BIOLOGY OF REPRODUCTION **70,** 473–480 (2004)
Published online before print 15 October 2003.
DOI 10.1095/biolreprod.103.016154

Involvement of Pro-Inflammatory Cytokines, Mediators of Inflammation, and Basic Fibroblast Growth Factor in Prostaglandin $F_{2\alpha}$-Induced Luteolysis in Bovine Corpus Luteum[1]

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ABSTRACT

The process of luteolysis requires very subtly modulated coordination of different factors and regulation systems. Immune cells and cytokines were shown to be relevant for bovine luteolysis. The aim of this study was to investigate the detailed pattern of mRNA expression of the pro-inflammatory cytokines tumor necrosis factor α (TNFα), TNF receptor type 1 (TNF-R1), interleukin 1β (IL-1β), and interferon γ (IFNγ), and of the inducible nitric oxide synthase (iNOS) and the basic fibroblast growth factor (FGF-2) during prostaglandin (PG) $F_{2\alpha}$-induced luteolysis in the bovine corpus luteum (CL). In addition, the mRNA expression for the LH-receptor (LH-R) and the steroidogenic enzyme P450scc was determined. Cows in the midluteal phase (Days 8–12) were injected with the $PGF_{2\alpha}$ analogue cloprostenol, and CL were collected by transvaginal ovariectomy before and 2, 4, 12, 48, and 64 h after $PGF_{2\alpha}$ injection. Conventional and real-time reverse transcription polymerase chain reaction RT-PCR (LightCycler) using SYBR Green I detection were employed to determine the mRNA expression for the investigated factors. All cytokines were significantly up-regulated during induced luteolysis. LH-R and P450scc mRNA were down-regulated ($P < 0.05$) during structural luteolysis (after 12 h), and P450scc in addition at 2 h after $PGF_{2\alpha}$ ($P < 0.05$). FGF-2 expression increased ($P < 0.001$) during functional luteolysis (until 12 h after $PGF_{2\alpha}$) and diminished thereafter. The mRNA expression for iNOS decreased ($P < 0.05$) after induction of luteolysis. In conclusion, cytokines may be involved not only in structural but also in functional luteolysis and the deprivation of luteal survival factors, leading to a situation where apoptosis can occur. FGF-2 may participate in the suppression of cytokine-induced iNOS mRNA expression and in the prevention of an inflammatory reaction in the surrounding tissues.

corpus luteum, corpus luteum function, cytokines, nitric oxide

INTRODUCTION

The regression of a cyclic corpus luteum (CL) is a very complex process, which involves major changes in tissue composition. At 24–48 h after induced luteolysis in the bovine CL, there is pronounced oligonucleosome formation with histological signs of luteal cell degeneration [1]. At this time, many cells show the characteristics of apoptotic cell death, but there is also evidence of some vacuolization and necrotic processes [2]. In the porcine CL, macrophages progressively increase around 24 h after induced luteolysis [3]. Invading macrophages were shown to ingest luteal cells by phagocytosis in the guinea pig CL [4]. In the bovine ovary, leucocytes, T-lymphocytes, and macrophages significantly increase during regression of the CL [5]. During late regression, 70% of all proliferating cells in the bovine CL are CD-14-positive macrophages [6], which is considered a characteristic of lipopolysaccharide-induced macrophage activation. The authors assume that the involution of the CL is an inflammatory-like condition.

Pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), and interferon γ (IFNγ) are involved in bovine luteolysis [7, 8]. In midcycle bovine luteal cells, TNFα, IL-1β, and IFNγ dose-dependently increase prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) production. TNFα and IFNγ are able to inhibit LH-stimulated progesterone synthesis [7]. Chronic exposure of bovine luteal cells to IL-1β also inhibits progesterone production [9]. IL-1β-converting enzyme mRNA expression is elevated in regressing CL and coincides with luteolysis [10]. There is also evidence that the combination of TNF and IFN is extremely cytotoxic [11, 12]. Furthermore, they stimulate the synthesis of monocyte chemoattractant protein-1 in endothelial cells of the bovine CL, which facilitates the attachment and migration of immune cells from the blood stream into sites of inflammation [13]. During both spontaneous and induced luteolysis, intraluteal TNFα increases significantly under in vivo conditions in microdialyzed bovine CL [14]. Endothelial cells from bovine CL express type 1 TNF receptors (TNF-R1) [15] and, in the absence of progesterone, undergo apoptosis in response to TNFα stimulation [16]. TNF peptide concentration significantly increases during the late luteal phase (Days 13–18) of the bovine estrous cycle [17].

The production of progesterone in the bovine CL is regulated by the activity of different steroidogenic enzymes, including the cytochrome P450scc. P450scc protein levels run parallel with plasma progesterone levels [18]. LH is the most important luteotropic hormone in domestic farm animals [19] and stimulates enzymes of luteal steroidogenesis, such as P450scc. In vitro, $PGF_{2\alpha}$ induces a marked down-regulation of LH-receptor (LH-R) mRNA expression [20]. The same is seen for P450scc expression in the late luteal phase of bovine CL and after induced luteolysis [16, 21].

