments with triplicate wells, including the one shown in the top panel, were measured by densitometric analysis and the relative densities were determined by normalization against G3PDH mRNA levels. Statistical significance: \* $P < 0.05$  and \*\* $P < 0.01$  vs 0 ng/ml VEGF.

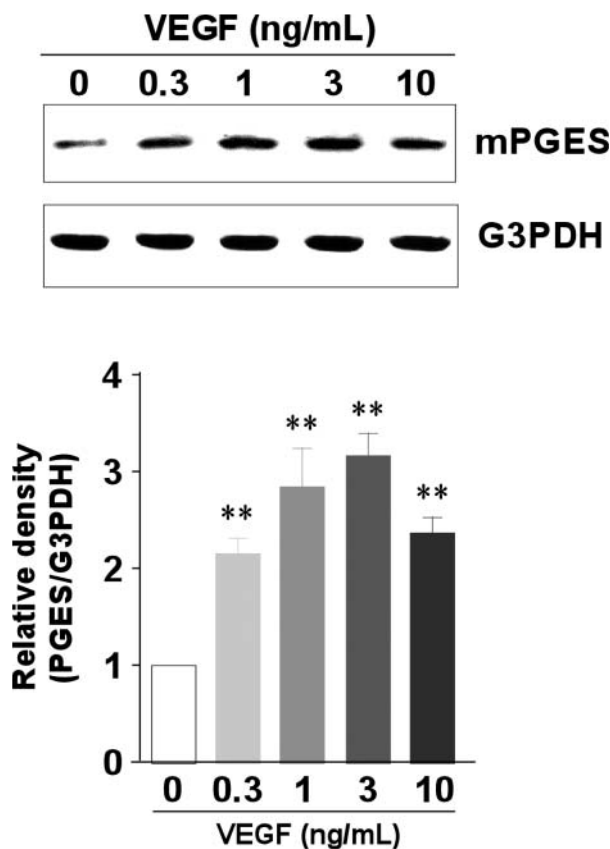
## Results

### *Effect of VEGF on COX mRNA levels in cultured luteal cells*

We determined whether treating cultured luteal cells with VEGF changes their expression of COX mRNA. As shown in Fig. 1, cultured luteal cells treated with 0.3–10 ng/ml VEGF showed a dose-dependent increase in their production of COX-II mRNA. In contrast, COX-I mRNA levels did not change. When we assessed the kinetics of the VEGF-induced enhancement of COX-II mRNA levels, we found that COX-II mRNA levels increased markedly within 6 h and that this level of stimulation was maintained for up to 12 h (data not shown). COX-II mRNA expression was also stimulated by treatment with 100 ng/ml LH (data not shown).

### *Effect of VEGF on mPGES mRNA levels in cultured luteal cells*

The effect of VEGF on the expression of mPGES mRNA in cultured luteal cells was also determined (Fig. 2). When

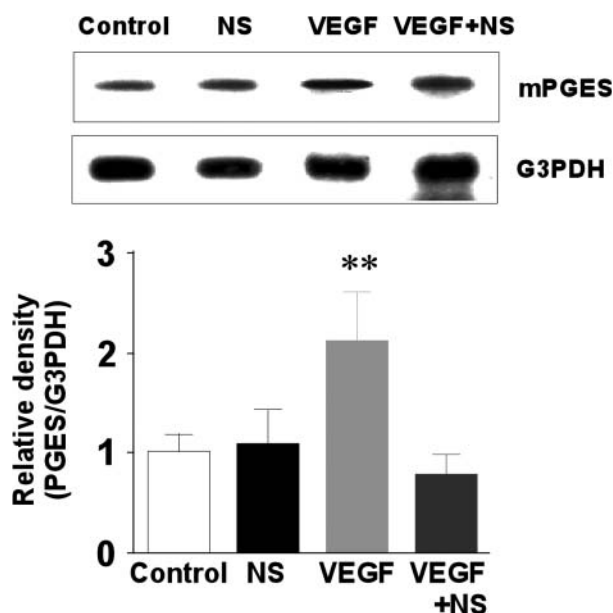


**Figure 2** Effect of VEGF on mPGES mRNA expression in cultured luteal cells. Luteal cells were obtained and treated with VEGF as described in the legend to Fig. 1. A representative blot showing the mPGES mRNA expression in these cells is shown in the top panel. The mPGES mRNA levels in four independent experiments with triplicate wells, including the representative data in the upper panel, were analyzed by densitometry and the relative densities of the blots were determined by normalization against G3PDH mRNA levels. Statistical significance: \*\* $P < 0.01$  vs 0 ng/ml VEGF.

