STUDIES ON TUMOR NECROSIS FACTOR-α AND ITS RECEPTOR IN BOVINE CORPUS LUTEUM

March 2000

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The Graduate School of Natural Science and Technology (Doctor Course) OKAYAMA UNIVERSITY
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PREFACE

The experiments described in this dissertation were carried out at the Graduate School of Natural Science and Technology (Doctor Course), Okayama University, Japan, from April 1996 to October 1999, under the supervision of Professor K. OKUDA. These studies are original works by the author and any assistance and collaboration from others are specially acknowledged.

This dissertation has not been submitted previously whole or in part to a council, a University or any other professional institution for degree, diploma or other professional qualification.

Ryosuke SAKUMOTO
March, 2000
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ABSTRACT

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The objective of this study was to investigate the physiological roles of tumor necrosis factor α (TNFα) in bovine corpus luteum (CL) function throughout the estrous cycle and the entire gestation period. In the first series of experiments, the expression of TNFα, the presence of functional TNFα receptors, and the expression of TNF receptor type I (TNF-RI) mRNA in the CL during different stages of the estrous cycle were examined. RT-PCR showed no difference in TNFα mRNA expression during the estrous cycle. Concentrations of TNFα in the CL tissue increased significantly from the mid- to the late luteal stage and decreased thereafter. A RT-PCR analysis showed higher levels of TNF-RI mRNA in CL of days 3-7 than in other stages. 125I-TNFα binding to the membranes of bovine CL was maximal after incubation at 38 C for 48 h. The binding was much greater for TNFα than for related peptides. A Scatchard analysis revealed the presence of a high-affinity binding site in the CL membranes collected at each phase of the estrous cycle (dissociation constant (Kd); 3.60±0.58 - 5.79±0.19 nM). In contrast to TNF-RI mRNA expression, the levels of receptor protein were similar at each stage in the estrous cycle. When cultured cells of all luteal stages were exposed to TNFα (0.06-6 nM), TNFα stimulated prostaglandin (PG) F2α and PGE2 secretion by the cells in a dose-dependent fashion, especially during the early luteal phase, although it did not affect progesterone secretion.

In the second series of experiments, the presence of TNFα mRNA and TNFα receptors in the bovine CL during the gestation period were investigated. The presence of TNFα mRNA and TNFα receptors on bovine CL from pregnant cows was investigated at three stages: trimesters I, II and III. TNFα mRNA was detected by an RT-PCR analysis in the CL of all stages of gestation. A Scatchard analysis revealed the presence of a high-affinity binding site (Kd; 5.1-6.9 nM) in the CL membranes collected at each stage of gestation. Furthermore, the concentrations of TNFα receptors in the CL of trimesters I (24.0±1.95 pmol/mg protein) and III (21.6±2.39 pmol/mg protein) of gestation were significantly higher than the concentration in trimester II (14.9±2.07 pmol/mg protein).

In the third series of experiments, I investigated the presence of functional TNFα receptors on the microvascular endothelial cells derived from developing bovine CL. TNFα receptors were analyzed by a radioreceptor assay using 125I-labeled TNFα on two types of cultured endothelial cells. One has a cobblestone appearance (CS cells), and the other has a tube-like structure (TS cells). 125I-labeled TNFα binding was maximal after incubation for 30
h at 37 C, and the specificity of binding was confirmed. A Scatchard analysis showed the presence of two binding sites (high- and low-affinity) for TNFα receptors on both CS and TS cells. The Kd values and concentrations of the high-affinity binding sites for TNFα receptors were similar between CS and TS cells. However, Kd values and concentrations of the low-affinity binding sites in CS cells were significantly higher than those in TS cells. The expression of TNF-RI mRNA was determined in both cell types. Furthermore, TNFα significantly stimulated PGE2 and endothelin-1 secretion by both CS and TS cells.

The final series of experiments were conducted to clarify the intracellular signaling pathway of TNFα to stimulate PGF2α production in cultured bovine luteal cells. Bovine luteal cells that were obtained from mid- (days 8-12 after ovulation) CL were incubated with TNFα (0.6 nM) and/or various compounds as follows: U-73122 (a phospholipase (PL) C inhibitor), ACA (a PL-A2 inhibitor), H-89 (a protein kinase (PK) A inhibitor), calphostin C (a PK-C inhibitor), L-NAME/L-NORG (a nitric oxide synthase inhibitor), PD98059 (a mitogen-activated protein kinase (MAPK) kinase inhibitor). U-73122 (0.1-10 μM), H-89 (0.1-10 μM), calphostin C (0.01-1 μM) and L-NAME/L-NORG (1-100 μM) did not affect TNFα-induced PGF2α secretion by the cultured cells. In contrast, ACA (1-100 μM) and PD98059 (0.1-100 μM) inhibited TNFα-stimulated PGF2α secretion by the cells in a dose-dependent fashion.

The overall results in the present study indicate the local production of TNFα and the presence of functional TNF-RI in bovine CL throughout the estrous cycle and entire gestation period, and suggest that TNFα plays some roles as a paracrine factor in regulating bovine CL function. Furthermore, the present results indicate the presence of two types of TNF receptors and the expression of TNF-RI mRNA in the endothelial cells derived from bovine CL, suggesting TNFα plays two or more roles in regulating the secretory function of the endothelial cells. Finally, the present study also showed that the stimulatory effect of TNFα on PGs secretion by bovine luteal cells might be mediated via activation of the MAPK and PL-A2 pathways.
INTRODUCTION

Tumor necrosis factor \(\alpha\) (TNF\(\alpha\)) is a nonglycosylated protein with a molecular weight of 17 kDa, which was first described as a tumoricidal factor produced by activated macrophages (Carswell et al., 1975). Extensive research during the last decade suggests that TNF\(\alpha\) plays one or more physiological roles in the corpus luteum (CL) in a variety of species. It has been demonstrated that TNF\(\alpha\) inhibits gonadotropin-supported progesterone production by murine (Adashi et al., 1990), porcine (Pitzel et al., 1993) and bovine (Benyo and Pate, 1992) luteal cells and stimulates prostaglandin synthesis by bovine luteal cells (Benyo and Pate, 1992). These findings imply that the actions of TNF\(\alpha\) on CL function are concerned with luteal regression. Indeed, the interaction of TNF\(\alpha\) and inflammatory cells has been postulated to promote the regression of the CL (Bagavandoss et al., 1990). Hence, if TNF\(\alpha\) has one or more roles in luteolysis, functional TNF\(\alpha\) receptors should be present in CL at least by that time.

It has been well demonstrated that TNF\(\alpha\) affects steroidogenesis and protein secretion in bovine granulosa and theca cells (Spicer and Alpizar, 1994, Brunswig-Spickenheier and Mukhopadhyay, 1993). Similar results have been observed in many species including rat (Emoto and Baird, 1988), pig (Tekpetey et al., 1993) and human (Terranova et al., 1991). Furthermore, Wang et al. (1992) demonstrated the presence of TNF\(\alpha\) in follicular fluid in human ovary, and demonstrated that TNF\(\alpha\) increases proliferation in granulosa-luteal cells taken from hCG-treated women before ovulation. These results strongly suggest that TNF\(\alpha\) plays physiological roles in follicular development and luteal development as well as in luteal regression.

On the other hand, the establishment of pregnancy is the result of a number of interactions between the conceptus and its dam. When pregnancy is established, the CL is saved and continuously produces progesterone to maintain the pregnancy. Hence, if TNF\(\alpha\) plays a key role in the mechanism of luteolysis, the CL would have a mechanism to protect itself from the luteolytic actions of TNF\(\alpha\) at the time of maternal recognition and during the gestation period. In pig, the degree of apoptotic cell death was found to be lower in the early pregnant CL than in the mid- and late CL of the estrous cycle, and the levels of TNF\(\alpha\) gene expression in the early pregnant CL were significantly lower than those in the late CL (Wuttke et al., 1997). Thus, I hypothesize that one or more mechanisms that protect against TNF\(\alpha\), e.g., a lack of TNF\(\alpha\) or TNF\(\alpha\) receptors, might be present in bovine CL in the gestation period.

Although, as mentioned above, TNF\(\alpha\) modulates the functions of steroidogenic cells in the bovine ovary and CL, the local production of TNF\(\alpha\) and the existence of TNF\(\alpha\) receptors in bovine ovary (CL as well as follicle cells) is not established. Therefore, the present study was conducted to determine the physiological meanings of TNF\(\alpha\) in regulating
bovine CL function with the following specific aims: 1) to identify the presence of TNFα and its specific binding sites in bovine cyclic and pregnant CL, 2) to examine the effects of TNFα on hormone secretion by cultured bovine luteal cells, 3) to investigate the presence of TNFα receptors and its roles in endothelial cells derived from bovine CL, and 4) to clarify the intracellular signaling pathways of TNFα in bovine luteal cells.
CHAPTER 1

GENERAL METHODOLOGY

Chemicals

Recombinant human TNFα (Lot. No. HF-13) and recombinant human interleukin-1α (IL-1α: Lot. No. HL-18) were kindly donated by Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Recombinant bovine interferon-α (IFNα) was kindly donated by Novartis Pharmaceutical Co. (Basel, Switzerland). Transforming growth factor-α (TGFα: #GE003) was purchased from Wakunaga Pharmaceutical Co., Ltd. (Osaka, Japan). Bovine LH (USDA-bLH-B6) was kindly donated by the USDA Animal Hormone Program. Prostaglandin (PG) F2α (#P7652), PGE2 (#P0409), calf serum (#C6278), dithiothreitol (DTT: #D0632), aprotinin (#A6279), NaN3 (#S2002) and phenylmethylsulfonyl fluoride (PMSF: #P7625) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Collection of Bovine Corpora Lutea

Ovaries with CL from Holstein cows were collected at a local abattoir within 10-20 min after exsanguination. The luteal stage was classified as early (days 2-3), developing (days 5-6), mid- (days 8-12), late (days 15-17) or regressed (days 19-21) stage by macroscopic observation of the ovary as described previously (Ireland et al., 1980, Okuda et al., 1988). The gestational ages were determined from fetal crown-rump length (Sweet et al., 1948; Eley et al., 1978) and classified as trimester I (6-20 cm), trimester II (25-45 cm) or trimester III (50-80 cm). After determination of the stages, corpora lutea were separated immediately from the ovaries, frozen rapidly in liquid nitrogen and then stored at -80 C until processed for studies of specific binding of TNFα and gene expression. For experiments involving cell culture, the ovaries with CL were submerged in ice-cold physiological saline, and transported to the laboratory.

