Antioxidant capacity is correlated with steroidogenic status of the corpus luteum during the bovine estrous cycle

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Abstract

The reactions of steroid hormone biosynthesis are accompanied by formation of oxygen radicals. We determined the levels of some antioxidants and antioxidative enzymes at different developmental stages of bovine corpora lutea to examine their correlation with steroidogenic status. Plasma progesterone concentrations of estrous cycle synchronized cows increased until day 16, and then decreased rapidly during luteal regression. The levels of steroidogenic cytochrome $P450$scc and adrenodoxin paralleled the changes in plasma progesterone. Among the antioxidative enzymes examined, the SOD and catalase activities showed patterns most similar to plasma progesterone. Catalase and SOD activities increased 6–8 fold from day 6 to 16 of the estrous cycle and then decreased during the luteal regression. Ascorbate and β-carotene showed low but significant correlation with $P450$scc and plasma progesterone levels. The profiles of two lipophilic antioxidants in corpora lutea were very different. β-carotene concentration increased by approximately 6 fold from day 6 to 16, and decreased in regressive tissue. α-tocopherol showed a 3 fold increase between days 6 and 9 followed by a rapid decrease. Thus, at the peak of steroidogenesis at mid-luteal phase α-tocopherol levels decreased, but β-carotene levels increased. The correlation between the levels of some antioxidant enzymes and compounds with progesterone levels indicates that antioxidative mechanisms are activated to cope with steroidogenesis dependent oxyradical formation in the bovine corpus luteum. © 1998 Elsevier Science B.V.

1. Introduction

Most of the steps in the biosynthesis of steroid hormones are catalyzed by cytochrome $P450$ type enzymes located in the mitochondria and endoplasmic reticulum. These reactions are dependent on the transfer of electrons from NADPH to $P450$ via electron transfer proteins adrenodoxin reductase and adrenodoxin in the mitochondria, and cytochrome $P450$ reductase in the endoplasmic reticulum [1,2]. In reconstituted enzyme systems, a significant portion of electrons may ''leak'' to other acceptors, such as O_2, and generate harmful oxy-radicals [3–5]. The degree of coupling of electron transfer to substrate hydroxylation varies among different $P450$ systems [3–5]. During steroidogenesis, the mitochondrial $P450$ sys-

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tems have been shown to produce oxygen radicals that may cause irreversible damage to molecules in the immediate surroundings such as cytochrome $P450$ and adrenodoxin [4–6].

The mitochondrial $P450scc$ system that catalyzes the first and the rate limiting step in steroidogenesis is expressed in highest concentrations in the adrenal cortex and the ovarian corpus luteum [7]. These steroidogenic tissues also contain high levels of antioxidants such as ascorbate, α-tocopherol, β-carotene and the enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione-S-transferase and glutathione reductase [8–12]. One function of these antioxidants may be protection against oxygen radicals produced during steroidogenesis [8,12]. Yet, oxygen radicals may also be functional in leading to luteolysis and apoptosis in corpus luteum during each reproductive cycle [12–14].

As the steroidogenic capacity of the corpus luteum shows major changes during the estrous cycle [7,15], we wished to determine whether protective mechanisms against oxidative damage are correlated with progesterone production in bovine corpus luteum at different stages of development. For this purpose we examined the levels of progesterone, $P450scc$, and selected antioxidants throughout the estrous cycle in the bovine corpora lutea.

2. Materials and methods

2.1. Synchronization of cows

The estrous cycle of Israeli-Holstein dairy cows ($n = 36$) from commercial herds was synchronized by intra-muscular injection of $625\,\mu g$ Cloprostenal (prostaglandin $F_{2\alpha}$ analog, Estrumate, Coopers, Burgwedel, Germany). Cows that manifested behavioral estrus (day 0) within 4 days following the injection were slaughtered and their ovaries were collected on ice on days 6, 9, 13, and 16 of the cycle. In addition, regressive corpora lutea were collected from 10 cows (not injected with $PGF_{2\alpha}$). These corpora lutea were characterized according to the criteria of Ireland et al. [16]. Corpora lutea were dissected from the surrounding tissue, processed immediately or stored at $-80°C$ until analysis.

