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Tumor Necrosis Factor- α (TNF α) Inhibits Progesterone and Estradiol-17 β Production from Cultured Granulosa Cells: Presence of TNF α Receptors in Bovine Granulosa and Theca Cells

Ryosuke SAKUMOTO^{1,2)}, Masami SHIBAYA¹⁾ and Kiyoshi OKUDA¹⁾

¹⁾Laboratory of Reproductive Endocrinology, Faculty of Agriculture, Okayama University, Okayama 700-8530, ²⁾Laboratory of Animal Neurophysiology, Department of Physiology and Genetic Regulation, National Institute of Agrobiological Sciences, Ibaraki 305-8602, Japan

Abstract. The aim of this study was to investigate whether functional tumor necrosis factor- α (TNF α) receptors are present in the granulosa cells and the cells of theca interna (theca cells), obtained from bovine follicles classified into one of three groups. Each group was defined as either small vesicular ovarian follicles (small follicles; 3–5 mm in diameter), preovulatory mature ovarian follicles (preovulatory follicles) or atretic follicles (12–18 mm) according to gross examination of the corpus luteum in the ipsilateral or contralateral ovary and the uterus (size, color, consistency and mucus), and the ratio of progesterone (P₄) and estradiol-17 β (E₂) concentrations in follicular fluid. A Scatchard analysis showed the presence of a high-affinity binding site on both granulosa and theca cells from all follicles examined (dissociation constant: 4.7 ± 0.15 to 6.9 ± 1.40 nM). Moreover, TNF α receptor concentrations in granulosa and theca cells obtained from atretic follicles were significantly higher than those in the cells from preovulatory follicles ($P < 0.05$). Exposure of cultured granulosa cells from small antral follicles to recombinant human TNF α (rhTNF α ; 0.06–6 nM) inhibited E₂ secretion in a dose-dependent fashion ($P < 0.01$), but did not affect P₄ secretion. In addition, rhTNF α inhibited follicle stimulating hormone-, forskolin- or dibutyryl cyclic AMP-induced P₄ and E₂ secretion by the cells ($P < 0.01$). These results indicate the presence of functional TNF α receptors in bovine granulosa and theca cells in small, preovulatory and atretic follicles, and suggest that TNF α plays a role in regulating their secretory function.

Key words: Tumor necrosis factor- α , Receptor, Ovary, Bovine

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Tumor necrosis factor- α (TNF α) has been identified in the follicles of many species, including rats [1], mice [2], rabbits [3] and humans [4]. Furthermore, it has been shown that TNF α modulates basal or gonadotropin-induced steroidogenesis and protein production in granulosa and theca cells *in vitro* [5]. Therefore, it is

likely that locally produced TNF α plays a physiological role in the regulation of ovarian function in several species.

Immunoreactive or bioactive TNF α is present in bovine follicles during follicular development [6]. It has been demonstrated that TNF α inhibits follicle stimulating hormone (FSH)-, insulin- or insulin-like growth factor-I (IGF-I)-induced estradiol-17 β (E₂) production by granulosa cells of the small antral follicles [7, 8]. In addition, TNF α inhibits luteinizing hormone (LH)-stimulated

androstenedione [8], and prorenin and renin [9] secretion by theca cells. These observations suggest that TNF α plays more than one physiological role in the regulation of bovine follicular cell function as seen in other species. Therefore, if TNF α is to play a role in bovine follicular function or development, functional TNF α receptors should be expressed by granulosa and theca cells during follicular development. Recently, a specific binding site for TNF α has been found in both bovine granulosa cells of the small antral follicles and theca cells of the large follicles [8], suggesting that TNF α affects cell function via these receptors, but the expression of TNF α receptors has yet to be determined in granulosa cells of the large follicles and in theca cells of the small follicles.

Therefore, the present study investigated whether TNF α receptors are present in granulosa and theca cells of bovine small vesicular follicles (small follicles), preovulatory mature follicles (preovulatory follicles) and atretic follicles. To determine whether functional TNF α receptors are present, both the effects of TNF α on progesterone (P₄) and E₂ production, and effects on intracellular signaling pathways of cultured granulosa cells of small antral follicles were investigated. A greater understanding of ovarian TNF α receptor function will provide support for the hypothesis that TNF α plays more than one role in bovine ovarian physiology.