Preparation of Luteal Cells

Luteal cells were prepared and cells were cultured as previously described (Okuda et al., 1992). Briefly, corpora lutea were perfused for 15 min with EGTA-buffer (0.1 mM EGTA (#E4378, Sigma), 10 mM Hepes (#H9136, Sigma), 140 mM NaCl, 7.1 mM KCl (pH 7.4)) to remove vascular blood and to loosen the connection between the vascular endothelial cells. Then, corpora lutea were perfused for 15 min with wash buffer (10 mM Hepes, 140 mM NaCl, 7.1 mM KCl, 5.0 mM CaCl2; pH 7.4). These perfusion buffers were bubbled with 5% CO2:95% O2 during perfusion. The dissociation of the cells was achieved by perfusing for 30 min with wash buffer containing 0.05% (w:v) collagenase (#C0130, Sigma)
and 0.1% (w:v) BSA (#735078, Boehringer Mannheim GmbH, Mannheim, Germany). The cells were dispersed from the CL matrix with steel combs to remove connecting tissues. Finally, the dissociated luteal cells were pooled and stirred for 30 min in Dulbecco’s Modified Eagle’s Medium (DMEM; #D1152, Sigma) containing 0.05% collagenase, 0.005% DNase I (#D5025, Sigma), and 0.1% BSA in a water bath at 37°C. After stirring, cells were filtered through metal wire meshes (200 μm, 150 μm, and 80 μm) to remove undissociated tissue fragments. The filtrate was washed three times by centrifugation for 10 min at 50 xg with the DMEM, supplemented with 60 μg/ml penicillin, 100 μg/ml streptomycin, and 0.1% BSA. The cells were counted with a hemocytometer, and cell viability was higher than 85% as assessed by trypan blue exclusion. The obtained cell suspension contained very few endothelial cells or fibrocytes (0-5%), and no erythrocytes.

RNA Isolation
Total RNA from bovine corpora lutea was isolated by the single-step method of Chomczynski and Sacchi (1987) using TRIzol™ reagent (Gibco BRL Life Technologies, Rockville, MD, USA). Total RNA from endothelial cells was isolated using the NucleoSpin RNA kit (Macherey-Nagel, Dueren, Germany). Finally, RNA was dissolved in water and spectroscopically quantified at 260 nm. Aliquots were electrophoresed on a 1% denaturing agarose gel to verify the quantity and quality of RNA by ethidium bromide staining.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)
Four micrograms of total RNA were used to generate single-strand cDNA in a 60 μl reaction mixture as described previously (Plath et al., 1997). Conditions for enzymatic amplification were optimized for each PCR as follows: the TNFα and TNF receptor type I (TNF-RI) PCR contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.6 μM of each primer and 0.5 units of thermostable polymerase PrimeZyme (Biometra, Göttingen, Germany) to 5 μl cDNA (final volume 25 μl). Ubiquitin PCR was performed under the same conditions as those for TNFα and TNF-RI, but a higher concentration of primer (1.5 μM) was used. Samples for TNFα and TNF-RI were amplified for 27 or 28 cycles, respectively (one single denaturation step at 94°C for 2 min, each cycle at 94°C for 1 min, at 60°C for 1 min and afterwards one additional elongation step at 72°C for 2 min). Samples for the house-keeping gene, ubiquitin, were amplified by 22 cycles (one single denaturation step at 94°C for 2 min, each cycle at 94°C for 45 sec, at 55°C for 45 sec and afterwards one additional elongation step at 72°C for 45 sec). To determine the optimal quantity of reverse transcriptase needed for PCR and to verify that the cDNA product depended on the amount of transcript, varying quantities of transcriptase were used in the PCR reaction. The RT product from 3 μl was in the linear range and produced a visible band when stained with ethidium bromide. To exclude the possibility of amplification of genomic DNA, all experiments included reactions in which the RT enzyme or cDNA template was
omitted. As a negative control, water was used instead of RNA for the RT-PCR to confirm that the buffers and tubes were not introducing any contaminants.

The primers were designed to encode the mRNA bovine sequences obtained from the EMBL database or were used as described elsewhere and commercially synthesized (Amersham-Pharmacia, Freiburg, Germany). The primers were chosen using the "Husar" online software package in Heidelberg (http://genome.dkfz-heidelberg.de).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>TNFα</td>
<td>for 5'-GAAGCTGGAAGACAACCA-3'</td>
<td>rev 5'-TCCCAAAGTAGACCTGCC-3'</td>
</tr>
<tr>
<td>(338 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-RI</td>
<td>for 5'-CACCACCACCATCTGCTT-3'</td>
<td>rev 5'-TCTGAACTGGGGTGCAGA-3'</td>
</tr>
<tr>
<td>(257 bp)</td>
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<tr>
<td>Ubiquitin</td>
<td>for 5'-ATGCAGATCTTTTGTAAGAC-3'</td>
<td>rev 5'-CTTCTGGATGTTGTAGTC-3'</td>
</tr>
<tr>
<td>(189 bp)</td>
<td></td>
<td>Gabler et al. (1997)</td>
</tr>
</tbody>
</table>

Aliquots of the PCR reaction products (5 μl) were added to 1 μl bromphenol blue glycerin and fractionated by electrophoresis through a 1.5% agarose gel containing ethidium bromide in a constant 60 V field. To determine the length of the products, a Mass Ladder and 100-bp marker were used. The ethidium bromide-stained gels were evaluated with a video documentation system (Amersham-Pharmacia). Band intensities were analyzed by computerized densitometry using the image master program (Amersham-Pharmacia). This method allowed only a relative quantification. To verify each PCR product, double-strand sequencing was performed directly or after subcloning (TopLab, Munich, Germany).

**Membrane Preparation**

Corpora lutea were thawed and minced with scissors in ice-cold 25 mM Tris-HCl containing 300 mM sucrose, 2 mM EDTA (#E6511), 3 mM DTT, 500 kIU/ml aprotinin and 0.5 mM PMSF (pH 7.4), and then homogenized in the same buffer with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) using three 10-sec bursts, separated by a 1-min cooling period in ice. For each luteal stage of the estrous cycle, four pools were prepared. Each pool was made from 5-17 CLs. In contrast, for each stage of the gestation period, individual CLs were homogenized for the radioreceptor assay.

The homogenate of luteal tissue was subsequently centrifuged at 800 xg for 10 min to remove tissue debris, and the supernatant was collected and centrifuged at 30,000 xg for 20 min to obtain the plasma membrane pellet. The pellets were resuspended and recentrifuged in the same buffer to dissociate TNFα from their binding sites. The pellets were then washed three times by centrifugation for 10 min at 30,000 xg, decanted and resuspended in 25 mM Tris-HCl containing 10 mM MgCl2 (pH 7.4). All steps of the luteal membrane preparations
were conducted at 4 C. The protein concentrations of the membrane preparations were
determined by the method of Lowry et al. (1951) using BSA as a standard. The preparation
was diluted to give a protein concentration of 10 mg/ml of luteal membrane with 25 mM Tris-
HCl containing 10 mM MgCl2 and 0.5% (w/v) BSA (pH 7.4).

Radioreceptor Assay (RRA)

Recombinant human TNFα was iodinated with carrier-free Na125I (IMS 30; Amersham International plc, Buckinghamshire, England) by the iodogen method as described
previously (Richards and Almond, 1994). The specific activity of 125I-TNFα ranged between 530 and 540 Ci/mmol and the maximum bindability was 30%.

Preliminary studies with mid-luteal (days 8-12) membranes were carried out to
establish the optimal conditions of incubation time and temperature for maximal binding of
125I-TNFα to the membranes. To reduce nonspecific binding, glass microtubes (12x75 mm; MLB Culture Tube, Ontario, Canada) were coated overnight with complete calf serum, and
then binding assays were initiated. Nonspecific binding was assessed for each level of tracer
through co-incubation with a 240-fold excess of unlabeled TNFα (120 nM; 10 µl). The
incubation mixture consisted of approximately 6x10^4 dpm (0.5 nM) 125I-TNFα (50 µl) and
50 µg protein (50 µl). The total volume of the mixture was 110 µl. The specificity of 125I-
TNFα binding was determined by incubating increasing amounts of various unlabeled
hormones (IFNa, IL-1α or TGFα) with a constant amount of 125I-TNFα (6x10^4 dpm/tube).
All reagents were prepared in 10 mM Tris-HCl containing 10 mM MgCl2 (pH 7.5), 3.0 mM
NaN3 and 0.1% (w/v) BSA.

The 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 xg for 40 min at 4 C. Supernatants were decanted immediately, and the
pellets were counted for 125I in a γ-counter (Pharmacia-Wallac 1282, Compugamma CS,
Turku, Finland) at an efficiency of 82%. Non-specific bindings accounted for <35% of total
binding.

Hormone Determination

Concentrations of progesterone were determined directly from the cell culture media
with an enzyme immunoassay (EIA) (Okuda et al., 1997). The samples for the progesterone
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 concentrations of PGF2α in the culture media were determined directly with a
double-antibody EIA as described previously (Uenoyama et al., 1997). The samples for the
PGF\textsuperscript{2\alpha} assay were diluted 50 times with assay buffer. The standard curves ranged from 15.6 to 4000 pg/ml, and the ED\textsuperscript{50} of the assay was 250 pg/ml. The intra- and interassay coefficients of variation were on average 8.8\% and 12.5\%, respectively.

PGE\textsubscript{2} concentrations were determined with an EIA using peroxidase-labeled PGE\textsubscript{2} and anti-PGE\textsubscript{2} serum as described previously (Miyamoto et al., 1998). The PGE\textsubscript{2} standard curve ranged from 0.11 ng/ml to 28.19 ng/ml and the ED\textsuperscript{50} of the assay was 0.97 ng/ml. The intra- and interassay coefficients of variation were on average 4.9\% and 8.6\%, respectively.

The concentrations of endothelin-1 (ET-1) were determined directly in the culture media by EIA as described previously (Miyamoto et al., 1997). ET-1 antiserum (1:10,000 final dilution) and biotin-labeled ET-1 (1:2,000 final dilution) were used in the assay. The standard curve ranged from 10 to 5000 pg/ml and the ED\textsuperscript{50} of the assay was 450 pg/ml. The intra- and interassay coefficients of variation were 8.7\% and 12.6\%, respectively.

TNF\textalpha concentrations were determined with a commercial EIA for human TNF\textalpha (IBL, Hamburg, Germany). The assay is a solid phase enzyme-linked immunosorbent assay based on the sandwich principle. Recombinant human TNF\textalpha was used as a standard and calibrated against WHO Standard 87/650. The sensitivity of the assay is 2.5 pg/ml. For testing the linearity of the assay in the bovine tissue samples (CL and liver), tissue extracts were diluted in sample dilution buffer. CL or liver tissue extracts diluted 1:2, 1:4, 1:8 or 1:2, 1:5, 1:10, 1:15 gave concentrations of 1420, 1471, 1535 and 1052, 991, 995, 1062 pg/g wet tissue, respectively. Tissue levels (pg TNF\textalpha/g wet tissue) were in the range 999-2464 for liver, 855-1902 for kidney, 36-193 for muscle, and <25-44 for lung. The percentage recovery for the human standard added to bovine CL or liver extracts was 85.54 ± 16\% (mean ± SD). The intra- and interassay coefficients of variation were on average 6.4\% and 9.1\%, respectively.

