Prolactin-Induced Activation of Nuclear Factor κ B in Bovine Mammary Epithelial Cells: Role in Chronic Mastitis

P. Boutet,^{*1} J. Sulon,^{*} R. Closset,^{*} J. Detilleux,[†] J.-F. Beckers,^{*} F. Bureau,^{*} and P. Lekeux^{*}

^{*}Department for Functional Sciences, and

[†]Department of Animal Production, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium

ABSTRACT

We sought to determine whether prolactin (PRL) could influence the neutrophilic inflammation that characterizes chronic mastitis. Most of the genes encoding inflammatory proteins depend on the nuclear factor κ B (NF- κ B) for their expression. We addressed the hypothesis that immunomodulatory activities of PRL might arise from an increase in NF- κ B activity. MAC-T cells, a bovine mammary epithelial cell line, were stimulated with increasing concentrations of bovine PRL (1, 5, 25, 125, and 1,000 ng/mL). Level of NF- κ B binding activity was measured and mRNA was evaluated for IL-1 β , IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- γ , and tumor necrosis factor (TNF)- α , cytokines known to require NF- κ B for their maximal transcription. Prolactin activated NF- κ B; maximal NF- κ B activation was weaker with PRL than with TNF- α at 30 or 180 min poststimulation. In addition, PRL significantly amplified, in a dose-dependent manner, mRNA expression of IL-1 β , IL-6, IL-8, GM-CSF, and TNF- α . We measured PRL concentrations in blood and milk from healthy and chronic mastitis-infected cows, and studied the relationship between the PRL concentration and the degree of inflammation in the mammary gland as indirectly assessed by somatic cell counts (SCC). Plasma PRL did not differ significantly between healthy and chronic mastitis-affected cows (63.7 and 67.5 ng/mL, respectively). Milk PRL concentration was significantly increased in chronic mastitis-affected quarters with the highest SCC, and had a positive significant correlation between SCC, as well as between the number of neutrophils present in milk samples. The present findings show that PRL promotes an inflammatory response in bovine mammary epithelial cells via NF- κ B activation, and suggest a role for PRL in the pathogenesis of chronic mastitis.

Key words: bovine mastitis, prolactin, nuclear factor κ B, cytokine

INTRODUCTION

Bovine mastitis, defined as an inflammatory reaction within the mammary gland, is usually caused by pathogenic bacteria. Chronic mastitis is a moderate inflammatory disease associated with a fluctuating and persistent increase in SCC. This increase is mainly due to the migration of polymorphonuclear neutrophils that are essential components of the innate immunity. The accumulation and activation of neutrophils at the site of infection require local expression of inflammatory proteins, such as interleukin (IL)-8, a potent chemotactic factor; IL-1 β , IL-6, and tumor necrosis factor (TNF)- α , cytokines that activate neutrophils; and granulocyte-macrophage colony-stimulating factor (GM-CSF), which increases neutrophil survival (Boutet et al., 2004). These inflammatory cytokines depend on the nuclear factor κ B (NF- κ B) for their expression (Blackwell and Christman, 1997). Numerous bacteria may directly activate NF- κ B in epithelial cells, which triggers the inflammatory response (Philpott et al., 2000). Moreover, bacteria-activated epithelial cells and macrophages release large amounts of proinflammatory cytokines, such as TNF- α , which are potentially able to activate NF- κ B in any cells present at the infection site, including the extravasated neutrophils (Sharma et al., 1998).

Parturition and the onset of lactation are characterized by important physiological changes influenced by hormonal and metabolic factors, which are responsible for a compromised immune system, especially by negatively affecting neutrophil functions (Diez-Fraile et al., 2003). Among those factors, sex steroids and glucocorticoids have negative effects on neutrophil functionality, whereas the effect of somatotropin, IGF-I, and prolactin (PRL) might be immunostimulatory (Vangroenweghe et al., 2005).

Prolactin exerts effects on reproduction and lactation, but also plays a role as an immunoregulatory hormone with inflammatory properties (Meli et al., 1997; Brand et al., 2004). Moreover, it appears that PRL can be anti- or proinflammatory, depending on the cell type, the tissue, and the physiological state of the organ (Yu-Lee, 2002). Indeed, PRL protected against trauma-hemor-

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¹Corresponding author: philippe.boutet@ulg.ac.be

rhage, which was associated with depressed immune function and increased risk of infection (Zellweger et al., 1996). Furthermore, the proinflammatory activity of PRL was shown in different experimental models of acute and chronic inflammation such as paw edema and carrageenan-induced pleurisy in the rat (Meli et al., 1993).