the luteal cells were treated with 0.3–10 ng/ml VEGF, mPGES mRNA levels were enhanced. When the kinetics of the VEGF-enhanced expression of mPGES mRNA over the 6–24 h after VEGF treatment were examined, it was found that enhanced mPGES mRNA levels were seen within 6 h and that these peaked around 6 h (data not shown). We next examined the effect of preincubating the luteal cells with NS-398, a selective COX-II inhibitor, on the VEGF-induced mPGES mRNA levels (Fig. 3). Preincubation with 10 µM NS-398 before VEGF (10 ng/ml) treatment abolished the VEGF-induced expression of mPGES mRNA.

### *Effect of VEGF on PGE<sub>2</sub> production by cultured luteal cells*

The PGE<sub>2</sub> concentrations in the culture media after the luteal cells were treated for 24 h with 10 ng/ml VEGF



**Figure 3** Effect of NS-398, a selective COX-II inhibitor, on VEGF-induced mPGES mRNA expression in cultured luteal cells. Luteal cells were treated with 10 ng/ml VEGF alone or with 10  $\mu$ M NS-398 (NS) for 6 h. The inhibitor was added 1 h before VEGF was introduced. A representative blot showing mPGES mRNA levels is shown in the top panel. The mPGES mRNA levels in three independent experiments with triplicate wells, including the representative data in the upper panel, were analyzed by densitometry and the relative densities of the blots were determined by normalization against G3PDH mRNA levels. Statistical significance: \*\* $P < 0.01$  vs control.

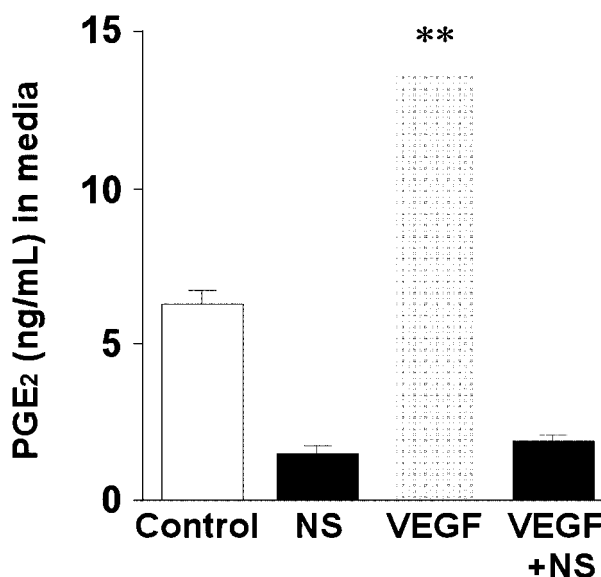
and/or 10  $\mu$ M NS-398 are shown in Fig. 4. The PGE<sub>2</sub> concentrations were approximately twofold higher in the VEGF-treated group compared with the control group. NS-398 treatment on its own tended to decrease the baseline PGE<sub>2</sub> levels but this effect was not statistically significant. However, when the cells were first treated with NS-398 and then subjected to VEGF stimulation, the VEGF-induced production of PGE<sub>2</sub> was significantly inhibited.

#### Effect of PGE<sub>2</sub> on VEGF mRNA expression in cultured luteal cells

Figure 5 shows the effect of treating luteal cells with PGE<sub>2</sub> on their expression of VEGF mRNA. The cells showed a dose-dependent increase in VEGF mRNA levels when they were treated with 0.003–3  $\mu$ M PGE<sub>2</sub> and then subjected to Northern blot analysis.

