Prostaglandin F_{2α} (PGF_{2α}) stimulates PTGS2 expression and PGF_{2α} synthesis through NFKB activation via reactive oxygen species in the corpus luteum of pseudopregnant rats

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Abstract

This study was undertaken to investigate how prostaglandin F_{2α} (PGF_{2α}) increases PGF_{2α} synthesis and PTGS2 expression in the corpus luteum of pseudopregnant rats. We further investigated the molecular mechanism by which PGF_{2α} stimulates PTGS2 expression. PGF_{2α} (3 mg/kg) or phosphate buffer as a control was injected s.c. on day 7 of pseudopregnancy. *Ptgs2* mRNA expression and PGF_{2α} concentrations in the corpus luteum were measured at 2, 6, and 24 h after PGF_{2α} injection. PGF_{2α} significantly increased *Ptgs2* mRNA expression at 2 h and luteal PGF_{2α} concentrations at 24 h. PGF_{2α} significantly decreased serum progesterone levels at all of the times studied. Simultaneous administration of a selective PTGS2 inhibitor (NS-398, 10 mg/kg) completely abolished the increase in luteal PGF_{2α} concentrations induced by PGF_{2α}. PGF_{2α} increased NFKB p65 protein expression in the nucleus of luteal cells 30 min after PGF_{2α} injection, and electrophoretic mobility shift assay revealed that PGF_{2α} increased binding activities of NFKB to the NFKB consensus sequence of the *Ptgs2* gene promoter. Simultaneous administration of both superoxide dismutase and catalase to scavenge reactive oxygen species (ROS) inhibited the increases of nuclear NFKB p65 protein expression, lipid peroxide levels, and *Ptgs2* mRNA expression induced by PGF_{2α}. In conclusion, PGF_{2α} stimulates *Ptgs2* mRNA expression and PGF_{2α} synthesis through NFKB activation via ROS in the corpus luteum of pseudopregnant rats.

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Introduction

The control mechanisms for functional luteolysis are complex and differ among animal species (Wiltbank & Ottobre 2003, Sugino & Okuda 2007). Prostaglandin F_{2α} (PGF_{2α}) is a critical factor for functional luteolysis in various species. PGF_{2α} increases in the corpus luteum during the regression phase (Olofsson & Selstam 1988, Olofsson *et al.* 1990) and inhibits progesterone production, suggesting that PGF_{2α} has a role in triggering and accelerating functional luteolysis. In fact, in PGF_{2α} receptor-deficient mice, serum progesterone concentrations do not decline, and parturition does not occur (Sugimoto *et al.* 1997).

Luteolytic PGF_{2α} is of uterine origin in various animal species including rodents and domestic animals, but not in humans and primates. Interestingly, the corpus luteum has also been found to produce PGF_{2α} in those species, indicating that luteal PGF_{2α} has an important role in the regulation of luteal function (Wiltbank & Ottobre 2003). PGF_{2α} is synthesized via PTGS2, which is the rate-limiting enzyme in PG biosynthesis. PTGS2 is induced

by a variety of stimulants such as cytokines and peptides. Interestingly, it has been reported that PGF_{2α} induces PTGS2 expression in the corpus luteum in various animal species (Narayansingh *et al.* 2002, Wiltbank & Ottobre 2003), suggesting the presence of a positive feedback pathway in the corpus luteum undergoing functional luteolysis. However, the molecular mechanism by which PGF_{2α} induces PTGS2 is not fully characterized.

Reactive oxygen species (ROS) are well known to be involved in functional luteolysis (Sugino 2006). ROS are increased in the corpus luteum during regression (Riley & Behrman 1991, Sugino *et al.* 1993a, Sawada & Carlson 1994, Shimamura *et al.* 1995) and inhibit progesterone production (Riley & Behrman 1991, Sawada & Carlson 1991, Sugino *et al.* 1993b, 1999). ROS, besides having detrimental effects such as DNA and protein damage and lipid peroxidation, also regulate cell function by activating bioactive substances associated with intracellular signaling pathways. In fact, ROS activate NFKB, a transcriptional factor in a variety of cells (Altavilla *et al.* 2001, Li & Engelhardt 2006,

