REPRODUCTIVE BIOLOGY

ORIGINAL RESEARCH

The influence of tumor necrosis factor α (TNF) on the secretory function of bovine corpus luteum: TNF and its receptors expression during the estrous cycle

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SUMMARY

Tumor necrosis factor α (TNF) inversely regulates the function of bovine corpus luteum (CL). Whereas the low doses of TNF induce luteolysis, the high doses prolong CL lifespan and prevent luteolysis *in vivo*. We suggest that the varying effects of TNF may be caused by its action exerted on CL *via* multiple signaling pathways involving two distinct receptors: TNFR-I (responsible for induction of the cell death) and TNFR-II (implicated in cell proliferation). In the study, we determined CL expressions of TNF, TNFR-I and TNFR-II mRNAs during the bovine estrous cycle using semi-quantitative RT-PCR. Specific transcripts for TNF, TNFR-I and TNFR-II were found in the CL with the highest ($p<0.05$) expression in the regressed CL. We also examined the TNF influence on the bovine CL function *in*

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vivo. On Day 15 of the estrous cycle, cows were infused (*via aorta abdominalis)* with saline, TNF (1 or 10 μg) or analogue of prostaglandin (PG)$F_{2\alpha}$ ($aPGF_{2\alpha}$, 500 μg; a positive control). Four hours after infusions, CLs were collected by colpotomy and luteal contents of progesterone (P_4), stable metabolites of nitric oxide (NO; nitrite/nitrate), leukotriene (LT)C_4, luteolytic $PGF_{2\alpha}$,and luteotropic PGE_2 were determined. Moreover, caspase-3 activity was measured in the CLs as an indicator of apoptosis induction. The luteal content of P_4 decreased ($p<0.05$) after infusion of 1 μg of TNF. TNF inversely affected PGs content in CL: the low dose increased ($p<0.01$) the $PGF_{2\alpha}$ level and the high dose increased ($p<0.05$) PGE_2 level. Contents of LTC_4 and nitrite/nitrate increased ($p<0.01$) after the low dose of TNF. Moreover, 1 μg of TNF induced apoptosis and increased ($p<0.05$) caspase-3 activity in the CLs collected during the late luteal phase. In conclusion, the high expressions of TNF and TNF receptors mRNAs were observed during or just after the luteolysis. A low concentration of TNF stimulated *in vivo* luteolytic factors such as $PGF_{2\alpha}$, LTC_4 and NO as well as induced apoptosis; whereas the high concentration of TNF stimulated a survival pathway in the bovine CL increasing luteal content of P_4 and PGE_2. *Reproductive Biology 2008* ***8*** *3:245-262.*

Key words: tumor necrosis factor α, progesterone, prostaglandins, luteolysis, cattle

INTRODUCTION

Corpus luteum (CL) is a heterogeneous gland which, in addition to steroidogenic luteal cells and endothelial cells, is composed of fibroblasts and immune cells [10]. The immune cells and their products - cytokines, are believed to be involved in the regulation of luteal function [32, 33]. Tumor necrosis factor (TNF), produced and secreted by immune cells of the CL (macrophages, mastocytes), might participate in functional and structural luteolysis in cattle [21, 31, 32, 34]. TNF inhibits gonadotropin-supported progesterone (P_4) production by murine [1, 5], porcine [8] and bovine ovarian cells [3] as well as stimulates prostaglandin (PG) synthesis

by bovine luteal cells [40]. Furthermore, it has been shown to induce a significant increase in major histocompatibility class 1 and 2 glycoprotein expression in cultured bovine luteal cells. These glycoproteins are recognized by cytotoxic T-cells which enable the luteal cells to be phagocytized [6, 32].