In the bovine, PRL is present in whole milk of postpartum cows at concentrations lower or equivalent to those in blood plasma or serum (Malven, 1977). An exception is noted near parturition when PRL in colostrum exceeds its concentration in plasma (Keller et al., 1977). Interestingly, incidence and severity of clinical mastitis are the highest around parturition (Burvenich et al., 2003), which suggests that PRL could play a potential role as an inflammatory activator. Because most of the genes encoding inflammatory proteins depend on NF- κ B for their expression, and because we previously suggested that NF- κ B could play a role in mastitis pathogenesis (Boulanger et al., 2003), we hypothesized that immunomodulatory activities of PRL might arise from an increase in NF- κ B activity. In an attempt to verify this hypothesis, we chose to stimulate bovine mammary epithelial cells with various doses of PRL and measure the level of NF- κ B binding activity and mRNA levels of IL-1 β , IL-6, IL-8, GM-CSF, IFN- γ , and TNF- α . Also, we measured PRL concentrations in blood and milk from healthy and chronic mastitis-affected cows to study the relationships between the PRL concentration and the degree of inflammation in the mammary gland, as indirectly assessed by SCC measurement.

MATERIALS AND METHODS

Animals

Holstein-Friesian cows from 4 herds were investigated. Cows with low and persistently elevated SCC were selected based on monthly SCC measurements and 3 consecutive microbiological analyses. Cows with low SCC (<200,000 cells/mL) and free of any mammary pathogen were considered "healthy" ($n = 7$). Cows with high SCC (>3 consecutive times of >200,000 cells/mL) and positive for a specific mammary pathogen were considered "chronic mastitis-affected" ($n = 28$). Chronic mastitis-affected cows were devoid of any clinical sign of the disease. Cows were between 2 and 8 yr old, had a lactation number ranging from 1 to 7, and were 3 to 9 mo postcalving when the milk samples were collected. Experimental cows did not receive treatment during the month preceding the experiments.

Milk and Plasma Sampling

Quarter milk samples were taken from all quarters of the 35 cows. Foremilk was collected using sampling procedures recommended by the National Mastitis Council (1999). Teat ends were vigorously disinfected with a solution containing 70% alcohol. A 50-mL vial was collected for total and differential cell counts, and RIA. Samples were placed in freezer packs immediately after collection. Blood samples were collected by venipuncture in the tail at the end of the milking. After collection into heparinized tubes, blood samples were chilled on ice until centrifugation for recovery of plasma. Plasma aliquots were stored at -20°C until hormones were assayed.

Total and Differential Cell Counts

Immediately after milk collection, 50 mL of each sample was shipped to a specialized laboratory (Comité du lait, Battice, Belgium) where SCC were performed with a Fossomatic Automatic Cell Counter (Foss Electric, Hillerød, Denmark).

To determine the somatic cell type, high SCC milk samples were diluted in PBS. Low SCC milk samples were centrifuged ($300 \times g$) for 20 min at 4°C . Pellets were then suspended in 1 mL of PBS. Cell differentials were performed on 300- μL cytopspin samples stained with May-Gründwald Giemsa (VWR, Leuven, Belgium). Neutrophils, lymphocytes, macrophages, and epithelial cells were differentiated according to their morphology. Three hundred cells were counted and the results of cell differentiation were expressed for each cell type as a proportion of the total number of cells counted.

Bacteriological Analysis

Each quarter milk sample was incubated overnight at 37°C . Then, aliquots (10 μL) of each sample were spread on 5% sheep blood agar, McConkey agar, M4 agar (selective for *Streptococcus* spp.), and Hektoen agar (Becton Dickinson, Erembodegem-Aalst, Belgium), and the plates were incubated for another 24 h at 37°C . Preliminary identification was by colony morphology, hemolysis, and Gram staining. Isolation of a minimum of 100 cfu was regarded as positive. Colonies were identified using classical procedures and appropriate API Sugar sets (BioMérieux, Marcy l'Etoile, France) and Crystal (Becton Dickinson). Because one negative sample does not signify the absence of infection, 3 consecutive samples were sometimes necessary for accurate diagnosis of infected quarters.

Mammary Epithelial Cell Culture and Stimulation Assays

MAC-T cells, an immortalized epithelial cell line isolated from bovine mammary tissue, were routinely cultured according to the recommended conditions (Huynh et al., 1991). The cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 5 μ g/mL bovine insulin, 10% fetal bovine serum, 50 μ g/mL of streptomycin, and 50 IU/mL of penicillin (Gibco Invitrogen, Merelbeke, Belgium). Cells were incubated at 37°C with 5% CO₂. For cell culture passage, adherent cells were released by using 0.25% trypsin (Difco, Erembodegem, Belgium).

