—Original Article—

Role of Nitric Oxide in the Regulation of Superoxide Dismutase and Prostaglandin F_{2α} Production in Bovine Luteal Endothelial Cells

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Abstract. Prostaglandin F_{2α} (PGF) induces a rapid reduction in progesterone production (functional luteolysis) followed by tissue degeneration and cell death (structural luteolysis). Reactive oxygen species (ROS) including nitric oxide (NO) play crucial roles in the luteolytic action of PGF. The local concentration of intraluteal ROS is controlled by superoxide dismutase (SOD), the main enzyme involved in the control of intraluteal ROS. To clarify the roles of NO in the regulation of SOD in luteolysis, we examined the effects of NO on SOD expression and activity in cultured bovine luteal endothelial cells (LECs) during short-term (2 h, mimicking functional luteolysis) and long-term (24 h, mimicking structural luteolysis) incubation. We also investigated whether NO modulates PGF production by LECs. LECs were isolated from mid-luteal phase CLs, and exposed to NONOate (a NO donor) for 2 or 24 h. SOD mRNA expression was stimulated by NONOate (10–100 μM) at 2 h ($P < 0.05$). Moreover, 10 μM NONOate stimulated SOD protein expression and SOD activity at 2 h ($P < 0.05$), whereas NONOate inhibited SOD mRNA and protein expressions at 24 h ($P < 0.05$). NONOate stimulated PGF biosynthesis at both incubation times. The overall findings suggest that NO differently regulates SOD in cultured LECs, depending on the exposure time. Acute elevation of SOD may represent a response of LECs to protect themselves against oxidative stress induced by PGF during functional luteolysis, whereas a later reduction of SOD levels by NO may facilitate an excess of intraluteal ROS during structural luteolysis.

Key words: Luteal endothelial cell, Nitric oxide, Superoxide dismutase

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The corpus luteum (CL) is a transient endocrine gland essential for the regulation of ovarian cycles as well as for establishment of pregnancy in mammals. If pregnancy does not occur, the CL regresses and loses its capacity to produce progesterone (P₄), so the animal returns to estrus and has another opportunity to become pregnant. Regression of the CL (luteolysis) is crucial to reset the ovarian cycle. Prostaglandin F_{2α} (PGF) produced by the uterus induces a rapid reduction in P₄ production (functional luteolysis) followed by tissue degeneration and cell death (structural luteolysis) [1–3]. Although PGF is regarded as a physiological luteolysin in the cow, the local mechanism involved in the luteolytic action of PGF remains unclear.

The CL formed from the wall of the ovulated follicle is one of the most highly vascularized organs in the body, and luteal endothelial cells (LECs) are the most abundant type of cells composing the CL [4]. Previous studies have demonstrated that endothelial cells produce reactive oxygen species (ROS) [5] including nitric oxide (NO) [6, 7] and that ROS inhibit P₄ production [8, 9] and induce apoptosis in bovine luteal steroidogenic cells (LSCs) [10, 11]. Therefore, ROS including NO in the LEC may play an important role for elucidating the local mechanisms of functional and structural luteolysis in the bovine CL.

ROS including hydrogen peroxide (H₂O₂), superoxide anion

(O₂^{•−}), oxygen (O₂) and NO have been implicated in the luteolytic process [12]. In cattle, we recently observed that an injection of PGF induces a transient (1–2 h) increase in the concentrations of NO and partial pressure of O₂ (pO₂) in ovarian venous blood [13] and that the pO₂ of venous blood is higher in the ovarian vein than in the jugular vein. Therefore, the luteal microenvironment seems to be exposed to high oxygen conditions, especially during a short period of time (1–2 h) following PGF treatment. Interestingly, PGF stimulates the NO generating system by increasing inducible NO synthase (iNOS) mRNA and protein expression and NOS activity in cultured LECs [7]. These findings suggest that NO plays crucial roles in the local mechanism of CL regression.

Superoxide dismutase (SOD) is an important antioxidant defense in nearly all cells exposed to oxygen. SOD catalyzes the dismutation of superoxide into oxygen and H₂O₂ [14, 15]. A decrease in intracellular SOD activity is accompanied by a decrease of P₄ production by the rat CL [16]. Previous studies from our laboratory demonstrated that NO produced by LECs directly stimulates PGF production and inhibits P₄ secretion in bovine LSCs [9, 17]. Thus, SOD seems to play an important role in regulating the local mechanisms controlling the luteolytic action of NO in the bovine CL.