Our *in vivo* study revealed that TNF changes the lifespan of bovine CL [43]. An infusion of 10 μg TNF increased P_4 and PGE_2 concentrations in the peripheral blood and prolonged the CL lifespan. A low concentration (1 μg) of TNF caused the stimulation of uterine $PGF_{2\alpha}$ and various luteolytic factors to complete a premature luteolysis [43]. The latter action was mainly caused by indirect effects of TNF on the bovine CL *via* the stimulation of uterine secretion/action of prostaglandins [46]. However, TNF is also released locally by bovine CL during $PGF_{2\alpha}$-induced and spontaneous luteolysis [27, 35, 40]. Moreover, expression of TNF mRNA rose in the bovine CL two hours after the start of $PGF_{2\alpha}$-induced luteolysis [27]. Therefore, it was suggested that TNF acts as an intraluteal mediator/modulator of luteolytic action of $PGF_{2\alpha}$ [45]. In the bovine CL, TNF interacts with $PGF_{2\alpha}$ and endothelin-1 directly inhibiting local release of P_4 [28]. During luteolysis, TNF together with other cytokines, may induce apoptosis of bovine steroidogenic CL cells *in vitro* [18, 48]. These findings imply that the TNF actions on CL function are strongly associated with luteolysis, particularly with the structural phase of the luteal regression.

On the other hand, there are data indicating the luteotropic role of TNF in cattle. During all stages of the luteal phase, TNF stimulated cultured bovine luteal cells to release luteotropic PGE_2 [38]. Moreover TNF and its receptors have been found in CL during early pregnancy in cattle [39]. These multiple, and sometimes even contradictory functions of TNF might be due to the activation of different TNF receptors (TNF-Rs).

The two identified [30] and immunologically distinct TNF-Rs (Type I; TNFR-I, 55 kDa and Type II; TNFR-II, 75 kDa) transduce different intracellular signaling pathways [30]. TNFR-I contains an intracellular death domain, which is required for apoptosis signaling. In contrast, TNFR-II was suggested to induce transcription of genes important for cell survival, growth and differentiation [30]. TNFR-I mRNA and the specific

binding sites for TNF are present in bovine CL throughout the estrous cycle [38, 39]. Moreover, the CL expressions of TNF protein and mRNA as well as TNFR-I mRNA [38] increased between Days 13 and 18 of the bovine estrous cycle. These data indicate that the direct influence of TNF on CL is possible by activation of TNFR-I.

The TNF effects exerted on the bovine CL may result from TNF local cooperation with other cytokines and/or nitric oxide (NO) and $PGF_{2\alpha}$. The TNF luteal effects may also depend on the TNF effects on endometrial secretion of PGs [23, 24, 46]. The aims of the study were: 1/ to examine the luteal expressions of TNF, TNFR-I and TNFR-II mRNA during the bovine estrous cycle; 2/ to study the effects of TNF and $PGF_{2\alpha}$ (a positive control) on luteal content of P_4, oxytocin (OT), stable metabolites of NO (nitrite/nitrate: NO^-_2/NO^-_3) as well as luteolytic ($PGF_{2\alpha}$ and LTC_4) and luteotropic (PGE_2) metabolites of arachidonic acid during the late luteal phase of the bovine estrous cycle; and 3/ to determine the effect of TNF and $PGF_{2\alpha}$ on caspase-3 activity in the bovine CL.

MATERIALS AND METHODS

Animals and surgical procedures

All procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (Agreement No. 4/2001/N). Normally cycling Holstein/Polish Black and White (75/25 %; respectively) cows (4-6 lactation; n=32), eliminated from the farm because of lower milk production, were used in the study. The estrus was synchronized by implants of P_4 analogue (Crestar, Intervet, Holland) with a subsequent injection of $PGF_{2\alpha}$ analogue ($aPGF_{2\alpha}$; dinoprost, Dinolytic, Upjohn- Pharmacia NVSA, Belgium). Follicular development, estrus symptoms and CL formation were monitored *via per rectum* by USG examination (Draminski Electronics in Agriculture, Olsztyn, Poland). The first day of estrus was assigned as Day 0. In the first experiment, the ovaries were collected from live cows through colpotomy using Hauptner's effeninator (Hauptner & Herberholz

GmbH & Co. KG, Solingen, Germany; [47]). The CL was removed from the ovary, rapidly frozen in liquid nitrogen and stored at -80°C.