#### Effect of VEGF on VEGF mRNA expression in cultured luteal cells

To explore a possible physiological mechanism by which VEGF induces luteal cells to produce PGE<sub>2</sub> and through

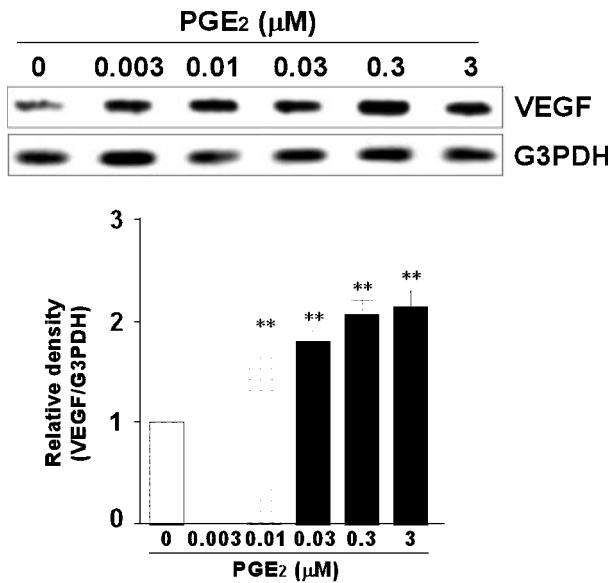


**Figure 4** Effect of NS-398 on the VEGF-induced PGE<sub>2</sub> production by luteal cells. Luteal cells were treated as described in the legend to Fig. 3 except that the incubation time was longer. Thus, cells were treated with 10 ng/ml VEGF alone or with 10  $\mu$ M NS-398 (NS) for 24 h. The levels of PGE<sub>2</sub> in the culture media were then evaluated by quantitative enzyme-linked immunosorbent assays. The results of four independent experiments are expressed as means  $\pm$  S.E.M. Statistical significance: \*\* $P < 0.01$  vs control.

which PGE<sub>2</sub> stimulates VEGF expression, the effect of VEGF on VEGF mRNA expression in cultured luteal cells was examined (Fig. 6). Thus, cells were cultured with 10 ng/ml VEGF and/or 10  $\mu$ M NS-398 for 30 h and their VEGF mRNA levels were examined. VEGF treatment for 30 h, but not for 6 h (data not shown), stimulated VEGF mRNA expression. However, pretreatment with NS-398 abolished the stimulatory effect of VEGF on VEGF mRNA expression.

## Discussion

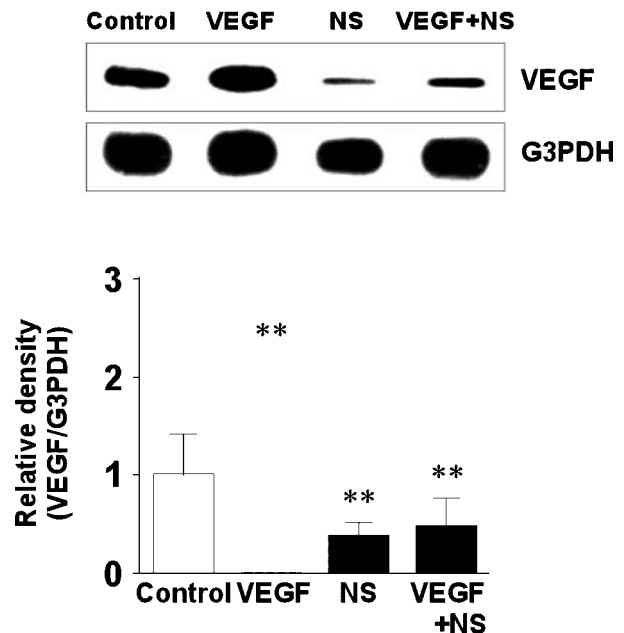
In the present study, we examined whether VEGF affects COX-II and mPGES mRNA expression in rat luteal cells and whether exposure of the cells to PGE<sub>2</sub> influences their expression of VEGF mRNA. Our data indicated that VEGF stimulates the expression of COX-II and mPGES mRNA in luteal cells derived from early developing corpus luteum. VEGF also increased the production of PGE<sub>2</sub> by these cells for 24 h after treatment. However, pretreatment of the cells with NS-398, a COX-II inhibitor, abolished the VEGF-induced PGE<sub>2</sub> production. This observation is consistent with an earlier report using human endothelial cells (Tamura *et al.* 2002). NS-398 also suppresses the mPGES mRNA expression that is induced by VEGF. This observation indicates that the