In the present study, to determine the physiological roles of NO in the local regulation of CL function in cows, we examined the influence of NO on the expressions of SOD mRNA and protein and SOD activity in cultured bovine LECs after two different incubation periods (2 and 24 h). We also investigated whether NO modulates PGF synthesis in the same culture system.

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Materials and Methods

Bovine LEC isolation and cell culture

LECs were isolated from five CLs at the mid-luteal phase (days 8–12 of the estrous cycle) [18, 19] using magnetic beads as described previously [20] and recently validated in our laboratory [7, 21]. Briefly, magnetic tosylactivated beads (Dynabeads M-450, 140.04; Dynal ASA, Oslo, Norway) were coated with 0.15 mg/ml lectin from *Bandeiraea simplicifolia* (BS-1; L2380; Sigma-Aldrich, St. Louis, MO, USA), which specifically binds the glycoproteins expressed by bovine LECs [20]. Luteal cell (LC) suspension was mixed with beads at a concentration of 4×10^8 beads/ml and incubated for 20 min at 4 °C on a rocking platform. The cells were cultured in endothelial cell (EC) growth medium (MV 2; C22121; Promo Cell, Heidelberg, Germany) at 37 °C in a humidified atmosphere of 5% CO₂ in air. Only colonies with a homogeneous cell population were removed with a pipette and cultured in collagen-coated 25-cm² culture flasks (690175; Greiner Bio-One, Frickenhausen, Germany). The cultures and passages were repeated until a homogeneous population of pure LECs was obtained. The LECs isolated in the present study were confirmed by immunocytochemical staining for rabbit anti-human von Willebrand factor (factor-VIII, F3520; Sigma-Aldrich) and CD31 expression as reported previously [7, 20, 21]. The bovine LECs used in the present study correspond to type 1 according to the previous classification reported by Spanel-Borowski and Bosch [22]. These cells formed a monolayer with a cobblestone pattern in confluent cultures and stained with cytokeratin antibody (data not shown). Experiments were performed on confluent cultures, and the cells (LECs) were from passages 2–5.

The LECs were seeded at a concentration of 1×10^5 viable cells/ml into 24-well plates (662160; Greiner Bio-One) for determination of SOD mRNA and PGF production and 1×10^6 viable cells/ml into 75-cm² culture flasks (658175; Greiner Bio-One) for determination of SOD protein expression and activity in culture cells. LECs were cultured in DMEM/F-12 (D/F; D8900; Sigma-Aldrich) supplemented with 10% calf serum (vol/vol, 16170-078; Life Technologies Inc., Grand Island, NY, USA), 20 µM gentamicin (G1397; Sigma-Aldrich) and 2 µM amphotericin B (A9528; Sigma-Aldrich). The cells reached confluence on the 5th day of culture. After the cells reached confluence, the medium was replaced with fresh D/F supplemented with 5 µM holo-transferrin (T3400; Sigma-Aldrich), 500 µM ascorbic acid (013-12061; Wako Pure Chemical Industries, Osaka, Japan), 5 nM sodium selenite (S5261; Sigma-Aldrich) and 0.1% (w/v) BSA (10 735 078 001; Roche Diagnostics, Mannheim, Germany). To examine whether SOD is differently regulated at the time of functional and structural luteolysis, LECs were cultured with NONOate (a NO donor; 82150;

Cayman Chemical, Ann Arbor, MI, USA) for 2 h (Exp. 1–3) or 24 h (Exp. 4) according to the protocol for each experiment.

Experiment 1: Dose-dependent effects of NONOate on SOD mRNA expression at 2 h

LECs were seeded into 24-well plates for determination of SOD mRNA expression. After reaching confluence, the cells were incubated with or without 1–100 µM NONOate (n=3 experiments).

Experiment 2: Effects of NONOate on SOD protein expression and SOD activity at 2 h

LECs were seeded into 75-cm² culture flasks for determination of protein expression and SOD activity. After reaching confluence, the cells were incubated with or without 10 µM NONOate. The samples were then used for analysis of SOD protein expression and enzyme activity (n=3 experiments).