Bonello *et al.* 2007, Kobayashi *et al.* 2008). Interestingly, we recently found that ROS activate NF κ B, which in turn increases PTGS2 expression and PGF $_{2\alpha}$ production in human endometrial stromal cells (Sugino *et al.* 2004). PGF $_{2\alpha}$ stimulates ROS production in the rat corpus luteum (Sawada & Carlson 1994, Shimamura *et al.* 1995, Aten *et al.* 1998, Motta *et al.* 2001). NF κ B stimulates PTGS2 expression by binding to its response element on the *Ptgs2* gene promoter (Nakao *et al.* 2000, 2002, Tsai *et al.* 2002, Nadjar *et al.* 2005). These findings, taken together, suggest that there is a close relationship among PGF $_{2\alpha}$, ROS, NF κ B, and PTGS2 in the corpus luteum. This study was undertaken to investigate how PGF $_{2\alpha}$ increases PTGS2 expression and PGF $_{2\alpha}$ synthesis in the corpus luteum during functional luteolysis.

Results

To investigate whether PGF $_{2\alpha}$ increases PGF $_{2\alpha}$ synthesis by inducing the expression of PTGS2 in the corpus luteum, PGF $_{2\alpha}$ was administered on day 7 of pseudopregnancy, and the expressions of *Ptgs2* and *Ptgs1* mRNA and PGF $_{2\alpha}$ concentrations in the corpus luteum were measured. PGF $_{2\alpha}$ significantly decreased serum progesterone concentrations compared with the control at 2 h ($P<0.01$), 6 h ($P<0.05$), and 24 h ($P<0.01$; Fig. 1A). There was no significant change in the weight of the corpus luteum by PGF $_{2\alpha}$ treatment at any of the times studied (data not shown). PGF $_{2\alpha}$ significantly increased PGF $_{2\alpha}$ concentrations in the corpus luteum at 24 h ($P<0.05$), but not at 2 and 6 h (Fig. 1B). *Ptgs2* mRNA levels were dramatically increased in the corpus luteum at 2 h ($P<0.01$) after PGF $_{2\alpha}$ administration, but not at 6 and 24 h (Fig. 1C), whereas there was no significant change in *Ptgs1* mRNA levels at any of the times studied (Fig. 1D).

To investigate whether the transient increase of PTGS2 expression by PGF $_{2\alpha}$ administration is responsible for the increase in PGF $_{2\alpha}$ concentrations in the corpus luteum at 24 h, a selective PTGS2 inhibitor, NS-398, was

administered simultaneously with PGF $_{2\alpha}$. NS-398 completely abolished the increase in PGF $_{2\alpha}$ concentrations in the corpus luteum induced by PGF $_{2\alpha}$ administration ($P<0.05$; Fig. 2). Serum progesterone concentrations in the control group, PGF $_{2\alpha}$ group, and PGF $_{2\alpha}$ NS-398 group were 101.7 ± 12.1 , 30.3 ± 5.4 , and 29.9 ± 6.7 ng/ml (mean $\pm$ S.E.M.) respectively.

To examine the transcriptional factor responsible for the increase in *Ptgs2* mRNA expression induced by PGF $_{2\alpha}$, we focused on NF κ B. Since NF κ B is rapidly activated within 30 min by some stimulation and is translocated into the nucleus, we decided to examine nuclear NF κ B protein contents 30 min after PGF $_{2\alpha}$ administration. NF κ B p65 protein expression was increased in the nucleus 30 min after PGF $_{2\alpha}$ administration (Fig. 3A). In addition, electrophoretic mobility shift assay (EMSA) revealed that PGF $_{2\alpha}$ increased the binding activity of NF κ B to the *Ptgs2* promoter region (Fig. 3B).