Endogenous nitric oxide (NO), which is predominantly generated by the NO synthases (NOS), endothelial NOS (eNOS), or inducible NOS (iNOS), is released at the site of acute inflammation and modulates edema formation [22]. Pro-inflammatory cytokines can induce acute inflammation,

[1]Supported by the German Research Foundation (DFG Scha 257/14). T.P.N. was supported by the H. Wilhelm Schaumann Stiftung.
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Received: 6 February 2003.
First decision: 22 February 2003.
Accepted: 14 October 2003.

ISSN: 0006-3363. http://www.biolreprod.org

TABLE 1. Forward (For) and reverse (Rev) primer sequences (5′ → 3′), RT-PCR product length, and reference of the investigated factors[a] or of the according accession number (Acc. No.) in the EMBL database.

Primer	Sequence (5′ → 3′)	Length (bp)	Reference
UBQ	For AGATCCAGGATA AGGAAGGCAT Rev GCTCCACCTCCAGGGTGAT	198	Acc. No. Z18245
LH-R	For GAT AGA AGC TAA TGC CTT TGA CAA C Rev CCA GAA TGA AAT TAA ATT CAG AGG AG	198	Acc. No. 420504
P450scc	For AAC GTC CCT CCA GAA CTG TAC C Rev CTT GCT TAT GTC TCC CTC TGC C	362	[20]
TNFα	For TAA CAA GCC GGT AGC CCA CG Rev GCA AGG GCT CTT GAT GGC AGA	277	Acc. No. AF011926
TNF-R1	For CAC CAC CAC CAT CTG CTT Rev TCT GAA CTG GGG TGC AGA	257	[15]
IL-1β	For TTC TCT CCA GCC AAC CTT CAT T Rev ATC TGC AGC TGG ATG TTT CCA T	198	Acc. No. M35589
IFNγ	For TTC AGA GCC AAA TTG TCT CC Rev AGT TCA TTT ATG GCT TTG CGC	205	Acc. No. M29867
iNOS	For ACC TAC CAG CTG ACG GGA GAT Rev TGG CAG GGT CCC CTC TGA TG	316	Acc. No. U14640
FGF-2	For TCA AAG GAG TGT GTG CGA AC Rev CAG GGC CAC ATA CCA ACT G	161	Acc. No. J04193

[a] UBQ, ubiquitin; LH-R, LH-receptor; P450scc, cytochrome P450 side-chain cleavage; TNFα, tumor necrosis factor α; TNF-R1, TNFα-receptor type 1; IL-1β, interleukin-1β; IFNγ, interferon γ; iNOS, inducible NO-synthase; and FGF-2, basic fibroblast growth factor.

and maximal promoter activation of iNOS occurs with synergistic combinations of TNFα, IL-1β, IFNγ, and lipopolysaccharide [23]. There is growing evidence that cytokine-induced iNOS mRNA expression may be blocked by basic fibroblast growth factor (FGF-2) [24, 25]. In bovine CL cell cultures, NO seems to have luteolytic effects, as it inhibits progesterone and stimulates prostaglandins [26]. The lifespan of the bovine CL can be prolonged with in vivo intraluteal applications of an NOS inhibitor (L-NAME) [27]. On the other hand, the cytokine-induced death of bovine luteal cells cannot be prevented by L-NMMA, another NOS inhibitor [11].

The aim of this study was to investigate the expression pattern of the pro-inflammatory cytokines TNFα, IFNγ, and ILβ-1, the NO producing inducible NOS and FGF-2 during well-defined stages of $PGF_{2\alpha}$-induced luteolysis in bovine CL, and to discuss possible interactions between these factors.

MATERIALS AND METHODS

Collection of Bovine CL

The investigations were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals. The experimental protocol was approved by the institutional care and use committee. In total, 28 cows (mainly Holstein Fresians) were used in this study. Cows at the midluteal phase (Days 8–12) were injected i.m. with 500 μg of the $PGF_{2\alpha}$-analogue cloprostenol (Estrumate, Intervet, Germany). Ovaries were then collected by transvaginal ovariectomy 2, 4, 12, 48, and 64 h (n = 4–5/group) after $PGF_{2\alpha}$ injection, and the CL were enucleated (1 ovary with 1 CL per cow). Control CL were obtained from cows at the midluteal phase (Days 8–12, n = 5) before $PGF_{2\alpha}$ injection. This is a classic model used to investigate the luteolytic process in cattle [1, 2, 5]. All CL were cut into small, equal pieces; immediately frozen in liquid nitrogen; and stored at −80°C until RNA extraction.

Progesterone Determination

Blood samples for progesterone determination were taken from the jugular vein. The concentration of progesterone in blood plasma was measured after extraction with petrol ether using an enzyme immunoassay applying the second antibody technique [28]. Progesterone-6-hydroxy-hemisuccinate horseradish peroxidase was used as enzyme solution. The effective dose for 50% inhibition (ED50) of the assay was 6 ng/ml. The intraassay coefficient of variation was 4%–5%, and the interassay coefficient of variation was 8%–9%, respectively.