For NF- κ B binding activity experiments and induction of cytokine expression using native purified bovine PRL (lot AFP7170E; gift from A. F. Parlow, NHPP-NIDDK, Torrance, CA), MAC-T cells were seeded on tissue culture plates (Greiner BioOne N.V./S.A., Wemmel, Belgium) and grown to 90% confluence in culture medium. Then, cells were treated with PRL concentrations of 1, 5, 25, 125, or 1,000 ng/mL for 30 and 180 min before protein and RNA extractions. As a positive control, MAC-T cells were also treated with 1.5 or 1 ng/mL of human TNF- α (150 or 100 U/mL; Roche Diagnostics, Brussels, Belgium).

Prolactin was tested for possible endotoxin (LPS) contamination (LAL Single Test Kit; Cambrex, Verviers, Belgium). The concentration was under the detection limit of the assay. The media were purchased LPS free.

Nuclear Protein Extraction

Protein extracts were prepared as previously described (Bureau et al., 2000). Cytoplasmic buffer contained 10 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.2% (vol/vol) Nonidet P-40, 1.6 mg/mL of protease inhibitors (Complete, Roche Diagnostics), and 3 mM of the serine protease inhibitor diisopropyl fluorophosphate (Sigma, Bornem, Belgium). Pelleted nuclei were resuspended in 20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.63 M NaCl, 25% (vol/vol) glycerol, 1.6 mg/mL protease inhibitors, and 3 mM diisopropyl fluorophosphate (nuclear buffer), incubated for 20 min at 4°C and centrifuged for 30 min at 12,000 $\times$ g. Protein amounts were quantified with the Micro BCA protein assay reagent kit (Pierce, Rockford, IL).

Nuclear Factor κ B Electrophoretic Mobility Shift Assays

Binding reactions were performed for 30 and 180 min at room temperature with 5 μ g of nuclear protein extracts in 20 mM HEPES, pH 7.9, 10 mM KCl, 0.2 mM

EDTA, 20% (vol/vol) glycerol, 1% (wt/vol) acetylated BSA, 3 μ g of poly(dI-dC) (Amersham Biosciences, Roosendaal, the Netherlands), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 100,000 cpm of ³²P-labeled double-stranded oligonucleotide probes. Probes were prepared by annealing the appropriate single-stranded oligonucleotides (Eurogentec, Liège, Belgium) at 65°C for 10 min in 10 mM Tris, 1 mM EDTA, and 10 mM NaCl, followed by slow cooling to room temperature. The probes were labeled by end-filling with the Klenow fragment of *Escherichia coli* DNA polymerase I (Roche, Mannheim, Germany), with [³²P]dATP and [³²P]dCTP (Dupont-New England Nuclear, Les Ulis, France). Labeled probes were purified by spin chromatography on Sephadex G-25 columns (Roche). Then, DNA-protein complexes were separated from unbound probe on 4% native polyacrylamide gels at 150 V in 0.25 M Tris, 0.25 M sodium borate, and 0.5 mM EDTA, pH 8.0. Gels were vacuum-dried and exposed to Fuji x-ray film (Tokyo, Japan) at -80°C for 12 h. To confirm specificity, competition assays were performed with a 50-fold excess of unlabeled wildtype and mutated probes. The sequence of the oligonucleotides used were as follows: wild-type palindromic κ B probe, 5'-TTG GCA ACG GCA GGG GAA TTC CCC TCT CTT TAG GTT-3'; and mutated palindromic κ B probe, 5'-TTG GCA ACG GCA GAT CTA TTC CCC TCT CCT TAG GTT-3'. Binding of the noninducible transcription factor OCT-1 was used as an internal standard (data not shown). The OCT-1 probe was as follows: 5'-TGT CGA ATG CAA ATC ACT AGA A-3'.

