Withdrawal of Ovarian Steroids Stimulates Prostaglandin F2α Production Through Nuclear Factor-κB Activation via Oxygen Radicals in Human Endometrial Stromal Cells: Potential Relevance to Menstruation

Norihiro SUGINO1, Ayako KARUBE-HARADA1, Toshiaki TAKETANI1, Aki SAKATA1 and Yasuhiko NAKAMURA1

1)Division of Obstetrics and Gynecology, Department of Reproductive, Pediatric and Infectious Science, Yamaguchi University School of Medicine, Minamikogushi 1–1–1, Ube 755–8505, Japan

Abstract. The present study was undertaken to investigate whether withdrawal of estrogen and progesterone (EP-withdrawal) stimulates prostaglandin F2α (PGF2α) production through oxygen radical (ROS)-induced NF-κB activation in human endometrial stromal cells (ESC). To study the EP-withdrawal, ESC that had been treated with estradiol (E, 10–8 M) and medroxyprogesterone acetate (MPA, 10–6 M) for 12 days were then incubated with or without E+MPA for a further 11 days. PGF2α concentrations in the medium and cyclooxygenase-2 (COX-2) mRNA levels were significantly increased after EP-withdrawal, while they were unchanged by the continuous treatment with E+MPA. When ESC were incubated with N-acetyl-L-cysteine (Nac, 50 mM), an antioxidant, during EP-withdrawal, Nac blocked the increases in PGF2α production and COX-2 mRNA expression caused by EP-withdrawal. Next, we examined whether ROS generated in response to EP-withdrawal acted through NF-κB activation. Electrophoretic mobility shift assay revealed that EP-withdrawal caused marked increases in NF-κB DNA binding activity, which was completely suppressed by Nac. Furthermore, when ESC were incubated with MG132 (3 µM), which inhibits NF-κB activation, during EP-withdrawal, MG132 blocked the increases in PGF2α production and COX-2 mRNA expression caused by EP-withdrawal. In conclusion, EP-withdrawal stimulates COX-2 expression and PGF2α production through ROS-induced NF-κB activation, suggesting a possible mechanism for menstruation.

Key words: Endometrial stromal cell, Prostaglandin F2α, NF-κB, Superoxide dismutase, Superoxide radical

We found that ROS stimulated prostaglandin F2α (PGF2α) production in human endometrial stromal cells (ESC) [12]. PGF2α is well known as a factor responsible for endometrial shedding [13]. PGF2α concentrations in the endometrium increase toward the late secretory phase and are highest at menstruation [14–16]. Therefore, we have hypothesized that there is a close relationship between SOD, ROS and PGF2α in the regulation of menstruation.

Withdrawal of ovarian steroids has been well accepted as a physiological stimulus for menstruation. We found recently in human ESC in vitro that withdrawal of ovarian steroids caused a decrease in copper-zinc SOD (Cu,Zn-SOD) located in cytosol [17], and that ROS stimulated PGF2α production through cyclooxygenase (COX), a rate-limiting enzyme for prostaglandin synthesis [12]. There are two types of COX. COX-1 is a constitutive enzyme involved in housekeeping functions, whereas COX-2 is an inducible enzyme that plays a role in various physiological conditions. It has been reported that COX-2 is involved in differentiation of human ESC [18–20] and that COX-2 is the main enzyme controlling the synthesis of prostaglandins by human decidual cells [21]. From these findings, there seems to be a possibility that withdrawal of ovarian steroids causes the decrease in Cu,Zn-SOD and the increase in ROS, which in turn induce menstruation by stimulating PGF2α production via COX-2.

Regarding the mechanism by which ROS stimulate COX-2 expression, it is of interest to note that ROS activate nuclear factor-κB (NF-κB), a transcription factor [9, 22–24]. NF-κB controls expression of a variety of genes involved in inflammatory responses [25]. The gene promoter of human COX-2 has the binding sites for NF-κB, and there are some reports that COX-2 expression is regulated by NF-κB [26–31]. Therefore, in order to investigate the mechanism for menstruation, we tested our hypothesis that withdrawal of ovarian steroids stimulates COX-2 expression and PGF2α production through NF-κB activation via ROS in human ESC.