2.2. Assays

For catalase and SOD assays, fresh (unfrozen) tissue was homogenized immediately in 1:11 (w/v) 50 mM K phosphate buffer, pH 7.4, 150 mM KCl, and $200\,\mu M$ EDTA. Protein concentration in the homogenate was determined by the biuret assay using bovine serum albumin as a standard [17]. Protein concentration in the homogenates was $12–22\,mg/ml$. The homogenate was centrifuged at $700 \times g$ for 20 min at 4°C. A portion of the cytoplasmic supernatant obtained was stored frozen for later assay of SOD.

For catalase assay a $3–20\,\mu l$ portion of the resulting supernatant was added to $700\,\mu l$ of 5 mM H_2O_2 solution in homogenization buffer kept at 37°C in a thermostated cuvette. The rate of decomposition of H_2O_2 was followed by determining the rate of decrease in absorbance at 240 nm ($\epsilon 240\,nm = 43.5\,M^{-1} \times cm^{-1}$), and the activity of catalase was calculated as described [18].

For SOD assay the frozen supernatant was thawed and processed as described [19]. SOD activity was measured using xanthine–xanthine oxidase system as a source of superoxide and nitroblue tetrazolium as a scavenger for this radical [20]. One unit of SOD activity was defined as the amount causing 50% inhibition of the initial rate of reduction of NBT [21].

The levels of adrenodoxin, and $P450scc$ were estimated by Western blot analysis [7,22–24]. The samples of purified proteins or homogenized tissue fractions ($20\,\mu g$ protein) were dissolved in gel sample buffer (final concentration: 60 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 10% 2-mercaptoethanol) and heated to 100°C for 2 min and electrophoresed on 8.5% or 15% (for adrenodoxin only) polyacrylamide gels with SDS. The blots were reacted with horseradish peroxidase linked-protein A followed by reaction with ECL-Western blotting kit (Amersham, England) (instead of reaction with ^{125}I-protein A). The resulting chemiluminescence was detected by short exposure to autoradiography film. The intensities of the bands were measured by densitometry. On each gel, in addition to tissue homogenates, four concentrations of purified enzymes were run to establish a standard curve for estimation of protein level. Protein levels were estimated in triplicate in tissue homogenates at different developmental stages of

corpus luteum on a dot-blot where 200 μl of diluted homogenates (of 2 μg protein content) were applied directly onto nitrocellulose paper using the same protocol for quantitation.

For measurement of lipids, 200–250 mg of thawed tissue was cut into thin slices and saponified with ethanolic KOH (freshly prepared by mixing 1:4 60% KOH with ethanol) by gently shaking at 65°C for 60 min. Unsaponifiable materials were extracted into petroleum ether, concentrated to 1 ml and stored at −20°C for later determinations of cholesterol, progesterone, β-carotene and α-tocopherol. Cholesterol was determined as described [25]. For tissue progesterone determination 100 μl of the organic phase was evaporated under nitrogen, dissolved in phosphate buffered saline, pH 7.5, with 0.8% gelatin, 0.1% NaN$_3$, for RIA [26]. The sensitivity of the assay was 7.8 pg/vial, the intra- and interassay coefficient of variation were 8.6% and 4.6% respectively. Plasma progesterone was assayed by the same method.

The saponified fraction was acidified with 8 N HCl and the fatty acids were extracted with petroleum ether. Total fatty acids were assayed gravimetrically [27].

For ascorbate assay about 200 mg of tissue was homogenized in 60% methanol [28]. The homogenate was centrifuged at 1000 × g and the supernatant was filtered through a 0.45 μm filter (Millipore). Ascorbate was determined by isocratic high-pressure liquid chromatography on an Econosil C-18 reversed phase

column (250 × 4.6 mm, Merck) eluted with 0.1% phosphoric acid, pH 3.0, and methanol (50:50), at a flow rate of 0.3 ml/min [29]. Absorbance was recorded at 265 nm.