Reverse Transcription-PCR

Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polyadenylated RNA was primed with oligo(dT) (Roche) and reverse transcribed with AMV reverse transcriptase (Roche) for 1 h at 42°C. The cDNA products were amplified by PCR using primers specific for IL-1 β , IL-6, IL-8, GMCSF, IFN- γ , TNF- α , and GAPDH as a control (Table 1). All primers were purchased from Eurogentec. A 50- μ L PCR reaction was set up containing 5 μ L of cDNA, 10 mM Tris-HCl, 25 pmol of each primer, 1.5 mM MgCl₂, 0.2 mM dNTP, and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer, Boston, MA). Amplification consisted of 1 cycle of 2 min at 94°C, followed by 27 (IL-6), 32 (TNF- α), 34 (IL-8, GMCSF), or 37 (IL-1 β , IFN- γ) cycles of 30 s of denaturation at 94°C, 30 s of annealing at 62°C (IL-6, IL-8, GMCSF, TNF- α) or 65°C (IL-1 β , IFN- γ), and 1 min of extension at 72°C. Amplification products were electrophoresed on 1.5% agarose gels, and PCR product quan-

Table 1. Sequences of semiquantitative reverse transcription PCR primers used to detected mRNA levels expression of bovine cytokines in MAC-T cells

Cytokine	Length, bp		Sequence 5' → 3'	Accession number ¹
IL-1 β	310	Sense	CTTGGGTATCAAGGACAAGAATC	NM_174093
		Antisense	GACTCCCTGGGTATGGCTTTC	
IL-6	303	Sense	ACTCCATTGCTGTCTCCCTG	NM_173923
		Antisense	TCGCCTGATTGAACCCAGATTG	
IL-8	310	Sense	ATGACTTCCAAGCTGGCTGTTG	NM_173925
		Antisense	TTTCATGGATCTTGCTTCTCAGC	
GMCSF	319	Sense	CTGAGCCTTCTGAACCACAGC	NM_174027
		Antisense	CTTCACTTCTGGGCTGGTTCC	
IFN- γ	304	Sense	AGAGCCAAATTGTCTCCTTCTAC	NM_174086
		Antisense	GGACCATTACGTTGATGCTCTC	
TNF- α	302	Sense	CGGGCTTTACCTCATCTACTCA	AF348421
		Antisense	CCAAAGTAGACCTGCCCAGAC	
GAPDH	356	Sense	GAGATGATGACCCTTTTGGC	NM_002046.2
		Antisense	GTGAAGGTCGGAGTCAACG	

¹Primers were designed from the published sequences in the GenBank database under the indicated accession numbers. GMCSF = granulocyte-macrophage colony-stimulating factor; TNF- α = tumor necrosis factor- α .

titles were assessed by densitometry (Gel Doc 2000; BioRad, Hercules, CA).

RIA for PRL Detection in Milk and Plasma Samples

The double-antibody RIA procedure described by Malven and McMurtry (1974) for measuring bovine PRL in milk was used with some modifications. Antiserum (R144) to bovine PRL was raised in a rabbit injected intradermally with 100 μ g of bovine native PRL (NIH-B5). The antigen was solubilized in PBS (500 μ L) and emulsified in an equal volume of complete Freund's adjuvant. The injections were repeated at 2-wk intervals for 4 mo. Blood was collected from the marginal vein of the ear. For assays, the antiserum was used at a final dilution of 1:150,000 in a total volume of buffer (Tris HCl, pH 7.6, BSA 0.1%) of 500 μ L. Bovine PRL (NIH-B5) was used as the standard and as the tracer. For this, 10 μ g of protein was iodinated using 1 mCi of ¹²⁵I by the chloramine T method (Hunter and Greenwood, 1962). Just after the iodination, the labeled hormone was separated from free ¹²⁵I reagents by chromatography on a Sephadex G 100 column equilibrated with 0.05 M phosphate buffer, pH 7.5, containing 0.1% BSA. Fractions of 1 mL were collected and monitored for radioactivity. The second antibody polyethylene glycol solution [0.83% vol/vol sheep antirabbit immunoglobulin, 0.17% vol/vol normal rabbit serum, 20 mg/mL polyethylene glycol 6000 (Vel, Leuven, Belgium), 0.05 mg/mL microcrystalline cellulose (Merck, Darmstadt, Germany), 0.4% (wt/vol) BSA (INC Biochemicals, Aurora, OH) diluted in Tris buffer] was added to assay buffer 30 min before centrifugation and decanting of supernatant. Frozen plasma samples were thawed, heated to

40°C, and assayed in duplicate at 25 μ L per assay tube. Milk samples were assayed fresh (i.e., without storage). They were warmed to 40°C, mixed, separated in 2 aliquots, centrifuged at 1,500 $\times g$ for 15 min, and finally assayed in the lipid-depleted phase at 50 μ L per assay tube. To test the reproducibility of the RIA, milk samples with low (mid lactation) and high concentrations of PRL (after calving) were tested. The precision of the RIA was determined by estimating the intra- and interassay coefficients of variation (CV). To determine the intraassay CV, the same sample was assayed 10 times using the same assay. Analyzing different samples in 5 consecutive assays assessed the interassay CV. The intraassay CV were below 5.8% and the interassay CV below 11.2%, when 25 μ L of plasma and 50 μ L of milk were assayed. The calculated detection limit of the assay was 0.8 ng/mL of milk and 2 ng/mL of plasma.

