Changes in activities of superoxide dismutase and lipid peroxide in corpus luteum during pregnancy in rats

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Activities of superoxide dismutase (SOD) and lipid peroxide (LPO) were studied in corpora lutea of pregnant rats. SOD activities, both Mn-SOD and Cu,Zn-SOD, gradually increased in the corpora lutea until day 15 of pregnancy and decreased thereafter until day 21 of pregnancy, in a similar manner to serum progesterone concentration. LPO activities remained low until day 15 of pregnancy, but increased rapidly after day 15 to day 21 of pregnancy. Incubation of the dispersed luteal cells from day 15 of pregnancy *in vitro* showed that FeSO₄ and ascorbic acid, which induce lipid peroxidation, significantly inhibited progesterone secretion. The inhibitory effects of FeSO₄ and ascorbic acid were blocked by the simultaneous addition of α -tocopherol. These results suggest important roles for SOD and LPO in regulating luteal function during pregnancy.

Introduction

It is well known that reactive oxygen species and their product lipid peroxide (LPO) damage the cell membrane (Tappel, 1973; Mead, 1976; Harman, 1982; Fridovich, 1983; O'Brien, 1984; Slater, 1984), by cross-linking in proteins and lipids, and by formation of the gel-phase lipid (Pauls and Thompson, 1980). Gatzuli *et al.* (1991) reported the anti-gonadotrophic and anti-steroidogenic actions of superoxide radical in rat luteal cells. Sawada and Carlson (1989) reported that the production of superoxide radical was increased in the plasma membrane of the corpus luteum during the regression phase in pseudopregnant rats. Lipid peroxidation, which is induced by superoxide radicals (Hochstein and Jain, 1981), also increases during the regression of the corpora lutea in pseudopregnant rats (Sawada and Carlson, 1985). Furthermore, luteal contents of superoxide dismutase (SOD), a scavenger of the superoxide radical, change during the ovulatory cycle in rats (Laloraya *et al.*, 1989) and humans (Shiotani *et al.*, 1991), or are even increased by luteinizing hormone in pseudopregnant rats (Laloraya *et al.*, 1988). It is therefore likely that superoxide radicals and their scavenging system play important roles in generating the luteal function. The present study was undertaken to investigate the involvement of SOD and LPO in the regulation of luteal function during pregnancy in rats.

Materials and Methods

Animals

Sprague–Dawley rats, weighing 220–270 g, were housed under controlled conditions (lights on from 05:00 to 19:00 h)

with free access to standard rat chow and water. Pro-oestrous rats were housed with males overnight, and day 1 of pregnancy was the day on which spermatozoa were found in the vaginal smear.

Treatments

Operations were carried out between 16:00 and 18:00 h on days 1, 7, 12, 15, 17, 19 or 21 of pregnancy. Rats were laparotomized under ether anaesthesia and blood samples were obtained from the portal vein. The ovaries were perfused with saline via the portal vein while blood samples were taken from the inferior vena cava. The ovaries were removed, and the corpora lutea were dissected and cleaned of adhering tissue in a watch glass. For assay of SOD, corpora lutea were homogenized with Tris HCl buffer (0.1 mol l⁻¹, pH 7.4), centrifuged at 800 g for 10 min at 4°C and the supernatant was stored at -70°C. For assay of LPO, corpora lutea were homogenized with 1.15% KCl and the supernatant was stored at -70°C. Serum samples were stored at -20°C for progesterone assay.