In the second experiment, infusions of saline (control group), $aPGF_{2\alpha}$, (cloprostenol; Bioestrophan, Biowet, Poland), or TNF (recombinant human TNF: HF-13; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) were performed on Day 15 of the estrous cycle *via* catheter inserted into the posterior *aorta abdominalis via* the coccygeal artery [15]. The animals were premedicated with xylazine (25-30 mg/animal, i.m.; Sedazin, Biowet, Pulawy, Poland) and local epidural anesthesia was induced by injecting 4 ml of 2% procaine hydrochloride (Polocainum Hydrochloricum, Biowet Drwalew, Poland) between the 1st and 2nd coccygeal vertebrae. The tip of the cannula was positioned in the aorta 60-65 cm ahead of the point of insertion, just cranial to the origin of the ovarian artery and caudal to the renal artery [15]. Such placement allowed infused reagents to be transported by the bloodstream directly into the reproductive tract. Ovaries were collected by colpotomy four hours after injection of treatments. The CLs were immediately separated from the ovaries, rapidly frozen in liquid nitrogen and stored at -80°C until used for hormone contents and caspase-3 activity assays.

Experiment 1. Expression of TNF, TNFR-I and TNFR-II mRNAs in the bovine CL during the estrous cycle

Corpora lutea were collected from live animals (n=4/each stage) by colpotomy at different stages of the estrous cycle: early-luteal (Days 2-3 after ovulation), mid-luteal (Days 8-9), late-luteal (Days 15-16) or follicular phase (Days 19-20). Total RNA was isolated from the luteal tissue using TRIZOL Reagent. Each sample of total RNA (1 μg) was reverse transcribed using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen; Carlsbad, USA). Semi-quantitative RT-PCR was carried out using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as an internal standard. G3PDH primer was added at the appropriate cycle number by the "primer-dropping method" [50]. The conditions for the PCRs were as follows: 27 (TNF), 28 (TNFR-I), 25 (TNFR-II) or 18 (G3PDH) cycles of reactions including denaturation

for 30 sec at 95°C, annealing for 1 min at 60°C, and extension for 1 min at 70°C, followed by an additional extension for 5 min at 72°C. The primers used and lengths of amplicons (bp) are depicted in Table 1.

Table 1. Sequences of forward (For) and reverse (Rev) primers and lengths of RT/PCR products

Primer	Sequence (5'→3')	Length (bp)	References
G3PDH	For TGT TCC AGT ATG ATT CCA CCC Rev TCC ACC ACC CTG TTG CTG TA	850	Tsai et al. [49]
TNF	For GAA GCT GGA AGA CAA CCA Rev TCC CAA AGT AGA CCT GCC	338	Sakumoto et al. [39]
TNF-RI	For CAC CAC CAC CAT CTG CTT Rev TCT GAA CTG GGG TGC AGA	257	Sakumoto et al. [39]
TNF-RII	For CTC GAC CAG CAG CAC GGA CA Rev GCG TCT GTG TCC CTC GTG GA	231	Kabeya et al. [13]

G3PDH: glyceraldehyde-3-phosphate dehydrogenase; TNF: tumor necrosis factor α: TNF-RI: 1 type of TNF receptor; TNF-RII: 2 type of TNF receptor

Aliquots of PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide with a known standard (100 bp Ladder; New England BioLabs Inc., MA, USA) and photographed under ultraviolet illumination. The band intensities were analyzed by computerized densitometry using the NIH image software (National Institutes of Health, USA). The amplified cDNA fragments were sequenced after being subcloned into pGEM3Zf (+). The nucleotide sequences were determined by the dideoxy chain termination method with an ABI310 sequencer (Applied Biosystems, CA, USA). Sequence analysis was carried out using GENETYX software and the homology of sequences to those received previously [13, 39] was 99%.