To examine whether PGF $_{2\alpha}$ activates NF κ B through the production of ROS, both superoxide dismutase (SOD) and catalase were administered just before PGF $_{2\alpha}$ administration. The increase in NF κ B p65 protein levels in the nucleus 30 min after PGF $_{2\alpha}$ administration was decreased by treatment with SOD and catalase (Fig. 4A). The increase in lipid peroxide (LPO) levels in the corpus luteum 30 min after PGF $_{2\alpha}$ administration was significantly ($P<0.05$) suppressed by SOD and catalase (Fig. 4B). The increase in *Ptgs2* mRNA levels in the corpus luteum 2 h after PGF $_{2\alpha}$ administration was also significantly ($P<0.05$) suppressed by SOD and catalase (Fig. 4C).

Discussion

This study showed that PGF $_{2\alpha}$ increased PGF $_{2\alpha}$ synthesis in the corpus luteum by stimulating PTGS2 expression. It has been reported that PGF $_{2\alpha}$ induces PTGS2 expression in the corpus luteum in various

Figure 1 Serum progesterone concentrations (A), PGF $_{2\alpha}$ concentrations in the corpus luteum (B), and *Ptgs2* mRNA expression (C) and *Ptgs1* mRNA expression (D) in the corpus luteum at 2, 6, and 24 h after PGF $_{2\alpha}$ administration. A luteolytic dose of PGF $_{2\alpha}$ (3 mg/kg body weight) was injected s.c. on day 7 of pseudopregnancy. The control rats received phosphate buffer. Values of *Ptgs1* and *Ptgs2* mRNA were normalized to those of *Sd1g1* (internal control) and expressed as a percent of the control. Each bar represents the mean $\pm$ S.E.M. for the number of animals given in the column. a; $P<0.01$ and b; $P<0.05$ compared with control.

Figure 2 Effects of a selective PTGS2 inhibitor on the increase in PGF_{2α} concentrations in the corpus luteum induced by PGF_{2α} administration. A selective PTGS2 inhibitor, NS-398, was administered simultaneously with PGF_{2α}. PGF_{2α} concentrations were measured in the corpus luteum 24 h after PGF_{2α} administration. Each bar represents the mean $\pm$ S.E.M. for the number of animals given in the column. a; $P < 0.05$ compared with the other groups.

species (Tsai & Wiltbank 1997, Narayansingh *et al.* 2002, Hayashi *et al.* 2003, Arosh *et al.* 2004, Zerani *et al.* 2007), suggesting the presence of a positive feedback pathway in which PGF_{2α} stimulates PGF_{2α} synthesis in the corpus luteum. This may contribute to accelerating and completing functional luteolysis to initiate the development of a new ovulatory follicle of the next reproductive cycle. There was a time lag between the increase in *Ptgs2* mRNA expression and luteal PGF_{2α} concentrations in this study. Since the increase in PGF_{2α} concentrations induced by PGF_{2α} administration was completely blocked by a selective PTGS2 inhibitor (NS-398), the transient increase of *Ptgs2* mRNA levels 2 h after PGF_{2α} administration seems to be responsible for the increase in PGF_{2α} concentrations at 24 h. Our result is also consistent with the report by Kobayashi *et al.* (2007), in which the inhibitory effect of the gene silencing of PTGS2 with siRNA on PGF_{2α} production appeared 24 h after the treatment in bovine granulosa cells *in vitro*. However, we cannot eliminate other potential intracellular mechanisms involved in PGF_{2α}-induced production of intraluteal PGF_{2α}, such as an activation of cytosolic phospholipase A2 mediated by MAP kinases or protein kinase C (Diaz *et al.* 2002).

In this study, although the increase in luteal PGF_{2α} contents caused by PGF_{2α} injection was inhibited 24 h after NS-398 treatment, serum progesterone levels were not affected by NS-398 treatment. This result may be due to that the effect of PGF_{2α} injection on serum progesterone levels lasted for 24 h.

Regarding a possibility that PGF_{2α} affects corpus luteum in an endocrine manner through some hormonal mediators, *in vivo* experiments always involve such a possibility. However, it is likely that PGF_{2α} directly affects the corpus luteum because NFKB activation was immediately observed 30 min after PGF_{2α} injection. Furthermore, although a well-established animal model was used in this study, we may be in a dilemma as to

whether the dose of PGF_{2α} used in this study is physiological. The dose of PGF_{2α} is based on a number of published reports, but we cannot completely neglect the pharmacological effects of PGF_{2α}.