Materials and Methods

Collection of bovine granulosa and theca cells

Bovine ovaries were obtained at a local abattoir within 10–20 min after exsanguination, and were transported to the laboratory in a thermos flask containing 0.85% (w/v) NaCl at 30–37 C. Follicles were carefully selected and classified into three groups. The groups were defined as small follicles (3–5 mm in diameter), preovulatory follicles or atretic follicles (10–18 mm) according to a gross examination of the corpus luteum in the ipsilateral or contralateral ovary and the uterus (size, color, consistency and mucus). Classification was also determined from the ratio of P₄ and E₂ concentrations in follicular fluid as described previously [10, 11]. The follicular fluid in the largest follicles, regarded as preovulatory, was E₂-inactive (post-gonadotropin surge). The granulosa

cells were collected and prepared as described previously [11]. The theca cells were prepared according to the following procedure. Follicles were cut into hemispheres, and the theca interna cell layer was microdissected away from the theca externa and cleaned of any adhering granulosa cells. The theca interna cell layers were minced into small pieces. The granulosa and theca cells were frozen and stored at –80 C until processed for the study of specific binding of TNF α . For experiments involving cell culture, the granulosa cells of small antral follicles were processed as described previously [11].

Membrane preparation

Granulosa and theca cells were thawed and minced with scissors in ice cold 25 mM Tris-HCl containing 300 mM sucrose, 2 mM EDTA, 3 mM dithiothreitol (#D0632, Sigma-Aldrich Co., St. Louis, MO, USA), 500 kIU/ml aprotinin (#A6279, Sigma-Aldrich) and 0.5 mM PMSF, pH 7.4 (#P7625, Sigma-Aldrich), and then homogenized in the same buffer with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) in three 10-sec bursts, separated by a one min cooling period in ice. For each group of follicles, four pools were prepared. Each pool contained from five to ten follicles.

The homogenate of these cells was subsequently centrifuged at 800 $\times$ g for 10 min to remove tissue debris, and the supernatant collected and centrifuged at 30,000 $\times$ g for 20 min to obtain the plasma membrane pellet. The pellets were then resuspended and centrifuged in the same buffer to dissociate TNF α from their binding sites. The pellets were then washed three times, and centrifuged for 10 min at 30,000 $\times$ g, decanted and resuspended in 25 mM Tris-HCl containing 10 mM MgCl₂ (pH 7.4). All steps in the membrane preparations were conducted at 4 C. The protein concentration of the membrane preparations was determined by the method described by Lowry *et al.* [12] with BSA (#735078, Boehringer Mannheim GmbH, Mannheim, Germany) as a standard. The preparation was diluted to give a protein concentration of 1 mg/ml of cell membrane with 25 mM Tris-HCl containing 10 mM MgCl₂ and 0.5% (w/v) BSA (pH 7.4).

Radioreceptor assay

Recombinant human TNF α (rhTNF α ; Lot. No. HF-13; kindly donated by Dainippon

Pharmaceutical Co., Ltd., Osaka, Japan) was iodinated with carrier-free Na¹²⁵I (IMS 30; Amersham International, Buckinghamshire, England) by the iodogen method as described previously [13]. The specific activity of ¹²⁵I-rhTNF α ranged between 390 and 472 Ci/mmol and the maximum bindability was 28%. Preliminary studies with granulosa membranes from small antral follicles were carried out to establish the optimal incubation time and temperature for maximum binding of ¹²⁵I-rhTNF α to the membranes. To reduce nonspecific binding, glass microtubes (12 $\times$ 75 mm; MLB Culture Tube, Ontario, Canada) were coated overnight with complete calf serum (#C6278, Sigma-Aldrich) and binding assays started. Non-specific binding was assessed for each level of tracer through co-incubation with a 240-fold excess of unlabeled rhTNF α (120 nM; 10 μ l). The incubation mixture consisted of approximately 5 $\times$ 10⁴ dpm (0.5 nM) ¹²⁵I-rhTNF α (50 μ l) and 15- μ g proteins (50 μ l). The total volume of the mixture was 110 μ l. The specificity of ¹²⁵I-rhTNF α binding was determined by incubating increasing amounts of unlabeled recombinant human interleukin-1 α (rhIL-1 α ; Lot. No. HL-18; kindly donated by Dainippon Pharmaceutical Co., Ltd.) or recombinant bovine interferon- α (rbIFN α ; kindly donated by Novartis Pharmaceutical Co., Basel, Switzerland) with a constant amount of ¹²⁵I-rhTNF α (5 $\times$ 10⁴ dpm/tube). All reagents were prepared in 10 mM Tris containing 10 mM MgCl₂ (pH 7.5), 3.0 mM NaN₃ (#S2002, Sigma-Aldrich) and 0.1% (w/v) BSA.