Statistical Analysis

The relation between PRL concentrations and milk SCC were analyzed using the MIXED procedure of SAS with the following mixed model:

$$y_{ijklmn} = \text{quarter}_i + \text{herd}_j + \text{age}_k + \text{stage}_l + \text{SCC}_m \\ + \text{cow}_n + e_{ijklmn}$$

where y_{ijklmn} is the PRL concentration, quarter_i is the fixed effect of quarter ($i = 1, 2, 3, 4$), herd_j is the fixed effect of herd ($j = 1, 2, 3, 4$), age_k is the fixed effect of sampling age in years ($k = 1, \dots, 8$), stage_l is the fixed

effect of the month in milk at sampling ($l = 1, \dots, 7$), SCC_m is the fixed effect for the class of quarter SCC ($m = 1, \dots, 5$), cow_n is the random cow effect ($n = 1, \dots, 35$), and e_{ijklmn} is the residual effect. The random cow effects were assumed normally distributed $N(0, I \sigma^2)$ and the covariance between residuals of repeated records on the same cow had compound symmetry as verified by the smallest Akaike information criterion (AIC) and Schwartz Bayesian information criterion (BIC) values. The correlation was computed between PRL and SCC using the SAS CORR procedure (SAS Institute, Inc., Cary, NC).

RESULTS

NF- κ B Activity and Inflammatory Cytokines mRNA Expression in Bovine Mammary Gland Epithelial Cells

Nuclear protein extracts from MAC-T cells treated with increasing doses of PRL were compared with those from either untreated (negative control), or $TNF-\alpha$ -treated (positive control; Figures 1A and B) cells to determine the effect of PRL on DNA-binding activity of NF- κ B in bovine mammary gland epithelial cells. Both PRL and $TNF-\alpha$ induced activation of NF- κ B complexes, albeit with different kinetics. Following $TNF-\alpha$ treatment, complexes formed rapidly (≤ 30 min; Figure 1A) and were still maintained with the same intensity 180 min after stimulation (Figure 1B). In contrast, upon PRL treatment, only weak complexes were detected at 30 min. However, at 180 min poststimulation, NF- κ B complexes were almost as strong as those observed after $TNF-\alpha$ stimulation. Administration of $TNF-\alpha$ + PRL did not further amplify NF- κ B DNA-binding activity (Figure 1C). A lower dose of $TNF-\alpha$ was chosen with the aim of avoiding overexpression to investigate the potential additive effect of PRL on $TNF-\alpha$ -stimulated MAC-T cells. Specificity of the NF- κ B binding activity was demonstrated by DNA binding competition experiments using a 50-fold excess of unlabelled wildtype and mutated probes (data not shown).

Levels of IL-1 β , IL-6, IL-8, GM-CSF, IFN- γ , and $TNF-\alpha$ mRNA were assessed by reverse transcription-PCR (RT-PCR) 3 h after stimulation with bovine PRL to determine whether PRL is able to stimulate transcription of NF- κ B-regulated cytokines in bovine MAC-T cells. As a positive control, MAC-T cells were also treated with $TNF-\alpha$. We found that $TNF-\alpha$ amplified ($P < 0.001$) mRNA levels of all cytokines, except for IFN- γ that was increased but not significantly (Figure 2). In response to PRL, we observed a dose-dependent increase in mRNA levels of all tested cytokines. At 5

Figure 1. Nuclear factor- κ B (NF- κ B) DNA-binding activity in mammary epithelial cells (MAC-T cells) stimulated with bovine prolactin (PRL). Electrophoretic mobility shift assays were performed with nuclear protein extracts prepared from MAC-T cells. Cells were treated for 30 min (A) and 180 min (B) with medium (lane 1), with the indicated concentrations of bovine PRL (lanes 2 to 6), or with 1.5 ng/mL of tumor necrosis factor ($TNF-\alpha$) (lane 7). (C) Cells were treated for 180 min with medium (Control, CTR) or with $TNF-\alpha$ (1 ng/mL) + PRL at the indicated concentrations. The solid arrows indicate specific NF- κ B complexes. This result is representative of 3 comparable experiments.

ng/mL of PRL, IL-6 and IL-8 mRNA levels were significantly higher than those of untreated transcripts (Figures 2B and 2C); a PRL dose of 1 ng/mL was sufficient to up-regulate ($P < 0.05$) IL-8 mRNA (Figure 2C). Prolactin from 25 ng/mL was able to significantly raise cytokine mRNA levels when compared with untreated