Statistical Analysis

All experimental data are shown as the mean ± SEM. The data on binding of TNF\textalpha to CL membranes were analyzed with the LIGAND program (Munson and Rodbard, 1980) using nonlinear iterative curve-fitting procedures (McPherson GA, 1980). The initial parameters were calculated by Scatchard analysis (Scatchard G, 1949) and were then iteratively refined until the weighted sum of squares was minimized. The goodness of fit for the selected model was analyzed by a runs test. Different models (one or two binding sites) were compared using 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 (Munson and Rodbard, 1980). The statistical significance of differences in mRNA expressions of TNF\textalpha and TNF-RI, TNF\textalpha concentrations in the CL tissue, the binding parameters of TNF\textalpha receptors and the concentrations of progesterone, PGF\textsuperscript{2\alpha}, PGE\textsubscript{2} and ET-1 in the culture media were assessed.
by ANOVA followed by Fisher's protected least significant difference procedure (PLSD) as a multiple comparison test.
CHAPTER 2

TUMOR NECROSIS FACTOR-α AND ITS RECEPTOR IN BOVINE CORPUS LUTEUM THROUGHOUT THE ESTROUS CYCLE

As mentioned in the INTRODUCTION, TNFα modulates the functions of steroidogenic cells in the bovine ovary and CL. However, it is not known whether TNFα is locally produced or whether TNFα receptors are present in bovine ovary (CL as well as follicle cells). Therefore, in the present study, bovine corpora lutea from different stages of the estrous cycle were examined to determine whether they express TNFα mRNA and produce TNFα, whether they express TNF-RI mRNA, whether they have specific binding sites for TNFα, and whether the TNFα receptors in bovine CL, if present, are functional. For the latter experiment, the effects of TNFα on progesterone, PGF2α and PGE2 secretion by cultured cells were studied.

Materials and Methods

Luteal Cell Culture

Luteal cells were prepared and cells were cultured as described in CHAPTER 1. Viable cells (5x10^5/well) of CL were cultured in a culture medium (DMEM and Ham's F-12 medium, 1:1 [v/v] (DMEM/Ham's F-12); #D8900, Sigma) supplemented with 10% calf serum and 20 μg/ml gentamicin (#15750-011, Gibco BRL) in 24-well culture plates (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 TNFα (0.06-6 nM) or bovine LH (USDA-bLH-B6; 10 ng/ml). The conditioned media were collected and stored at -30 C until assayed for progesterone, PGF2α and PGE2.

Tissue Extraction of TNFα

Tissue (1 g wet weight) was transferred into 10 ml of an acidic buffer (pH 2.8) containing 2.54 mg orthophosphoric acid, 22.64 mg NaH2PO4·H2O, 18.6 mg EDTA, 70.1 mg NaCl, 2 mg NaN3, 20 mg BSA, 1 ml Triton X-100 and was homogenized in an ice bath with an Ultra Turrax Homogenizer (Janke and Kunkel, Staufen, Germany). Five bursts of 15 sec at maximum speed with 45 sec intervals of cooling between each burst were applied. The homogenate was subsequently centrifuged at 2000 xg for 15 min at 4 C. The supernatant was directly used for the TNFα determination.
Results

Expression of mRNA for TNFα and TNF-RI

Specific transcripts for TNFα and TNF-RI were detected in bovine CL. Each PCR product showed 100% homology to the known bovine genes after sequencing. To confirm the integrity of the mRNA templates and RT-PCR protocol, the housekeeping gene ubiquitin was examined in all samples. A representative sample for the ubiquitin-specific RT-PCR products (189+417 bp) is shown in Fig. 1A. The relative signal intensities for PCR products specific for TNFα and TNF-RI were assessed after correction based on the ubiquitin signal intensities. The ubiquitin was found to be stably expressed in the bovine corpora lutea during the estrous cycle. A representative example for the TNFα RT-PCR is given in Fig. 1B. There was no obvious difference in the expression between the samples. The results of the densitometric analysis of TNFα mRNA in the CL tissue during the estrous cycle are shown in Fig. 2. There was no statistically significant difference between any of the stages examined.

A representative example for the TNF-RI RT-PCR is shown in Fig. 1C. There are clear differences for the receptor during days 3-7 and days 13-18 of the luteal phase. The results of the densitometric analysis of TNF-RI mRNA are shown in Fig. 3. The mRNA expression was high at the very early stage (days 1-2) followed by a further significant increase during days 3-7 with a significant decrease thereafter (P<0.05).

Tissue Concentration of TNFα

The TNFα concentrations in the CL tissue are given in Fig. 4. TNFα in CL tissue was very low during the early and mid-luteal phases (average range between 71-116 pg/g wet tissue), and then increased significantly during the late luteal phase (days 13-18) followed by a decrease after regression (P<0.05).

Binding Characteristics

A preliminary assay of the binding of TNFα to bovine CL membranes was carried out to test the conditions for the radioreceptor assay described in CHAPTER 1. It was confirmed that maximal binding was reached after 48 h at 38 C (Fig. 5A). Specific binding increased with increasing protein concentration. A linear relationship was established in the amount of binding from 1 to 100 μg/50 μl (Fig. 5B). For this reason, further assays were carried out under these conditions.

Figure 5C shows the displacement curves of 125I-TNFα with three related peptides. The binding was highly specific for TNFα. There was little or no competition for TNFα binding sites by IFNα, IL-1α or TGFα. Scatchard plots of the binding data were linear (Fig. 5D). Analysis with the LIGAND program revealed that the concentrations of TNFα receptors were constant in the estrous cycle except in the late stage (days 15-17) (Table 1; P<0.05).
dissociation constant (Kd) values of the CL membranes in the mid- and late stages and regressed stage (days 19-21) were significantly higher than those in the early stages (Table 1; P<0.05).

Effects of TNFα on Progesterone, PGF2α and PGE2 Secretion by Bovine Luteal Cells

Bovine LH stimulated progesterone secretion by cultured luteal cells of all stages of the estrous cycle, indicating that the cells cultured with the present experimental design were reactive (Fig. 6A). As shown in Fig. 6A, progesterone secretion by the cells of all luteal stages was not affected by any dose of TNFα (0.06-6 nM). In contrast, TNFα significantly stimulated PGF2α and PGE2 secretion by the cells of the early, mid- and late stages in a dose-dependent fashion (Figs. 6B and 6C; P<0.01). The stimulatory effects of TNFα (6 nM) on PGF2α and PGE2 secretion by the early luteal cells (330-790% vs. control) were higher than those of the other stages (180-230% vs. control).
Figure 1. Representative sample of specific RT-PCR products for (A) Ubiquitin (189+417 bp), (B) tumor necrosis factor α (TNFα; 338 bp) and (C) TNFα receptor type I (TNF-RI; 257 bp); 1) DNA mass ladder (200 and 400 bp), 2, 3) bovine luteal tissue (days 3-7), 4, 5) bovine luteal tissue (days 13-18), separated by agarose gel electrophoresis.
Figure 2. Relative levels of tumor necrosis factor α (TNFα) mRNA (RT-PCR, 27 cycles, arbitrary units) in bovine corpus luteum (CL) during the estrous cycle. Results represent means ± SEM from 4 CLs/stage.
Figure 3. Relative levels of tumor necrosis factor α receptor type I (TNF-RI) mRNA (RT-PCR, 28 cycles, arbitrary units) in bovine corpus luteum (CL) during the estrous cycle. Results represent means ± SEM from 4 CLs/stage. Different superscript letters indicate significant differences (P<0.05).
Figure 4. Concentrations of tumor necrosis factor α (TNFα) in the bovine luteal tissue during the estrous cycle. Values represent mean ± SEM from 4-6 corpora lutea/stage. Different superscript letters indicate significant differences (P < 0.05).
Figure 5. Characteristics of binding of $^{125}$I-tumor necrosis factor α (TNFα) to bovine corpus luteum membranes. (A) Relationship between the binding of $^{125}$I-TNFα and incubation time at 4 C, 22 C or 38 C. The difference in the binding of $^{125}$I-TNFα bound in the presence of 120 nM TNFα and in the absence of TNFα was used to calculate the specific binding, expressed as a percentage of total $^{125}$I-TNFα (6x10$^4$ dpm/tube; 0.5 nM) added. (B) Relationship between the binding of $^{125}$I-TNFα and membrane concentrations of corpus luteum from the mid-luteal stage. (C) Competitive binding of $^{125}$I-TNFα and various unlabeled peptides on bovine luteal membranes from the mid-luteal stage. IFNα = Interferon-α, IL-1α = Interleukin-1α, TGFα = Transforming growth factor-α. (D) Representative Scatchard plots for competitive binding of $^{125}$I-TNFα and unlabeled TNFα on bovine luteal membranes obtained from each stage of the estrous cycle. Each line represents the means of duplicate determinations from one of three independent experiments.
Table 1. Binding affinities and concentrations of receptors for tumor necrosis factor α (TNFα) on bovine luteal membranes obtained from each stage of the estrous cycle.*

<table>
<thead>
<tr>
<th>Days of the estrous cycle</th>
<th>Binding affinity (Kd; nM)#</th>
<th>Receptor concentration (pmol/mg protein)#</th>
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<tr>
<td>Days 2-3</td>
<td>3.6±0.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.0±1.91&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Days 5-7</td>
<td>3.6±0.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.2±0.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Days 8-12</td>
<td>5.8±0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.6±5.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Days 15-17</td>
<td>4.2±0.57&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>17.1±0.69&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Days 19-21</td>
<td>5.5±0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.4±2.30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*The data are shown as the means±SEM (n=3).
#Different superscript letters indicate significant differences (P<0.05 or lower) as determined by ANOVA followed by Fisher's PLSD as a multiple comparison test.
Figure 6. Effects of tumor necrosis factor α (TNFα) on (A) progesterone, (B) prostaglandin F2α (PGF2α) and (C) prostaglandin E2 (PGE2) secretion by bovine luteal cells from the early (days 5-7), mid- (days 8-12) and late (days 15-17) stages of the estrous cycle. The cells were cultured for 24 h, TNFα was added, and then the cells were cultured for another 24 h. Values represent mean ± SEM for 4 separate experiments, each run in triplicate. Different superscript letters indicate significant differences (P<0.01).
Discussion

The present study describes a combined approach incorporating TNFα concentrations by an EIA, mRNA expressions of TNFα and TNF-RI by an RT-PCR analysis, and the specific binding for TNFα by an RRA to provide information on TNFα and its receptor in bovine CL. In addition, the fact that TNFα stimulated PGF2α as well as PGE2 secretion by cultured bovine luteal cells of all luteal stages in a dose-dependent fashion confirmed that the receptors for TNFα in bovine CL are functional. It is well known that there are two types of TNF receptors, i.e., TNF-RI and type II receptor (Tartaglia and Goeddel, 1992). However, since the cDNA sequence of the bovine TNFα receptors has been reported only for TNF-RI (Lee et al., 1998), the expression of mRNA for the receptor in bovine CL was examined by use of RT-PCR in the present study. The mRNA of TNF-RI was clearly expressed in the bovine CL of all stages during the estrous cycle. It is therefore likely that the TNF-RI might contribute to the specific binding of 125I-TNFα to membrane preparations of the bovine CL, as was observed in the present study. Collectively, these findings provide evidence for a local action of TNFα within the bovine CL by receptor-mediated mechanisms.