Incubation was terminated by transferring the tubes into ice-cold water and by adding the same buffer into the assay tube. Bound and free tracers were separated by centrifugation at 3,000 $\times$ g for 40 min at 4 C. Supernatants were decanted immediately, and ¹²⁵I measured in the pellets with a γ -counter (Pharmacia-Wallac 1282, Compugamma CS, Turku, Finland) at an efficiency of 82%. Non-specific bindings accounted for less than 25% of total binding.

Granulosa cell culture

Viable cells (2.5 $\times$ 10⁵/well) from small antral follicles were cultured in a culture medium (Dulbecco's Modified Eagle's Medium and Ham's F-12 medium, 1:1 [v/v]; #D8900, Sigma-Aldrich) supplemented with 10% calf serum and 20 μ g/ml gentamicin (#15750-011, Gibco BRL, Rockville, MD,

USA) in 24-well culture plates (#3526, Costar, Cambridge, MA, USA) for up to 48 h in a humidified atmosphere of 5% CO₂ in air at 37.5 C. In the final 24 h of culture, the cells were exposed to varying concentrations of rhTNF α (0.06–6 nM) with or without ovine FSH (0.03–3 nM), bovine LH (0.03–3 nM), forskolin (0.01–10 μ M; #F-105, Research Biochemicals International, Natick, MA, USA), or dibutyryl cyclic AMP (dbcAMP, 1–1,000 μ M; #CN-125, BIOMOL[®]; Research Laboratories Inc., Plymouth Meeting, PA, USA). The conditioned media were collected and stored at –30 C until assays were performed for P₄ and E₂. Since granulosa cells proliferated in these culture conditions, the DNA content of the granulosa cells was estimated and used to standardize the results [14].

Enzyme immunoassay

The concentration of P₄ was determined directly from the cell culture media with an enzyme immunoassay [11]. The samples for the P₄ assay were diluted 200 times with assay buffer. The standard curve ranged from 0.39 to 100 ng/ml and the effective dose for 50% inhibition (ED₅₀) of the assay was 9.56 ng/ml. The intra- and interassay coefficients of variation were on average 6.8 and 9.6%, respectively.

The concentration of E₂ was determined directly from the cell culture media with an enzyme immunoassay [15] with a minor modification with peroxidase-labeled E₂ and E₂ antiserum (GDN#244; kindly donated by Dr. Niswender, Colorado State University). Cross-reactivities of the E₂ antiserum, validated by comparison of the inhibition of binding of peroxidase-labeled E₂ were: 100% for E₂, 1.06% for estriol, 0.26% for estrone, 0.016% for androstenedione, 0.0013% for androsterone, 0.047% for testosterone, 0.0002% for pregnenolone, 0.007% for P₄, <0.0002% for cortisol, and <0.0004% for deoxycorticosterone. The E₂ standard curve ranged from 31.25 to 8,000 pg/ml, and the ED₅₀ of the assay was 750 pg/ml. The intra- and interassay coefficients of variation were on average 5.8 and 8.6%, respectively.

Statistical analysis

All experimental data are shown as the mean $\pm$ SEM. The data on rhTNF α binding to granulosa and theca membranes were analyzed with the LIGAND program [16] and nonlinear iterative

curve-fitting procedures [17]. The initial parameters were calculated by Scatchard analysis [18] and were then iteratively refined until minimization of the weighted sum of squares. The goodness of fit for the selected model was analyzed by a runs test. Different models (one or two binding sites) were compared with F-test statistics to determine whether a change in the model resulted in a significant reduction in the weighted sum of squares. The criteria for rejecting or accepting a particular model were based on the calculated probability values [16]. The statistical significance of differences in the binding parameters of TNF α receptors and the concentrations of P₄ and E₂ in culture media were assessed by an ANOVA followed by a Fisher's protected least significant difference procedure (PLSD) as a multiple comparison test.

Results

Binding characteristics

A preliminary assay of rhTNF α binding to bovine granulosa membranes was carried out to test the conditions for the radioreceptor assay described in Materials and Methods. Maximum binding was reached after 48 h at 38 C (Fig. 1a). Specific binding increased with higher protein concentrations with a linear relationship for the amount of binding from 1 to 25 μ g per 50 μ l (Fig. 1b). Figure 1c shows the displacement curves of ¹²⁵I-rhTNF α with two related peptides. The binding was highly specific for rhTNF α with little or no competition for rhTNF α binding sites by rbIFN α or rhIL-1 α . Scatchard plots of the binding data were linear, and analysis with the LIGAND program revealed the expression of TNF α receptors on both granulosa and theca membranes obtained from each group of follicles. The dissociation constant (K_d) values were similar for the membranes from all groups. The concentrations of TNF α receptor on bovine granulosa and theca membranes contained fewer preovulatory follicles than those in small antral follicles (Fig. 2). The TNF α receptor concentrations of granulosa and theca membranes from atretic follicles were similar to those in the small follicles, but were significantly higher than those in the preovulatory follicles (Fig. 2; $P < 0.05$).