Cell preparation for incubation *in vitro*

On day 15 of pregnancy, ovaries were removed under light ether anaesthesia and the corpora lutea were dissected free from the ovary into the medium (Dulbecco's Modified Eagle's Medium: HAM's nutrient mixture F12 1:1, containing 30 µg glutamine ml⁻¹). The corpora lutea were minced and incubated in the medium containing 0.125% collagenase (type I, Sigma Chemical Co., St Louis, MO) and hyaluronidase 0.125% (type V, Sigma Chemical Co.) for 40 min at 37°C under an atmosphere of 95% O₂:5% CO₂ in a shaking water bath. The medium

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was replaced with fresh medium containing 0.25% trypsin (DIFCO laboratories, Detroit, MI) and incubation was continued for 40 min under the same atmosphere. After incubation, the tissue was placed in the fresh medium containing 0.1% trypsin inhibitor (type II-S, Sigma Chemical Co.), and dispersed to cells by flushing through a Pasteur pipette. The cell suspension was filtered through a nylon mesh (150X-T2), and spun at 800 *g* for 5 min at 22°C. The cells were suspended in the culture medium, containing 10% fetal calf serum (Bioproducts Inc., Walkersville, MD), 50 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. The cell number and viability were tested by the trypan blue exclusion method, and the number of cells was adjusted to 1 × 10⁵ viable cells ml⁻¹. The cell suspension (100 µl) was distributed into each well of a Falcon 96-well tissue culture plate (Nippon Becton Dickinson Co. Ltd, Tokyo) which contained FeSO₄ (40 µmol l⁻¹), ascorbic acid (100, 200 or 400 µmol l⁻¹) (Nakarai Co. Ltd, Tokyo), and hCG (0.2 U ml⁻¹) (Sigma Chemical Co.) in 100 µl of the medium. The incubation was continued for 2 h at 37°C under an atmosphere of 95% air: 5% CO₂. At the end of incubation, the culture plates were centrifuged at 800 *g* for 5 min, and the supernatant was stored at -20°C for progesterone assay. The specificity of FeSO₄-ascorbic acid reaction to induce lipid peroxidation was tested by adding 100 µl of DL- α -tocopherol (50 or 100 µmol l⁻¹) (Nakarai), an inhibitor of lipid peroxidation, to several wells containing 40 µmol FeSO₄ l⁻¹, 100 µmol ascorbic acid l⁻¹ and 1 × 10⁴ cells.

Determination of SOD activity

Total SOD activity was measured by the nitrite method reported by Oyanagi (1984), using the following reagents: (1) buffer solution: 60.6 mmol Na₂B₄O₇ · 10 H₂O l⁻¹ and 86.8 mmol KH₂PO₄ l⁻¹ (pH 8.2); (2) reagent A: 2.0 mmol hypoxanthine l⁻¹ (pH 8.2) (Nakarai Co. Ltd) dissolved in buffer solution; (3) reagent B: 10.9 mmol hydroxylamine HCl l⁻¹ (Nakarai Co. Ltd) and 8.8 mmol hydroxylamine-*O*-sulfonic acid l⁻¹ (Merck-Schuchardt, München); (4) enzyme reagent: 1.33 × 10⁻² U xanthine oxidase ml⁻¹ (Boehringer Mannheim BmbH Biochemica, Germany) and 0.8 mmol EDTA · 2 Na l⁻¹ (Sigma Chemical Co.) dissolved in buffer solution; (5) colouring reagent: 33.2 µmol *N*-1-naphthylethylenediamine l⁻¹ (Nakarai Co. Ltd) and 2.6 mmol sulfonic acid l⁻¹ (Nakarai Co. Ltd) and 25% acetic acid. Reagent A (0.1 ml), reagent B (0.1 ml) and 0.1 ml of the test sample were added to 0.5 ml distilled water. After preincubation for 5 min at 37°C, 0.2 ml of enzyme reagent was added, and the mixture was incubated for 30 min at 37°C, followed by the addition of 2.0 ml of colouring reagent. The final mixture was left for 30 min at room temperature and optical absorption was then measured at 550 nm. The Mn-SOD assay was performed in exactly the same way as the assay for total SOD activity, except that 0.5 ml of 2 mmol KCN l⁻¹ was added instead of 0.5 ml distilled water. Usually, dose-response curves with serial dilutions of the samples were linear between 2 and 40 µg protein per tube. The amount of protein required for 50% inhibition was defined as one unit (Nitrite Unit; NU) of SOD activity. All data were expressed in Nitrite Unit (NU) of SOD activity per mg protein. Protein concentration was measured by the method reported by Lowry *et al.* (1951). Cu,Zn-SOD activity was determined by subtracting Mn-SOD

activity from the total SOD activity. The intra- and interassay coefficients of variation were 3.8% and 9.6% for the total SOD assay, and 4.7% and 6.4% for the Mn-SOD assay, respectively.

