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Reactive oxygen species stimulate prostaglandin F2α production in human endometrial stromal cells *in vitro*

Norihiro Sugino[1], Ayako Karube-Harada, Shiro Kashida, Shuji Takiguchi and Hiroshi Kato

Department of Obstetrics and Gynecology, Yamaguchi University School of Medicine, Minamikogushi 1–1–1, Ube 755-8505, Japan

[1]To whom correspondence should be addressed. E-mail: obgyn@po.cc.yamaguchi-u.ac.jp

BACKGROUND: The present study was undertaken to investigate the effect of reactive oxygen species on prostaglandin F2α (PGF2α) production by human endometrial stromal cells (ESC). METHODS AND RESULTS: Isolated ESC were incubated with hydrogen peroxide, which induces lipid peroxidation. Hydrogen peroxide increased both intracellular and medium concentrations of PGF2α (P < 0.01). A time course study showed that hydrogen peroxide significantly increased PGF2α concentrations in the medium after 6 h incubation (P < 0.01), after which no further increase was observed. To study whether the increase in PGF2α production caused by hydrogen peroxide was mediated by cyclooxygenase, ESC were incubated with indomethacin (0.5 μg/ml), an inhibitor of cyclooxygenase, in the presence of hydrogen peroxide. Indomethacin significantly blocked the increases in PGF2α production caused by hydrogen peroxide (P < 0.01). Hydrogen peroxide also increased PGF2α production by decidualized ESC (P < 0.01), induced by the incubation with medroxyprogesterone acetate (10^{-6} mol/l) and oestradiol (10^{-8} mol/l). CONCLUSIONS: Reactive oxygen species stimulate PGF2α production in ESC, suggesting that they might influence endometrial function by regulating PGF2α production.

Key words: endometrial stromal cell/human/hydrogen peroxide/prostaglandin F2α/reactive oxygen species

Introduction

It has been reported that both reactive oxygen species (ROS) and superoxide dismutase (SOD), an enzyme that scavenges superoxide ions, play important roles in the regulation of endometrial function (Narimoto *et al.*, 1990; Sugino *et al.*, 1996, 2000a,b). Reactive oxygen species are increased in the late secretory phase endometrium, prior to menstruation, suggesting that ROS may be involved in endometrial shedding by causing tissue damage (Sugino *et al.*, 1996). It has also been reported that prostaglandin F2α (PGF2α) is locally produced in the endometrium and regulates the endometrial function (Baird *et al.*, 1996; Novaro *et al.*, 1996). The level of PGF2α in the human endometrium increases toward the late secretory phase and is the highest at menstruation (Downie *et al.*, 1974; Ishihara *et al.*, 1986). It has therefore been well accepted that PGF2α is involved in endometrial shedding by its vasoconstriction effect (Baird *et al.*, 1996). It is noted that ROS can produce substances that have biological activities (Morrow *et al.*, 1990; Wu *et al.*, 1992; Schenk *et al.*, 1994; Pratico and FitzGerald, 1996). Since there are several reports in other tissues that prostaglandin synthesis can be stimulated by ROS (Hemler *et al.*, 1979; Hemler and Lands, 1980; Cherouny *et al.*, 1988; Karayalcin *et al.*, 1990; Kodaman *et al.*, 1994; Feng *et al.*, 1995), functions of ROS in the endometrium may be mediated through PGF2α production. Thus, it is of interest to know whether ROS can influence human endometrial function by regulating PGF2α production.

PGF2α can be produced by endometrial stromal cells (Lumsden *et al.*, 1984; Chen *et al.*, 1995; Skarzynski *et al.*, 2000), and cyclooxygenase, the rate-limiting enzyme in the biosynthesis of prostaglandins, is also expressed in endometrial stromal cells (Han *et al.*, 1996; Jones *et al.*, 1997). We recently reported close association of ROS or SOD with PGF2α in decidual cells (Sugino *et al.*, 2000b). Therefore, we focused on endometrial stromal cells and studied whether ROS could stimulate PGF2α production to influence endometrial function.