Fig. 1. Relationship between the binding of ¹²⁵I-rhTNF α and (a) incubation time of 22 C or 38 C, (b) membrane concentrations of the granulosa cells from bovine small follicles. The difference in the binding of ¹²⁵I-rhTNF α bound in the presence of 120 nM rhTNF α and in the absence of rhTNF α used to calculate the specific binding, was expressed as a percentage of total ¹²⁵I-rhTNF α (6×10^4 dpm/tube; 0.5 nM) added. (c) Competitive binding of ¹²⁵I-rhTNF α and various unlabeled peptides on the membranes of granulosa cells from bovine small follicles. rbIFN α = recombinant bovine interferon- α , rhIL-1 α = recombinant human interleukin-1 α .

Fig. 2. Concentrations of TNF α receptor on granulosa and theca membranes obtained from bovine small, preovulatory or atretic follicles. Different superscript letters indicate significant differences ($P < 0.05$ or lower) as determined by an ANOVA followed by a Fisher's PLSD as a multiple comparison test.

Effects of TNF α on P_4 and E_2 secretion by bovine granulosa cells

Basal and FSH-stimulated E_2 secretion by the cultured granulosa cells was inhibited by rhTNF α (Fig. 3; $P < 0.05$). Although rhTNF α did not affect basal P_4 secretion by the cells, FSH-induced P_4 secretion was significantly inhibited by rhTNF α ($P < 0.05$). Secretion of E_2 and P_4 was not altered by the addition of LH, but rhTNF α inhibited basal and LH-stimulated E_2 secretion by these cells (Fig. 4; $P < 0.05$). To determine the intracellular signaling pathway activated by rhTNF α in bovine granulosa cells, we investigated the effects of rhTNF α on forskolin- or dbcAMP-induced P_4 and E_2 secretion. Recombinant human TNF α significantly inhibited both forskolin- and dbcAMP-induced P_4 and E_2 secretion in a dose-dependent fashion (Figs. 5 and 6; $P < 0.01$).

Discussion

The present study demonstrates that specific

Fig. 3. Effects of rhTNF α on basal and FSH-induced P_4 and E_2 secretion by cultured granulosa cells obtained from bovine small antral follicles. The cells were exposed to rhTNF α in the final 24 h of culture. Different superscript letters indicate significant differences ($P < 0.05$ or lower), as determined by an ANOVA followed by a Fisher's PLSD as a multiple comparison test.

Fig. 4. Effects of rhTNF α on basal and LH-induced P₄ and E₂ secretion by cultured granulosa cells obtained from bovine small antral follicles. The cells were exposed to rhTNF α in the final 24 h of culture. Different superscript letters indicate significant differences ($P < 0.05$ or lower), as determined by an ANOVA followed by a Fisher's PLSD as a multiple comparison test.

Fig. 5. Effects of rhTNF α on forskolin-induced P₄ and E₂ secretion by cultured granulosa cells obtained from bovine small antral follicles. The cells were exposed to rhTNF α in the final 24 h of culture. Different superscript letters indicate significant differences ($P < 0.05$ or lower), as determined by an ANOVA followed by a Fisher's PLSD as a multiple comparison test.

binding sites for TNF α are present in granulosa and theca cells of bovine small, preovulatory and atretic follicles. In addition, the expression of functional TNF α receptors in granulosa cells was confirmed, since rhTNF α inhibited both basal and FSH-stimulated E₂ and P₄ secretion by cultured granulosa cells in a dose-dependent fashion. The affinities and concentrations of TNF α receptors in bovine granulosa and theca cells from all follicles examined in this study ranged between 4.7–6.9 nM and 13.7–31.3 pmol/mg proteins, respectively. These values are comparable with the

characteristics of TNF α receptors in the granulosa cells of pigs [19] and cows [8], and in the corpus luteum of pigs [20] and cows [13]. Previous studies have shown that bovine follicular fluid contains TNF α -like activity, and that the levels of TNF α measured in bovine follicular fluid [21] and serum [22] are comparable with the effective doses of TNF α and the affinity of TNF α receptors described in the present study. Collectively, locally produced TNF α in bovine follicles may act as an autocrine and/or paracrine factor regulating follicular cell function.