Experiment 2. Direct effect of TNF on bovine CL function during the late luteal phase

Twenty ml of saline (control; n=4), a putative luteolytic dose of TNF (1 μg/animal; n=4 cows) or a putative luteotropic dose of TNF (10 μg/animal;

n=4 cows) in 20 ml of saline was infused for 30 minutes into the *aorta abdominalis* of cows on Day 15 of the estrous cycle [15]. a$PGF_{2\alpha}$ (500 µg) was infused as a positive control (n=4 cows). Four hours after infusions, the CL were collected and frozen in liquid nitrogen. The luteal tissue was homogenized in a Vibratory Mill (Retsch MM-2) and dissolved in phosphate buffer. CL concentrations of P_4, OT, PGE_2, $PGF_{2\alpha}$ and LTC_4 were measured by enzyme immunoassay (EIA). In addition, the concentrations of nitrite/nitrate and activity of caspase-3 were measured colorimertrically.

Hormone concentration assays

The luteal content of P_4, following homogenization and extraction with diethyl ether (extraction efficiency: 82.7%), were assayed by EIA [41, 51]. The antibodies had been characterized previously [16]. The P_4 standard curve ranged from 0.78 ng/ml to 100 ng/ml and the effective dose for 50% inhibition (ID50) of the assay was 2.85 ng/ml. The intra- and inter-assay coefficients of variation averaged 6.6% and 8.4%, respectively. Oxytocin was extracted from homogenized luteal tissue using acetone (POCH SA, Gliwice, Poland) with extraction efficiency averaged to 89.7% [14] and then assayed by EIA [41]. The OT standard curve ranged from 1.95 to 500 pg/ml, and the ID50 of the assay was 34.7 pg/ml. The intra- and inter-assay coefficients of variation were 7.8% and 11.7%, respectively.

Luteal contents of $PGF_{2\alpha}$ and PGE_2 were determined by EIA [17, 42] using commercial anti-$PGF_{2\alpha}$ and anti-PGE_2 sera (Sigma; St. Louis, USA), respectively. Luteal tissue (0.2 g) was homogenized and vortexed for 20 min in 1 ml of ice-chilled diethyl ether (POCH SA, Gliwice, Poland) with an addition of 1N HCl (45 µl/1 ml). The $PGF_{2\alpha}$ and PGE_2 extraction efficiencies were 87.4% and 86.9%, respectively. The $PGF_{2\alpha}$ standard curve ranged from 0.07 to 20 ng/ml, and the ID50 of the assay was 1.82 ng/ml. The intra- and inter-assay coefficients of variation were 7.4% and 11.6%, respectively. The PGE_2 standard curve ranged from 0.07 ng/ml to 20 ng/ml and the ID50 of the assay was 1.25 ng/ml. The intra - and inter-assay coefficients of variation were 6.9% and 9.7%, respectively.

Luteal content of LTC_4 was determined after CL homogenization and extraction (extraction efficiency=85.7%) by diethyl ether with an addition of 1 N HCl (45 µl/1 ml) using an EIA kit (Cayman Chemical Co., Ann Arbor, USA; [19]). The LTC_4 standard curve ranged from 0.98 pg/ml to 500 pg/ml and the ID50 of the assay was 1.85 pg/ml. The intra- and inter-assay coefficients of variation were on average 4.9% and 7.4%, respectively.

Nitrite/nitrate concentration and caspase-3 activity assays

Luteal tissue content of nitrite/nitrate, the stable metabolites of NO, was measured colorimetrically [9] with our modifications [11]. The assay sensitivity was 0.065 µg/ml and the standard curve ranged from 0.05 µg/ml to 6.9 µg/ml. The intra- and inter-assay coefficients of variation were on average 7.4% and 11.2%, respectively. Caspase-3 activity was measured using a commercially available caspase-3 colorimetric assay kit (Sigma, USA) according to the manufacturer's instructions.

Statistical Analysis

All experimental data are shown as means ±SEM. The luteal expressions of the examined mRNAs as well as the effect of TNF or $PGF_{2\alpha}$ on luteal concents of P_4, OT, $PGF_{2\alpha}$, PGE_2, LTC_4, stable metabolites of NO (NO_2^- /NO_3^-) or caspase-3 activity were analyzed by one-way ANOVA followed by Bonferroni's post-hoc test (ANOVA; GraphPAD PRISM version 4.00, GraphPad Software, San Diego, USA).