Materials and Methods

This project was reviewed and approved by the committee of investigations involving human subjects of Yamaguchi University School of Medicine. Informed consent from patients was obtained before collection of any tissue samples for this study.

Materials

Phenol Red-free Dulbecco’s modified Eagle’s medium (DMEM) and glutamine were purchased from ICN Biomedical Inc (Aurora, OH, USA). Streptomycin, penicillin, 1 × trypsin-EDTA, deoxyribonucleotide triphosphate and Moloney murine leukemia virus reverse-transcriptase were from Life Technologies Inc (Grand Island, NY, USA). Collagenase, estradiol, medroxyprogesterone acetate (MPA), N-acetyl-L-cysteine, benzamidine, aprotinin, leupeptin and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co (St. Louis, MO, USA), MG132 from BIOMOL Research Laboratories Inc (Plymouth Meeting, PA, USA). Acetonitrile (high performance liquid chromatography grade) was obtained from Nacalai Tesque Co Ltd (Kyoto, Japan). Fetal calf serum (FCS) was obtained from PAA Laboratories GmbH (Linz, Austria); tissue flasks and nylon mesh from Becton Dickinson Co (Franklin lakes, NJ, USA); random hexamer and Taq DNA polymerase from Perkin-Elmer Co (Foster City, CA, USA); [α-32P]-dCTP and [α-32P]-ATP from Amersham (Arlington Heights, IL, USA); isogen from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

ESC isolation

Human endometrium was obtained at hysterectomy from patients with a normal menstrual cycle, aged 39–43 years, who underwent surgery for myoma uteri. Endometrial samples were histologically diagnosed as late proliferative phase according to the criteria of Noyes et al. [32]. Tissue samples were washed with Phenol Red-free DMEM containing 4 mM glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin, and minced into small pieces of <1 mm³. ESC were isolated as reported previously [33]. In brief, after the enzymatic digestion of minced tissues with 0.2% collagenase in a shaking water bath for 2 h at 37°C, stromal cells were separated by filtration through a 70 μm nylon mesh. The filtrates were washed three times, and the number of viable cells was counted by Trypan Blue dye exclusion. The homogeneity of the stromal cell preparation was verified by...
immunocytochemistry using the specific antibody against stromal cells, vimentin (data not shown). Cells were seeded at 10^4 cells/cm^2 in 75 cm^2 tissue culture flasks and incubated in Phenol Red-free DMEM containing glutamine, antibiotics and 10% dextran-coated charcoal-stripped FCS at 37°C, 95% air and 5% CO₂. After three passages (7–10 days after plating) by standard methods of trypsinization with 1 × trypsin-EDTA, cells were distributed into 25 cm^2 tissue culture flasks. At >80% confluence (3–5 days after the distribution), the cell culture medium was changed to the treatment medium.

