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Effect of inhibition of oxygen free radical on ovulation and progesterone production by the in-vitro perfused rabbit ovary*

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Summary. The potential role of oxygen free radicals in hCG-induced ovulation was investigated using the free radical scavenging enzymes superoxide dismutase (SOD) and/or catalase with the in-vitro perfused rabbit ovary preparation. SOD (25 μg/ml) and SOD + catalase (25 μg/ml) significantly reduced the % of large follicles that ovulated during perfusion ($P < 0.005$). Neither maturity nor degeneration of ovulated ova and follicular oocytes was affected by SOD and/or catalase. Progesterone concentration in the perfusate was significantly increased in the SOD + catalase treatment group ($P < 0.01$). These results indicate a significant role for oxygen free radicals in the process of ovulation.

Keywords: free radicals; ovulation; progesterone; superoxide dismutase

Introduction

The mechanism of ovulation has been compared to an inflammatory reaction (Espey, 1980). Components of inflammation that are also found in the process of ovulation include synthesis and release of prostaglandins (Armstrong, 1981), the action of proteolytic enzymes (Strickland & Beers, 1976; Yoshimura *et al.*, 1987a), and appearance of other mediators such as bradykinin (Yoshimura *et al.*, 1988) and histamine (Kitai *et al.*, 1985). Increased vascular permeability and apical thrombosis are visible in the follicular capillaries of rabbits induced to ovulate with hCG (Kanzaki *et al.*, 1982).

Toxic metabolites of oxygen, including the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH\cdot$), are important mediators of inflammatory tissue injury (McCord, 1974; Del Maestro *et al.*, 1980; Weiss, 1986) (Fig. 1). Moreover, these highly toxic oxygen metabolites have been found to be the final common mediator of tissue damage in a large number of disparate processes, including inflammation and post-ischaemic re-perfusion injury (Bulkley, 1983; Weiss, 1986; Bulkley, 1987). There are therefore striking similarities between many known actions of oxygen-derived free radicals and the events leading to follicle rupture.

Because of their highly reactive nature, free radicals have extremely short half-lives (often measured in nanoseconds) and consequently do not accumulate in tissues at levels that can be readily detected. Many investigators have therefore used indirect means, at least in initial studies, to characterize free radical mechanisms in physiological processes (Bulkley, 1987). An indirect approach frequently employed is the use of the highly specific inhibitor of free radicals, superoxide dismutase (SOD: EC 1.15.1.1).

Ovulation can be consistently induced by administration of hCG in an isolated in-vitro perfused rabbit ovary system (Kobayashi *et al.*, 1981). This in-vitro system offers certain advantages for the

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investigation of the effects of scavenging enzymes such as SOD, for their half-life is less than 10 min *in vivo* due to renal excretion. The objective of this study was to use the in-vitro perfusion system to determine whether the inhibition of toxic oxygen metabolites with SOD, catalase (an enzyme catalysing the breakdown of H_2O_2), or both would affect hCG-induced follicle rupture, oocyte maturation, and progesterone production.

Materials and Methods

Animals. Sexually mature New Zealand White female rabbits were isolated for a minimum of 3 weeks before the experimental procedure. The rabbits were caged individually under controlled light and temperature conditions and given free access to Purina Rabbit chow (Ralston-Purina, St Louis, MO, USA) and water.

In-vitro perfusion. Rabbits weighing at least 3·5 kg were anaesthetized with intravenous pentobarbitone sodium (32 mg/kg), given heparin sulphate (120 units/kg) for anticoagulation and then subjected to laparotomy. Ovaries were excluded from study if they appeared immature or if 50% or more of the surface follicles appeared haemorrhagic. Each ovarian artery was cannulated *in situ*, and the ovary with its ovarian artery and vein and supportive adipose tissue was removed and perfused *in vitro* with 150 ml of tissue culture Medium 199 (M. A. Bioproducts, Walkerville, MD, USA) supplemented with insulin (20 U/l), heparin sulphate (200 U/l), streptomycin (50 mg/l), and pencillin G (75 mg/ml). The cannulation procedure and perfusion apparatus have been previously described in detail (Lambertsen *et al.*, 1976; Kobayashi *et al.*, 1981). A capillary membrane oxygenator gassed with a mixture of 95% O_2 and 5% CO_2 was used to oxygenate the medium. One ovary of each animal was perfused with medium containing highly purified, pharmaceutical grade bovine erythrocyte SOD (kindly supplied by Dr Leopold Flohe, Grunenthal, GmbH, Aachen, FRG) at 25 µg/ml and/or bovine liver catalase (Worthington Biochemical, Freehold, NJ, USA) at 25 µg/ml, while the contralateral ovary was perfused in a separate chamber with medium alone and thereby served as a control. The enzyme doses were chosen because they had been successfully used previously in other systems (Kuehl *et al.*, 1979; Smolen & Weissmann, 1980) and we found them to be the lowest effective doses (data not shown). Four ovaries were perfused at one time. The left ovary of the first rabbit received treatment and the right ovary was the control. The order was reversed for the second rabbit. At 30 min after the onset the perfusion, both ovaries were stimulated by addition of 50 i.u. hCG (Organon Inc., West Orange, NJ, USA) to induce ovulation, and perfusion was continued for 12 h thereafter.