Figure 2. Induction of cytokine mRNA up-regulation in bovine mammary epithelial cells by prolactin (PRL). RNA was prepared from MAC-T cells cultured for 3 h in medium (Control, CTR), with indicated concentrations of PRL, or with tumor necrosis factor (TNF)- α . Expression of IL-1 β , IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, IFN- γ , and TNF- α was performed by reverse transcription-PCR. As a control for quantification, GAPDH was amplified. Columns show the ratio between cytokine and GAPDH mRNA as determined by densitometric analyses. Data are presented as means $\pm$ SE; *Different from values obtained with untreated cells at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. S = values of PRL-treated cells different from values obtained with 1 ng/mL of PRL. These results are representative of at least 3 comparable experiments.

controls, except for IFN- γ . Between PRL concentrations of 25 and 1,000 ng/mL, levels of cytokine mRNA reached a plateau (Figure 2). The expression level of the

housekeeping gene GAPDH was unaffected by the treatment, and remained stable during the different stimulation conditions (Figure 2).

Table 2. Cellular characteristics of milk (mean $\pm$ SEM) recovered from quarters categorized according to the SCC measured at sampling time

Trait	SCC groups ($\times 1,000/\text{mL}$)				
	Group 1 <200 (n = 63)	Group 2 200 to 399 (n = 22)	Group 3 400 to 999 (n = 21)	Group 4 1,000 to 1,999 (n = 13)	Group 5 >2,000 (n = 16)
SCC ($\times 1,000/\text{mL}$)	85.6 $\pm$ 6.9	279.7 $\pm$ 11.7	661.7 $\pm$ 37.5	1,369.1 $\pm$ 64.3	5,314.9 $\pm$ 742.4
Macrophages, %	26.5 $\pm$ 1.6	20.6 $\pm$ 4.7	20.7 $\pm$ 3.9	17.3 $\pm$ 3.1	15.7 $\pm$ 2.9
Lymphocytes, %	5.1 $\pm$ 1.1	1.3 $\pm$ 0.9	0.4 $\pm$ 0.4	0.5 $\pm$ 0.3	0.3 $\pm$ 0.3
Neutrophils, %	42.4 $\pm$ 3.1	63.5 $\pm$ 5.6	65.9 $\pm$ 4.1	71.9 $\pm$ 2.7	77 $\pm$ 3.8
Epithelial cells, %	26 $\pm$ 2.6	14.6 $\pm$ 3.2	13.0 $\pm$ 2.2	10.3 $\pm$ 2.9	7.0 $\pm$ 1.1

These results indicate that stimulation of bovine mammary epithelial cells with PRL 1) activates the transcription factor NF- κ B, and 2) promotes up-regulation of NF- κ B-dependent proinflammatory cytokines in a dose-dependent manner.

PRL Levels in Blood Plasma and Milk from Healthy and Chronic Mastitis-Affected Cows

Mean plasma PRL from healthy and chronic mastitis-affected cows was not significantly different (healthy cows: 63.7 $\pm$ 13.6 ng/mL; chronic mastitis-affected cows: 67.5 $\pm$ 6.9 ng/mL). Quarter samples were categorized into 5 groups according to the SCC measured at sampling time (group 1 was $<2 \times 10^5$ SCC; group 2 was 2 to 4×10^5 ; group 3 was 4×10^5 to $<10^6$ SCC; group 4 was 10^6 to $<2 \times 10^6$ SCC; and group 5 was SCC $\geq 2 \times 10^6$ cells/mL). Cellular characteristics of milk samples recovered from the 5 groups are provided in Table 2 and bacterial species isolated from samples obtained in those groups are in Table 3. All quarters from group 1 were free of udder pathogens, and therefore, group 1 was considered "healthy." Prolactin from groups 1 and 2 was lower ($P < 0.01$) than those of groups 4 and 5 (Figure 3). Groups 1 and 5 had PRL concentrations of

6.98 $\pm$ 0.53 and 8.43 $\pm$ 0.57 ng/mL, respectively. The difference in milk PRL concentrations between healthy and chronic mastitis-affected cows represented 20.8%. The PRL concentrations were not influenced by other factors, such as quarter, herd, age, and stage of lactation. A positive significant correlation was found between milk PRL concentrations and SCC ($r = 0.39$), as well as between milk PRL and the number of neutrophils present ($r = 0.42$) in milk samples. No significant correlation was observed between PRL and pathogen or the percentage of each cellular type. However, a significant relationship was observed between pathogen type and SCC group. The quarters from groups 4 and 5 were infected by a higher proportion of major pathogens, namely, *Staphylococcus aureus* and *Streptococcus uberis*, and the quarters from groups 2 and 3 were dominated by CNS.