The basal level of PGF_{2α} in the corpus luteum despite low luteal PTGS2 expression suggests that it is from the other organs than from corpora lutea. It is most likely of uterine origin. This may be supported by the finding that intraluteal PGF_{2α} levels after NS-398 treatment tended to be lower than the control, although it was not significant. In addition, PTGS1 may also contribute to PGF_{2α} synthesis in the uterus in rodents (Stocco *et al.* 2007). However, further studies are needed regarding how much basal luteal PGF_{2α} is from the uterus or what extent of basal PGF_{2α} is synthesized via PTGS2 in the corpus luteum.

This study showed the molecular mechanism by which PGF_{2α} induces PTGS2 expression in the corpus luteum. The *Ptgs2* gene has several consensus sequences for binding of transcriptional factors, including NFKB, C/EBP, CRE, and E-box in the promoter region (Sirois *et al.* 1993). NFKB stimulates PTGS2 expression in a variety of cells (Nakao *et al.* 2000, 2002, Tsai *et al.* 2002, Sugino *et al.* 2004, Nadjar *et al.* 2005). In this study, PGF_{2α} increased nuclear NFKB p65 protein expression and binding activities of NFKB to the *Ptgs2* promoter region. Our results suggest that PGF_{2α} induced PTGS2 expression through NFKB activation in the corpus luteum. On the other hand, an *in vitro* study with ovine luteal cells showed that E-box in the *Ptgs2* promoter was critical for PGF_{2α}-induced *Ptgs2* gene transcription besides C/EBP and CRE (Wu & Wiltbank 2001). In addition, it is reported that C/EBP plays an

Figure 3 Effects of PGF_{2α} on NFKB p65 protein expression in the nucleus (A) and on the binding activities of NFKB to the *Ptgs2* promoter (B). (A) Corpora lutea were collected 30 min after PGF_{2α} administration on day 7 of pseudopregnancy. The NFKB p65 protein expression in the nucleus was analyzed by western blotting. The immunoblot is a representative of three independent experiments. Histone H1 was used as an internal control. (B) NFKB binding activities were analyzed by EMSA. Unlabeled AP-1 oligonucleotides and nuclear extracts of HeLa cells were used as a nonspecific competitor (NSC) and a positive control (PC) respectively. The NFKB binding was increased by PGF_{2α} (lane 2) and was competed by the addition of the excess cold (lane 3), but not by NSC (lane 4). The autoradiogram is a representative of three independent experiments.

Figure 4 Effects of superoxide dismutase (SOD) and catalase (CAT) on the increases in NFKB p65 protein expression in the nucleus (A), lipid peroxide (LPO) concentrations (B), and *Ptgs2* mRNA expression in the corpus luteum (C) induced by PGF_{2α}. Both SOD (50 000 U/kg body weight) and CAT (100 000 U/kg body weight) were administered just before PGF_{2α} administration on day 7 of pseudopregnancy. The control rats received phosphate buffer. The NFKB p65 protein expression in the nucleus 30 min after PGF_{2α} administration was analyzed by western blotting. LPO concentrations and *Ptgs2* mRNA levels were measured in the corpus luteum 30 min and 2 h after PGF_{2α} administration respectively. The immunoblot is a representative of three independent experiments. Values of *Ptgs2* mRNA were normalized to those of *Sd1g11* (internal control) and expressed as a percent of the control. Each bar represents the mean $\pm$ S.E.M. for the number of animals given in the column. a; $P < 0.01$ compared with control, b; $P < 0.05$ compared with PGF_{2α}.

important role in the increase in PTGS2 expression of rat granulosa cells during the ovulatory process (Sirois & Richards 1993). However, in our unpublished data, PGF_{2α} treatment did not influence the binding activity of C/EBP to the *Ptgs2* promoter region using EMSA with an oligonucleotide sequence corresponding to the C/EBP site (−283 to −264 bp) of the rat *Ptgs2* promoter. Further studies are needed to know the detailed transcriptional mechanism regulating PGF_{2α}-induced PTGS2 expression.