Total RNA Extraction and Reverse Transcription

Small slices of the frozen CL were cut and weighed. Total RNA from CL was extracted with peqGOLD TriFast (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. RNA was dissolved in water and spectroscopically quantified at 260 nm. The integrity of RNA was verified by an optical density (OD) absorption ratio OD_{260nm}:OD_{280nm} between 1.8 and 2.0, and by electrophoresis with ethidium bromide staining on a 1% denaturating agarose gel. Either 1 or 2 μg of total RNA (for real-time or conventional polymerase chain reaction [PCR], respectively) were reverse-transcribed to cDNA with 200 U of M-MLV Reverse Transcriptase (Promega, Madison, WI) according to the manufacturer's instructions.

Real-Time PCR Quantification

Primers were designed using the EMBL database (Ubiquitin, TNFα, IL-1β, IFNγ, LH-R, FGF-2) or used according to the literature [15, 29]. Their sequences and expected PCR product length are shown in Table 1. A master mix of the following reaction components was prepared to the indicated end-concentrations: 6.4 μl water, 1.2 μl $MgCl_2$ (4 mM), 0.2 μl forward primer (0.2 μM), 0.2 μl reverse primer (0.2 μM), and 1.0 μl LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). Nine microliters of the master mix were filled in glass capillaries, and 1 μl PCR template containing 25 ng reverse transcribed total RNA was added. To ensure an accurate quantification of the desired product, a high temperature fluorescence measurement in a fourth segment of the PCR run was performed [30]. The elevated temperature for fluorescence acquisition results in melting unspecific products (e.g., primer dimers) and eliminating nonspecific fluorescence signals. The following general real-time PCR protocol was employed: denaturation for 10 min at 95°C, 40–50 cycles of a four segmented amplification and quantification program (factor-specific conditions are summarized in Table 2), a melting step by slow heating from 60°C to 99°C with a rate of 0.1°C/sec and continuous fluorescence measurement, and a final cooling down to 40°C. Crossing point (CP) values were acquired by using the second derivative maximum method of the LightCycler software 3.3 (Roche). All CPs of the 32 samples (n = 4–5/group) per investigated factor were detected in one run to eliminate interassay variance. Real-time PCR efficiencies were acquired by amplification of a standardized dilution series, and slopes were determined using LightCycler Software 3.3 (Roche). The corresponding efficiencies (E) were then calculated according to the equation: $E = 10^{(-1/slope)}$ [31]. The specificity of the desired products in bovine CL was documented using a high-resolution gel electrophoresis and analysis of the melting temperature, which is product-specific. For specific melting temperatures and PCR efficiencies, see Table 3.

TABLE 2. Factor-specific conditions for LightCycler real-time PCR amplification and quantification of the investigated factors.[a]

Factor	Denaturation 15 s (°C)	Annealing 10 s (°C)	Elongation 20 s (°C)	Fluorescence acquisition 5 s (°C)	Cycle number
UBQ	95	60	72	86	40
LH-R	95	61	72	80	40
P450scc	95	62	72	86	40
TNFα	95	66	72	87	40
TNF-R1	95	68	72	90	40
IL-1β	95	60	72	83	40
IFNγ	95	60	72	79	45
iNOS	95	62	72	88	45
FGF-2	95	60	72	77	40

[a] UBQ, ubiquitin; LH-R, LH-receptor; P450scc, cytochrome P450 side-chain cleavage; TNFα, tumor necrosis factor α; TNF-R1, TNFα-receptor type 1; IL-1β, interleukin-1β; IFNγ, interferon γ; iNOS, inducible NO-synthase; and FGF-2, basic fibroblast growth factor.

TABLE 3. Product-specific melting temperature, real-time PCR efficiency in bovine CL, mean (n = 31) coefficient of variance in percentage (CV%), and range of crossing points (CP) of the investigated factors.[a]

Factor	Melting temperature (°C)	PCR efficiency	Mean CV%	CP range
UBQ	88.5	1.72	1.57	19.22–23.19
LH-R	83.1	1.73	6.99	22.76–34.06
P450scc	88.4	1.62	11.0	17.64–30.41
TNFα	89.7	1.83	2.60	26.63–32.08
TNF-R1	92.4	2.22	1.67	26.77–30.80
IL-1β	85.6	1.69	6.48	23.18–30.00
IFNγ	83.3	1.89	4.23	30.83–37.03
iNOS	90.5	1.87	1.66	27.31–31.87
FGF-2	80.7	1.90	3.02	21.95–27.99

[a] UBQ, ubiquitin; LH-R, LH-receptor; P450scc, cytochrome P450 side-chain cleavage; TNFα, tumor necrosis factor α; TNF-R1, TNFα-receptor type 1; IL-1β, interleukin-1β; IFNγ, interferon γ; iNOS, inducible NO-synthase; and FGF-2, basic fibroblast growth factor.

Conventional PCR

Conventional PCR was performed for iNOS and FGF-2 to confirm real-time PCR data of the LightCycler and to investigate the comparability of the two methods.