Experiment 3: Dose-dependent effects of NONOate on PGF production at 2 and 24 h

LECs were seeded into 24-well plates for determination of PGF production in culture medium. After reaching confluence, the cells were exposed to NONOate (1–100 µM). After culture, the conditioned media were collected in 1.5-ml tubes containing 5 µl of a stabilizer solution (0.3 M EDTA, 1% (w/v) acid acetyl salicylic, pH 7.3) and frozen at –30 °C until the PGF assay (n=3 experiments).

Experiment 4: Effects of NONOate on SOD mRNA and protein expressions at 24 h

LECs were seeded into 24-well plates for mRNA and into 75-cm² culture flasks for protein expression. After reaching confluence, the cells were incubated for 24 h with or without 10 µM NONOate. After culture, the cells were used for SOD mRNA and protein expression, respectively (n=3 experiments).

Reverse transcription-PCR

Total RNA was extracted from the LECs using Trizol Reagent according to the manufacturer's directions (15596-026; Invitrogen, Carlsbad, CA, USA). One microgram of each sample of total RNA was reverse transcribed using a ThermoScript™ RT-PCR System (11146-016; Invitrogen), and the reaction mixture was used in each PCR together with the appropriate oligonucleotide primer pairs. The primers for the SOD and *beta-actin* (ACTB) were designed and characterized as described previously (Table 1). The RT-PCRs were conducted with the housekeeping gene ACTB as an internal standard. ACTB primer was added at the appropriate cycle number by the “primer-drop-ping method” as described by Wong *et al.* [23] with our own modification [24]. The PCRs were carried out using TaKaRa Taq (R001A; Takara Bio, Shiga, Japan) and a thermal

Table 1. Primers used in semi-quantitative RT-PCR

Gene	Primer	Sequence (5'–3')	Accession no.	Product (bp)
SOD	Forward	AAGGCCGTCTGCGTGCTGAA	M81129	240
	Reverse	CAGGTCTCCAACATGCCTCT		
ACTB	Forward	CGGCATTACGAAACTACC	AY141970	536
	Reverse	ATCAAGTCCTCGGCCACAC		

cycler (iCycler; Bio-Rad Laboratories, Hercules, CA, USA). The PCR conditions were as follows: activation of DNA polymerase for 20 sec at 95 °C followed by the appropriate number of cycles (see below) of denaturation for 30 sec at 95 °C, annealing for 1 min at 60 °C, and extension for 1 min at 70 °C, followed by final extension for 5 min at 72 °C. The number of cycles was 27 for *SOD* and 25 for *ACTB*.

SOD protein expression

SOD protein expression in cultured bovine LECs was assessed by Western immunoblotting analysis. The cells were lysed in 150 μ l lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 [T8787; Sigma-Aldrich], 10% glycerol [G7757; Sigma-Aldrich], Complete [11 697 498 001; Roche Diagnostics, Basel, Switzerland], pH 7.4). The protein concentrations in the lysates were determined by the method of Osnes *et al.* [25], using BSA as a standard. The proteins were then solubilized in SDS gel-loading buffer (10% glycerol, 1% β -mercaptoethanol [137-06862; Wako Pure Chemical Industries], pH 6.8) and heated at 95 °C for 10 min. Samples (50 μ g protein) were electrophoresed on a 15% SDS-PAGE for 1.5 h at 30 mA. The separated proteins were electrophoretically transblotted to a 0.2- μ M nitrocellulose membrane (LC2000; Invitrogen) at 300 mA V for 3 h in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). The membrane was washed in TBS-T (0.1% Tween 20 in TBS [25 mM Tris-HCl, 137 mM NaCl, pH 7.5]), incubated in blocking buffer (5% nonfat dry milk in TBS-T) for 1 h at room temperature, incubated at 4 °C with a primary antibody specific to each protein (*SOD* antibody [23 kDa; 1:500 in TBS-T, overnight; sc-8637; Santa Cruz Biotechnology, Santa Cruz, CA, USA] and *ACTB* antibody [internal standard, 42 kDa; 1:4000 in TBS-T, overnight; A2228; Sigma-Aldrich]). The membrane was washed three times for 5 min in TBS-T at room temperature, incubated with secondary antibody (for *SOD* [1:10000 in TBS-T], anti-goat Ig, HRP-linked whole antibody produced in donkey, sc-2020, Santa Cruz; for *ACTB* [1:40000 in TBS-T], anti-mouse Ig, HRP-linked whole antibody produced in sheep, NA931, Amersham Biosciences, Buckinghamshire, UK) for 1 h and washed three times in TBS for 5 min at room temperature. The signal was detected using a ECL Western immunoblotting detection system (RPN2109; Amersham Biosciences). The intensity of the immunological reaction was estimated by measuring the optical density in the defined area by computerized densitometry using NIH Image (National Institutes of Health; Bethesda, MD, USA).