It is well recognized that bovine CL consists of several cell types, e.g., large and small luteal cells, endothelial cells and fibroblasts (O'Shea et al., 1989). Since I used membrane homogenates that were obtained from whole CL for the RRA in this study, it is not possible to say which cell types have TNFα receptors. Kull et al. (1985) showed the presence of high-affinity binding sites for TNFα on bovine aortic endothelial cells. In addition, it has been demonstrated that TNFα stimulated phospholipase A2 activity in bovine aortic endothelial cells (Clark et al., 1988), which provides indirect evidence that functional TNFα receptors are present on endothelial cells derived from CL. Therefore, the results of the mRNA analysis and the binding test that were obtained in this study might indicate the expression of TNFα receptors on endothelial cells in bovine CL. However, most of the endothelial cells as well as fibroblasts and erythrocytes would have been removed with use of our cell culture techniques (Okuda et al., 1992). Furthermore, TNFα clearly stimulated PGF2α and PGE2 secretion by the cultured luteal cells in the present study. I assume that functional TNFα receptors are present at least in the luteal cells in the bovine CL, although the distribution of TNFα receptors in luteal cells (large or small cells) remains unknown.

It is interesting to note that high-affinity TNFα receptors and TNF-RI mRNA expression were found in bovine CL in the early stage, and that TNFα dose-dependently stimulated both PGF2α and PGE2 secretion by the cells of the early stage. It is well demonstrated that luteal PGF2α and PGE2 have a luteotropic effect, e.g., they stimulate progesterone secretion by bovine CL in vitro (Miyamoto et al., 1993, Okuda et al., 1998). Moreover, it is well known that macrophages (Carswell et al., 1975) and endothelial cells (Hehnke-Vagnoni et al., 1995) are sources of TNFα, and that these cells infiltrate into newly
formed CL concomitant with vascular angiogenesis (Lobel and Levy, 1968, Reynolds et al., 1994). Therefore, I postulate that TNFα contributes to the production of PGF2α and PGE2 by early CL, and may partly promote the formation of CL. I expected to find that TNFα indirectly stimulates progesterone output by the TNFα-promoted PGs from the cultured luteal cells. However, no changes in progesterone secretion were observed. I could not find an appropriate explanation for this phenomenon. Further studies are needed to clarify the roles of TNFα in the early luteal stage.

Local secretion of TNFα in bovine CL of the late stage was higher than that of the mid-CL (Shaw and Britt, 1995), and TNFα concentrations in the CL were dramatically increased from the mid- to the late luteal phase in this study. However, the levels of TNFα mRNA expression were similar in the CL throughout the estrous cycle, and similar results were recently reported (Petroff et al., 1999). The discrepancy between the expression of TNFα mRNA and the concentrations of TNFα in the CL during the estrous cycle could be explained by the fact that macrophages infiltrate into the CL tissue at the time of luteolysis (Bagavandoss et al., 1990, Paavola LG, 1979, Hehnke-Vagnonni et al., 1994b). Furthermore, another explanation for the discrepancy might be due to post-translational processing. Since it has been well demonstrated that TNFα production is regulated by rate-limiting steps that involve transcription, translation, protein storage, membrane insertion and ultimate secretion (Beutler and Cerami, 1989, Beutler et al., 1992), the post-translational processing for TNFα could be controlled by unknown factor(s). Hence, I assume that post-translational processing in the cells might be restricted during the early luteal phase, and then start rapidly in the mid- and late phases. A related phenomenon has been reported concerning oxytocin and its mRNA expressions in bovine CL. Oxytocin mRNA expression was maximal in early CL, although the maximum level of oxytocin protein was observed in mid-CL (Wathes and Denning-Kendall, 1992).

It has been postulated that TNFα plays some roles in luteolysis (Pate JL, 1995, Terranova PF, 1997). Indeed, in the present study, the maximum concentrations of TNFα were observed in the CL of the late stage (days 13-18). However, the concentration of TNFα receptors in the late CL was significantly lower than the concentrations of the other stages. Since it has been demonstrated that the expression of TNFα receptors was down-regulated by TNFα (Douvdevani et al., 1996), the low expression of TNF-RI in the late CL in this study might be due to a down-regulation by locally produced TNFα in the CL. On the other hand, high-affinity binding sites (Kd: 5.5 ± 0.2 nM, concentration: 30.4 ± 2.3 pmol/mg protein) for TNFα were found in the CL of the regressed stage in the present study. It has been clearly demonstrated that TNFα induces a significant increase in the expression of major histocompatibility (MHC) class 1 glycoproteins in cultured bovine luteal cells and that these glycoproteins are recognized by cytotoxic T cells in order for the T cells to devour the luteal cells (Benyo and Pate, 1992). Moreover, TNFα was shown to induce apoptosis of cultured mouse luteal cells (Jo et al., 1995). All of these findings suggest that TNFα may play an
important role in luteolysis of cattle, in particular at the phase of luteal regression (death and destruction of luteal cells) at which time the CL has high concentrations of TNFα and its specific receptor.

In conclusion, the overall results of the present study indicate the production and the presence of local TNFα as well as functional TNFα receptors (at least TNF-RI) in bovine CL during the estrous cycle, and suggest that TNFα plays physiological roles in regulating bovine CL function not only at the time of luteal regression but throughout all luteal phases.

Summary

The objective of this study was to investigate TNFα expression, the presence of functional TNFα receptors, and expression of TNF-RI mRNA in the bovine CL during different stages of the estrous cycle. RT-PCR showed no difference in TNFα mRNA expression during the estrous cycle. The concentrations of TNFα in the CL tissue increased significantly from the mid- to the late luteal stage and decreased thereafter (P<0.05). An RT-PCR analysis showed higher levels of TNF-RI mRNA in CL of days 3-7 than in other stages (P<0.05). 125I-TNFα binding to the membranes of bovine CL was maximal after incubation at 38 C for 48 h. The binding was much greater for TNFα than for related peptides. A Scatchard analysis revealed the presence of a high-affinity binding site in the CL membranes collected at each phase of the estrous cycle (Kd: 3.60 ± 0.58 - 5.79 ± 0.19 nM). In contrast to TNF-RI mRNA expression, the levels of receptor protein were similar at each stage in the estrous cycle. When cultured cells of all luteal stages were exposed to TNFα (0.06-6 nM), TNFα stimulated PGF2α and PGE2 secretion by the cells in a dose-dependent fashion (P<0.01), especially during the early luteal phase, although it did not affect progesterone secretion. These results indicate the local production of TNFα and the presence of functional TNF-RI in bovine CL throughout the estrous cycle, and suggest that TNFα plays some roles in regulating bovine CL function throughout the estrous cycle.
CHAPTER 3

TUMOR NECROSIS FACTOR-α AND ITS RECEPTOR IN THE CORPUS LUTEUM OF PREGNANT COWS

The possible roles of TNFα and the presence of TNFα and its receptor have been well demonstrated in the pregnant uterus, placenta, oviduct and embryo (for reviews, see Terranova et al., 1995; Hunt et al., 1996). However, the physiological roles of TNFα in the bovine CL throughout the gestation period are not known. Therefore, in the present study, the bovine CL from three stages of the gestation period were examined to determine whether there are any changes in TNFα mRNA expression and whether specific binding sites for TNFα are present. These experiments were carried out with an RT-PCR analysis and an RRA, respectively.

Materials and Methods

RT-PCR

Isolation of RNA and an RT-PCR were conducted as described in CHAPTER 1 with a slight modification. Briefly, one μg of total RNA was used to generate single-strand cDNA in a 15 μl reaction mixture using a First-Strand cDNA synthesis kit (Amersham International plc., Buxinghamshire, UK). Conditions for enzymatic amplification were optimized for the PCR as follows: the TNFα contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 4 μM of each primer and 1.5 units of Taq polymerase (AmpliTaq™ Gold, The Perkin-Elmer Co., Norwalk, CT, USA) to 1 μl cDNA (final volume 25 μl). Beta-actin PCR was performed under the same conditions as those for TNFα. Samples for TNFα were amplified by 30 cycles each consisting of 1 min at 94 C, 1 min at 60 C and 2 min at 72 C followed by a single denaturation step at 94 C for 7.5 min. Samples for β-actin were amplified by 22 cycles (1 cycle; 94 C for 45 sec, 55 C for 45 sec and 72 C for 45 sec) followed by a single denaturation step at 94 C for 2 min.

Aliquots of the PCR reaction products (5 μl) were added to 1 μl bromphenol blue glycerin and fractionated by electrophoresis through a 1.5% agarose gel (Nacalai Tesque, Kyoto, Japan) containing ethidium bromide in a constant 100 V field. To determine the length of the products, a Mass Ladder and 100-bp marker were used. The ethidium bromide-stained gels were evaluated with a UV transilluminator. Band intensities were analyzed by computerized densitometry using the NIH image program.
Results

Expression of mRNA for TNFα

Specific transcripts for TNFα were detected in bovine corpora lutea of each stage of gestation. The PCR product showed 100% homology to the known bovine gene after sequencing (data not shown). To confirm the integrity of the mRNA templates and RT-PCR protocol, the β-actin was examined in all samples. The β-actin was found to be stably expressed in the bovine corpora lutea during the gestation period. A representative example for the TNFα RT-PCR is given in Fig. 7A. There was no significant difference in the level of expression between the samples. The results of the densitometric analysis of TNFα mRNA in the CL tissue during the gestation period are shown in Fig. 7B. There was no significant difference between any of the stages examined.

TNFα Receptors in Bovine CL during Pregnancy

Scatchard plots of the binding data were linear (Fig. 8). Analysis with the LIGAND program revealed that the concentrations of TNFα receptors in the CL of trimesters I and III were significantly higher than those of trimester II (Table 2; P<0.05). The Kd values of the CL membranes were similar in the CL of trimesters I, II and III (Table 2).
Figure 7. (A) Representative sample of specific RT-PCR products for tumor necrosis factor α (TNFα) (338 bp); 1) DNA mass ladder, 2) bovine luteal tissue (trimester I), 3) trimester II, 4) trimester III, separated by agarose gel electrophoresis. (B) Relative levels of TNFα mRNA (RT-PCR, 30 cycles, arbitrary units) in bovine corpus luteum (CL) during the gestation period. Results represent means ± SEM from 4 CLs/stage.
Figure 8. Representative Scatchard plots for competitive binding of $^{125}$I-tumor necrosis factor α (TNFα) and unlabeled TNFα on bovine luteal membranes obtained from each stage of the gestation period. Each line represents the means of duplicate determinations from one of four independent experiments.
Table 2. Binding affinities and concentrations of receptors for tumor necrosis factor α (TNFα) on bovine luteal membranes obtained from each stage of the gestation period.*

<table>
<thead>
<tr>
<th>Pregnancy</th>
<th>Binding affinity (Kd; nM)</th>
<th>Receptor concentration (pmol/mg protein)#</th>
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<tr>
<td>Trimester I</td>
<td>5.6±1.36</td>
<td>24.0±1.95a</td>
</tr>
<tr>
<td>II</td>
<td>5.1±1.66</td>
<td>14.9±2.07b</td>
</tr>
<tr>
<td>III</td>
<td>6.9±3.49</td>
<td>21.6±2.39a</td>
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*The data are shown as the means±SEM (n=4).