Our finding that SOD and catalase inhibited the increase in expression of nuclear NFKB p65 protein and *Ptgs2* mRNA in the corpus luteum after PGF_{2α} administration indicates that PGF_{2α}-induced PTGS2 expression was mediated by NFKB activation via ROS. NFKB activation via ROS is further supported by our finding of concurrent rapid increases of NFKB p65 protein in the nucleus and ROS production following PGF_{2α} administration. Our hypothesis is also supported by previous reports that ROS activate NFKB in a variety of cells (Altavilla *et al.* 2001, Li & Engelhardt 2006, Bonello *et al.* 2007, Kobayashi *et al.* 2008) and increase PTGS2 expression in bovine luteal cells and human endometrial stromal cells (Nakamura & Sakamoto 2001, Sugino *et al.* 2004).

Interestingly, SOD and catalase only partly decreased LPO concentrations in the corpus luteum in this study, indicating that they only partly scavenged ROS. This partial inhibition of ROS by SOD and catalase suggests the possible source of ROS in the corpus luteum. If SOD and catalase do not enter luteal cells because of their large molecular weight, it is possible that ROS scavenged by SOD and catalase are derived from the cells other than luteal cells, e.g. phagocytic leukocytes such as macrophages or neutrophils, and vascular endothelial cells. In fact, there are a number of macrophages in the corpus luteum of pseudopregnant rats (Sugino *et al.* 1996), and PGF_{2α} stimulates superoxide radical production by nonsteroidogenic cells in the corpus luteum of the rats (Aten *et al.* 1998). It is also reported that ROS such as hydrogen peroxide produced by activated neutrophils can enter the luteal cell and affect luteal function (Pepperell *et al.* 1992). Furthermore, the importance of vascular endothelial cells and ROS produced by them by PGF_{2α} stimulation in functional luteolysis has been reported in the bovine corpus luteum (Shirasuna *et al.* 2008, Acosta *et al.* 2009, Lee *et al.* 2010). Therefore, PGF_{2α} may have stimulated ROS production by vascular endothelial cells in the corpus luteum in this study.

On the other hand, the partial inhibition of ROS also suggests another source of ROS. ROS that were not scavenged by SOD and catalase may be derived from luteal cells. In fact, superoxide radicals are generated within the rat luteal cells 7–15 min after PGF_{2α} administration *in vivo* (Sawada & Carlson 1994). Since SOD and catalase do not enter luteal cells, ROS generated in the luteal cells by PGF_{2α} stimulation may have not scavenged in this study.

This study suggested the presence of a positive feedback pathway in which PGF_{2α} stimulates PGF_{2α} synthesis in the corpus luteum. Although this system may not be a main mechanism for the increase in PGF_{2α} in the regressing corpus luteum, it may, at least, in part, contribute to accelerating and completing functional luteolysis. Furthermore, our results suggested the intracellular signaling pathway through which PGF_{2α} stimulates PTGS2 expression in the corpus luteum: PGF_{2α}-induced PTGS2 expression is mediated by NFKB activation via ROS.

Materials and Methods

Animals

Sprague–Dawley rats (Japan SLC, Hamamatsu, Japan), weighing 220–270 g, were housed at 24 °C under controlled conditions (lights on from 0500 to 1900 h) with free access to standard rat chow and water. Vaginal smears were obtained daily, and only the rats showing at least two consecutive 4-day estrous cycles were used. Pseudopregnancy was induced by mechanical stimulation of the uterine cervix for 1 min with

a glass rod at 1800 h on proestrus and at 0900 h on estrus. The last day on which the rat exhibited an estrous smear was designated as day 1 of pseudopregnancy. Vaginal smears were checked every day. The experiments were reviewed and approved by the Committee for the Ethics on Animal Experiment in Yamaguchi University Graduate School of Medicine.