SOD activity assay

SOD activity was determined in the LECs at the end of a 2-h incubation period using an *SOD* Assay Kit-WST (S311-08; Dojindo Laboratories, Kumamoto, Japan). *SOD* activity was calculated using a concurrently run *SOD* standard curve, and expressed as percentage of control (raw data on *SOD* activity was normalized based on protein concentrations in units per mg of cellular protein).

Determination of the PGF concentration

The concentration of PGF in the culture medium was determined by enzyme immunoassay (EIA) as described previously [17]. The

PGF standard curve ranged from 15.625 to 4000 pg/ml, and the median effective dose (ED_{50}) of the assay was 250 pg/ml. The intra- and interassay coefficients of variation were 7.4 and 11.6%, respectively. The DNA content, estimated using the spectrophotometric method of Labarca and Paigen [26], was used to standardize the PGF concentrations.

Statistical analysis

Data for *SOD* mRNA, protein level and *SOD* activity were obtained in three separate experiments. Determination of the PGF concentration was performed in triplicate samples for each experimental group. The statistical significances of differences in the amounts of *SOD* mRNA, *SOD* protein levels, activity of *SOD* and differences in PGF concentrations were analyzed using two-way analysis of variance (ANOVA) with repeated-measures or one-way ANOVA followed by Fisher's protected least-significant difference (PLSD) procedure as multiple comparison tests. All values were expressed as means $\pm$ SEM of three separate experiments. A level of $P < 0.05$ was considered to be statistically significant.

Results

Dose-dependent effect of NONOate on SOD mRNA at 2 h

NONOate increased the expression of *SOD* mRNA (240 bp) in a dose-dependent manner ($P < 0.05$, Fig. 1). NONOate at concentration 10 and 100 μ M increased the expression of *SOD* mRNA ($P < 0.05$). Therefore, a concentration of 10 μ M of NONOate was used in the following experiments.

Effect of NONOate on SOD protein and activity at 2 h

NONOate significantly stimulated *SOD* protein expression ($P < 0.05$, Fig. 2A) and *SOD* activity ($P < 0.05$, Fig. 2B).

Fig. 1. Dose-dependent effects of NONOate on *SOD* mRNA expression in cultured bovine LECs. The cells were cultured with or without NONOate (1, 10 and 100 μ M) for 2 h. Photographs show the expression of *SOD* (240 bp) and *ACTB* (536 bp) mRNA. The amount of *SOD* is expressed relative to the amount of *ACTB* mRNA. Asterisks indicate significant differences compared with untreated cells (Control, $P < 0.05$). All values represent means $\pm$ SEM of three separate experiments.

Fig. 2. Effects of NONOate on the expression of SOD protein and SOD activity in cultured bovine LECs. The cells were cultured with or without 10 μ M NONOate for 2 h. (A) Photograph shows the protein expression of SOD (23 kDa) and ACTB (42 kDa). (B) SOD activity was determined by a colorimetric method. Asterisk indicates significant differences compared with untreated cells ($P < 0.05$). All values represent means $\pm$ SEM of three separate experiments.

Effect of NONOate on PGF production

PGF production was simulated by high concentrations, but not by low concentrations of NONOate at 2 and 24 h ($P < 0.05$, Fig. 3).

Effect of NONOate on SOD mRNA and protein expression at 24 h

NONOate decreased SOD mRNA ($P < 0.05$, Fig. 4A) and SOD protein expression ($P < 0.05$, Fig. 4B) in cultured LECs at 24 h.

Discussion

The present study demonstrated that NO has an acute stimulatory effect on the expression of SOD mRNA, protein and SOD activity in cultured bovine LECs. In addition, NO increased PGF production by LECs. These results suggest that NO has a physiological role in regulating SOD in the function of the bovine CL.