#Different superscript letters indicate significant differences (P<0.05 or lower) as determined by ANOVA followed by Fisher’s PLSD as a multiple comparison test.
Discussion

The present study describes a combined approach incorporating an mRNA analysis and an RRA to provide information on the presence of TNFα and its receptor in bovine CL in the gestation period. High affinity binding sites for TNFα were present throughout the gestation period, and the concentrations of TNFα receptors changed during the gestational stages. Specificity for the PCR products was based on size (338 bp, including primers) and hybridization to the specific sequence; furthermore, I confirmed that the PCR product sequences were identical to the corresponding region of bovine TNFα cDNA. Moreover, the densitometric analysis (Fig. 7B) showed that the relative levels of TNFα mRNA were similar during the gestation period. These findings indicate that TNFα is locally produced in the bovine CL and that the action of TNFα within the CL is mediated via its specific receptor. Although the possible production of TNFα from the large luteal cells (Wuttke et al., 1993) and the endothelial cells (Hehnke-Vagnoni et al., 1995) has been demonstrated in porcine CL, it is still unclear which cell types (macrophages, large and small cells, endothelial cells, fibroblasts, etc.) in the bovine CL produce TNFα.

It is well known that there are two types of TNF receptors, and these receptors have different intracellular signaling pathways (Tartaglia and Goeddel, 1992; Beutler et al., 1992). In this study, Scatchard plots of binding data were linear, suggesting that only one type of TNFα receptor is present in pregnant CL. In CHAPTER 2, I demonstrated that TNF-RI mRNA was present in the bovine cyclic CL, and that the affinity of the TNFα receptor in the cyclic CL is similar to that of the pregnant CL observed in the present study. Thus, it could be speculated that TNFα receptors in bovine pregnant CL are TNF-RI.

At the end of the gestation period of women, macrophages infiltrate into the endometrial tissue (Chen et al., 1991) and produce large amounts of TNFα (Vince et al., 1992). Furthermore, the concentration of TNFα is markedly elevated in the amniotic fluid of women whose condition is associated with preterm labor (Romero et al., 1989). The present data show a high concentration of TNFα receptors as well as the expression of TNFα mRNA in the CL of the late gestation period. Thus, TNFα seems to play one or more roles in the bovine CL as well as in various other organs at the time of parturition. I assume that TNFα locally produced in the CL might contribute to the luteal resorption in order to induce parturition.

In the present study, specific binding sites for TNFα were also found in the bovine CL of the early and mid-gestational stages. Furthermore, the affinity and the concentration of the receptor in the pregnant CL are comparable with those in the CL of the regressed stage (see CHAPTER 2). These findings suggest that TNFα acts on CL function in the early and mid-gestational stages. However, since TNFα is recognized as a luteolytic factor in a variety of species (Terranova PF, 1997), the present data do not support the hypothesis that only low
concentrations of TNFα and/or TNFα receptors should be present in the pregnant CL. TNFα may play roles in regulating CL function during the entire gestation period as well as in the estrous cycle of cows. TNFα, in addition to having a cytotoxic action, has been demonstrated to stimulate PGE2 as well as PGF2α secretion by cultured bovine luteal cells in a dose-dependent fashion (CHAPTER 2). Both luteal PGE2 and PGF2α in the CL are known to be luteotropic agents (Miyamoto et al., 1993; Okuda et al., 1998). Therefore, it could be assumed that the luteal TNFα contributes to maintain pregnancy by stimulating the production of PGF2α and PGE2 by the pregnant CL as well as by the cyclic CL, indirectly resulting in an increase of progesterone output from the pregnant CL. Recently, it has been shown that the corpora lutea from Day 200 pregnant cows secreted more PGEs and PGF2α in vitro than those of Day 14 of the estrous cycle (Weems et al., 1998a). Furthermore, progesterone secretion in vitro by Day 200 CL of pregnancy was increased by PGE2 but not by LH (Weems et al., 1998b). These findings appear to support the above supposition that TNFα indirectly contributes to produce progesterone by stimulation of luteal PGs in bovine CL during the gestation period. However, it remains to be determined how TNFα switches its role from a luteotropic agent to a luteolytic agent in the gestation period.

The present study demonstrated that the concentration of TNFα receptors in the CL of trimester II of gestation was significantly lower than that of trimesters I and III. Interestingly, the transitional pattern of TNFα receptor concentration in the bovine pregnant CL is similar to that of the progesterone concentration in bovine CL during the gestation period (Stormshak and Erb, 1961). Moreover, it has been demonstrated that TNF-RI mRNA expression in ovariecetomized mouse uteri increased by the treatment of progesterone (Roby et al., 1996). Therefore, I presume that progesterone is one of the regulators of the expression of TNFα receptors in the bovine CL, and that the low concentration of TNFα receptors in the CL of trimester II of gestation in the present study is due to the decrease of progesterone concentration in the CL at that time. Furthermore, the facts that the concentration of TNFα in the bovine cyclic CL was elevated after the decrease of progesterone concentration (Shaw and Britt, 1995), and that progesterone inhibited TNFα-induced PGF2α secretion by bovine luteal cells (Pate JL, 1995), seem to support the above hypothesis.

Based on the above findings, the present results demonstrate the presence of TNFα mRNA and TNFα receptors in bovine CL during the gestation period, and suggest that TNFα plays one or more roles in regulating bovine CL function in the entire gestation period as well as in the estrous cycle.

Summary

The objective of this study was to investigate the presence of TNFα mRNA and TNFα receptors in the bovine CL during the gestation period. TNFα mRNA and TNFα...
receptors were determined on bovine CL from pregnant cows at three stages: trimester I (fetal crown-rump length: 6-20 cm), trimester II (25-45 cm) and trimester III (50-80 cm). TNFα mRNA was detected by an RT-PCR analysis in the CL of all stages of gestation. A Scatchard analysis revealed the presence of a high-affinity binding site (Kd; 5.1-6.9 nM) in the CL membranes collected at each stage of gestation. Furthermore, the concentrations of TNFα receptors in the CL of trimesters I (24.0±1.95 pmol/mg protein) and III (21.6±2.39 pmol/mg protein) of gestation were significantly higher than the concentration in trimester II (14.9±2.07 pmol/mg protein)(P<0.05). These results indicate that TNFα is locally produced and that TNFα receptors are present in bovine CL during the gestation period, and suggest that TNFα plays one or more roles as a paracrine factor in regulating bovine CL function during the entire gestation period.
CHAPTER 4

TUMOR NECROSIS FACTOR-α RECEPTORS IN MICROVASCULAR ENDOTHELIAL CELLS FROM BOVINE CORPUS LUTEUM

In CHAPTERS 2 and 3, I provided evidence that TNFα and its TNF receptors are present in bovine CL throughout the estrous cycle and the gestation period, and suggested that the actions of TNFα in regulating bovine CL function are mediated by its specific receptors. However, since I used membrane homogenates that were obtained from whole CL for the RRA in the previous study, it was not possible to say which cell types (large or small luteal cells, endothelial cells or fibroblasts, etc.) have TNF receptors. Recently, it has been shown that specific binding sites for TNFα were present on both granulosa and theca cells in bovine ovary (Spicer LJ, 1998), and that TNFα clearly stimulated PGF2α and PGE2 secretion by highly purified bovine luteal cells (CHAPTER 2, Schams et al., 1995). Based on these latest findings, I assume that TNF receptors are present at least on luteal cells in bovine CL, although it is still unclear whether CL cells other than luteal cells contain TNF receptors.

On the other hand, it is well recognized that endothelial cells constitute the major proportion (53.5%) of the bovine CL (O'Shea et al., 1989). The microvascular endothelial cells in several tissues produce PGE2 (Chung-Welch et al., 1988) and endothelin-1 (ET-1) (Yanagisawa et al., 1988). These hormones have been demonstrated to affect steroidogenesis of bovine CL in vitro (Godkin et al., 1977, Miyamoto et al., 1997, Girsh et al., 1996). The presence of high-affinity binding sites for TNFα has been shown in bovine aortic endothelial cells (Kull et al., 1985). In addition, it has been demonstrated that TNFα stimulated phospholipase A2 activity in bovine aortic endothelial cells (Clark et al., 1988), which suggests that functional TNF receptors are also present on endothelial cells derived from CL. Hence, in addition to TNFα’s action on the function of the luteal cells, we hypothesized that it also acts as a paracrine regulator in regulating the function of the endothelial cells in bovine CL via its specific receptors.

Therefore, in the present study, two types of cultured microvascular endothelial cells that were obtained from developing bovine CL (Spanel-Borowski and van der Bosch, 1990) (one has a cobblestone appearance, and the other has a tube-like structure) were examined for the expression of TNF-RI mRNA by RT-PCR analysis, and the TNFα binding characteristics of these cells were investigated by RRA. Furthermore, the possible effects of TNFα on PGE2 and ET-1 secretion by cultured endothelial cells were studied.
Materials and Methods

Culture of Microvascular Endothelial Cells

Cytokeratin-negative endothelial cells, type 3, derived from the microvascular bed of the developing bovine CL (Spanel-Borowski and van der Bosch, 1990, Mayerhofer et al., 1992, Fenyves et al., 1993) were kindly provided by Dr. Spanel-Borowski (University of Leipzig, Germany). These cytokeratin-negative cells are known to occur in two forms in confluent culture. One form has a cobblestone appearance (CS cells), and the other form spontaneously expresses a tube-like structure (TS cells). The CS and TS cells were separately cultured for 8 passages. Both CS and TS cells were then stored in liquid nitrogen. I confirmed that these cells retain a stable form during culture for at least 17 passages (data not shown). The cells at 11 passages were used in the present study.

The cells were cultured in flasks (80 cm², 260 ml, #178905; Nunc, Roskilde, Denmark) until they were confluent. The flasks were pre-coated with 1% collagen type I (Vitrogen 100®; Collagen Corp., Palo Alto, CA, USA) for 2 h at 37 C. For the culture medium, DMEM and Ham’s F12 medium were mixed 1:1 (v/v) with 15 mM Hepes, 22 mM NaHCO₃, 5% fetal calf serum (FCS; #S0115, Biochrom Beteiligungs GmbH & Co., Berlin, Germany) and 20 µg/ml gentamicin. When the cells were confluent, 0.02% trypsin (1:250; #T0646, Sigma) solution was added to the cells for 7 min at 37 C. The cells were removed and counted with a hemocytometer and assessed for viability by trypan blue dye exclusion. Cell viability was higher than 90%.

Experiment 1. Two types of microvascular endothelial cells were examined for the presence of TNFα receptors by an RRA using a ¹²⁵I-labeled recombinant human TNFα. The cells were resuspended in fresh culture medium containing 5% FCS and were plated in 24-well cluster dishes (Falcon® #3047; Becton Dickinson & Co., Lincoln Park, NJ, USA) (3x10⁵ cells/well) in a humidified atmosphere of 5% CO₂ in air at 37 C. Confluence of the cells was generally observed after 6-7 days in culture.