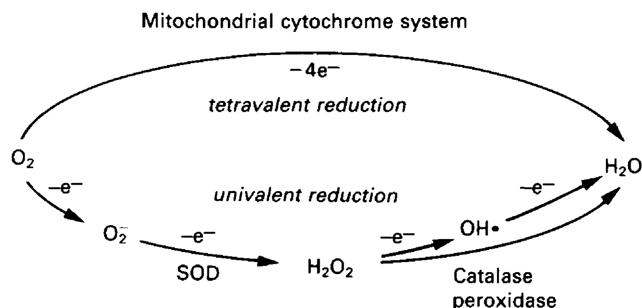

Fig. 1. Actions of oxygen free radicals within the cell. Diagram of 1–5% of oxygen that is metabolized by the cell is reduced univalently, producing the toxic oxygen metabolites, the superoxide and hydroxyl free radicals and hydrogen peroxide. A number of intracellular enzyme systems have evolved to deal with these toxic species, including the superoxide dismutases (SOD), the catalases and the peroxidases, as well as glutathione and other scavenging systems.

Ovaries were observed every 15 min for evidence of follicle growth and rupture. A follicle was considered to be ruptured when the cumulus containing an ovum was observed protruding from the ovarian surface. Ovulation time, the elapsed time between addition of hCG and follicle rupture, was recorded. The ovulated ovum, surrounded by its cumulus mass, was carefully recovered from the ovarian surface. Follicular oocytes were recovered from mature, but unruptured follicles (≥ 1·5 mm in diameter) after perfusion. Both ovulated ova and follicular oocytes were assessed for stage of maturity and signs of degeneration, as previously described (Kobayashi *et al.*, 1981). The degree of ovum maturity was expressed as the percentage of oocytes which had achieved germinal vesicle breakdown (GVBD). Ovulatory efficiency, defined as the total number of ova released divided by the total number of large follicles (≥ 1·5 mm), was calculated for each group. In a random group of rabbits, the number of preovulatory follicles per ovary that may potentially ovulate vary before treatment. During the 12 h of perfusion, perfusate samples (2 ml) were

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withdrawn from the perfusion chamber at 0, 1, 2, 4, 6, 8, 10 and 12 h after hCG administration and replaced with an equal volume of fresh medium. The samples were frozen and stored at −20°C for later measurement of progesterone. Three experimental groups were defined and 4 rabbits were used in each group.

Progesterone radioimmunoassays. Progesterone concentration in the perfusate was measured using a solid-phase kit (Diagnostic Products Co., Los Angeles, CA, USA) (Yoshimura *et al.*, 1987b) in which the progesterone antibody is bound covalently to the inner surface of polypropylene assay tubes. All samples and progesterone standards (100 µl) were assayed in duplicate. The sensitivity was 0·05 ng/ml, and the intra- and inter-assay variations were 7·5% and 6·6%, respectively.

Statistical analysis. ANOVA, followed by the Student–Newman–Keuls test, was used to analyse ovulation time and a 2 × 4 contingency table was used to analyse ovulatory efficiency. Progesterone data were evaluated by ANOVA with repeated measures. A probability of <0.05 was considered to be significant.

Results

Effect of SOD and/or catalase on hCG-induced ovulation

The ovulatory efficiency was significantly reduced by SOD and SOD + catalase compared with the control groups. Ovulatory efficiency fell from 82 to 30% ($P < 0.005$) with SOD treatment and from 96 to 29% ($P < 0.001$) with SOD + catalase treatment, but there was no significant difference between the two treatments. In addition, the mean ovulation time was significantly prolonged in the SOD + catalase-treated group ($P < 0.005$) (Table 1). Catalase alone did not significantly affect ovulation.