In cows, ROS including NO seems to play crucial roles in the luteolytic action of PGF. NO has been shown to decrease P₄ biosynthesis [9, 11, 17] and to induce apoptosis in LSCs [11]. In

Fig. 3. Effects of NONOate on PGF production by bovine LECs. The cells were cultured with or without NONOate (1, 10 and 100 μ M) for 2 and 24 h. All values represent means $\pm$ SEM of five separate experiments. Asterisks indicate significant differences compared with untreated cells ($P < 0.05$).

addition, intraluteal SOD regulates luteal ROS production [16]. In the present study, NO stimulated SOD expressions and SOD activity at 2 h in cultured bovine LECs. Increased SOD protects cells from excessive ROS generation [14]. Moreover, ROS increased SOD activity and mRNA expression in human endothelial progenitor cells [27]. Also, an increase in SOD activity and a decrease in catalase activity have been demonstrated during the loss of luteal function in mice [28]. Previous studies have shown that PGF stimulates NO synthesis in the bovine *in vivo* [13] and in LECs *in vitro* [7]. The above findings suggest that a local increase in NO production induced by PGF within the bovine CL affects the function of the CL and that the acute elevation of SOD represents a response of LECs to protect themselves against oxidative stress induced by PGF treatment at the beginning of functional luteolysis.

In the present study, NO stimulated PGF production in bovine LECs. These results were consistent with our previous reports [9]. NO produced locally has been shown to decrease P₄ in the bovine CL during the late luteal phase [29]. NO has been demonstrated to be a potent mediator of the luteolytic action of PGF *in vivo* [29–31]. A possible interrelationship between PGF and ROS in the bovine ovary has been suggested by our previous *in vivo* [13] and *in vitro* [7] studies, in which PGF increased NO and pO₂ in bovine ovarian venous blood and NO production in cultured LECs, respectively.

Fig. 4. Effects of NONOate on the expression of SOD mRNA and protein in cultured bovine LECs. The cells were cultured with or without 10 μ M NONOate for 24 h. (A) Photograph shows the expression of SOD (240 bp) and ACTB (536 bp) mRNA and (B) the protein expression of SOD (23 kDa) and ACTB (42 kDa). The amount of SOD is expressed relative to the amount of ACTB. Asterisks indicate significant differences between untreated and treated cells ($P < 0.05$). All values represent means $\pm$ SEM of three separate experiments.

The above evidences strongly suggest that NO has the ability to increase intraluteal PGF production in a paracrine and/or autocrine manner.

Although luteolytic PGF is derived from the uterus, in many species, including women [32], cows [33], ewes [34] and rats [35], a considerable amount of PGF is also synthesized by the CL [32–36]. Some studies have proposed that endometrial/extraluteal PGF initiates functional luteolysis, whereas luteal PGF may contribute to structural luteolysis [36, 37]. Thus, a local increase of luteal PGF within the CL seems to facilitate regression of the CL. Furthermore, because abundant NOS enzymes have been found in ECs [38], the endothelium is recognized to be one of the main sources of NO. PGF and NO may interact and activate LECs and non-LECs to initiate functional and structural luteolysis, as both are produced by LECs. Interestingly, PGF stimulates NO production in cultured

bovine LECs [7]. On the other hand, NO increases PGF production in both LSCs [9] and LECs (the present study). The above findings strongly suggest the presence of a positive feedback loop between PGF and NO in bovine LECs when luteolysis occurs.

The present study demonstrated that NO increased PGF production but reduced the expression of SOD mRNA and protein in bovine LECs cultured for 24 h. The inhibitory effects of NO on SOD expression at 24 h contrast with those at 2 h. The increase in SOD observed at 2 h seems to be the result of an acute stimulatory effect of PGF on ROS production by LECs. However, the mechanism by which NO inhibits the expression of SOD at 24 h of incubation is unclear. SOD catalyzes the dismutation of superoxide into H_2O_2 and oxygen to maintain low-state levels of superoxide [14]. Therefore, a reduction of SOD by NO at 24 h may enhance intraluteal ROS or superoxide radical accumulation for the promotion of structural luteolysis [10, 39–41]. In support of such an idea, accumulation of superoxide radicals and decrease in SOD levels are associated with the inhibition of luteal P_4 secretion [16] and apoptotic cell death [39, 40].

In conclusion, NO differently regulates SOD in cultured LECs, depending on the exposure time. Acute elevation of SOD may represent a response of LECs to protect themselves against oxidative stress induced by PGF during functional luteolysis, whereas a later reduction of SOD levels by NO may facilitate an excess of intraluteal ROS during structural luteolysis.

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