**Cell culture**

We first examined the effect of withdrawal of estrogen and progesterone (EP-withdrawal) on PGF2α production, COX-1 and COX-2 mRNA expression in human ESC. ESC that had been treated with culture medium (Phenol Red-free DMEM supplemented with glutamine, antibiotics, 2% stripped FCS) containing estradiol (10^{-8} M) and MPA (10^{-6} M) for 12 days were washed and then incubated with or without estradiol (10^{-8} M) and MPA (10^{-6} M) [EP (+) group and EP (-) group, respectively], for a further 11 days at 37°C, in 95% air and 5% CO₂. To further examine whether the changes in PGF2α production and COX-2 mRNA expression caused by EP-withdrawal are mediated by ROS, ESC that had been treated with estradiol and MPA for 12 days were then incubated with N-acetyl-L-cysteine (50 mM), an antioxidant [EP (+) group and EP (-) group, respectively], from day 4 to day 11 after EP-withdrawal, because Cu,Zn-SOD activities in ESC were significantly decreased from 4 days after EP-withdrawal in our previous study [17]. Secondly, we studied whether ROS generated in response to EP-withdrawal activate NF-κB, using the electrophoretic mobility shift assay described below. For this purpose, cells from EP (+) group, EP (-) group, and EP (+) + Nac group were used for extraction of nuclear fraction. Furthermore, we studied whether NF-κB activated by EP-withdrawal actually stimulates PGF2α production and COX-2 mRNA expression. Activation of NF-κB is brought about after the degradation of an inhibitor of NF-κB (IκB) by proteasome. Therefore, ESC that had been treated with estradiol and MPA for 12 days were then incubated with MG132, a proteasome inhibitor, 3 µM, (EP (-) + MG132 group) from day 4 to day 11 after EP-withdrawal. The concentrations of Nac and MG132 were based on our previous reports [34, 35]. MG132 caused no significant effect on cell viability. The medium was changed every other day. A single incubation was performed in triplicate on cells from a single hysterectomy sample. The samples from three individuals were used and, hence, three different incubations were performed in a single experiment.

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed as reported previously [35]. We obtained the nuclear extracts using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (PIERCE, Rockford, IL, USA). After incubation, cells were washed with PBS, resuspended in cytoplasmic extraction reagent supplemented with benzamidine (0.5 mg/ml), aprotonin (2 µg/ml), leupeptin (2 µg/ml) and PMSF (0.75 mM), and centrifuged at 16,000 × g for 5 min. The supernatant was removed, and the pellet was resuspended in nuclear extraction reagent supplemented with benzamidine (0.5 mg/ml), aprotonin (2 µg/ml), leupeptin (2 µg/ml) and PMSF (2 mM), and centrifuged at 16,000 × g for 10 min. The supernatant was concentrated by a centrifugal filter device (Microcon; Millipore Co., Bedford, MA, USA) and used as a nuclear fraction. The protein concentrations were about 2 mg/ml. EMSA was performed using Gel Shift Assay Systems (Promega Co., Madison, WI, USA). NF-κB consensus oligonucleotides (5’-AGTTGAGGGGAC TTTCCAGGC-3′; 5’-GCCCTGGGAAAGTCCCCTC AACT-3’) (3.5 pmol) were radiolabeled with [γ-32P]-ATP (3,000 Ci/nmol, 10 mCi/ml) using T4 polynucleotide kinase. The labeled oligonucleotides were separated from unincorporated nucleotides by chromatography through a G-25 spin column (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The radiolabeled probe was incubated with 4 µg nuclear extracts prepared from the treated cells in gel shift binding buffer. To verify the specificity of the binding reaction, an excess of unlabeled oligonucleotide was added to the reaction before adding the radiolabeled probe. The DNA-protein complexes were analyzed on 4% non-denaturing acrylamide gels.