RESULTS

Experiment 1. Expression of TNF, TNFR-I and TNFR-II mRNAs in bovine CL during the estrous cycle

Specific TNF (fig.1A), TNFR-I (fig. 1B) and TNFR-II (fig. 1C) transcripts were detected in the bovine CL throughout the bovine estrous cycle. The expression levels of the three examined mRNAs were significantly ($p<0.05$) higher in the regressed CL (Days 19-21) than in CLs collected during earlier stages of the estrous cycle.

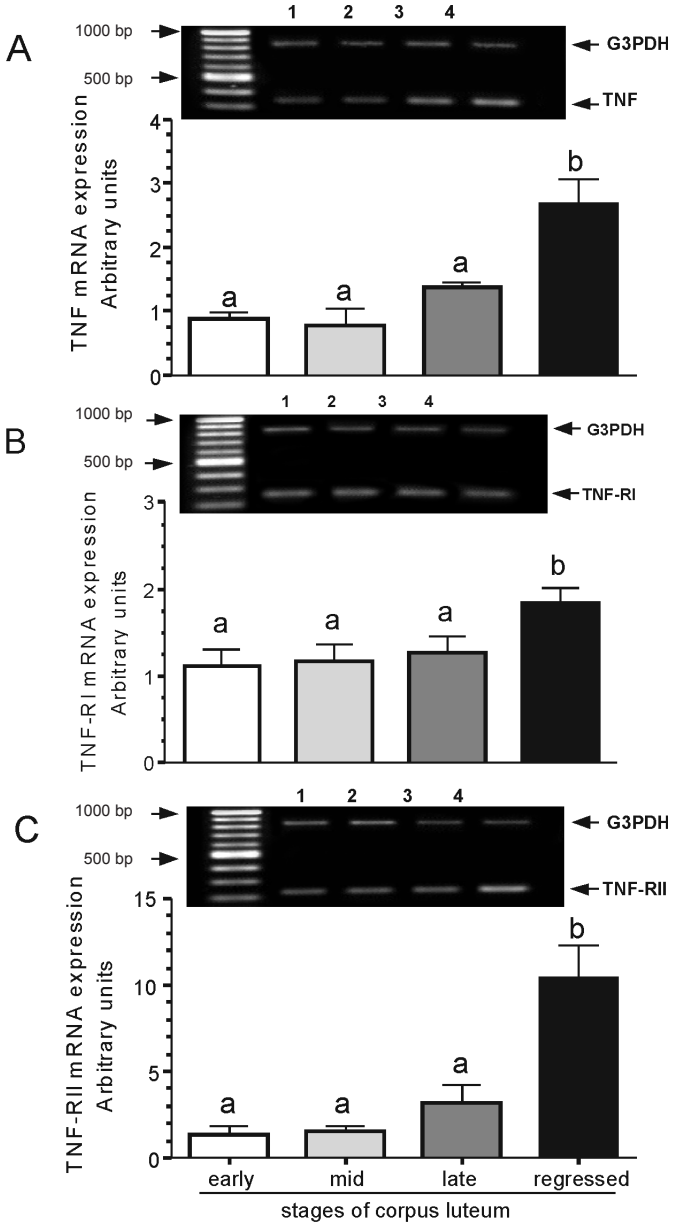

Figure 1. The luteal expressions of tumor necrosis factor α (TNF; A) and TNF receptors (TNFR-I; B, TNFR-II; C) mRNA during the bovine estrous cycle (n=4). Upper panels: representative samples of specific RT-PCR products for TNF, TNFR-I, TNFR-II and glyceraldehyde-3-phosphate dehydrogenase (G3PDH; 850 bp). Lower panels: densitometric values of mRNA luteal levels of TNF, TNFR-I, TNFR-II mRNA (mean±SEM) shown in relation to G3PDH mRNA level. Different letters indicate significant differences ($p<0.05$).