For measurement of α-tocopherol and β-carotene, 2 μg of retinyl-acetate was added as an internal standard to an aliquot of the unsaponifiable fraction. The lipids were separated by isocratic high-pressure liquid chromatography on an Econosil C-18 reversed phase column (250 × 4.6 mm, Merck) eluted with methanol:ethyl-acetate (70:30), at a flow rate of 0.3 ml/min [30]. The fluorescent detector was programmed with excitation at 330 nm and emission at 470 nm from 0 to 7.5 min for retinyl acetate detection, and at excitation 290 nm, and emission 330 nm from 7.5 to 25 min for α-tocopherol detection. β-carotene was detected with the UV–VIS detector at 450 nm.

Unless otherwise specified, the concentrations in all assays were expressed per wet weight of tissue.

2.3. Statistical analysis

Difference between treatments were examined by analysis of variance with two tailed *t*-tests between each pair of treatments using the SAS general linear model procedures (SAS 1986). Significance was $P < 0.05$ unless otherwise noted. Results are the means ± SE of 6 individual (unpooled) corpora lutea from 6 different cows per day. The value for each corpus
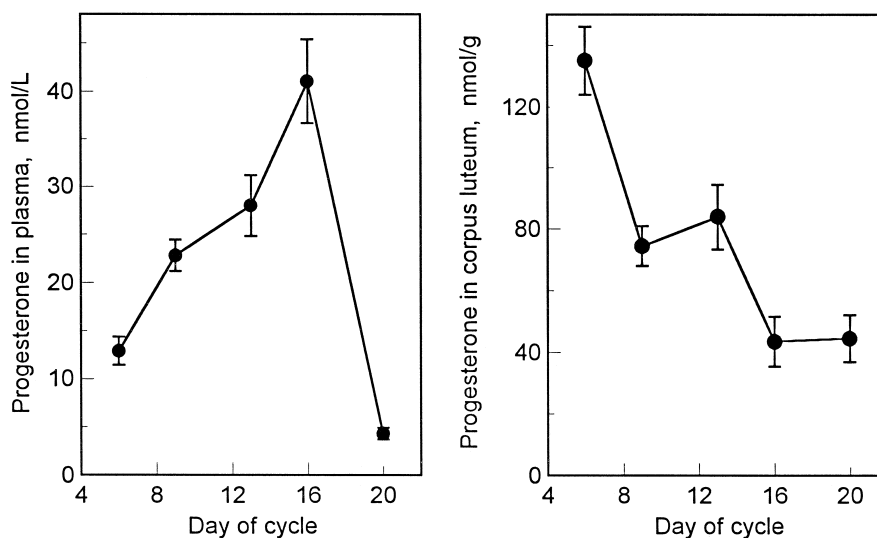

Fig. 1. Progesterone levels in plasma and in corpus luteum during the synchronized estrous cycles of cows. Day 0 is the day of estrus.
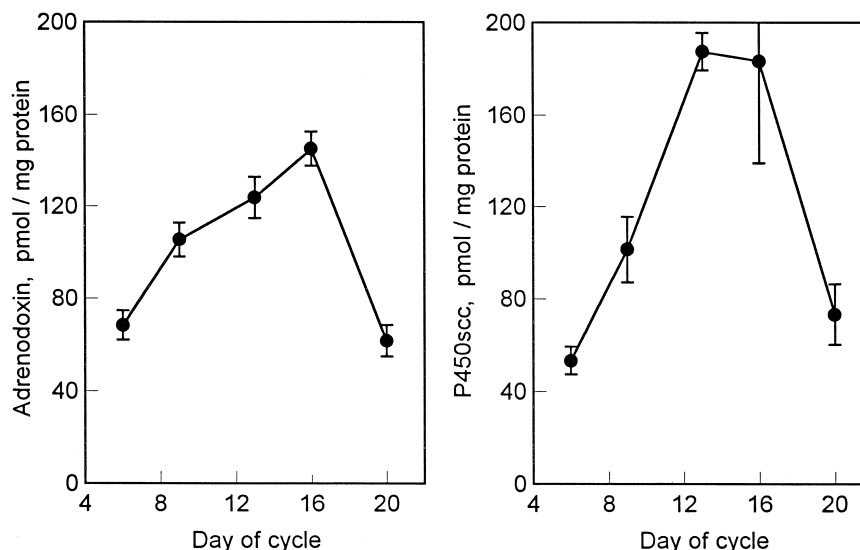
Fig. 2. Levels of adrenodoxin and P450scc in corpus luteum.

luteum was the average of three independent measurements.