**PGF2α assay**

After incubation, the medium was stored at –20°C until PGF2α assay. After the medium was stored...
removed, cells were washed twice, resuspended in PBS (0.01 M) and sonicated. Protein concentrations in the sonicated samples were determined by a published method [36]. Prostaglandins were extracted as reported previously [12, 37]. In brief, the medium was applied to a C18-LRC solid phase extraction cartridge (Bond-Elut, Varian Co., Harbor City, CA, USA), and the cartridge was rinsed by distilled water and 10% acetonitrile. Prostaglandins were then eluted with methanol and evaporated under nitrogen. The dried extract was dissolved with ethanol and the kit assay solution, and PGF2α concentrations were determined by a PGF2α enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI, USA). The results were expressed as ng of PGF2α per cellular mg protein per day. The sensitivity of the assay was 4.6 pg/ml. The intra- and inter-assay coefficients of variation were 7.0% and 7.8%, respectively.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the cultured cell with Isogen by the method provided by the manufacturer. For mRNA analysis, RT-PCR was performed with the oligonucleotide primers for COX-1 (5'-TGCCCAGCTCCTGGCCCGCCGCTT-3' and 5'-GTCCATCAACACAGGCGCTTCTTC-3') and COX-2 (5'-AGATCATCTCTGCTTGA GTATCTT-3' and 5'-TTCAATGAGATTTG TGGGAAAATTGC-3') reported by Merritt et al. [38] and Critchley et al. [39], respectively. Two oligonucleotide primers (5'-CTGAGGTCAAG GGAATTG-3' and 5'-GGACAGAGTCTTG ATGATCTC-3') were also used to amplify ribosomal protein L19 as an internal control as reported previously [40]. Briefly, 3 µg of total RNA were reverse-transcribed at 42 C in a reaction mixture (single-strength PCR buffer, 2.5 µM deoxynucleotide triphosphates, 5 µM random hexamer primer, 1.5 µM MgCl₂, and 200 IU Moloney murine leukemia virus reverse transcriptase). The RT product was divided into two equal aliquots (one tube was for L19 primers), and PCR was performed. For PCR amplification, a mixture containing the oligonucleotide primers (50 pmol), [α-³²P]-dCTP (2 mCi at 3000 Ci/mmol), and Taq DNA polymerase (2.5 IU) was added to each reaction. Amplification was carried out for 30 cycles consisting of 94 C (1 min), 55 C (1 min) and 72 C (1 min) for COX-1, and 35 cycles consisting of 94 C (1 min), 60 C (1 min) and 72 C (1 min) for COX-2, followed by 10 min of final extension at 72 C in a programmed temperature control system PC-800 (ASTEC, Fukuoka, Japan). The predicted sizes of the PCR-amplified products were 303 bp for COX-1, 350 bp for COX-2 and 194 bp for L19. Reaction products were electrophoresed on an 8% polyacrylamide non-denaturing gel. After autoradiography, band intensities were analyzed using a bioimaging analyzer BAS2000 (Fuji Photo Film Co, Tokyo, Japan). For quantification, the density of the signals of COX-2 was normalized to that of the internal control L19.

SOD assay

After incubation, cells were washed with PBS, resuspended in Tris-HCl buffer (0.01 M, pH 7.4) and sonicated. Cu,Zn-SOD activity was determined as reported previously [41]. The amount of protein required for 50% inhibition in the absorbance at 550 nm was defined as one unit (nitrite unit=NU) of SOD activity. All data were expressed in NU of SOD activity per mg protein. Protein concentrations were determined by a published method [36].

Statistical analysis

Data were examined by analysis of variance and Duncan’s new multiple range test. Differences were considered significant at P<0.05.

Results

We first examined whether EP-withdrawal stimulates PGF2α production in human ESC. PGF2α concentrations in the medium increased after EP-withdrawal and there was a significant difference between the EP (–) and EP (+) groups on days 8 and 11 after EP-withdrawal (Fig. 1). PGF2α is synthesized through COX, and there are two types of COX, COX-1 and COX-2. To study which type of COX is responsible for the increase in PGF2α production caused by EP-withdrawal, we examined COX-1 and COX-2 mRNA expression in ESC after EP-withdrawal. There was no significant change in COX-1 mRNA levels after EP-withdrawal (Fig. 2B). However, COX-2 mRNA levels increased after EP-withdrawal, and there was a significant difference between the EP (–) and
PG PRODUCTION BY PROGESTERONE WITHDRAWAL

EP (+) groups on days 8 and 11 after EP-withdrawal (Fig. 2A). This change in COX-2 mRNA levels was similar to that in PGF2α production as shown in Fig. 1.