DISCUSSION

In cows, plasma PRL peaks 1 d before parturition, whereas milk PRL concentrations increase throughout the prepartum period to 400 ng/mL within the hours that follow parturition. Milk PRL sharply decreases thereafter and become equivalent to levels in blood,

Table 3. Bacterial species isolated (number and %) from quarter milk samples categorized according to the SCC measured at sampling time

Bacterial species isolated, no. (%)	SCC groups ($\times 1,000/\text{mL}$)				
	Group 1 <200 (n = 63)	Group 2 200 to 399 (n = 22)	Group 3 400 to 999 (n = 21)	Group 4 1,000 to 1,999 (n = 13)	Group 5 >2,000 (n = 16)
Negative	63 (100)	4 (18.2)	2 (9.5)	0 (0)	0 (0)
CNS	0 (0)	14 (63.6)	10 (47.6)	1 (7.7)	1 (6.2)
<i>Streptococcus uberis</i>	0 (0)	2 (9.1)	3 (14.3)	5 (38.5)	9 (56.3)
<i>Streptococcus agalactiae</i>	0 (0)	0 (0)	1 (4.8)	1 (7.7)	1 (6.2)
<i>Staphylococcus aureus</i>	0 (0)	2 (9.1)	5 (23.8)	6 (46.1)	5 (31.3)

around 10 to 35 ng/mL according to the studies (Malven, 1977; Accorsi et al., 2005). Because parturition and early lactation are periods during which mastitis is more severe and incident, we postulated that PRL could have inflammatory activity able to influence immune responses in the bovine mammary gland.

As the contribution of mammary epithelial cells to leukocyte recruitment has still not been thoroughly studied, MAC-T cells, an immortalized epithelial cell line from bovine mammary tissue, were stimulated with increasing concentrations of bovine PRL. We found that PRL activated the transcription factor NF- κ B in a dose-dependent manner. Nuclear factor- κ B played a role in bovine mastitis pathogenesis, because its activity was increased in milk cells from acute mastitis and varied from low to high in chronic mastitis (Boulanger et al., 2003).

Prolactin-dependent NF- κ B activation was detected by 30 min and reached a maximal level by 180 min. In contrast, TNF- α -dependent NF- κ B activation was faster with a maximal level by 30 min, consistent with previous reports (Ozes et al., 1999). By 180 min after stimulation, the level of PRL-dependent NF- κ B activity was almost as intense as observed with TNF- α . In addition, PRL promoted up-regulation of several cytokines, namely IL-1 β , IL-6, IL-8, GM-CSF, and TNF- α , which require NF- κ B for their maximal transcription (Blackwell and Christman, 1997). These proteins play important roles in clinical and subclinical/chronic mastitis, but their response differs according to the infectious pathogen.

The proinflammatory role of PRL was found in animal models of inflammatory diseases. In experimental carageenan pleurisy in rat, repeated administration of ovine PRL provoked a significant increase in the leukocytes number in the exudate (Meli et al., 1993). Neutrophils obtained from inflammatory exudates displayed a marked increase in TNF- α production that was reduced after bromocriptine treatment (Meli et al., 1997). Moreover, it was demonstrated that PRL increases cytokine secretion, including IL-1 β , IL-6, and IFN- γ , from various cell types (Cesario et al., 1994; Tseng et al., 1997). Here, in bovine mammary epithelial cells, PRL induced a significant increase in the mRNA of TNF- α in a dose-dependent manner. Moreover, PRL up-regulated expression of IL-1 β , IL-6, and IL-8, but this up-regulation varied with PRL concentrations used. A PRL dose of 1 ng/mL was sufficient to significantly increase mRNA expression of IL-8. But, up-regulation of IL-6 and IL-1 β needed at least 5 and 25 ng/mL, respectively. Although PRL significantly enhanced IFN- γ production in peripheral human blood mononuclear cells (Cesario et al., 1994), we did not observe a significant increase in IFN-

Figure 3. Prolactin (PRL; $\pm$ SEM) in milk from healthy and chronic mastitis-affected quarters. Quarters were categorized according to the SCC measured at sampling time. 1) SCC $<2 \times 10^5$; 2) 2×10^5 to $<4 \times 10^5$; 3) 4×10^5 to $<10^6$; 4) 10^6 to $<2 \times 10^6$; and 5) $\geq 2 \times 10^6$ cells/mL. ^aDifferent from groups 1 and 2 at $P < 0.01$; ^bDifferent from groups 1, 2, and 3 at $P < 0.01$.