Primers were designed using the EMBL database (iNOS) or as used previously in the literature [32]: ubiquitin forward 5′-ATG CAG ATC TTT GTG AAG AC-3′, reverse 5′-CTT CTG GAT GTT GTA GTC-3′ (189 + 417 bp); iNOS forward 5′-TAG AGG AAC ATC TGG CCA GG-3′, reverse 5′-ATG CTG CCA TCT GGC ATC TG-3′ (355 bp); FGF-2 forward 5′-GAA CGG GGG CTT CTT CCT-3′, reverse 5′-CCC AGT TCG TTT CAG TGC C-3′ (288 bp).

A master mix of the following reaction components was prepared: 18.9 μl of water; 2.5 μl of 10× PCR reaction buffer (Roche); 0.5 μl of deoxynucleoside triphosphates (25 μmol, Roche); 0.5 μl forward primer (20 μM); 0.5 μl reverse primer (20 μM); and 0.1 μl Taq-DNA-Polymerase (5 U/μl, Roche). We used 10× PCR-reaction buffer containing 1.5 mM $MgCl_2$ for iNOS and 1.0 mM $MgCl_2$ for FGF-2. Twenty-three microliters of the master mix were filled in 0.2-ml cups, and 2 μl cDNA template containing 66.6 ng reverse transcribed total RNA were added. PCR conditions were optimized for $MgCl_2$ concentration and annealing temperature. As conventional PCR only allows end-point quantification, the reactions were stopped in the log linear portion of amplification after having optimized the cycle number.

The following general PCR protocol was employed: initial denaturation step at 94°C for 2 min, followed by 20 (ubiquitin), 30 (FGF-2), and 32 (iNOS) cycles, respectively, of the amplification step beginning with denaturation at 94°C for 45 sec; annealing at 55°C (ubiquitin) and 60°C (iNOS), respectively, for 45 sec; and extension at 72°C for 45 sec. For FGF-2, denaturation and annealing (60°C) lasted 1 min, and there was no additional extension step. The amplification step was followed by a final elongation step at 72°C for 2 min and subsequent cooling to 4°C.

To exclude amplification of genomic DNA, all experiments included controls lacking the reverse transcription enzyme. To rule out contamination from buffers and tubes, a negative control with water instead of the cDNA template was used.

Five microliters of the PCR product were mixed with 1 μl bromophenol-blue-glycerine and fractionated by electrophoresis through a 1.8% agarose gel containing ethidium bromide in a constant 70-V field. To determine the length of the products, a 100 bp DNA ladder (New England BioLabs Inc., Beverly, MA) was fractionated in the same gel. All samples were run and stained at the same time for minimizing between-run variability. The ethidium bromide-stained gels were evaluated by a video documentation system (Image Master VDS, Pharmacia, Freiburg, Germany), and band intensities were analyzed by computerized densitometry using the Image Master-1D-software (Pharmacia) for relative quantification. The expression data of the investigated factors were normalized against the equivalent PCR products for ubiquitin (189 bp). The identity and specificity of all PCR products were confirmed by sequencing (TopLab, Munich, Germany).

Statistical Analysis

The statistical significance of differences in mRNA expression for the examined factors was analyzed by the Relative Expression Software Tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR [33]. This software calculates an expression ratio relative to the control group (before $PGF_{2\alpha}$) and is normalized by a reference gene (ubiquitin). The mRNA expression data for ubiquitin showed no significant changes compared with the control group during any of the investigated stages of induced luteolysis. Thus it was considered suitable as a reference gene. REST also indicates coefficients of variance in percentages (mean values for CV% in Table 3) and standard deviations based on CPs of the target gene. The data are shown as the mean difference (Δ) ± SEM of CPs between the control group and the following times after $PGF_{2\alpha}$-induced luteolysis. A positive ΔCP means an earlier increase of fluorescence, and therefore a higher concentration of the target gene. As the PCR amplification is a process with exponential character, a difference of two CPs signifies a change in regulation by a factor of approximately $E^{\Delta CP}$ (with E = efficiency) and is indicated in the text according to the expression ratio calculated by REST.

Statistical significance of differences in mRNA expression of the conventional PCR was calculated with one-way ANOVA followed by the Fisher least significant difference test as a multiple-comparison test. The mRNA expression of the target gene was normalized against the reference gene ubiquitin by generating the ratio OD target gene:OD reference gene. As in the LightCycler PCR, the mRNA expression data for ubiquitin in conventional PCR showed no significant changes compared with the control group during any of the investigated stages of induced luteolysis. Here it was considered suitable as a reference gene. The experimental data are shown as mean OD ± SEM.

RESULTS

Progesterone Blood Levels During Induced Luteolysis

Peripheral blood levels of progesterone before $PGF_{2\alpha}$ injection averaged (mean ± SEM) 5.10 ± 1.38 ng/ml plasma and decreased 12 and 48 h after $PGF_{2\alpha}$ application to 1.6 ± 0.65 and 0.55 ± 0.43 ng/ml, respectively. Progesterone levels < 1.0 ng/ml are basal levels and are considered to reflect luteolysis. Thus, the measured progesterone levels demonstrate the efficiency of induced luteolysis.