3. Results and discussion

3.1. Steroidogenic status

The concentration of plasma progesterone showed a normal pattern according to the day of the estrous cycle [15,31], increasing until day 16, and then decreasing rapidly during luteal regression (Fig. 1). The peak level of blood progesterone was associated with a decrease in luteal tissue level of progesterone (Fig. 1). The levels of both adrenodoxin and P450scc paralleled the changes in plasma progesterone (Fig. 2), showing correlation coefficients of 0.66 and 0.72 respectively (Table 1).

The tissue concentration of cholesterol increased during the early luteal phase and then decreased when progesterone levels in plasma were high. (Fig. 3). Since cholesterol is the precursor of ovarian steroids, its decrease may be a result of enhanced utilization by steroidogenesis. The changes in cholesterol levels were not accompanied by a change in the levels of the total lipids which showed a small but not significant tendency to increase until day 22 (Fig. 3).

3.2. Correlation of antioxidant levels with steroidogenesis

Among the antioxidants examined, the concentrations of SOD and catalase showed patterns similar to those of steroidogenic enzymes and plasma progesterone, peaking at day 16 and decreasing during the luteal phase (Fig. 4). Catalase and SOD activities increased 6–8 fold from day 6 to 16; catalase decreased to about 40% of the maximal value in regressive tissue and SOD to about 60%. Both activities were significantly correlated with plasma progesterone (Table 1).

In the corpus luteum of pregnant rats and in pseudopregnant rabbits, SOD activity was also found to parallel plasma progesterone levels [32–34]. Overall

Table 1
Correlations between plasma progesterone, steroidogenic enzyme, and antioxidant levels in the bovine corpus luteum during estrous cycle. Only results with $P < 0.1$ are shown

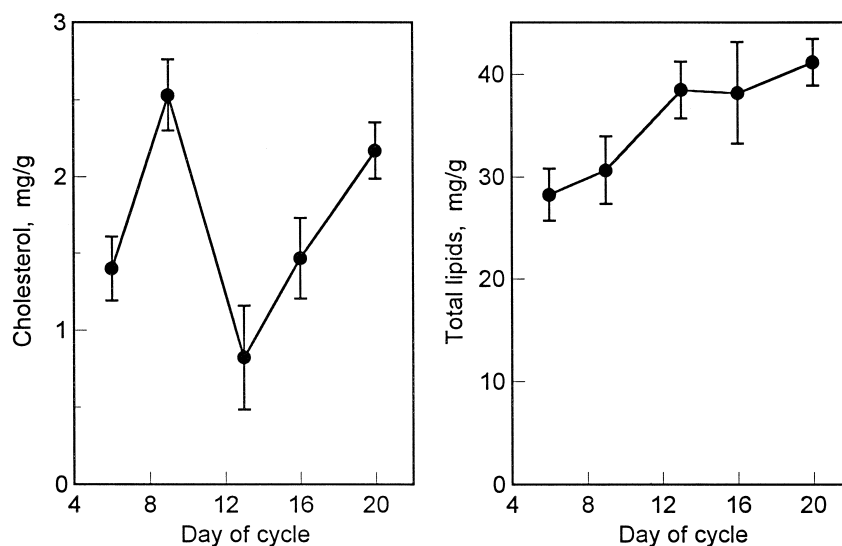| Dependent	Independent	r	$P <$
Progesterone	P450scc	0.72	0.09
Progesterone	Adrenodoxin	0.66	0.07
Progesterone	Catalase	0.50	0.001
Progesterone	β-carotene	0.43	0.09
Progesterone	SOD	0.66	0.001
Ascorbate	P450scc	0.56	0.05
Catalase	Adrenodoxin	0.42	0.10

Fig. 3. Concentration of cholesterol and total lipids in corpus luteum.

these findings indicate that SOD activity is coregulated with steroidogenesis in the corpora lutea of animals with widely differing profiles of estrous cycles.