Experimental procedures

Experiment 1

A luteolytic dose of PGF_{2α} (3 mg/kg body weight) was injected s.c. on day 7 of pseudopregnancy. The dose of PGF_{2α} used in this study was based on our previous study (Shimamura *et al.* 1995) and other published reports (Sawada & Carlson 1991, Aten *et al.* 1998, Motta *et al.* 2001, Narayansingh *et al.* 2002). PGF_{2α} was the kind gift of Ono Pharmaceutical Co. Ltd (Osaka, Japan). Control rats received phosphate buffer (vehicle). Each group of rats underwent laparotomy under light ether anesthesia 2, 6, and 24 h later. After blood sampling from the portal vein, the ovaries were perfused with saline via the portal vein during draining of the inferior vena cava to remove the blood and to avoid the contamination of the blood as reported previously (Sugino *et al.* 1993b). The ovaries were removed, and the corpus luteum was dissected, cleaned of adhering tissue in a watch glass, and weighed. Corpora lutea were stored at −80 °C. Expressions of *Ptgs2* and *Ptgs1* mRNA and intraluteal PGF_{2α} concentrations were measured as described below. The serum samples were stored at −20 °C for progesterone assay.

Experiment 2

A selective PTGS2 inhibitor, NS-398 (10 mg/kg body weight, Cayman Chemical Co., Ann Arbor, MI, USA), was administered simultaneously with PGF_{2α} on day 7 of pseudopregnancy. The dose of NS-398 used in this study was based on previous studies (Futaki *et al.* 1993, Mikuni *et al.* 1998). The control rats received phosphate buffer. Each group of rats was autopsied 24 h later as described in Experiment 1, and PGF_{2α} concentrations in the corpus luteum were measured. NS-398 had no effects on *Ptgs2* mRNA expression in our preliminary experiments. It is also verified by a manufacturer that NS-398 administration *in vivo* causes no significant effects on mRNA and protein expression of PTGS2 in the rats (Uno *et al.* 2004).

Experiment 3

To examine the protein expression of NFκB p65 in the nucleus and the binding activities of NFκB to the promoter of *Ptgs2* after PGF_{2α} administration, corpora lutea were collected 30 min after PGF_{2α} administration on day 7 of pseudopregnancy. The control rats received phosphate buffer. The NFκB p65 protein expression and NFκB binding activities in the nucleus were analyzed by western blotting assay and EMSA respectively.

Experiment 4

Both SOD (50 000 U/kg body weight, Sigma–Aldrich Co.), a specific scavenger of superoxide radicals, and catalase

(100 000 U/kg body weight, Sigma–Aldrich Co.), a scavenger of hydrogen peroxide, were administered in the tail just before PGF_{2α} administration on day 7 of pseudopregnancy. The doses of SOD and catalase used in this study were based on our previous study (Sugino *et al.* 1993a). The control rats received phosphate buffer. The NFκB p65 protein expression in the nucleus and concentrations of LPOs (byproducts of ROS) in the corpus luteum were measured 30 min after PGF_{2α} administration. *Ptgs2* mRNA levels in the corpus luteum were measured 2 h after PGF_{2α} administration.

Real-time RT-PCR

Total RNA was isolated from corpora lutea using TRIzol reagent (Invitrogen, Inc.) according to the manufacturer's protocol. For PCR amplification, 3 µg total RNA were reverse transcribed at 42 °C in 20 µl reaction mixture (single strength PCR buffer, 2.5 mmol/l dNTPs, 5 µmol/l random hexamer, 1.5 mmol/l MgCl₂, and 200 U Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, Roche Molecular Systems Inc., Branchburg, NJ, USA)). Real-time PCR was performed as reported previously (Asada *et al.* 2008) using LightCycler (Roche Diagnostics) with the oligonucleotide primers for *Ptgs2* (5'-GATTGACAGCCCACCAACTT-3' and 5'-CGGGATGAAGTCTCTCCTCA-3'; 174 bp amplified products), for *Ptgs1* (5'-GTACTATCCCTGAGATCTGGAC-3' and 5'-TGAGTACTTCTCGGATGAAGG-3'; 132 bp amplified products), and for *Sdtg11* (5'-CTGAAGGTCAAAGGGAATGTG-3' and 5'-GGACAGAGTCTTGATGATCTC-3'; 194 bp amplified products) on the basis of the rat *Ptgs2*, *Ptgs1*, and *Sdtg11* cDNA sequences (Chan *et al.* 1987, Kelsen *et al.* 2006). *Sdtg11* was used as reference gene for normalization. The reaction mixture contained 10 µl SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan), 0.2 µmol/l each of primer sets of *Ptgs2*, *Ptgs1*, or *Sdtg11* and 2 µl cDNA (a product of RT reaction) in a total volume of 20 µl. The thermocycling program was 40 cycles of 96 °C for 5 s and 60 °C for 20 s with an initial cycle of 96 °C for 10 s.