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 the patient 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 Biomedicals Inc. (Aurora, OH, USA). Streptomycin, penicillin and trypsin-EDTA were from Life Technologies Inc. (Grand Island, NY, USA). Collagenase, indomethacin, 6-α-methyl-17OH-hydroxyprogesterone acetate (MPA), and oestradiol were from Sigma Chemical Co. (St Louis, MO, USA). Hydrogen peroxide was from Wako Pure Chemical Industries Ltd (Osaka, Japan). High performance liquid chromatography grade acetonitrile was obtained from Nacalai Tesque Co Ltd (Kyoto, Japan). Tissue flasks, culture plates and nylon mesh were from Becton Dickinson Co. (Franklin Lakes, NJ, USA).

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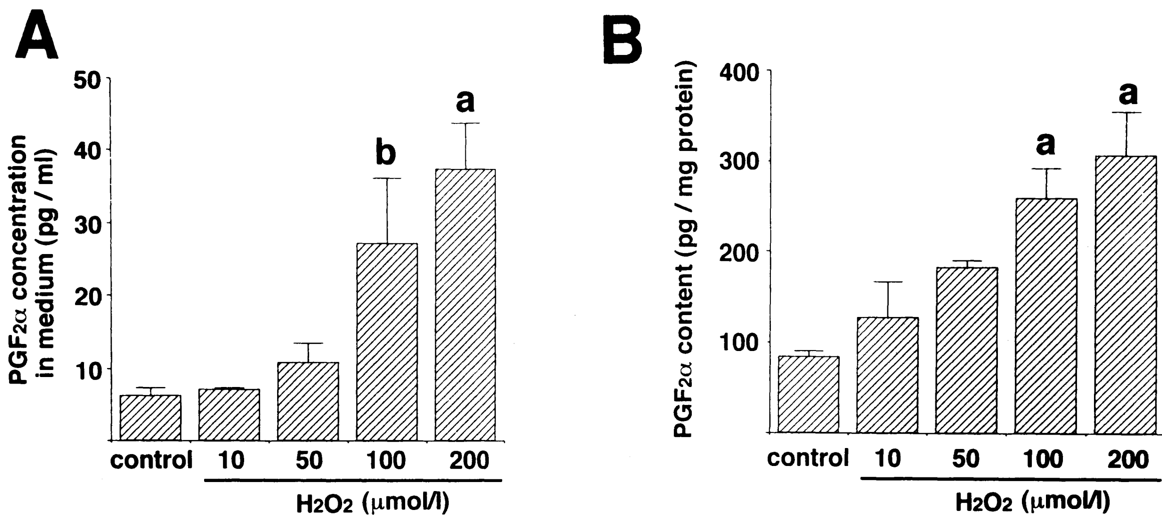

Figure 1. Effects of hydrogen peroxide on PGF2α production by human endometrial stromal cells. Isolated endometrial stromal cells were incubated with hydrogen peroxide (H2O2; 10, 50, 100, 200 μmol/l) for 6 h at 37°C, 95% O_2 and 5% CO_2. (**A**) PGF2α concentrations in the medium and (**B**) PGF2α content in the cell. A single incubation was performed in triplicate on cells from a single hysterectomy sample. The samples from three individuals were used in a single experiment. Therefore, three different incubations were performed in a single experiment. An incubation-related mean was calculated for each incubation and the data are mean ± SEM of three incubation-related means. $^{a}P < 0.01$ and $^{b}P < 0.05$ versus control.

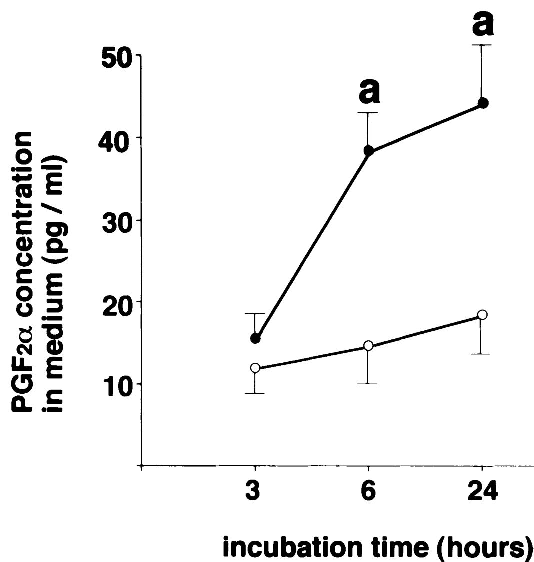

Figure 2. Time course of PGF2α release by endometrial stromal cells treated with hydrogen peroxide. Endometrial stromal cells were incubated with hydrogen peroxide (200 μmol/l) for 3, 6 or 24 h. PGF2α concentrations in the medium were determined. Values are mean ± SEM of three incubations with different samples as described in the legend to Figure 1. Hydrogen peroxide; ●, control; ○, $^{a}P < 0.01$ versus control.