Expression of LH-R and P450scc During Induced Luteolysis

The LH-R mRNA expression was different from control at 12 ($P < 0.05$), 48, and 64 h ($P < 0.01$) after $PGF_{2\alpha}$-induced luteolysis, with a maximal down-regulation at 48 h by a factor of 50 (Fig. 1). The P450scc mRNA expression was down-regulated at 2 h ($P < 0.05$) after $PGF_{2\alpha}$ by a

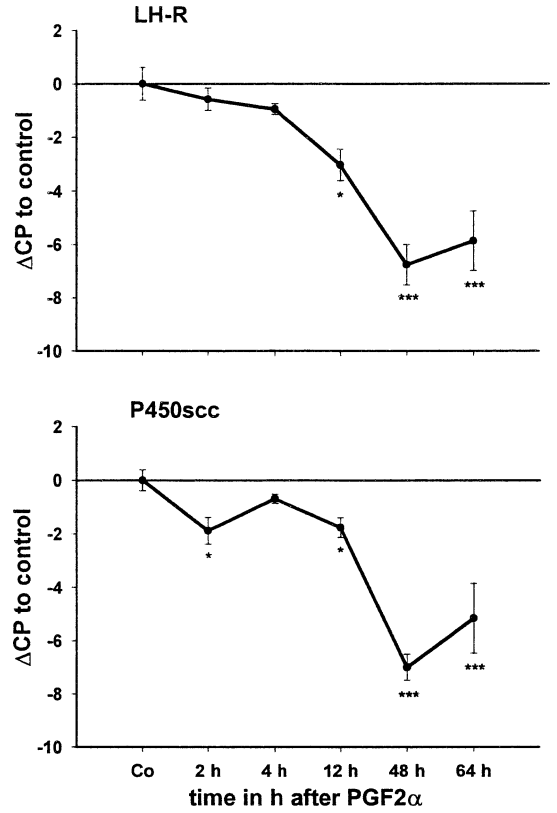

FIG. 1. Expression data (mRNA) for LH-R and P450scc during induced luteolysis; data are shown as mean of crossing point difference (ÄCP) ± SEM between the control group (Co = Days 8–12, before $PGF_{2\alpha}$) and the following times in hours after $PGF_{2\alpha}$ administration (n = 4–5/stage). Significances are indicated in relation to the control group: $*P < 0.05$, $***P < 0.001$.

factor of 3. The expression data slightly increased at 4 h, but did not reach the expression level of the control group. At 12 h P450scc decreased ($P < 0.05$) to a maximal 25-fold down-regulation at 48 h ($P < 0.001$). The down-regulation at 64 h was a little bit less than at 48 h, but still highly significant ($P < 0.001$).

Expression of the Cytokines TNFα, IL-1β, and IFNγ and TNF-R1 During Induced Luteolysis

During the whole investigated period, TNFα mRNA expression was up-regulated ($P < 0.05$ and $P < 0.01$, respectively) when compared with the control group (Fig. 2). As early as 2 h after $PGF_{2\alpha}$ administration there was a significant ($P < 0.01$) and maximal up-regulation by a factor of 10. At 4 h the expression data remained approximately of the same level, after which there was a further decrease to a lower level at 12 h after $PGF_{2\alpha}$. From 12 to 64 h the up-regulation was approximately 3-fold in comparison to the control group. TNF-R1 mRNA showed no significant difference from the control during induced luteolysis. Maximal regulation occurred at 48 h after $PGF_{2\alpha}$ and was decreased 2-fold.

Messenger RNA expression for IL-1β proceeded similar to that for TNFα. There was maximal up-regulation by a factor of 15 ($P < 0.01$) at 2 h after induced luteolysis, with this level persisting until 4 h after $PGF_{2\alpha}$. From 12 to 64 h IL-1β mRNA expression then decreased to a lower level of a 2- to 3-fold up-regulation ($P < 0.01$ and $P < 0.05$, respectively) during the remaining period.