PGF_{2α} assay

PGs were extracted as reported previously (Sugino *et al.* 2000). Corpora lutea were homogenized on ice with PBS (pH 3.5). The homogenate was applied to a C18-LRC solid phase extraction cartridge (Bond-Elute, Varian Co., Harbor City, CA, USA), and the cartridge was rinsed with distilled water and 10% (v/v) acetonitrile. PGs were then eluted with methanol and evaporated under nitrogen. The dried extract was dissolved with ethanol and the kit assay solution, and the PGF_{2α} concentrations were determined by a PGF_{2α} enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI, USA). The results were expressed as pg of PGF_{2α} per cellular mg protein. The protein concentrations in the extracted sample were determined by the Lowry method (Lowry *et al.* 1951). The sensitivity of the assay was 4.6 pg/ml. The intra- and interassay coefficients of variation (CV) were 7.0 and 7.8% respectively.

Western blot analysis

The nuclear fraction was extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA) according to the manufacturer's protocol. Forty micrograms protein of the nuclear extract were separated by 10% (w/v) SDS-PAGE and transferred to the PVDF membranes (ATTO, Tokyo, Japan). The membranes were blocked with 5% (w/v) skim milk in Tween 20-PBS (PBS-T) and incubated with the primary antibodies including anti-rabbit NF- κ B p65 antibodies (1:500; Upstate, Lake Placid, NY, USA), and anti-histone H1 antibodies (1:1000; MBL, Nagoya, Japan) as nuclear internal control. The membranes were then incubated with HRP-conjugated secondary antibodies. The reacted band was developed on a film with an ECL kit (Amersham Pharmacia Biotech).

EMSA

EMSA was performed using Gel Shift Assay Systems (Promega Co.) as reported previously (Sugino *et al.* 2002). The oligonucleotide sequence corresponding to the NF- κ B binding site (−548 to −528 bp) of the rat *Ptgs2* promoter (5′-GGCAAGGGGATCCCTTAGTT-3′; Goren *et al.* 2004) was annealed with the complementary sequence. Double strand NF- κ B oligonucleotides (10 pmol) were radiolabeled with [γ -³²P]ATP (3000 Ci/mmol, 10 mCi/ml) using T4 polynucleotide kinase. The labeled oligonucleotides were separated from unincorporated nucleotides by chromatography through a G-25 spin column (GE Healthcare, Amersham, UK). The radiolabeled probe was incubated with 10 μ g nuclear extracts in gel shift binding buffer. To verify the specificity of the binding reaction, an excess of unlabeled oligonucleotides ($\times 100$; cold) were added to the reaction before adding the radiolabeled probe. The DNA–protein complexes were separated on native 6% (w/v) polyacrylamide gels in 0.5% (w/v) Tris–borate–EDTA buffer.

LPO assay

Concentrations of LPOs in the corpus luteum were measured by the thiobarbituric acid method as reported previously (Ohkawa *et al.* 1979). The results were expressed as nmol of malondialdehyde per gram wet tissue weight.

Progesterone assay

Progesterone concentrations in the serum were determined by a specific RIA described as reported previously (Kato *et al.* 1982). The sensitivity of the assay was 100 pg/ml, and the intra- and interassay CV were 7.0 and 14.4% respectively.

Statistical analyses

Statistical significance was determined by one-way ANOVA. After ANOVA, the Tukey–Kramer test was applied to analyze differences among the groups. As for the comparisons between the two groups, unpaired *t*-test was performed. All statistical analyses were performed using SPSS for Windows version 11 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P < 0.05$.

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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