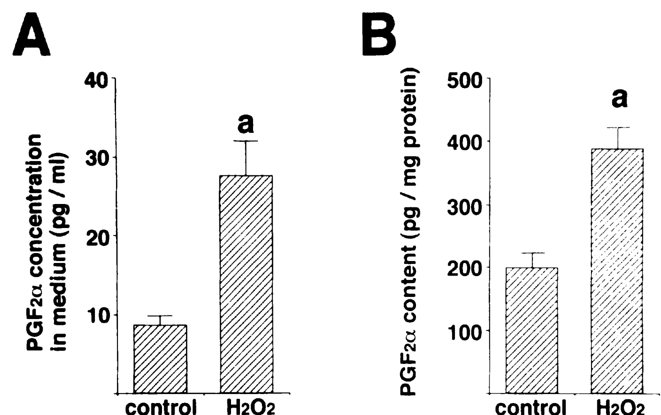

Figure 3. Effects of hydrogen peroxide on PGF2α production in decidualized endometrial stromal cells. Endometrial stromal cells were decidualized by the incubation with MPA (10^{-6} mmol/l) and oestradiol (10^{-8} mmol/l) for 18 days, and then incubated with hydrogen peroxide (H_2O_2; 200 μmol/l) for 6 h. (**A**) PGF2α concentrations in the medium and (**B**) PGF2α content in the cell. Values are mean ± SEM of three incubations with different samples as described in the legend to Figure 1. $^{a}P < 0.01$ versus control.

Endometrial stromal cell isolation

Human endometrium was obtained at hysterectomy from normally cycling premenopausal women, aged 40–42 years, who underwent surgery for myoma uteri. Endometrial samples were histologically diagnosed as late proliferative phase according to the criteria of Noyes *et al.* (Noyes *et al.*, 1950). Tissue samples were washed with Phenol Red-free DMEM containing 200 mmol/l glutamine, 100 mg/ml streptomycin and 50 IU/ml penicillin, and minced into small pieces of <1 mm^3. Endometrial stromal cells were isolated as reported previously (Sugino *et al.*, 2000a). 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 for the stromal cell-reacting antibody (vimentin) (data not shown). Cells were seeded at 10^5 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 fetal calf serum (FCS) at 37°C, 95% air and 5% CO_2. At confluence, cells were treated with 1×trypsin-EDTA and subcultured into 25 cm^2 tissue culture flasks. At ~80% confluence after the first passage, the cell culture medium was changed to the treatment medium.