**Figure 5** Effect of PGE<sub>2</sub> on VEGF mRNA expression in cultured luteal cells. Luteal cells were incubated with 0.003–3 μM PGE<sub>2</sub> for 2 h. A representative blot showing the VEGF mRNA levels is shown in the top panel. The VEGF mRNA levels in three independent experiments with triplicate wells, including the representative data in the upper panel, were analyzed by densitometry and the relative densities of the blots were determined by normalization against G3PDH mRNA levels. Statistical significance: \*\**P*<0.01 vs 0 μM PGE<sub>2</sub>.

VEGF-induced mPGES mRNA expression requires COX-II activity in luteal cells. mPGES is an inducible enzyme that is highly expressed in the reproductive organs and is coordinately induced and functionally coupled with COX-II (Jakobsson *et al.* 1999, Murakami *et al.* 2000). The COX-II inhibitor reduces PGE<sub>2</sub> generation in rat carrageenin-induced pleurisy more profoundly than other PGs (Harada *et al.* 1996). Ovarian follicular fluid after the ovulatory LH surge shows a distinct increase in PGE<sub>2</sub> production (Brown & Poyser 1984) and PGE<sub>2</sub> treatment restores anovulation in COX-II-deficient mice (BenEzra 1978). Our present data suggest that the functional coupling of COX-II and mPGES may be important for the secretion of PGE<sub>2</sub> by luteal cells, and that VEGF stimulates the expression of both molecules resulting in the production of a large amount of PGE<sub>2</sub>.

What could be the physiological role of the VEGF-stimulated PGE<sub>2</sub> production of luteal cells? Luteal PGE<sub>2</sub> may directly promote the angiogenesis in the corpus luteum, which is usually induced during the early luteal phase, because it is known that PGE<sub>1</sub> and PGE<sub>2</sub> have a particularly enhanced pro-angiogenic effect (Ziche *et al.* 1982, Form & Auerbach 1983). It has been reported that PGE<sub>2</sub> participates in angiogenesis by several mechanisms (Peterson 1983, Leahy 2000, Salcedo *et al.* 2003). For example, Jones *et al.* (1999) indicated that the non-steroidal anti-inflammatory drug (NSAID)-induced inhi-