Experiment 2. Cells, prepared as described above, were plated in 48-well cluster dishes (1.5x10⁵ cells/well) in a humidified atmosphere of 5% CO₂ in air at 37 C. Confluence of the cells was generally observed after 6-7 days in culture. When the cells were confluent, they were washed two times with 250 µl of fresh culture medium containing 0.1% BSA (#A7888, Sigma) and 20 µg/ml gentamicin. After washing, the cells were incubated in fresh medium containing 0.1% BSA, 10 µM arachidonic acid (#A8798, Sigma), 2 µg/ml insulin (#977420, Boehringer Mannheim), 5 µg/ml transferrin (#T3400, Sigma), 5 ng/ml sodium selenite (#S5261, Sigma), 20 µg/ml gentamicin and varying concentrations of TNFα (0.06-3 nM) for 24 h at 37 C. After incubation, conditioned media were collected in tubes with 5 µl of a
stabilizer [0.3 M EDTA, 1% aspirin (#A2093, Sigma), pH 7.3] and stored at -20°C until assayed for PGE2 and ET-1.

**DNA Assay**

At the end of each experiment, the DNA content of the endothelial cells was estimated spectrophotometrically as described by Labarca and Paigen (1980). Briefly, the cells were washed 2 times with 250 μl of phosphate-saline buffer (50 mM NaH2PO4, 140 mM NaCl, 2 mM EDTA, pH 7.4), and completely destroyed by ultrasonication for 20 sec. The samples and standard were dispensed in a 96-well plate (#7655077; Greiner GmbH, Frickenhausen, Germany), and then 40 μl of bis-benzimide (8.43 μM, #B2883, Sigma) was added into each well of the plate. After 10 min at 4°C, the fluorescence was evaluated by FLUOROSKAN II (Flow Laboratories GmbH, Meckenheim, Germany). The DNA from calf thymus (#D3664, Sigma) was used for a standard, and the standard curve was determined for concentrations in the range from 0.09 to 12.5 μg/ml. Since DNA content of the cultured cells was not altered by TNFα treatment, it was used to standardize the results.

**RRA**

When the cells were confluent, they were washed 2 times with 500 μl of modified Ca2+- and Mg2+-free Hanks' Balanced Salt Solution (mHBSS; #H2387, Sigma). The cells were then incubated with 50,000 disintegrations per minute (0.1 nM) 125I-labeled TNFα and 0-42 nM unlabeled TNFα. All incubations were performed in 560 μl of mHBSS containing 10 mM MgCl2 and 0.1% BSA (#11930; Serva Feinbiochemica GmbH & Co., Heidelberg, Germany) for 30 h at 37°C. The incubation was terminated by rapid washing of the cells with ice-cold mHBSS. After washing two times, the cells were removed from the cluster dishes with 250 μl of 0.02% trypsin, and the bound radioactivity was counted in a γ-counter. The difference in the 125I-TNFα binding in the presence and the absence of unlabeled TNFα (42 nM) was used to calculate the specific binding. To determine the ligand specificity of the receptor, a competitive binding assay was performed with TNFα, IL-1α, and IFNα.

**Results**

**Receptor Assay Conditions and Binding Characteristics**

The conditions for the RRA on the endothelial cells were initially validated. It was confirmed that maximal binding was reached after 24 h at 37°C (Fig. 9A). On the basis of these results, further assays were carried out under these conditions.

Figure 9B shows that the competition curves of 125I-labeled TNFα with three cytokines in the cultured endothelial cells. The binding was highly specific for TNFα, while IL-1α and IFNα did not display any competition with 125I-labeled TNFα.
Scatchard plots of the competitive binding of $^{125}$I-labeled TNFα and unlabeled TNFα on both CS and TS cells were curvilinear (Figs. 10A and 10B), indicating that there are two binding sites in each cell type. Mean values of the Kd and the concentration of these receptors were analyzed by the LIGAND program (Table 3). Kd values and concentrations of high-affinity binding sites were similar in both CS and TS cells. On the other hand, Kd values and concentrations of low-affinity binding sites of CS cells were significantly higher than those of TS cells (P<0.05).

Expression of mRNA for TNF-R1
Specific transcripts for TNF-R1 were detected in cultured endothelial cells and bovine CL. The PCR product showed 100% homology to the known bovine genes after sequencing. To confirm the integrity of the mRNA templates and RT-PCR protocol, the housekeeping gene ubiquitin was examined in all samples. A representative sample for the ubiquitin-specific RT-PCR products (189+417 bp) is shown in Fig. 11A. The relative signal intensities (in arbitrary units; n=3) for PCR products specific for TNF-R1 were assessed after correction based on the ubiquitin signal intensities. We repeated the PCR analyses using three sets of separate RNA samples and obtained similar results. A representative example for the TNF-R1 RT-PCR is given in Fig. 11B. There were clear differences in the expression between the samples (1.52 ± 0.14, 0.62 ± 0.05 and 0.42 ± 0.06 in CL, and CS and TS cells, respectively). The expression of TNF-R1 was lower in endothelial cells than in CL.

Effects of TNFα on PGE2 and ET-1 Secretion by Cultured Endothelial Cells
TNFα stimulated PGE2 secretion by both CS and TS cells (P<0.05, Fig. 12). The mean concentration of PGE2 in the culture media of CS cells (Fig. 12A) was 10 times higher than that of TS cells (9.20 ± 0.65 and 0.82 ± 0.04 pmol/μg DNA in CS and TS cells, respectively) (Fig. 12B).

ET-1 secretion by both CS and TS cells also increased as a result of treatment with TNFα (P<0.05 or lower, Fig. 12C, D). The mean concentrations of ET-1 in the culture media of CS and TS cells without TNFα were 1.4 ± 0.13 and 0.66 ± 0.26 pmol/μg DNA, respectively, and these values were not significantly different.
Figure 9. A. Relationship between the binding of $^{125}$I-labeled tumor necrosis factor α (TNFα) and incubation time at 37 C (circles) or 20 C (squares) on cultured microvascular endothelial cells, that have a cobblestone appearance (CS cells), obtained from developing bovine corpora lutea. B. Competitive binding on the cultured microvascular endothelial cells (solid circles: CS cells; gray circles: tube-like structure (TS) cells) of $^{125}$I-labeled TNFα and unlabeled TNFα (circles), interleukin-1α (solid squares) and interferon-α (solid triangles).
Figure 10. Scatchard plots for competitive binding of $^{125}$I-labeled tumor necrosis factor α (TNFα) and unlabeled TNFα on endothelial cells, (A) CS and (B) TS cells, obtained from developing bovine corpora lutea. Different colored symbols represent the data of three separate experiments.
Table 3. $^{125}$I-TNFα binding affinities and concentrations of the high- and low-affinity binding sites of the cultured endothelial cells obtained from bovine corpus luteum.*

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Binding affinity (Kd; nM)#</th>
<th>Receptor concentration (fmol/μg DNA)#</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>1.34±0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.24±0.66&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low</td>
<td>108.00±7.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58.24±4.79&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TS cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>0.21±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Low</td>
<td>56.53±8.91&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.97±5.10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*The data are shown as the means±SEM (n=3).
#Different superscript letters indicate significant differences (P<0.05 or lower) as determined by ANOVA followed by Fisher's PLSD as a multiple comparison test.
Figure 11. Specific RT-PCR products for A) Ubiquitin (189+417 bp), and B) tumor necrosis factor α receptor type I (TNF-RI) (257 bp) separated by agarose gel electrophoresis; 1) DNA mass ladder, 2) bovine luteal tissue, 3) CS cells, and 4) TS cells.
Figure 12. Effects of tumor necrosis factor α (TNFα) on prostaglandin E2 (A and B) and endothelin-1 (ET-1) (C and D) secretion by two forms of endothelial cells obtained from developing bovine corpora lutea. The cells were exposed to TNFα in the final 24 h of culture. All values are expressed as a percentage of the control value. The concentrations of PGE2 in the control were 9.20 ± 0.65 or 0.82 ± 0.04 pmol/μg DNA in (a) CS or (b) TS cells, respectively. The concentrations of ET-1 in the control were 1.40 ± 0.13 or 0.66 ± 0.26 pmol/μg DNA in (c) CS and (d) TS cells, respectively. Different superscript letters indicate significant differences (P<0.05 or lower), as determined by analysis of variance followed by Fisher's protected least significant difference procedure as a multiple comparison test. Bars marked "ab" are not significantly different from either bars marked "a" or bars marked "b".
Discussion

This study presents strong evidence for the existence of TNFα binding sites and TNF-RI mRNA expression in two different forms of endothelial cells derived from developing bovine CL. In addition, the fact that TNFα stimulated PGE2 as well as ET-1 secretion by the cultured endothelial cells used in this study confirmed that the receptors for TNFα on the cells are functional.

In the present study, the Scatchard analyses clearly demonstrated that two binding sites (high-affinity binding site: Kd=0.2-1.4 nM, low-affinity binding site: Kd=56.5-108 nM) for TNFα are present on both CS and TS cells. It is well known that there are two types of TNF receptors (Tartaglia and Goeddel, 1992). Furthermore, it has been shown that endothelial cells which were derived from human umbilical vein have both p55 and p75 receptors for TNF (Paleolog et al., 1994, Bradley et al., 1995). Thus, the results of the binding test raise the possibility that these two receptors (p55 and p75) are expressed on endothelial cells in the bovine CL. Moreover, the concentrations of TNFα (0.6-3 nM), that increased PGE2 and ET-1 secretion in the present study, are comparable with the affinity of high-affinity binding sites for TNFα on cultured endothelial cells, which were found in the present study. Therefore, the stimulatory effects of TNFα on PGE2 and ET-1 secretion might be mediated at least by the high-affinity TNFα receptors present in endothelial cells.

With the technique of RT-PCR, I was able to demonstrate the expression of TNF-RI mRNA in the endothelial cells. The intensity of expression did not differ between CS and TS cells, and was in agreement with the data for binding affinities and concentrations of binding sites. When compared with the mRNA from total CL tissue, the intensity of expression was lower in endothelial cells. Although a direct comparison in the intensities of mRNA between freshly prepared CL and cultured endothelial cells is not possible, it is possible that the luteal cells are the main target cells for TNFα in the bovine CL.

Although the endothelial cells from bovine CL have been well characterized morphologically (Spanel-Borowski and van der Bosch, 1990, Mayerhofer et al., 1992, Fenyves et al., 1993), the function of these cells is still not understood. The present study demonstrated that the basal secretion of ET-1 from CS cells tended to be higher than that from TS cells. Moreover, the basal secretion of PGE2 from CS cells was 10-times higher than that of TS cells. The type 3 endothelial cells are thought to be derived from postcapillary venules (Spanel-Borowski and van der Bosch, 1990). Normally, cell growth (angiogenesis) occurs at the postcapillary venules. Moreover, tubular forms of the endothelial cells correspond to angiogenesis. In general, proliferating cells are thought to have a poor ability to produce hormones. Thus, it is likely that TS cells mainly participate in angiogenesis and have a poor ability to produce hormones, including PGE2.

The present study clearly demonstrated that PGE2 secretion by cultured endothelial cells was
significantly stimulated by treatment with TNFα. It is well known that macrophages (Carswell et al., 1975) and endothelial cells (Hehnke-Vagnoni et al., 1995) are sources of TNFα, and that these cells infiltrate into newly formed CL concomitant with vascular angiogenesis (Lobel and Levy, 1968, Reynolds et al., 1994). Since it has been clearly demonstrated that both TNFα (Leibovich et al., 1987) and PGE2 (Form and Auerbach, 1983) affect the proliferation of endothelial cells, I assume that TNFα, and PGE2 which is induced by TNFα, play a role in inducing vascular angiogenesis as autocrine and/or paracrine regulators. In addition, PGE2 is known to stimulate progesterone production from bovine CL in vitro as a luteotropic agent (Godkin et al., 1977, Miyamoto et al., 1993).