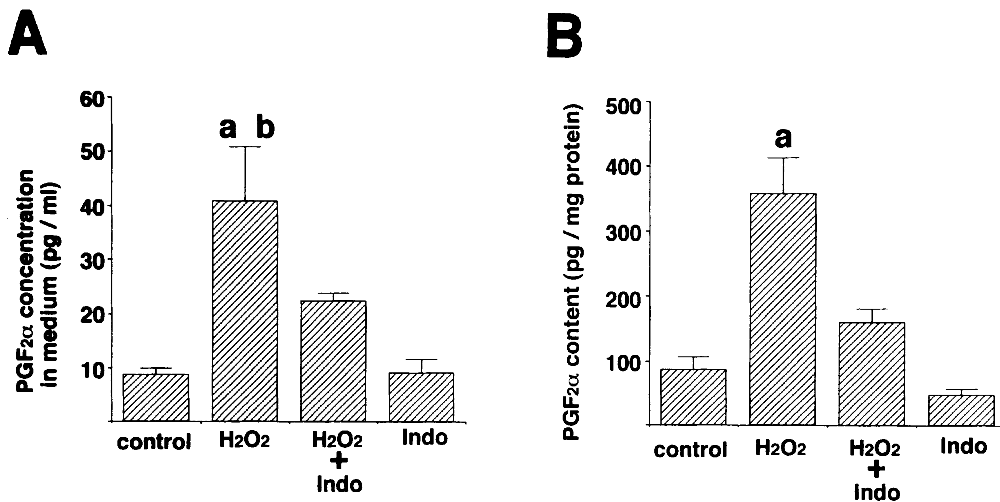

Figure 4. Inhibition of hydrogen peroxide-stimulated PGF2α production by indomethacin. Endometrial stromal cells were incubated with indomethacin (Indo; 0.5 μg/ml) in the presence of hydrogen peroxide (H_2O_2; 200 μmol/l) for 6 h. (**A**) PGF2α concentrations in the medium and (**B**) PGF2α content in the cell. Values are mean ± SEM of three incubations with different samples as described in the legend to Figure 1. (**A**) $^{a}P < 0.01$ versus control, $^{b}P < 0.05$ versus H_2O_2 + Indo, (**B**) $^{a}P < 0.01$ versus control or H_2O_2 + Indo.

Cell culture

To examine the effect of lipid peroxidation on PGF2α production by endometrial stromal cells, cells were incubated with hydrogen peroxide (10, 50, 100, 200 μmol/l) in the medium (Phenol Red-free and serum-free DMEM supplemented with glutamine and antibiotics) for 6 h at 37°C, 95% air and 5% CO_2.

To study the time course, endometrial stromal cells were incubated with hydrogen peroxide (200 μmol/l) for 3, 6, or 24 h under the same condition as described above.

To examine whether hydrogen peroxide is also effective in decidualized endometrial stromal cells, endometrial stromal cells were decidualized by incubation with phenol red-free DMEM supplemented with glutamine, antibiotics, 2% stripped FCS, MPA (10^{-6} mmol/l) and oestradiol (10^{-8} mmol/l) for 18 days at 37°C, 95% air and 5% CO_2, and then incubated with hydrogen peroxide (200 μmol/l) for 6 h under the same condition as described above. Decidualization was confirmed by the mRNA expression of insulin-like growth factor-binding protein-1 (IGFBP-1), which is a specific marker of decidualization (Giudice *et al.*, 1992; Kim *et al.*, 1998; Sugino *et al.*, 2000a).

To study whether the effect of hydrogen peroxide on PGF2α production is mediated by cyclooxygenase, endometrial stromal cells were incubated with indomethacin (0.5 μg/ml), an inhibitor of cyclooxygenase, in the presence of hydrogen peroxide (200 μmol/l) for 6 h under the same condition as described above. After cell incubation, PGF2α concentrations in the medium and cells were determined. A single incubation was performed in triplicate on cells from a single hysterectomy sample. The samples from three individuals were used in a single experiment. Therefore, three different incubations were performed in a single experiment. To examine the effect of hydrogen peroxide and/or indomethacin on cell viability, cells were seeded at 10^5 cells/ml into each well of a 24-well culture plate and incubated. After incubation, cell viability was tested by the Trypan Blue dye exclusion method. Three different incubations were performed in triplicate. Hydrogen peroxide (200 μmol/l) and/or indomethacin had no effect on cell viability after 6 h incubation: control: 75.3 ± 3.3%, H_2O_2: 73.5 ± 3.7%, indomethacin: 72.9 ± 2.3%, H_2O_2 + indomethacin: 71.3 ± 5.3%; mean ± SEM of three different incubations.

PGF2α assay

After incubation, the cells were washed twice, resuspended in PBS (0.01 mmol/l, pH 3.0) and sonicated. Prostaglandins were extracted as reported previously (Sugino *et al.*, 2000b) based on a previously reported method (Olofsson *et al.*, 1990). In brief, sonicated samples and the medium were applied to a C18-LRC solid phase extraction cartridge (Bond-Elut, Varian Co., Harbor City, CA, USA), and the cartridge was rinsed with distilled water and 10% acetonitrile. Prostaglandins were then eluted with methanol and evaporated under nitrogen. The dried extract was dissolved in 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 sensitivity of the assay was 4.6 pg/ml. The intra- and inter-assay coefficients of variation were 7.8 and 7.0% respectively. The results were expressed as ng PGF2α per mg protein for cellular concentrations and as ng per ml for medium concentrations. Protein concentrations in the sonicated samples were determined by the method described by Lowry *et al.* (Lowry *et al.*, 1951).

Statistical analysis

Data were examined by analysis of variance (ANOVA) and Duncan's new multiple range test. Where appropriate, Student's *t*-test was employed. Differences were considered significant at $P < 0.05$.