Table 1. Characteristics of rabbit ovaries perfused with SOD and/or catalase

	SOD	Catalase	SOD + catalase	Control
No. of ovaries perfused	4	4	4	12
No. of ovaries ovulating	2	4	4	12
Total no. of follicles	20	15	31	63
Total no. of ovulations	6	10	9	54
Ovulation time (h)†	6·78 ± 0·98	6·80 ± 0·86	9·13 ± 0·93*	5·92 ± 0·37

Values are mean ± s.e.m.
†Time from addition of hCG to follicle rupture for those follicles that ruptured.
*$P < 0.005$ compared with control.

No significant difference was noted in percentage of ovulated ova or follicular oocytes achieving GVBD and degeneration in any of the groups examined (Table 2).

Table 2. Stage of maturity and degeneration of ovulated ova and follicular oocytes

	SOD	Catalase	SOD + Catalase	Control
Ovulated ova				
Total no.	6	10	9	54
% GVDB	100	90·0	88·9	88·9
% Degeneration	16·7	10·0	33·3	20·4
Follicular oocytes				
Total no.	14	5	22	9
% GVDB	100	100	100	100
% Degeneration	21·4	0	9·1	33·3

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Progesterone concentrations

Progesterone production by the control ovary of each experimental group reached its maximum at 4 h after hCG administration. Progesterone production in the SOD + catalase treated group was significantly increased ($P < 0.01$) compared to the contralateral control. This increase was evident by 2 h after hCG administration (Fig. 2). The concentration of progesterone in the perfusate of ovaries treated with SOD alone and catalase alone increased in a similar manner, and was not different from respective controls.

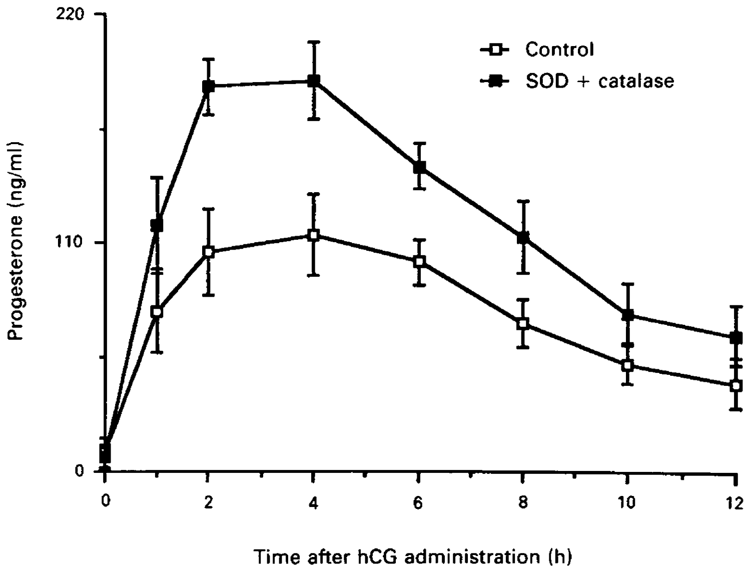

Fig. 2. Concentrations of progesterone in the perfusate of ovaries treated with SOD + catalase and the contralateral controls. Data points are the mean ± s.e. of 4 perfused ovaries from at least 2 different experiments. Progesterone was significantly ($P < 0.01$) increased by treatment, as measured by ANOVA with repeated measures.

Discussion

Many factors may result in the formation of toxic metabolites of oxygen in tissues. These include mitochondrial, endoplasmic reticular and nuclear membrane electron transport systems, oxidant enzymes such as cytochrome P_{450}, xanthine oxidase, and lipoxygenase, and the NAD(P)H oxidase of phagocytic cells (Weiss, 1986; Cross *et al.*, 1987) (Fig. 1). Although these experiments were not designed to identify the precise source of radical generation, the fact that SOD blocked ovulation in an asanguineous (medium-perfused) preparation argues against that source being circulating neutrophils, a common biological source of radical generation. Regardless of the source, the scavenging of O_2^- with SOD significantly inhibited follicle rupture in these perfused rabbit ovaries, while the removal of H_2O_2 with catalase had no apparent effect. These findings suggest that O_2^-, or a secondary radical species dependent for its production on O_2^- generation, plays an essential role in follicle rupture. In these experiments we have used SOD to determine that the process of ovulation was dependent upon the activity of O_2^-. Although these approaches have been used commonly in the past by others in the field of free radical biology (Bulkley, 1987), they offer only indirect evidence for O_2^- generation. SOD is a very specific enzyme; in the 20 years since its description by McCord & Fridovich (1969), no other biological action of this compound has been