Cu,Zn-SOD activities in the EP (–) group (13.2 ± 1.5 NU/mg protein, mean ± SEM of three different incubations) were significantly lower than those in the EP (+) group (32.6 ± 3.5 NU/mg protein) on day 11 after EP-withdrawal. We also reported recently that Cu,Zn-SOD activities gradually decreased after EP-withdrawal and there was a significant difference between the EP (–) and EP (+) groups on days 8 and 11 after EP-withdrawal [17]. Therefore, we decided to examine whether the increase in PGF2α production and COX-2 mRNA levels caused by EP-withdrawal is mediated by ROS. For this purpose, ESC were incubated in the presence of N-acetyl-L-cysteine, an antioxidant, from day 4 to day 11 after EP-withdrawal, and PGF2α concentrations in the medium and COX-2 mRNA levels were measured on day 11 after EP-withdrawal. The increase in PGF2α production and COX-2 mRNA expression as a result of EP-withdrawal were completely suppressed by the addition of N-acetyl-L-cysteine (Fig. 3).

Next, we examined by EMSA whether ROS generated in response to EP-withdrawal activate NF-κB. As shown in Fig. 4, EP-withdrawal caused marked increases in NF-κB DNA binding activity (lane 2), whereas nuclear extracts from the cells in the EP (+) group showed negligible binding activity (lane 1). The binding activity induced by EP-withdrawal was clearly suppressed by addition of N-acetyl-L-cysteine (lane 3). To verify the specificity of the binding reaction, an excess of unlabeled oligonucleotide (excess cold) was added to the reaction before adding the radiolabeled probe. The complexes of the EP (+) and EP (–) groups were competed by addition of the excess cold (lane 4 and lane 5, respectively).

Furthermore, we studied whether NF-κB activated by EP-withdrawal actually stimulates PGF2α production and COX-2 mRNA expression. NF-κB is activated after the degradation of IκB by proteasome followed by translocation of NF-κB into the nucleus. Therefore, ESC were incubated in the presence of MG132, a proteasome inhibitor that inhibits NF-κB activation, during EP-withdrawal. The increase in PGF2α production and COX-2 mRNA expression as a result of EP-withdrawal were significantly inhibited by the addition of MG132 (Fig. 5).

Discussion

The present study has shown that withdrawal of ovarian steroids stimulates COX-2 mRNA expression and PGF2α production via ROS in human ESC. This result is consistent with our recent finding that ROS stimulated PGF2α production through COX in human ESC in vitro [12]. ROS generation by withdrawal of ovarian steroids could be due to the decreased Cu,Zn-SOD activity, also shown in the present study, which is also consistent with our recent report [17]. Withdrawal of ovarian steroids has been well accepted as a physiological stimulus for menstruation. However, it has not been clarified how the vague phenomenon, such as the
withdrawal of ovarian steroids, gives the endometrium a signal for menstruation. The present study, here, we propose a possible mechanism for onset of menstruation: withdrawal of ovarian steroids causes a decrease in Cu,Zn-SOD activities, which in turn stimulates COX-2 mRNA expression and PGF2α production through ROS. In fact, Cu,Zn-SOD activities are low and ROS levels are high in the late secretory phase endometrium, just before menstruation [8]. In addition, increased COX-2 expression and PGF2α concentrations are observed in the late secretory phase endometrium [14–16, 42], and significant elevation of both COX-2 mRNA expression and PGF2α production in response to withdrawal of progesterone has been reported in the human endometrium [39].

Recently, much attention has been focused on reports that ROS regulate cell function by controlling production or activation of substances that have biological activities [12, 9–11]. The present study raises the possibility that withdrawal of ovarian steroids activates NF-κB via ROS, which in turn stimulates the COX-2 and PGF2α system in human ESC. It has been reported that ROS activate NF-κB by stimulating IκB degradation in a variety of cell types [9, 22–24]. The gene promoter of human COX-2 has binding sites for NF-κB, and there are, in fact, some reports showing that COX-2 expression is regulated by NF-κB [26–31]. Recent evidence has shown the existence of the NF-κB signaling pathway in the human endometrium [35, 43–45], and it is of special interest to note that NF-κB signaling is activated in the endometrium during the premenstrual phase [44].