Fig. 6. Effects of rhTNF α on dibutyl cyclic AMP (dbcAMP)-induced P₄ and E₂ secretion by cultured granulosa cells obtained from bovine small antral follicles. The cells were exposed to rhTNF α in the final 24 h of culture. Different superscript letters indicate significant differences ($P < 0.05$ or lower), as determined by an ANOVA followed by a Fisher's PLSD as a multiple comparison test.

TNF α has been shown to inhibit basal and FSH-stimulated E₂ secretion in rat [23], human [24] and bovine [7, 8] granulosa cells. Furthermore, TNF α suppresses aromatase activity in rat [23] and human [24] granulosa cells. As expected, rhTNF α inhibited basal, FSH-, forskolin- or dbcAMP-induced E₂ secretion by cultured granulosa cells in the present study, suggesting that TNF α inhibits aromatase activity in bovine granulosa cells, as has been seen in other species. In contrast, rhTNF α inhibited FSH-, forskolin- or dbcAMP-stimulated P₄ secretion by granulosa cells (Figs. 3, 5 and 6), but

not basal and LH-stimulated P₄ secretion (Fig. 4). Similar findings have been observed in a previous study showing TNF α inhibited gonadotropin-dependent P₄ production in rat [25], porcine [19] and human [26] granulosa cells. Since TNF α inhibits cAMP accumulation and protein kinase A activity in rat granulosa cells [25], TNF α may inhibit FSH-induced adenylate cyclase-protein kinase A activity as well as aromatase activity, resulting in reduced E₂ and P₄ secretion by bovine granulosa cells.

It has been demonstrated that TNF α inhibits E₂ secretion by granulosa cells from small follicles, but not from large follicle cells [7]. In the present study, we found that the concentrations of TNF α receptors in the granulosa cells from preovulatory follicles were lower than those in the cells from small follicles. The lack of the effects of TNF α observed by Spicer and Alpizar [7] might be explained by the smaller number of TNF α receptors expressed by granulosa cells in large follicles. TNF α has been implicated in the mechanisms of ovulation in rats [27] and ewes [28]. TNF α conceivably plays a role in the mechanisms of ovulation in cows as well as in rats and ewes. For the present study, the largest follicles regarded as preovulatory were first divided into two groups by the criteria of Junglas and Luck [10]: E₂-active (pre-gonadotropin surge) or E₂-inactive (post-gonadotropin surge). We then selected the E₂-inactive group of follicles. Nevertheless, we have shown that TNF α receptor concentrations in the follicular cells are low in preovulatory follicles. One possible explanation for this phenomenon is the reduction of TNF α receptors. Bioactive TNF α in bovine follicles increased during Days 3 to 20 of the estrous cycle [21], and the expression of TNF α receptors was reduced by TNF α itself [29], so that, as shown in this study, the low levels of TNF α receptor expression in the cells of preovulatory follicles might arise from a local TNF α -mediated reduction.

Two immunologically distinct TNF α Receptors of approximately 55 kDa (Type I) and 75 kDa (Type II) have now been identified [30]. Type I and II receptors have different intracellular signaling pathways [31]. Type I receptor contains an intracellular death domain, which is required for signaling pathways associated with apoptosis. In contrast, type II receptor can induce gene transcription for cell survival, growth, and differentiation. In this study, TNF α receptor

concentrations in granulosa and theca cells of atretic follicles were higher than those in preovulatory follicles. It is generally accepted that TNF α induces apoptosis in various cell types by activating type I receptor, and that cytokines, including TNF α , stimulate apoptosis in follicular cells to establish ovarian atresia in many species [32, 33]. Since TNF α is present in bovine atretic follicles [6], the results of our binding study support the idea that TNF α mediates apoptosis at the time of follicle atresia mediated by TNF α receptors present on the plasma membrane.

In conclusion, the overall results of the present study indicate the presence of functional TNF α receptors in granulosa and theca cells of bovine small, preovulatory and atretic follicles, and suggest that TNF α plays a role in regulating the function of granulosa and theca cells via its specific receptors. Since TNF α receptor concentrations in granulosa and theca cells from small and atretic

follicles were higher than the concentration in these cells from preovulatory follicles, TNF α may suppress early follicular growth and/or induce follicle atresia in cows.

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