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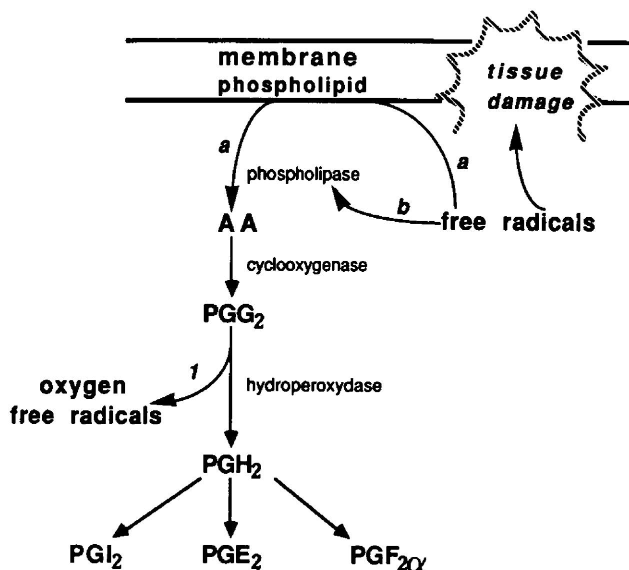

Fig. 3. Scheme for free radical interactions with prostaglandins. Free radicals are produced during reduction of PGG-2 to PGH-2 (1). They may also increase PG production by (a) directly releasing arachidonic acid (AA) from membranes, and/or (b) activating phospholipase.

demonstrated (Bulkley, 1987). It is therefore unlikely that non-specific effects of the highly purified enzymes used in these experiments could account for our observations, but the possibility cannot be disproved without the use of inactivated enzymes.

Our observations do not indicate a role for H_2O_2 in ovulation, although the presence of both SOD and catalase prolonged the ovulation time of those follicles that ovulated. While these data appear to suggest a more important role of O_2^- than H_2O_2 in follicle rupture, H_2O_2 has been found to inactivate SOD slowly and irreversibly (Hodgson & Fridovich, 1975). The addition of catalase may have protected SOD from inactivation by H_2O_2.

In this study, SOD and/or catalase did not alter the percentage of ovulated ova or follicular oocytes undergoing GVBD or degeneration. These data suggest that oxygen free radicals may not play a role in the processes of oocyte maturation or degeneration.

Progesterone concentration significantly increased in those ovaries treated with SOD + catalase. The reason for this increase is not clear, but SOD + catalase might protect steroid-producing cells from damage due to the secondary generation of OH· from $O_2^- + H_2O_2$. Alternatively, treatment may have shifted steroid metabolism, for instance changing the ratio between progesterone and 20α-dihydroprogesterone, or altered hormone secretion.

The interaction between oxygen free radicals and PGs, both of which are intimately involved in the inflammatory process, is complex. Oxygen free radicals are not only formed during PG biosynthesis, but may themselves initiate the arachidonic acid cascade (Fig. 3), many of the steps of which are radical-dependent lipid peroxidations. Oxygen free radicals may cause follicle rupture by activating phospholipase (Baud *et al.*, 1981) and by stimulating direct release of arachidonic acid from biomembranes (Seeger *et al.*, 1982) (Fig. 3). Furthermore, it has been found that SOD decreases PG production (Baud *et al.*, 1981) and inhibits cyclooxygenase and phospholipase activity (Rahimtula & O'Brien, 1976; O'Brien & Hulett, 1980; Parente, 1982). Free radicals may affect follicle rupture through prostaglandins, by releasing proteolytic enzymes from lysosomes, and/or by direct attack on the follicle wall.

In conclusion, the data derived using an in-vitro perfusion model clearly demonstrated that the oxygen free radical, superoxide, plays a role in the mechanical process of ovulation. Future studies

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will be needed, however, to clarify further the precise role of each particular radical species in the process.

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