Results

To examine the effect of lipid peroxidation on PGF2α production, isolated endometrial stromal cells were incubated with hydrogen peroxide. Hydrogen peroxide appeared to increase both intracellular and medium concentrations of PGF2α in a dose-dependent manner (Figure 1). A time course study showed that hydrogen peroxide (200 μmol/l) significantly ($P < 0.01$) increased PGF2α concentrations in the medium after 6 h incubation (Figure 2) but did not increase it further after 24 h. We next examined whether decidualization affected the responsiveness of endometrial stromal cells to hydrogen peroxide. Hydrogen peroxide also significantly ($P < 0.01$)

increased intracellular and medium concentrations of PGF2α in the decidualized endometrial stromal cell (Figure 3). To study whether the increase in PGF2α production caused by hydrogen peroxide is mediated by cyclooxygenase, endometrial stromal cells were treated with indomethacin (0.5 μg/ml), an inhibitor of cyclooxygenase, in the presence or absence of hydrogen peroxide (200 μmol/l). As shown in Figure 4, hydrogen peroxide significantly ($P < 0.01$) increased intracellular and medium concentrations of PGF2α, and indomethacin significantly ($P < 0.05$ and $P < 0.01$) blocked the increases in PGF2α production caused by hydrogen peroxide. Indomethacin alone caused no significant effect (Figure 4).

Discussion

It has been reported that ROS and SOD play important roles in the regulation of endometrial function (Narimoto *et al.*, 1990; Sugino *et al.*, 1996, 2000a,b). The role of ROS in human endometrial function has not been fully clarified. We previously suggested that accumulation of ROS may be involved in the shedding of the endometrium by causing tissue damage (Sugino *et al.*, 1996). Recently, much attention has been focused on the observation that ROS can produce substances that have biological activities (Morrow *et al.*, 1990; Wu *et al.*, 1992; Schenk *et al.*, 1994; Pratico and FitzGerald, 1996). The present study has demonstrated that ROS stimulate PGF2α production in human endometrial stromal cells. This is consistent with previous reports in other tissues that prostaglandins synthesis can be stimulated by ROS (Hemler *et al.*, 1979; Hemler and Lands, 1980; Cherouny *et al.*, 1988; Karayalcin *et al.*, 1990; Kodaman *et al.*, 1994; Feng *et al.*, 1995). It is well accepted that PGF2α is locally produced in the endometrium and regulates endometrial function (Baird *et al.*, 1996; Novaro *et al.*, 1996). PGF2α has been implicated as a vasoconstrictor in the modulation of blood vessel tone prior to and during menstruation and is responsible for endometrial shedding (Baird *et al.*, 1996). In fact, the increase in PGF2α concentrations and lipid peroxide concentrations was observed in the late secretory human endometrium, just before menstruation (Downie *et al.*, 1974; Ishihara *et al.*, 1986; Sugino *et al.*, 1996). Secretory endometrium has also been reported to release more PGF2α than proliferative endometrium (Abel and Baird, 1980). Thus, the present study suggests that ROS influence human endometrial function by regulating PGF2α production.

In contrast, it has been reported that synthetic capacity of prostaglandins is very low after conception in human decidua (Maathuis and Kelly, 1978; Ishihara *et al.*, 1986). We also found that ROS were low in the decidua of early pregnancy (Sugino *et al.*, 1996). These findings are in agreement with the present result and seem reasonable for the maintenance of pregnancy. In spontaneous abortion, expulsion of the uterine content occurs to terminate pregnancy, usually accompanied by uterine contraction. However, in the case of the missed abortion, dead products of conception are retained in the uterus without bleeding for several weeks. Thus, the exact mechanism responsible for spontaneous expulsion is not precisely clarified. We recently reported that the concentrations of lipid peroxide and PGF2α of the decidua of missed abortion were the same as those of normal pregnancy and much lower than those of incomplete abortion with uterine contraction and uterine bleeding (Sugino *et al.*, 2000b). Therefore, increased lipid peroxides in the decidua could be responsible for the increased PGF2α production, eventually to induce uterine contraction and expulsion of the uterine content (Cherouny *et al.*, 1988; Norman *et al.*, 1991).

In conclusion, the present study has shown that ROS might influence endometrial function by regulating PGF2α production in human endometrial stromal cells.

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