Determination of LPO activity

Concentrations of LPO in the corpus luteum were measured by the thiobarbituric acid method of Ohkawa *et al.* (1979). Dose-response curves with serial dilutions of the sample were linear between 5 and 20 µg protein per tube. The results were expressed as nmol of malondialdehyde (MDA) per g wet weight of tissue.

Progesterone assay

Serum progesterone concentrations were determined by the specific radioimmunoassay reported by Kato *et al.* (1982). The sensitivity of the assay was 100 pg per tube, and the intra- and interassay coefficients of variation were 7% and 14.4%, respectively.

Statistical analysis

Data were analysed by analysis of variance and the new Duncan's multiple range test. Differences were considered to be significant if *P* < 0.05.

Results

Total SOD and Mn-SOD activities, as well as Cu,Zn-SOD activity, changed in parallel during pregnancy; activities increased at day 12 of pregnancy and gradually decreased thereafter (Fig. 1).

A positive correlation was found between serum progesterone concentrations and total SOD activity (*r* = 0.63, *P* < 0.01) (Fig. 2). In contrast, LPO concentrations increased rapidly from day 15 to day 21 of pregnancy. LPO activities showed negative correlations with serum progesterone concentrations (*r* = -0.74, *P* < 0.01), or total SOD activity (*r* = -0.46, *P* < 0.05).

Progesterone production of dispersed luteal cells was significantly (*P* < 0.05) inhibited by FeSO₄ and ascorbic acid in a dose dependent manner (Fig. 3). Progesterone production in the absence of hCG was not significantly inhibited by FeSO₄ and ascorbic acid (data are not shown). The inhibitory effect of FeSO₄ plus ascorbic acid on progesterone secretion was blocked by the simultaneous addition of α -tocopherol (Fig. 4).

Discussion

This study shows that the marked decrease in serum progesterone concentration after day 15 of pregnancy coincided with the rapid increase in lipid peroxide activity in the corpus luteum. These data are consistent with the previous findings that superoxide radicals and lipid peroxidation increase during the regressing phase of corpus luteum (Sawada and Carlson, 1985,

Fig. 1. Changes in Cu,Zn-SOD (◆), Mn-SOD (●) and total SOD (□) activities in the corpus luteum of rats during pregnancy. Values are means $\pm$ SEM for the number of animals given in parentheses. NU: nitrate unit.

Fig. 2. Changes in serum progesterone concentration (●), total SOD activity (□), and LPO concentrations (◆) in the corpus luteum of rats during pregnancy. Values are means $\pm$ SEM for the number of animals given in parentheses. NU: nitrate unit.

1989), and inhibit luteal function (Gatzuli *et al.*, 1991). This is also confirmed by the present data showing that FeSO_4 and ascorbic acid inhibited the progesterone production of the dispersed luteal cells. Lipid peroxidation, induced by free radicals (Hochstein and Jain, 1981), caused membrane damage such as changing membrane fluidity (Pauls and Thompson, 1980). Behrman and Preston (1989) reported that hydrogen peroxide caused rapid inhibition of luteinizing hormone-stimulated cAMP accumulation in the membrane of the rat corpus luteum that resulted in the decrease of progesterone production. Behrman and Aten (1991) further demonstrated that peroxide inhibited the luteinizing hormone-dependent component of steroidogenesis, which agrees with our present data showing that FeSO_4 plus ascorbic acid inhibited the hCG-stimulated production of progesterone, but did not affect the basal production

of progesterone in the absence of hCG. Anti-gonadotrophic actions of hydrogen peroxide were also reported in rat ovarian cells (Margolin *et al.*, 1990). Since it has been clearly demonstrated that hydrogen peroxide blocked the transport of cholesterol from cytosol to mitochondria (Behrman and Aten, 1991), it would be reasonable to suppose that the free radical system plays a crucial role in the luteolytic process, probably by inhibiting the production of progesterone.