Experiment 2. Direct TNF effect on bovine CL function during the late luteal phase

The effects of TNF on luteal contents of P_4, OT, arachidonic acid metabolites and nitrite/nitrate differed between the infused doses (1 and 10 μg; fig. 2). Four hours after TNF infusion, the P_4 luteal content was lower ($p<0.05$; fig. 2A) in animals infused with 1 μg of TNF than those in control cows, and the cows infused with 10 μg of TNF. Although 10 μg of TNF did not affect

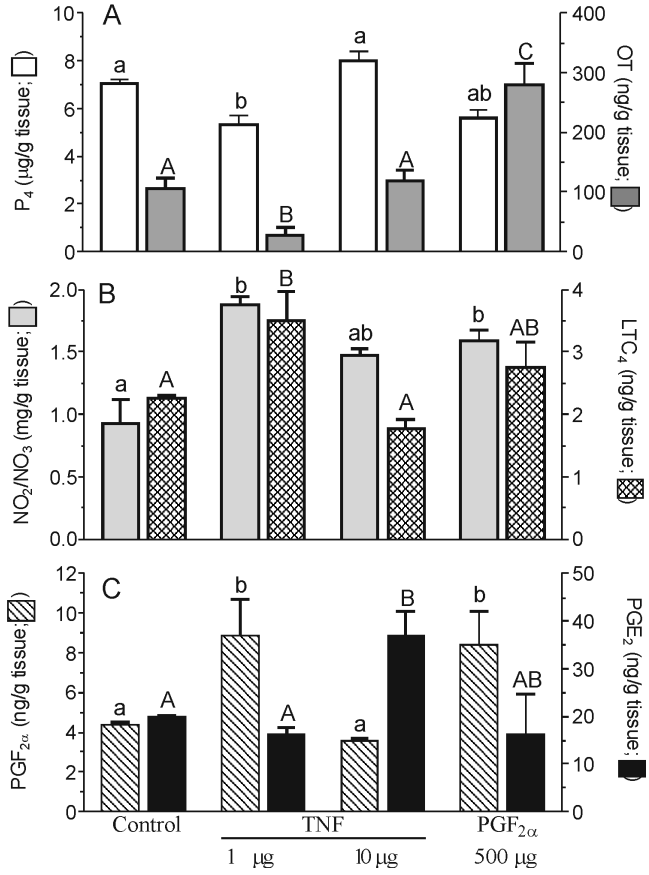

Figure 2. The effects of tumor necrosis factor α (TNF) and prostaglandin (PG) $F_{2\alpha}$ analogue ($aPGF_{2\alpha}$; a positive control) on luteal content of progesterone (P_4) and oxytocin (OT; A), stable metabolites of NO (NO_2^-/NO_3^-) and leukotriene C_4 (LTC_4; B), $PGF_{2\alpha}$ and PGE_2 (C) during the bovine late luteal phase. Different lowercase letters and capital letters indicate significant differences ($p<0.05$) for parameters depicted on the left and right OY axes, respectively.

($p>0.05$) OT content in bovine CL, the OT content decreased ($p<0.01$; fig. 2A) by 24% after the infusion of 1 μg of TNF. Luteal contents of LTC_4 and stable NO metabolites increased ($p<0.01$; fig. 2B) after a lower dose of TNF. TNF inversely affected the CL content of PGs (fig. 2C): while the lower dose increased ($p<0.01$) $PGF_{2\alpha}$ and did not alter PGE_2 ($p>0.05$), the higher TNF dose increased ($p<0.05$) PGE_2 and did not alter ($p>0.05$) $PGF_{2\alpha}$ luteal content. The analogue of $PGF_{2\alpha}$ (500 μg) increased ($p<0.05$) OT, nitrite/nitrate and $PGF_{2\alpha}$ luteal levels and did not affect ($p>0.05$) levels of P_4, LTC_4 and PGE_2.

The strongest stimulatory TNF effect on caspase-3 activity was observed in CLs after infusions of 1 μg of TNF and $aPGF_{2\alpha}$ (increase by 349% and 292% compared to controls; $p<0.001$). 10 μg of TNF did not affect caspase-3 activity ($p>0.05$; fig. 3).