On the other hand, TNFα also stimulated ET-1 secretion by the cultured endothelial cells in this study. Since some previous studies showed that ET-1 has a luteolytic effect in bovine CL in vitro (Miyamoto et al., 1997, Girsh et al., 1996), TNFα-induced ET-1 from the endothelial cells may act on luteal cells as a luteolytic agent in the CL concomitant with endometrial PGF2α. Furthermore, it has been postulated that TNFα directly acts on luteal cell function at the time of luteolysis (Pate JL, 1995, Terranova PF, 1997). Thus, it could be assumed that TNFα affects the function of the endothelial cells as well as the luteal cells at the time of luteolysis to complete the luteal regression. However, the physiological roles of ET-1, which is secreted throughout the estrous cycle (Ohtani et al., 1998), have not been clearly defined. On the other hand, it has been well demonstrated that TNFα plays a role as a potent stimulator of luteal PGs including PGF2α, PGE2 and PGI2 (CHAPTER 2, Benyo and Pate, 1992, Schams et al., 1995). The stimulatory effects of TNFα on luteal PGF2α and PGE2 production in the early luteal stage (days 2-4) were much higher than those in latter stages (Schams et al., 1995). Moreover, these luteal PGs are known to stimulate progesterone production from bovine CL in vitro as luteotropic agents (Godkin et al., 1977, Miyamoto et al., 1993, Okuda et al., 1998). Since TNFα receptors in whole CL are present throughout the estrous cycle (CHAPTER 2), it is possible that TNFα acts on bovine CL function as either a luteotropic or luteolytic agent depending on the stages of the estrous cycle.

In conclusion, the overall results of the present study indicate the presence of functional TNFα receptors (high- and low-affinity receptors) on the endothelial cells in bovine CL. Since my previous work demonstrated that functional TNFα receptors are present on bovine CL throughout the estrous cycle, these results strongly suggest that TNFα plays two or more roles in regulating bovine CL function as an autocrine and/or paracrine regulator throughout the estrous cycle.

**Summary**

The purpose of the present study was to identify the presence of functional TNFα receptors on the microvascular endothelial cells derived from developing bovine CL. TNFα
receptors were analyzed by an RRA using $^{125}$I-labeled TNFα on two types of cultured endothelial cells. One has a cobblestone appearance (CS cells), and the other has a tube-like structure (TS cells). $^{125}$I-labeled TNFα binding was maximal after incubation for 30 h at 37°C, and the specificity of binding was confirmed. A Scatchard analysis showed the presence of two binding sites (high- and low-affinity) for TNFα receptors on both CS and TS cells. The Kd values and concentrations of the high-affinity binding sites for TNF receptors were similar between CS and TS cells. However, Kd values and concentrations of the low-affinity binding sites in CS cells were significantly higher than those in TS cells (P<0.05 or lower). The expression of TNF-RI mRNA was determined in both cell types. Furthermore, TNFα significantly stimulated PGE2 and ET-1 secretion by both CS and TS cells (P<0.05 or lower). These results indicate the presence of two types of TNF receptors and the expression of TNF-RI mRNA in the endothelial cells derived from bovine CL, and suggest that TNFα plays two or more roles in regulating the secretory function of the endothelial cells.
CHAPTER 5

INTRACELLULAR SIGNALING PATHWAYS OF TUMOR NECROSIS FACTOR-α IN BOVINE LUTEAL CELLS

It has been demonstrated that TNFα clearly stimulates PGF2α and/or PGE2 secretion by cultured bovine luteal cells and endothelial cells derived from CL (in CHAPTERS 2 and 4). Furthermore, it has been shown that the stimulatory effects of TNFα on PGF2α production were mediated via the activation of phospholipase (PL) A2 but not by the activation of PL-C (Benyo and Pate, 1992; Townson and Pate, 1996). On the other hand, multihormonal effects of TNFα have also been observed in various reproductive tissues. For example, it has been shown that TNFα acts through the protein kinase (PK) C and PK-A pathways in rat theca-interstitial cells (Zachow and Terranova, 1993, Zachow et al., 1993), and the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway in bovine theca cells (Brunswig-Spickenheier and Mukhopadhyay, 1997). Hence, it could be speculated that TNFα activates not only the PL-A2 pathway but also other intracellular signaling pathways in bovine luteal cells. Therefore, the present study was conducted to determine the effects of various compounds (PL-A2, PL-C, PK-A, PK-C, NO synthase (NOS) and mitogen-activated protein kinase (MAPK) inhibitors) on TNFα-induced PGF2α production in cultured bovine luteal cells.

Materials and Methods

Chemicals

U-73122 {1-[6-((17B-3-Methoxyestra-1,3,5(10)-tri-en-17-y1)amino)hexyl]-1H-pyrrole-2,5-dione} (#662035; PL-C inhibitor) and ACA {N-(p-Amylcinnamoyl) anthranilic acid} (#104550; PL-A2 inhibitor) were purchased from Calbiochem®-Novabiochem Co., San Diego, CA, USA. L-NAME {N⁰-Nitro-L-Arginine Methyl Ester Dihydrochloride} (#A161; inducible NOS inhibitor) and L-NORG {N⁰-Nitro-L-Arginine} (#A160; constitutive NOS inhibitor) were purchased from RBI®, Natick MA, USA. H-89 {N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide-2HCl} (#EI196; PK-A inhibitor), calphostin C (#EI198; PK-C inhibitor) and PD98059 {2'-Amino-3'-methoxyflavone} (#EI360; MAPK kinase inhibitor) were purchased from BIOMOL® Research Laboratories, Inc., Plymouth Meeting, PA, USA.
**Luteal Cell Culture**

Luteal cells were prepared and cells were cultured as previously described in CHAPTER 1. Viable cells (3x10^5/well) of mid-CL (days 8-12) were cultured in a DMEM/Ham’s F-12 medium supplemented with 10% calf serum and 20 μg/ml gentamicin in 48-well culture plates 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 TNFα (0.6 nM) with or without U-73122 (0.1-10 μM), ACA (1-100 μM), H-89 (0.1-10 μM), calphostin C (0.01-1 μM), L-NAME (1, 100 μM), L-NORG (1, 100 μM) or PD98059 (0.1-100 μM). The conditioned media were collected and stored at -30 C until assayed for PGF2α. Furthermore, to determine whether the cultured cells in the present experiments were reactive, the cells were treated with bovine LH (10 ng/ml) for 24 h. I confirmed that bovine LH stimulates progesterone secretion by cultured luteal cells in all experiments.

**Results**

**Effects of PL-C and -A2 Inhibitors on TNFα-induced PGF2α Secretion**

As shown in Fig. 13A, U-73122 did not alter the stimulatory effect of TNFα on PGF2α secretion by the cultured luteal cells. In contrast, ACA dose-dependently inhibited the TNFα-induced PGF2α secretion by the cells (Fig. 13B; P<0.05 or lower).

**Effects of PK-A and -C Inhibitors on TNFα-induced PGF2α Secretion**

The stimulatory effect of TNFα on PGF2α secretion by the cultured luteal cells was not affected by treatment with either H-89 or calphostin C (Fig. 14).

**Effects of NOS Inhibitors and MAPK Kinase Inhibitor on TNFα-induced PGF2α Secretion**

Neither L-NAME nor L-NORG affected TNFα-stimulated PGF2α secretion by cultured luteal cells (Fig. 15A). PD98059, a selective MAPK kinase inhibitor, inhibited TNFα-induced PGF2α secretion by the cells in a dose-dependent fashion (Fig. 15B; P<0.05 or lower).

**Possible Intracellular Signaling Pathways of TNFα to Stimulate PGF2α Secretion by Bovine Luteal Cells**

Figure 16 shows a diagram of a possible intracellular signaling pathway of TNFα in order to stimulate PGF2α production in bovine luteal cells. TNFα may activate the MAPK and PL-A2 pathways, resulting in the release of arachidonic acid and the subsequent production of PGF2α.
Figure 13. Effects of tumor necrosis factor α (TNFα) on prostaglandin F2α (PGF2α) secretion by cultured luteal cells of mid-stage treated with (A) U-73122 (a phospholipase C inhibitor) or (B) ACA (a phospholipase A2 inhibitor). All values are expressed as a percentage of the respective control values. Different superscript letters indicate significant differences (P<0.05), as determined by ANOVA followed by Fisher's PLSD multiple comparison test.
Figure 14. Effects of tumor necrosis factor α (TNFα) on prostaglandin F2α (PGF2α) secretion by cultured luteal cells of mid-stage treated with (A) H-89 (a protein kinase A inhibitor) or (B) calphostin C (a protein kinase C inhibitor). All values are expressed as a percentage of the respective control values. Different superscript letters indicate significant differences (P<0.05), as determined by ANOVA followed by Fisher's PLSD multiple comparison test.
Figure 15. Effects of tumor necrosis factor α (TNFα) on prostaglandin F2α (PGF2α) secretion by cultured luteal cells of mid-stage treated with (A) L-NAME and L-NORG (an NOS inhibitor) or (B) PD98059 (a MEK inhibitor). All values are expressed as a percentage of the respective control values. Different superscript letters indicate significant differences (P<0.05), as determined by ANOVA followed by Fisher's PLSD multiple comparison test.
Figure 16. Diagram of a possible intracellular signaling pathway of tumor necrosis factor α (TNFα) to stimulate prostaglandin F2α production in bovine luteal cells.
Discussion

The preceding results clearly demonstrated that the treatment of ACA (a PL-A2 inhibitor) and PD98059 (a MAPK kinase inhibitor) inhibits TNFα-stimulated PGF2α secretion in a dose-dependent fashion. This finding suggests that the stimulatory effect of TNFα on PGF2α production in bovine luteal cells is mediated by the MAPK and PL-A2 intracellular signaling pathway.

In the present study, a selective inhibitor of PL-A2 (ACA) completely stopped the actions of TNFα, whereas the inhibitors of PL-C, PK-A and PK-C did not significantly inhibit TNFα-induced PGF2α production. This result suggests that TNFα induces the activity of PL-A2 but not other phospholipases and protein kinases in order to produce PGF2α in the luteal cells. It is now generally accepted that PL-A2 stimulates intracellular arachidonic acid accumulation. Furthermore, Townson and Pate (1996), who used a PL-A2 inhibitor (aristolochic acid) and a PL-C inhibitor (compound 48/80), demonstrated that the TNFα-stimulated PGF2α secretion by bovine luteal cells is dependent on the stimulation of PL-A2 but not on the stimulation of PL-C. Thus, the present study strongly supports the idea that the TNFα/TNF-RI complex may activate the PL-A2 pathway and stimulate the production of arachidonic acid and PGs in bovine luteal cells.