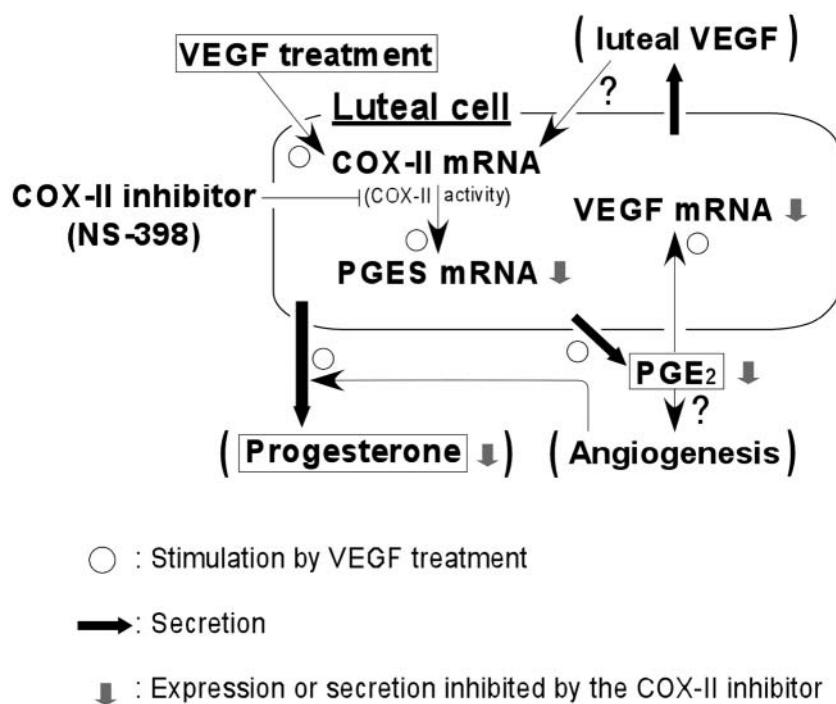


**Figure 6** Effect of VEGF on VEGF mRNA expression and of NS-398 pretreatment on VEGF-enhanced VEGF mRNA levels in cultured luteal cells. The cells were treated as indicated in the legend to Fig. 3 except that the incubation time after VEGF treatment was 30 h. The poly (A)<sup>+</sup> RNA (0.5 μg RNA) was then extracted from the cultured cells for Northern blot analysis. The relative densities were determined by normalization against the G3PDH mRNA levels (triplicate wells in each case). Statistical significance: \*\**P*<0.01 vs control.

bition of the *in vitro* angiogenesis of human microvascular endothelial cells is partially reversed by the addition of PGE<sub>2</sub>. Addition of PGE<sub>2</sub> to a luteal cell culture increased the levels of P4 in the culture media (data not shown). The results suggest that high levels of PGE<sub>2</sub>, which may be induced by gonadotropin or inflammatory cytokines *in vivo*, may directly stimulate the production of P4 by rat luteal cells. This observation is consistent with the reports of Horvath *et al.* (1986) and Elvin *et al.* (2000).

One particularly interesting finding in the present study was that PGE<sub>2</sub> enhanced VEGF mRNA expression. Several mechanisms are involved in regulating VEGF gene expression in the ovary (Ravindranath *et al.* 1992, Neulen *et al.* 1995, Christenson & Stouffer 1997, Pai *et al.* 2001). PGE<sub>2</sub>-elicited cAMP is known to be one of the mediators of VEGF expression (Lopez Bernal *et al.* 1995). We found that exogenous PGE<sub>2</sub> (30 pM) significantly induced VEGF mRNA expression and that PGE<sub>2</sub> concentration in the culture media 24 h after VEGF treatment approximated 30 pM (12 ng/ml), as shown in Fig. 4. There is a possibility that endogenous PGE<sub>2</sub> may induce VEGF mRNA expression in luteal cells. Supporting our observations are reports that show a similar PGE<sub>2</sub>-mediated enhancement of VEGF expression in rat gastric microvascular endothelial cells (Pai *et al.* 2001) and human





**Figure 7** A possible mechanism by which VEGF induces PGE<sub>2</sub> secretion, and the roles of PGE<sub>2</sub> in the functions of luteal cells during the early luteal phase in rats.

granulosa-luteal cells (Laitinen *et al.* 1997). The addition of NS-398 alone inhibits VEGF mRNA levels. The cause of this inhibition might be due to the abolishment of the stimulatory effects of endogenous PGE<sub>2</sub> secreted from luteal cells. Taken together, our findings suggest that VEGF, COX-II, PGES and PGE<sub>2</sub> may interact in luteal cells during the early stage of corpus luteum development in rats (Fig. 7). An increase in VEGF expression may induce COX-II and mPGES mRNA expression, which together stimulate PGE<sub>2</sub> production. The increase in PGE<sub>2</sub> levels might affect P4 production, possibly by enhancing angiogenesis in the corpus luteum, and further enhancing VEGF mRNA expression. Thus, there may be a positive feedback loop between VEGF expression and PGE<sub>2</sub> production.

The present study shows that VEGF stimulates PGE<sub>2</sub> production and VEGF mRNA expression in luteal cells but that this effect is abolished by NS-398 pretreatment. This demonstrates that ovarian COX-II activity is associated with enhanced VEGF expression, which is considered to be essential for angiogenesis in the corpus luteum. Both VEGF and PGE<sub>2</sub> are produced at high levels around the time of ovulation and luteinization in the ovary. This autocrine feedback loop may contribute to the increases in the levels of these factors and thus may be associated with angiogenesis and blood vessel permeability that promotes the development of corpus luteum function.

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