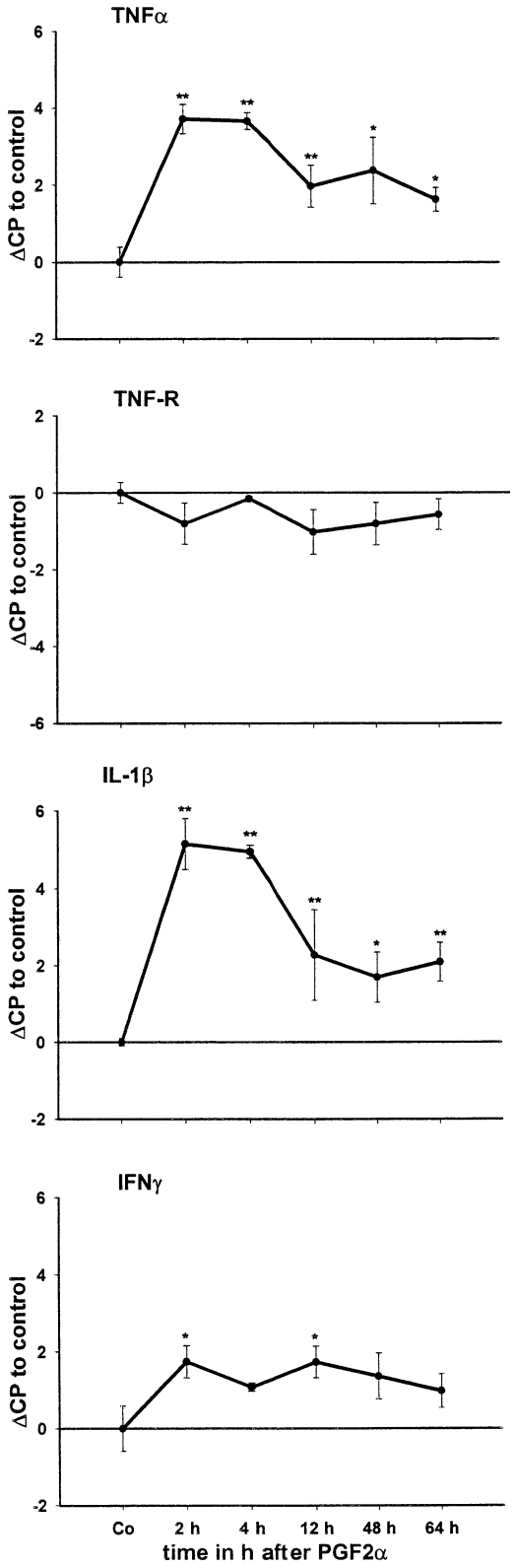

FIG. 2. Expression data (mRNA) for TNFα, TNF-R1, IL-1β, and IFNγ during induced luteolysis; data are shown as mean of crossing point difference (ΔCP) ± SEM between the control group (Co = Days 8–12, before $PGF_{2\alpha}$) and the following times in hours after $PGF_{2\alpha}$ administration (n = 4–5/stage). Significances are indicated in relation to the control group: $*P < 0.05$, $**P < 0.01$.

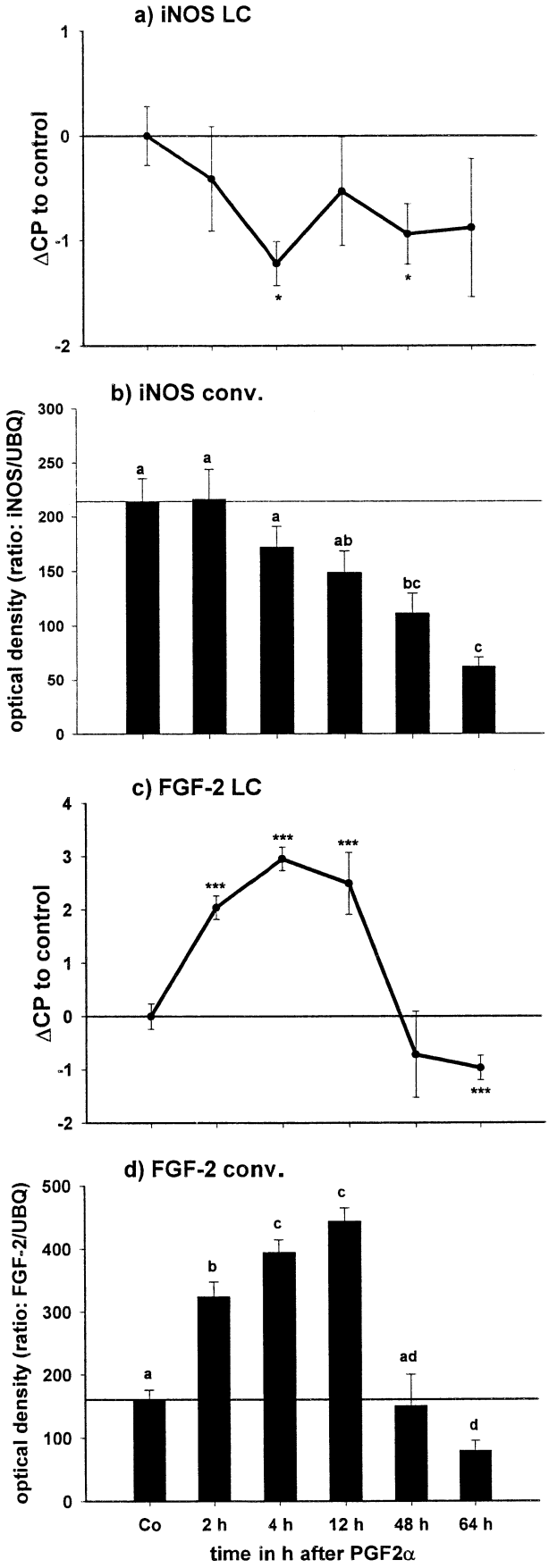

FIG. 3. Expression data (mRNA) for iNOS (**a** and **b**) and FGF-2 (**c** and **d**) during induced luteolysis. **a**) and **c**) Data were acquired by real-time PCR (LightCycler) and are shown as mean of crossing point difference (ΔCP) ± SEM between the control group (Co = Days 8–12, before $PGF_{2\alpha}$) and the following times in hours after $PGF_{2\alpha}$ administration (n = 4–5/stage). Significances are indicated in relation to the control group: $^{*}P < 0.05$, $^{***}P < 0.001$. **b**) and **d**) Data were acquired by conventional PCR and are shown as mean of optical density (with ratio target gene:ubiquitin) ± SEM (n = 4–5/stage). Different superscripts indicate statistically significant differences between results ($P < 0.05$).