In the early part of the estrous cycle SOD is expected to play a protective antioxidant role. The superoxide producing xanthine oxidase system was demonstrated to inhibit progesterone synthesis in rat luteal cells [35] and to disrupt rat luteal cell membranes [36]. The high levels of catalase and SOD at day 16, when progesterone synthesis is maximal,

most probably have a protective role against locally produced radicals.

In the luteolytic phase, the balance of the activities of the antioxidant enzymes may determine the dispensation of oxygen radical species. Thus, the smaller decrease in SOD activity relative to catalase (Fig. 4) may lead to an accumulation of H_2O_2 in tissue from rapid dismutation of superoxide radicals. Superoxide and/or H_2O_2 may be involved in the luteolytic process by mediating phospholipase A_2 activation which is the rate-limiting enzyme in the synthesis of
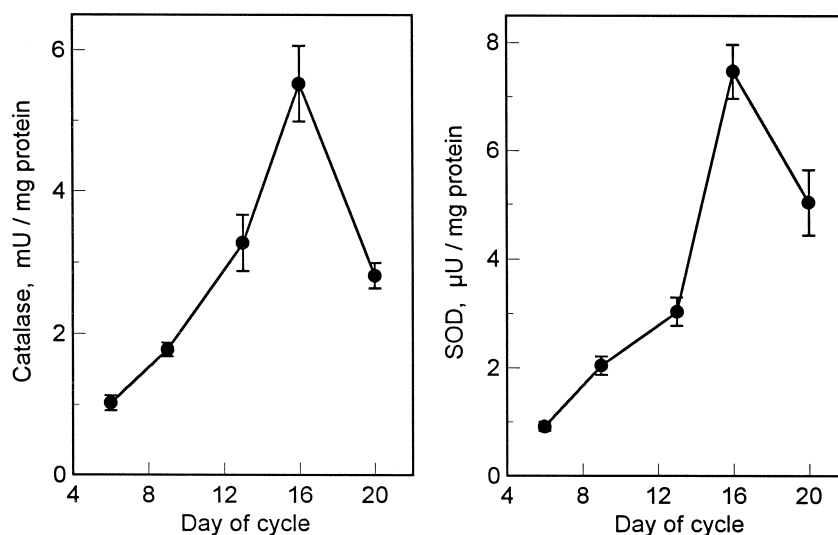
Fig. 4. Activities of antioxidant enzymes, catalase and SOD, in corpus luteum.
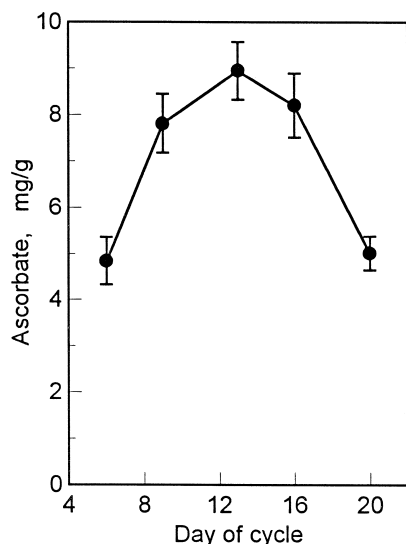
Fig. 5. Concentration of ascorbate in corpus luteum.

PGF2α that acts as a luteolytic signal [13,35,37–39]. Catalase can reverse the luteolytic action of superoxide, supporting the idea that H_2O_2, and not superoxide, may have a dominant role in activating phospholipase A_2 [37].

Examination of other antioxidant compounds revealed that ascorbate and β-carotene showed a low but significant correlation with parameters of steroidogenic status (Table 1). Ascorbate levels in tissue are much higher than those of β-carotene. Ascorbate levels increased nearly 2 fold and then decreased (Fig. 5). The profiles of the two lipophilic antioxidants studied were different (Fig. 6). β-Carotene concentration increased by approximately 6 fold from day 6 to 16, and decreased in regressive tissue. In contrast, α-tocopherol levels showed a 3 fold increase between days 6 and 9, decreased on days 13–16 and increased again in regressive tissue. Thus, at the peak of steroidogenesis at mid-luteal phase α-tocopherol levels decreased, but β-carotene levels increased.