γ mRNA levels after PRL treatment of bovine mammary epithelial cells.

Cows suffering from subclinical mastitis with a persistently increased SCC were defined as chronic mastitis-affected cows. We measured PRL concentrations in blood and milk from healthy and chronic mastitis-affected cows to determine the relationships between PRL and the degree of inflammation in the udder by using SCC measurements. Plasma PRL concentrations did not differ between healthy and chronic mastitis-affected cows. But, in contrast to previous results (Malven, 1977), PRL levels were higher in blood than in milk. In milk, we expected a positive correlation between SCC and milk PRL concentrations. In mastitis-affected quarters with SCC $>10^6$ cells/mL, PRL concentrations were significantly higher than those in healthy quarters with low SCC, supporting our hypothesis. Nevertheless, our results do not allow us to determine whether PRL is responsible for higher SCC or whether a persistently high SCC induces elevated PRL concentrations.

It is well recognized that, although the pituitary gland is the main source of PRL, the hormone is produced by a variety of other tissues, including the brain, placenta, cells of the immune system, and mammary epithelial cells (Freeman et al., 2000), which supports the second observation. Moreover, transcytosis of plasma PRL across mammary epithelial cells might be facilitated in the case of mastitis. Another hypothesis

explaining higher PRL found in quarters with high SCC could be due to differences in the distribution of pathogens. Indeed, it appears that quarters with SCC $>10^6$ cells/mL are infected by a higher proportion of major pathogens, namely *Staph. aureus* and *Strep. uberis*, whereas quarters with 200×10^3 to 999×10^3 cells/mL are dominated by CNS. Interestingly, the pathogenesis and the innate immune response to IMI with major pathogens are markedly different than that developed with CNS. Further studies are needed to confirm the higher PRL found in mastitic quarters. Meanwhile, the present findings suggest that PRL might modulate the inflammatory response in the bovine mammary gland by acting as an autocrine or paracrine factor.

Persistent accumulation of inflammatory cells at the site of inflammation requires both continuous neutrophil influx and increased survival of extravasated granulocytes. Accordingly, IL-8 plays an important role as a potent neutrophil chemoattractant (Baggiolini et al., 1989), whereas GM-CSF delays neutrophil apoptosis (Boutet et al., 2004). These cytokines are crucial for triggering and maintaining the neutrophilic inflammation that characterizes chronic mastitis. We found that IL-8 was the only cytokine up-regulated at any dose of PRL. Granulocyte-macrophage colony-stimulating factor mRNA levels were increased, but from a 25 ng/mL dose of PRL. Therefore, these results suggest that PRL could indirectly participate in the persistent accumulation of neutrophils in milk from chronic mastitis-affected cows. Although milk PRL concentrations in mastitis-affected quarters were 20.8% higher than in healthy quarters, this does not explain the significant effect on SCC. Furthermore, PRL up-regulated TNF- α at doses as low as 5 ng/mL and TNF- α increased the expression of all cytokines studied here, suggesting that both factors contribute to a synergistic effect influencing local inflammation and SCC.

Recently, Brand et al. (2004) demonstrated, in human whole blood cultures, that PRL by itself or in combination with LPS caused an increase in the binding activity of NF- κ B. Here, we demonstrate that PRL activates NF- κ B in bovine mammary epithelial cells, and induces a dose-dependent increase in the mRNA of inflammatory cytokines and chemokines, notably involved in neutrophil activation and migration. Therefore, these findings let us hypothesize that mammary epithelial cells could amplify the neutrophilic inflammation that characterizes mastitis by an indirect mechanism involving PRL. This PRL-activated NF- κ B signaling pathway could contribute to autoregulatory feedback loops perpetuating inflammation. Further investigations are needed to confirm the relevance of potential synergistic mechanisms such as the virulence of the pathogen and the host response mobilization.

CONCLUSIONS

This study shows that PRL is endowed with proinflammatory activity in bovine mammary epithelial cells. By activating NF- κ B, PRL may trigger up-regulation of cytokines, such as IL-1 β , IL-6, IL-8, GM-CSF, and TNF- α in a dose-dependent manner. Because milk PRL levels were increased in chronic mastitis-affected quarters and positively correlated to SCC and the number of neutrophils, this study suggests that PRL might modulate the inflammatory response in the bovine mammary gland and play a role in mastitis pathogenesis.

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