Fig. 2. Effects of withdrawal of estrogen and progesterone (EP-withdrawal) on (A) cyclooxygenase-2 (COX-2) and (B) COX-1 mRNA expression in human endometrial stromal cells (ESC). COX-1 and COX-2 mRNA levels of the EP (+) group and EP (−) group, which are indicated in the legend to Fig. 1, were measured on days 4, 8 and 11 after EP-withdrawal. For mRNA analysis, total RNA was isolated and subjected to RT-PCR. The intensity of the signals of COX-1 and COX-2 was normalized to that of the internal control L19. The quantification data (the ratio of COX-1/COX-2 to L19) represent the mean ± SEM of three different incubations. a: p<0.01 vs EP (+).
observations may be supported by the present result that NF-κB activation is induced by withdrawal of ovarian steroids.

The cascade in which withdrawal of ovarian steroids stimulates PGF2α production through the redox system, may also be crucial for understanding the mechanisms for maintenance of pregnancy, especially uterine quiescence. When implantation is successful and progesterone levels...
are normally maintained, Cu,Zn-SOD activities in the endometrium are high, therefore ROS generation and subsequent PGF2α production would be suppressed. In fact, Cu,Zn-SOD activities are high and ROS levels are low in the decidua of early pregnancy [8], and the synthetic capacity of prostaglandins is very low after conception in human decidua [15, 16]. In addition, we found that the concentrations of ROS and PGF2α were lower and Cu,Zn-SOD activities were higher in the decidua of normal pregnancies than those in failed pregnancies accompanied by uterine contraction and uterine bleeding [37]. Thus, Cu,Zn-SOD may contribute to uterine quiescence by preventing the accumulation of ROS leading to PGF2α synthesis and uterine contraction.

The role of endometrial epithelial cells in the production of PGF2α has been reported in the human endometrium [13]. However, since the endometrium apparently consists of a considerable number of stromal cells, PGF2α from ESC could, at least in part, play a role in the regulation of menstruation. It has also been reported that initial events of menstruation may be triggered by stromal cells [45].

COX-2 is an inducible enzyme that quickly responds to some inducers. However, in the present data, it seems that COX-2 expresses constitutively, and the increase in COX-2 lasts for a long time after withdrawal of ovarian steroids. This is not surprising because there are many reports showing that COX-2 is constitutively expressed in normal tissues and may even be the dominant form [46–49]. These findings suggest that regulation of COX-2 expression may be more complex than originally thought. It has been suggested that some basal physiological stimulation causes basal COX-2 expression, and accordingly, in some cells, COX-2 is continuously stimulated by a transcription factor that is activated by the basal stimulation. Also, since it has been reported that other prostanoids such as PGE2 or PGI2 and other enzymes for PGF2α production such as PGF synthase or PG dehydrogenase are involved in endometrial shedding [13, 14], further studies are needed to better understand the regulation by prostaglandins.

Menstruation is initiated by the decrease in the circulating concentrations of ovarian steroids and has been shown to involve cytokines, proteinases and prostaglandins [13, 50–52]. The present study has shown the signaling pathway to PGF2α production in response to withdrawal of ovarian steroids in human ESC, proposing a possible mechanism for menstruation. Since a number of lymphocytes and macrophages that could influence ESC are present in the human endometrium, the present in vitro study may not exactly mimic a
normal physiological change. We do not, therefore, ignore the mechanisms for menstruation that have been proposed so far [50–52].

Acknowledgements

This work was supported in part by Grant-in-Aid for Scientific Research (13671721 and 15591753) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

20. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK. Multiple female reproductive failures in cyclooxygenase 2-deficient


45. Kelly RW, King AE, Critchley HOD. Cytokine