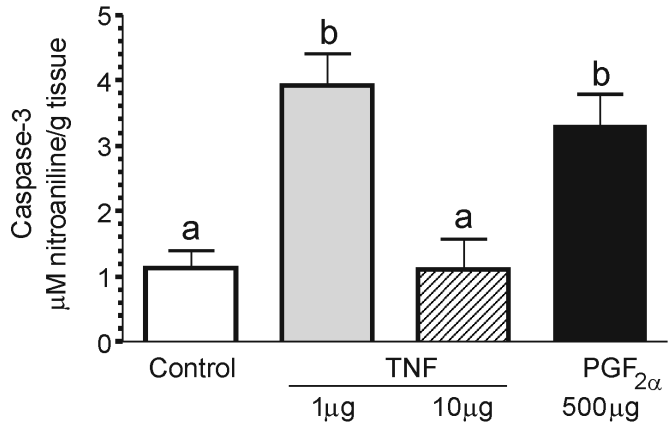

Figure 3. The effects of TNF and prostaglandin (PG) $F_{2\alpha}$ analogue ($aPGF_{2\alpha}$; a positive control) on caspase-3 activity in bovine corpus luteum (CL) during the late luteal phase. Different letters indicate significant differences ($p<0.05$).

DISCUSSION

In this study, the luteal expressions of TNF, TNFR-I and TNFR-II mRNAs were demonstrated throughout the bovine estrous cycle. We confirmed that TNF is one of the crucial, local factors involved in the regulation of

the CL function in cattle. In contrast to TNFR-I [7, 27, 38], there was limited information concerning the presence of TNFR-II in the bovine ovary. Recently, TNFR-II was found in granulosa cells of porcine ovaries [26]. TNFR-II involvement in the regulation of CL function has not yet been proven. Neuvians et al. [27] showed that TNF mRNA expression was up-regulated after a luteolytic dose of $PGF_{2\alpha}$ *in vivo* but TNFR-I was stable by this treatment in the cows. We have shown the highest mRNA expressions of TNFR-I, TNFR-II and TNF in the regressed CL, which suggests TNF role as luteolytic factor/mediator. However, Sakumoto et al. [38] reported opposite results concerning expression of TNFR-I mRNA in the CL during the bovine estrous cycle. They found the highest level of the receptor expression between Days 3 and 7 [38]. On the other hand, our results agree with those of Friedman et al. [7] who described the highest TNFR-I expression in regressing bovine CL.

We showed that in contrast to the higher dose, an infusion of 1 μg of TNF inhibited P_4 luteal content during the late luteal phase of the bovine estrous cycle. In the cows infused with 10 μg TNF, the luteal concentration of P_4 was elevated, which suggests that TNF participates in the two opposite processes in the CL: directly and/or indirectly stimulates steroidogenesis and mediates luteolysis. The *in vivo* effects of TNF on CL functions are strictly dose dependent. However, under *in vitro* conditions, none of the tested TNF doses affected P_4 production by pure steroidogenic luteal cells harvested during the early, mid and late luteal stages [38]. Only Liebermann et al. [20] showed that TNF increased P_4 secretion by microdialyzed *in vitro* CL during the early luteal stage. However, during $PGF_{2\alpha}$-induced luteolysis, TNF decreased P_4 release by microdialyzed ovine CL pretreated with $PGF_{2\alpha}$ and/or endothelin-1 (ET-1; [29]). At the same time TNF was able to stimulate ET-1 and $PGF_{2\alpha}$ release thereby establishing a local positive feedback, which could accelerate the luteolytic cascade in the CL [28, 29]. Such results confirm that TNF acts in a dose dependent manner. It appears that in order to fully recognize and comprehend the TNF *in vivo* action, it is necessary to create a new *in vitro* experimental model using a modern *in vitro* culture system with a mixture of different CL cell types characterized by preserved cell-to-cell contact.

In our study, luteal OT content decreased following the infusion of the putative luteolytic dose of TNF (1 μg). Studies are scarce concerning the direct influence of TNF on OT secretion. One available study revealed that during each stage of the estrous cycle, TNF increased OT secretion by *in vitro* microdialysed CL [20]. It should be emphasized, however, that OT was found to directly stimulate P_4 secretion by luteal tissue and cells in bovine CL [22, 37, 41]. Moreover, the highest reactivity of bovine CL to OT, with regard to P_4 stimulation *in vitro*, was found on Days 8-12 of the cycle [22, 41]. Thus, the observed decrease in luteal content of OT after a luteolytic dose of TNF is compatible with the hypothesis concerning the luteoprotective role of OT in the bovine CL [22].