The mRNA expression for IFNγ was increased by a factor of 3 at 2 and 12 h ($P < 0.05$). At the other times during the investigated period, the expression data were about 2-fold up-regulated, although this was not significant.

Expression of iNOS and FGF-2 During Induced Luteolysis

The iNOS mRNA expression showed a decrease during induced luteolysis ($P < 0.05$) at 4 and 48 h after $PGF_{2\alpha}$ (Fig. 3a). The maximal down-regulation was about 2-fold. There was a slight increase to be seen at 12 h, but this increase of mRNA expression did not reach the level of the control group. The expression data of the conventional PCR confirmed the decrease ($P < 0.05$) of iNOS mRNA during induced luteolysis (Fig. 3b).

The mRNA expression for FGF-2 markedly rose ($P < 0.001$) at 2 h after $PGF_{2\alpha}$ to a maximal 7-fold increase ($P < 0.001$) at 4 h (Fig. 3c). At 12 h, FGF-2 mRNA expression was still elevated ($P < 0.001$) by a factor of 5. Later, there was a rapid drop of FGF-2 mRNA to below the level of the control group. At 64 h after induced luteolysis, FGF-2 mRNA expression was around 2-fold lower ($P < 0.001$) than before $PGF_{2\alpha}$ administration. The same pattern of mRNA expression could be seen in the conventional PCR ($P < 0.05$; Fig. 3d).

DISCUSSION

The decrease in progesterone blood levels confirmed the occurrence of induced luteolysis. In the subsequent discussion, we define the time when progesterone levels fall below the basal level (<1.0 ng/ml) as the beginning of structural luteolysis (in this study, between 12 and 48 h after $PGF_{2\alpha}$). This coincides with pronounced oligonucleosome formation and histological signs of the degeneration of luteal cells [1], which are signs for the beginning of irreversible CL regression. Functional luteolysis is characterized by the decrease of peripheral progesterone levels and is potentially reversible. The expression data for LH-R and P450scc were selected to reflect and confirm the luteolytic process at the mRNA level. Our results agree with the findings in several publications [16, 20, 21], where the mRNA expression of LH-R and P450scc was markedly down-regulated during regression of the bovine CL and after induced luteolysis.

Until now, no major differences in cytokine expression during induced luteolysis in bovine CL have been detected using conventional RT-PCR [5]. In a study by Petroff et al. [34], the expression of TNFα and IL-1β transcripts seem to be elevated at 1 h and at 1 and 4 h, respectively, after induced luteolysis, but are not significantly different from the mRNA expression at Days 11–12 of the estrous cycle. In this study, the mRNA expression for IFNγ is significantly decreased at 1 h after induced luteolysis, although at subsequent times—4 and 24 h after $PGF_{2\alpha}$—the expression of IFNγ mRNA is not significantly different from that of midcycle CL. In contrast to these findings, we found a permanent increase (maximal 3-fold) of IFNγ expression in comparison with CL at Days 8–12, which is significant at

2 and 12 h after induced luteolysis. Real-time RT-PCR is highly sensitive and allows quantification of rare transcripts and detection of small differences in mRNA expression. High accuracy and reproducibility can be reached using the LightCycler PCR method [30], as applied in the present investigation. The use of different methods may be the reason why our results differ from those in the cited studies.

In our study, cytokine expression, especially of TNFα and IL-1β, is significantly augmented throughout the period of induced luteolysis and shows a maximal increase during functional luteolysis (until 12 h after $PGF_{2\alpha}$). Thus, they already seem to play an important role at the beginning of the luteolytic process. The amount of lymphocytes in the bovine CL already increases from Day 16 of the estrous cycle onward [5]. These lymphocytes could be the source for cytokine production at the very beginning of luteolysis. Moreover, cytokines are not only produced by activated macrophages and lymphocytes, but also by fibroblasts and endothelial and epithelial cells [35], which are abundant in bovine CL [36]. Cytokines are able to inhibit LH-stimulated progesterone production in bovine luteal cells [7]. It was demonstrated that TNFα [37], IL-1β [38], and IFNγ [39] can directly inhibit the mRNA expression of P450scc in rat and porcine Leydig cells of the testis. This may be the reason for the significant decrease of P450scc mRNA as early as 2 h after induced luteolysis in our study. Furthermore, Lin et al. [37] determined that TNF can inhibit insulin-like growth factor I (IGF-I) mRNA expression in a dose-dependent manner. IGF-I is an important luteotropic factor in the bovine CL and stimulates progesterone secretion [40]. In Hep G2 (hepatoma) cells, TNFα and IL-1β directly increase mRNA expression and protein concentration of the IGF binding protein 1 (IGFBP-1) [41]. IGFBP-1 is markedly elevated during induced luteolysis. It is assumed that it inhibits the luteotropic actions of IGF-1 in the bovine CL [42]. Thus, there may be several mechanisms for cytokines to reduce progesterone production. By decreasing luteal survival factors like IGF-I and progesterone, Fas-mediated cell death in bovine luteal cells is no longer suppressed [43], and cytokine-induced apoptosis via the Fas-Fas ligand system may occur [12]. In addition, cytokines induce monocyte chemoattractant protein-1 mRNA expression, which may promote further immune cell recruitment and influx of macrophages into the CL to facilitate luteal regression [13, 44].