The hydrophobic α-tocopherol and β-carotene are located in the membranes and in the lipid cytoplasmic droplets [40] while the water soluble ascorbate is expected to be in the cytosol and the mitochondrial matrix. Since side chain cleavage of cholesterol is catalyzed by P450scc located in the inner membrane, the lipophilic antioxidants may be more functional in preventing oxidative damage at this first step of steroidogenesis. The decrease of α-tocopherol at the peak of steroidogenesis may reflect its consumption by P450 generated oxygen radicals. Thus, this lipophilic antioxidant may ''absorb'' oxygen radicals first because of its proximity and high reactivity. The repletion of bovine luteal cells with β-carotene but not with α-tocopherol inhibited presumably oxy-radical mediated cross-linking between P450scc and adrenodoxin in [41,42]. Thus, it was concluded that the high levels of β-carotene in corpora lutea may protect against damage due to oxygen free radicals generated in the course of progesterone synthesis [41,42].
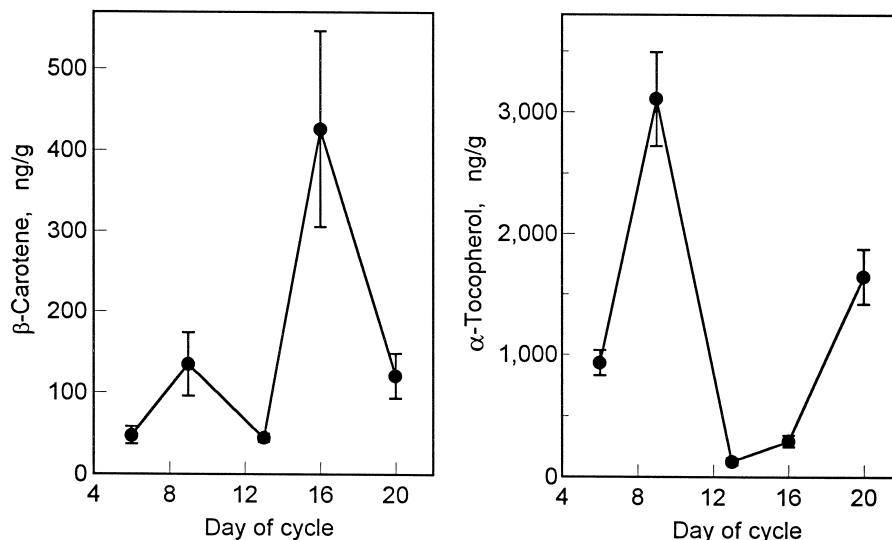
Fig. 6. Concentrations of β-carotene and α-tocopherol in corpus luteum.

The levels of the antioxidants may also be influenced by reciprocal interactions. During luteal regression, β-carotene decreased while α-tocopherol increased (Fig. 6). Based on similar observations in rats which have a very different estrous cycle, it has been suggested that α-tocopherol may be replenished through depletion of ascorbate [38] whereas there is no evidence that β-carotene may be recycled.

The changes in the levels of the compounds examined may be ascribed to additional functions. β-carotene in addition to being an antioxidant serves as a precursor of retinol that exerts effects on cellular differentiation and homeostasis through a receptor mediated pathway [43,44]. Retinol, retinoic acid and β-carotene were also reported to stimulate progesterone secretion by porcine luteal cells in vitro [45]. With purified enzymes we observed that β-carotene stimulated *P*450scc activity (unpublished observations). This effect of β-carotene may be partly mediated by its lipophilic character rather than its antioxidant properties. The depletion of ascorbate during functional luteolysis may at least in part be associated with structural luteolysis as ascorbate plays an essential role in collagen synthesis [46].

Overall, the present study has revealed a complex pattern of regulation and possible function of the antioxidant enzymes and compounds in the bovine corpus luteum. The correlation of the levels of some of these antioxidants with steroidogenesis indicates that antioxidative mechanisms are activated to cope with oxidative stress that is associated with steroid hormone synthesis.

Acknowledgements

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