TNF also affected, in a dose-dependent manner, local, luteal content of PGs: the lower dose (1 μg) increased the $PGF_{2\alpha}$ and did not change the PGE_2 level. In the contrary, the infusion of the higher dose of TNF (10 μg) increased the PGE_2 and did not change the $PGF_{2\alpha}$ level. Being a mediator of PGs secretion/action both during CL development and in the process of CL regression, TNF may play multiple roles in the regulation of CL function during the bovine estrous cycle [21, 37, 44, 46]. In addition to known luteolytic action of TNF within the CL (induction of luteolytic $PGF_{2\alpha}$ production and output), a luteotropic role of this cytokine was also reported [37, 38]. TNF induced the production and output of luteotropic PGE_2 in cultured bovine luteal cells [39]. Modulation of PGF-converting enzymes may be a mechanism by which TNF switches prostanoid metabolism from production of luteotropic PGE_2 to luteolytic $PGF_{2\alpha}$ [52].

TNF affects bovine CL during the time of luteolysis and its luteolytic action may be mediated by other factors such as $PGF_{2\alpha}$, NO and LTs [19, 45]. In this study, we showed that a luteolytic dose of TNF (1 μg) increased the luteal contents of NO metabolites and LTC_4. Both *in vivo* [45] and *in vitro* [11, 17, 18] studies revealed that NO was involved in the regulation of luteolysis. Moreover, several products of the lipoxygenase pathway, particularly LTB_4 and C_4, were found to play roles in luteolysis [4]. Luteal peaks of $PGF_{2\alpha}$ and luteolytic leukotrienes were reported on Day 18 of the cycle in heifers undergoing spontaneous luteolysis. These peak frequencies rose within a12 h-period during which P_4 decline occurred [4]. The LTC_4

release may result from action of luteolytic factors including TNF [19]. These findings support the concept that TNF plays an important role in functional luteolysis, especially with regard to the stimulation/activation of some luteolytic mediators.

The proapoptotic effect of TNF, similar to other luteolytic factors (i.e. cytokines, NO), was demonstrated by several *in vitro* studies. This TNF effect was found usually when TNF acted synergistically with interferon-γ (IFN; [18, 32, 34, 48]). However, under *in vivo* conditions independent proapoptotic action of TNF in bovine CL has not yet been proven. Our study is the first to show that TNF infused into bovine female reproductive organs induces local changes involved in the process of structural CL regression. In the present study, the luteolytic dose of TNF stimulated luteal caspase-3 activity determined four hours after the TNF infusion into the *aorta abdominalis*. Steroidogenic CL cells were found to undergo apoptosis during a later stage of luteal regression, when a massive degeneration of capillaries had already occurred [2, 12]. It has been shown that, potentiated by IFN, TNF-induced apoptosis acts mainly on microvascular endothelial cells of the bovine CL [37]. Thus, it is possible that TNF action during the structural regression of CL begins with immune cells, but the main apoptotic action of TNF is directed to endothelial cells and might be amplified by $PGF_{2\alpha}$, IFN and NO [21, 25, 36, 43, 45, 48]. Therefore, the proapoptotic action of TNF showed in our study, may reflect early TNF effects on endothelial cells of CL mediated by both types of TNF-Rs.

In summary, we demonstrated that TNF action on bovine CL depends on TNF dose and receptor type. The multiple effects of TNF may be affected by the stage of the luteal phase but at the same time the TNF actions are dose-dependent. The expressions of two types of TNF receptors were showed in the bovine CL with their highest level during or just after luteolysis. TNF at a low dose increased the CL level of the luteolytic factors such as $PGF_{2\alpha}$, LTC_4, and NO, and may be responsible for inducing apoptosis. On the other hand, TNF at a high dose may be a stimulator of a survival pathway in the bovine CL *via* increasing the CL level of P_4 and PGE_2.

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