It is unclear how lipid peroxidation is increased after day 15 of pregnancy in rats. It is interesting to note that lipid peroxidation occurs during the ageing process of cells (Tappel, 1973). SOD activity decreases with age in rats (Reiss and Gershon, 1976), and the auto-oxidation of polyunsaturated fatty acids, which stimulates the production of lipid peroxides (Tappel, 1980; Harman, 1981), increases during the ageing process

Fig. 3. Effects of FeSO_4 and ascorbic acid on progesterone production from rat luteal cells. (Control: ■; FeSO_4 plus 50 μmol ascorbic acid l^{-1} : ▨; FeSO_4 plus 100 μmol ascorbic acid l^{-1} : ▩; FeSO_4 plus 200 μmol ascorbic acid l^{-1} : □). Each bar represents the mean $\pm$ SEM of five incubations from one of two similar experiments. * $P < 0.05$ compared with the control group (new Duncan's multiple range test).

Fig. 4. Effects of α -tocopherol on progesterone production from luteal cells. (Control: ■; FeSO_4 plus ascorbic acid: ▨; FeSO_4 plus ascorbic acid plus 25 μmol α -tocopherol l^{-1} : ▩; FeSO_4 plus ascorbic acid plus 50 μmol α -tocopherol l^{-1} : □). Each bar represents the mean $\pm$ SEM of six incubations from one of two similar experiments. * $P < 0.01$ compared with the control group; † $P < 0.01$ compared with FeSO_4 plus ascorbic acid groups (new Duncan's multiple range test).

(Logani and Davies, 1980). Reduction of blood flow, one of the typical characteristics of ageing, occurs during regression of the corpus luteum (Pang and Behrman, 1979) and stimulates the conversion of xanthine dehydrogenase to xanthine oxidase which stimulates the production of superoxide radical and cell damage (McCord, 1985). Decreases in SOD and increases in lipid peroxides may be related to the ageing phenomena of the corpus luteum.

Although the involvement of prostaglandins in the luteolytic process has been clearly indicated (Rothchild, 1981), the initial trigger of prostaglandin synthesis at the beginning of luteolysis is unknown. There is some evidence that the production of superoxide radicals is stimulated by arachidonic acid (Clark et

al., 1987) or prostaglandin $\text{F}_{2\alpha}$ (Sawada and Carlson, 1991; Aten et al., 1992), whereas superoxide radical also activates phospholipase A_2 activity (Sawada and Carlson, 1991) or prostaglandin synthesis (Rahimtula and O'Brien, 1976; Hemler et al., 1979; Hemler and Lands, 1980; Band et al., 1981). Further studies are needed regarding the interrelation in the biosynthesis process between prostaglandins and superoxide.

The present data also showed that the change in SOD activity in the corpus luteum was parallel to that of serum progesterone concentrations, decreasing rapidly after day 15 of pregnancy (Fig. 2). SOD converts superoxide radical to hydrogen peroxide, which should certainly have luteolytic activity (Behrman and Preston, 1989). However, lipid peroxide remained low during mid-pregnancy and began to rise after day 15 of pregnancy. This may be due to the rapid conversion of hydrogen peroxide to H_2O . Hydrogen peroxide is converted to H_2O by catalase and glutathione peroxidase, and glutathione peroxidase is increased by luteotrophins (Agrawal and Laloraya, 1977). Since placental luteotrophins are increased during mid-pregnancy in rats (Greenwald and Rothchild, 1968), it is likely that hydrogen peroxide is rapidly converted to H_2O at about day 15 of pregnancy. Our previous findings that the luteal function in rats with one conceptus was influenced by environmental stress, for example ACTH or corticosterone treatments, much more than that in rats with full complements of conceptuses (Kato et al., 1988; Sugino et al., 1991) suggest that placental luteotrophins play a role in the regulation of the superoxide radical system in the corpus luteum.

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