For iNOS and FGF-2, conventional PCR was additionally performed. The results show a good comparability between the two PCR methods, and the real-time PCR data of the LightCycler could be confirmed. It was surprising to see that iNOS mRNA expression, which is maximally induced by the stimulation of pro-inflammatory cytokines [23], did not increase during induced luteolysis and was even down-regulated, despite the marked increase in the expression of TNFα, IL-1β, and IFNγ. Although NO has been shown to be potentially involved in luteolysis [27], cell culture experiments have shown that TNFα- and IFNγ-induced DNA fragmentation in bovine luteal cells is not mediated by NO or arachidonic metabolism [11], but involves the Fas-Fas ligand system [12]. This would support the assumption of a predominantly apoptotic, not necrotic, pattern of cell death during luteolysis. Davis and Rueda [8] mention some discrepancies between in vitro and in vivo studies concerning the role of NO in luteolysis. They discuss the possibility that NO might not be obligatory, but serve to make luteal regression more efficient. Skarzynski et al. [26] showed in bovine luteal cell cultures that the NO donor S-nitroso-N-acetylpenicillamine inhibits progesterone and stimulates $PGF_{2\alpha}$. In contrast, Weems et al. [45] observed no $PGF_{2\alpha}$ increase and no change in progesterone levels in bovine CL slices when incubated with different NO donors. Skarzynski et al. [46] found the strongest immunolabeling of iNOS and eNOS in the late CL (Days 14–17) and further showed that L-NAME blocked the luteolytic action of $PGF_{2\alpha}$ in vivo. Considering this, in our study we may have missed (CL of the midluteal phase and first measurement of mRNA expression at 2 h after $PGF_{2\alpha}$ administration) the potential increase of eNOS or iNOS mRNA, which could initiate functional luteolysis. Over 50% of the CL is comprised of endothelial cells and pericytes [36], so eNOS activity is probably a major source of NO. In the vascular system NO is a mediator of vasodilatation. Indeed Acosta et al. [47] observed an initial increase in blood flow at 0.5–2 h after $PGF_{2\alpha}$-induced luteolysis, which may be caused by the release of NO or prostaglandins. But in the following time period the luteal blood flow significantly decreases in comparison to the initial value and correlates with the decreasing CL volume. NO is not only a vasoactive agent, but also an effector molecule of activated macrophages and leads to tissue injury [35]. It is one of the characteristics of luteolysis that this high potential endocrine gland is completely regressing without any major inflammatory reaction in the surrounding tissue. Two of the cardinal signs of inflammation are swelling and vasodilatation in the inflamed region. These signs are missing in structural regression of the CL [47]. There is some evidence that cytokine-induced iNOS mRNA expression can be suppressed by FGF-2 in rat and bovine tissues [48, 49], and that FGF-2 is involved in the prevention of cytokine-induced inflammation, permitting tissue repair and remodeling [24]. IL-1β induces FGF-2 transcription, which reaches its maximum 2–4 h after IL-1β exposure in human vascular smooth muscle cells [50]. Beyond this, FGF-2 can be stimulated by angiotensin II [51], which is an important peptide in the initial phase of bovine luteolysis [2, 52]. FGF-2 and cytokines stimulate prostaglandin secretion in bovine luteal cells [7, 53], so that there may be positive feedback on local $PGF_{2\alpha}$ production to ensure that luteolysis occurs. In the late luteal stage and after regression of the bovine CL, FGF-2, which is a nonsecretory protein, accumulates in the cytoplasm of luteal cells [54]. When phagocytosis of luteal tissue emerges, this might be the factor that prevents the induction of iNOS expression and NO production by activated macrophages.

In conclusion, we demonstrated in our study a significant increase in the mRNA expression of the pro-inflammatory cytokines TNFα, IL-1β, and IFNγ during induced luteolysis. They may already play a role during functional luteolysis by decreasing luteal survival factors like progesterone, and thereby facilitate and induce the onset of apoptotic processes and phagocytosis in the regressing bovine CL. Significantly augmented FGF-2 may suppress a cytokine-induced iNOS increase, so that major inflammatory reactions of the surrounding tissues may be prevented. The beginning of luteolysis seems to be an inflammatory-like process, but instead of causing necrotic tissue injury, mainly apoptosis (of an unusually high number of cells) occurs. The results obtained reflect the very well-organized process of luteal regression, which requires the cooperation of many different factors and regulation systems.

ACKNOWLEDGMENTS

We would like to thank Ch. Fochtmann and Dr. W. Kraetzl for the surgical help in collecting the CL, G. Schwentker for technical assistance,

and Dr. R. Ivell for critical reading of the manuscript. We would also like to thank the Animal Experimental Section Thalhausen (Prof. Dr. H.R. Fries and Prof. Dr. H. Eichinger) for the generous support with dairy cows for the experiments.

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