A common pathway for the action of TNFα in cells is the induction of NOS, resulting in the generation of NO and the subsequent production of cGMP (Moncada et al., 1991, Davies et al., 1995). It has been assumed that NO directly influences the activity of heme-containing enzymes such as cyclooxygenase (Salvemini et al., 1993) and modulates PGF2α production in reproductive organs including CL (Novaro et al., 1996, Motta et al., 1997, Motta and Gimeno, 1997). Therefore, I expected to find that the TNFα-induced PGF2α production is mediated by induction of NOS activity and the subsequent generation of NO-cGMP. However, in the present study, the treatment of NOS inhibitors (L-NAME and L-NORG) did not significantly affect TNFα-stimulated PGF2α production. Thus, it could be postulated that the NO-cGMP pathway is independent of the PL-A2 pathway, and that the inhibitory effects of L-NAME and L-NORG on TNFα-induced NO-cGMP generation and subsequent PGF2α production is overcome by PGF2α release by activation of the PL-A2 pathway.

Recent studies have shown that the MAPK cascade is involved in the transduction of diverse extracellular signals into nuclear signals that regulate gene expression. In the classical MAPK cascade, the serine/threonine kinase Raf and Ras (MAPK kinase kinases) activate MAPK kinase that, in turn, activates MAPK by phosphorylation of both tyrosine and threonine residues (Cobb and Goldsmith, 1995, Watanabe et al., 1995). MAPK transmits extracellular signals into the nucleus (Chen et al., 1992), and phosphorylates various
transcription factors, e.g., c-Jun (Baker et al., 1992) and c-Myc (Gupta and Davis, 1994) that are involved in regulating gene expression. PD98059 (effective doses; 10-100 µM) which inhibits the MAPK kinase activation (Alessi et al., 1995, Pang et al., 1995) clearly suppressed TNFα-induced PGF2α secretion by cultured bovine luteal cells in the present study. Hence, it could be speculated that MAPK activation is needed to stimulate PGF2α secretion in response to TNFα. Moreover, inhibition of MAPK kinase activity by treatment with PD98059 resulted in the decrease of TNFα-induced PGF2α secretion, suggesting that the MAPK cascade may couple with the PL-A2 pathway. Supporting this idea, several reports have proposed that MAPK, in part, regulates cytoplasmic PL-A2 activation in a variety of cells (Sa et al., 1995, Lin et al., 1993, Pyne et al., 1997). Furthermore, it has been demonstrated that bovine luteal cells possesses Raf protein, and that PGF2α activates the Raf/MAPK kinase/MAPK pathway in bovine luteal cells (Chen et al., 1998). Based on the above findings, I assume that TNFα activates the Raf/MAPK kinase/MAPK signaling cascade and subsequently activates the PL-A2 pathway in bovine luteal cells to produce arachidonic acid and PGF2α.

In summary, the present study is the first to demonstrate the relevance of TNFα and the MAPK/PL-A2 intracellular signaling pathway in bovine luteal cells. Thus, TNFα plays one or more physiological roles in regulating bovine corpus luteum function, and in part, stimulates PGs production in bovine luteal cells via the MAPK/PL-A2 pathway.

Summary

The objective of the present study was to clarify the intracellular signaling pathway of TNFα to stimulate PGF2α production in cultured bovine luteal cells. Bovine luteal cells of mid- (days 8-12) CL were incubated with TNFα (0.6 nM) and/or various compounds. U-73122 (a PL-C inhibitor; 0.1-10 µM), H-89 (a PK-A inhibitor; 0.1-10 µM), calphostin C (a PK-C inhibitor; 0.01-1 µM) and L-NAME/L-NOR (an NOS inhibitor; 1-100 µM) did not affect TNFα-induced PGF2α secretion by the cultured cells. In contrast, ACA (a PL-A2 inhibitor; 1-100 µM) and PD98059 (a MAPK kinase inhibitor; 0.1-100 µM) inhibited TNFα-stimulated PGF2α secretion by the cells in a dose-dependent fashion (P<0.05 or lower). These findings indicate that TNFα may activate the MAPK and PL-A2 pathways in bovine luteal cells to stimulate PGF2α secretion.
CONCLUSION

The present study is composed of four series of experiments. The first series of experiments demonstrated the presence of local TNFα as well as functional TNFα receptors (at least TNF-RI) in bovine CL during the estrous cycle by using a combined approach incorporating an RT-PCR analysis and an RRA. In addition, the fact that TNFα stimulated hormone secretion by cultured luteal cells of all luteal stages confirmed that the receptors for TNFα in bovine CL are functional. Furthermore, the presence of TNFα mRNA and TNFα receptors in bovine CL during the gestation period were demonstrated in a second series of experiments. The third series of experiments indicated the presence of functional TNFα receptors (high- and low-affinity receptors) on the endothelial cells in bovine CL, suggesting that TNFα affects not only luteal cell function, but also endothelial cell function in bovine CL. The final series of experiments demonstrated the relevance of TNFα and the MAPK/PL-A2 intracellular signaling pathway in bovine luteal cells.

The overall results indicate the local production of TNFα and the presence of functional TNFα receptors in bovine CL throughout the estrous cycle and entire gestation period. These findings strongly suggest that TNFα plays two or more physiological roles in regulating bovine CL function as an autocrine and/or paracrine regulator.
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ABSTRACT IN JAPANESE

ウシ黄体における腫瘍壞死因子 (TNFα) ならびにそのレセプターに関する研究

作本亮介

腫瘍壞死因子 (TNFα) は主にマクロファージで産生されることの知られるサイトカインの一つである。近年、雌性生殖機構、特に、黄体退行機構への TNFα の関与が注目されているが、その詳細は明らかでない。本研究では、ウシ黄体における TNFα の生理的役割を知るための基礎研究として、発情周期ならびに妊娠期におけるウシ黄体の TNFα およびその特異的なレセプターについて多角的に検討するとともに、局所機能調節因子としての TNFα の生理的役割を知るためにウシ黄体の内分泌機能におよぼす TNFα の影響について調べた。その結果、以下のような新しい知見を得た。

1）周期性黄体における TNFα とそのレセプター

ウシ黄体を、肉眼的所見から形成初期 (days 2-3)、形成後期 (days 5-6)、中期 (days 8-12)、後期 (days 15-17) ならびに退行期 (days 19-21) に分類した。黄体組織から total RNA を抽出した後、reverse transcriptase polymerase chain reaction (RT-PCR) 法を用いて、TNFα mRNA ならびに TNF レセプター・タイプ I (TNF-R1) mRNA 発現を調べた。また、黄体組織中の TNFα 濃度は、ヒト TNFα ELISA キットを用いて検討した。さらに、黄体組織から常法に従い作成した膜分画について、125I-標識 TNFα を用いてラジオレセプター・アッセイ (RRA) を行い、Scatchard 解析により TNFα レセプターの濃度ならびに親和性を求めた。その結果、黄体内の TNFα mRNA 発現量は発情周期を通じて有意な変化は見られなかったが、TNFα の濃度は発情周期にともなって増加し、後期ならびに退行期に高い値を示した (P<0.05)。また、発情周期を通じて黄体に TNF-R1 mRNA の発現が見られ、さらに TNFα に特異的な結合部位 (Kd; 3.6-5.8 nM) の存在することが明らかとなった。次に、黄体に存在する TNFα レセプターが機能的であるかどうかを知る目的で、形成後期、中期ならびに後期の黄体から灌流法により採取した黄体細胞を培養し、培養 24 時間後に培養液を交換した後、TNFα (0.06-6 nM) で 24 時間処理した。培養液中のプロゲステロン (P4)、プロスタグランジン (PG) F2α ならびに PGE2 濃度をエンザイムイムノアッセイにより測定した。その結果、形成後期、中期および後期のいずれの黄体より採取した培養細胞においても、PGE2α ならびに PGE2 分泌は、TNFα によって有意に促進されたが、P4 分泌には影響が見られなかった。

2）妊娠黄体における TNFα とそのレセプター

ウシ黄体のステージを、胎児の頭尾体長より妊娠初期 (<90 日)、中期 (90-180 日) ならびに後期 (>180 日) に分類した (各 n=6)。黄体組織から total RNA を抽出した後、RT-PCR を行い、TNFα mRNA 発現を調べた。一方、黄体組織から作成した膜分画について RRA を行い、Scatchard 解析によりレセプターの濃度ならびに
親和性を求めた。その結果、いずれのステージの妊娠黄体にも TNFα mRNA の発現が見られたが、その発現量にステージによる差は認められなかった。また、いずれのステージの妊娠黄体にも TNFα に特異的な結合部位が存在し、その濃度は妊娠初期 (24.0±1.9 pmol/mg protein) ならびに妊娠後期 (21.6±2.4 pmol/mg) に高く、中期 (14.9±2.1 pmol/mg) に低かった。

3）黄体由来血管内皮細胞における TNFα とそのレセプター
実験 1）、2）より、ウシ黄体に TNFα ならびにその特異的なレセプターの存在することが明らかとなった。しかし、黄体を構成する細胞は黄体細胞だけでなく、血管内皮細胞、繊維芽細胞といったその他の細胞も多く、上記の研究では黄体細胞以外の細胞での TNFα の生理作用について明確にできなかった。そこで、本研究では黄体の約 50% 以上を構成する血管内皮細胞における TNFα レセプターならびにその生理的意義について検討した。ウシ黄体から単離した 2 種類の血管内皮細胞 (CS cells と TS cells) を培養し、RRA ならびに RT-PCR により、TNFα レセプターの存在について検討した。その結果、CS および TS cells ともに 2 つの異なる親和性をもつ TNFα レセプターならびに TNF-R1 mRNA の存在することが示された。さらに、細胞培養法による刺激試験から、TNFα が血管内皮細胞のエンドセリン-1 ならびに PGE2 分泌を濃度依存的に増加させることが明らかとなった。

4）TNFα の黄体細胞内シグナル伝達機構
実験 1）において、TNFα が培養黄体細胞の PGF2α 分泌を促進することが明らかになった。TNFα は様々な細胞内シグナル伝達機構を介して作用することが知られているが、黄体細胞での機構には不明な点が多い。本研究では、種々の細胞内シグナルに関与する物質の特異的阻害剤を用いて、TNFα の黄体細胞内シグナル伝達機構について検討した。ウシ中期黄体細胞を常法に従い培養し、種々の細胞内シグナル伝達阻害剤と TNFα を組み合わせて添加した後の、PGF2α 分泌量の変化を調べた。その結果、TNFα による黄体細胞の PGF2α 分泌促進効果は、phospholipase (PL) A2 ならびに mitogen activated protein kinase (MAPK) の特異的阻害剤により濃度依存的に抑制された。このことから、TNFα は PL-A2 および MAPK を介して PGF2α 分泌を促進する可能性が示唆された。

以上の研究から、発情周期だけでなく妊娠期を通じてウシ黄体に TNFα ならびにその特異的なレセプターの存在することが明らかとなり、TNFα は局所機能調節因子として、黄体進行機構だけでなく、発情周期、妊娠期を通じてのウシ黄体機能調節にも関与することが示唆された。また、ウシ黄体由来血管内皮細胞にも特異的な TNFα レセプターの存在することが明らかとなったことから、TNFα は黄体内局所調節因子として、黄体細胞のみならず血管内皮細胞の内分泌機能にも作用して、オートクレインならびにパラクレイン的にウシ黄体機能調節に関与することが示唆された。