Bovine mammary epithelial cells (bMECs) synthesize lactoferrin, which is secreted into milk. Our results suggest that prolactin stimulated secretion of lactoferrin in primary bMECs and their clonal cell line under serum-free conditions. Prolactin also stimulated mRNA expression of lactoferrin in the clonal cell line. This effect was reduced by AG-490, suggesting that the prolactin-stimulated mRNA expression of lactoferrin was mediated by Janus kinase (JAK)2.

Key words: lactoferrin; prolactin; mammary epithelial cells; bovine

Lactoferrin (LF), an 80-kDa iron-binding glycoprotein belonging to the transferrin family, exhibits potent antimicrobial and immunomodulatory activities.1) In addition to these effects, accumulating evidence indicates that LF has several pleiotropic effects, such as regulation of cell migration,2) differentiation, proliferation,3) and cytokine secretion.4) Furthermore, experiments in animal models as well as clinical studies in humans have shown that the oral administration of bovine LF has various protective effects against infection (as reviewed by Yamacha et al.).5) It has been reported that lipopolysaccharides (LPS) and fetal calf serum (FCS) stimulate LF synthesis in bovine mammary epithelial cells (bMECs),6,7) a well-known type of LF-producing cells.8) In the present study, we investigated to determine whether PRL affects LF synthesis in bMECs, and if so, the mechanism underlying LF synthesis.

bMECs were prepared as previously described, with several modifications.10) In brief, mammary tissue from lactating Holstein cows was dispersed with 120 U/ml of collagenase. The cell suspension was filtered through a 150-μm nylon mesh filter and centrifuged at 80 g for 10 min. The centrifuged cells and cell clumps were washed twice with RPMI 1640 medium (Sigma Chemical, St. Louis, MO) containing 10% FCS. The bMECs were then seeded into plastic dishes in RPMI 1640 medium containing 10% FCS, 5 μg/ml of insulin, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The LF in the medium was measured using a bovine lactoferrin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX). Subconfluent bMECs were cultured in RPMI 1640 medium containing 5% FCS, 5 μg/ml of insulin, and 5 μg/ml of dexamethasone for 48 h, and further cultured in the medium alone for 48 h. The cells were then stimulated with 8 μg/ml of ovine PRL (Sigma Chemical) or the vehicle for the indicated periods. When it was indicated, the cells were pretreated with alpha-cyano-(3,4-dihydroxy)-N-benzylcinnamamide (AG-490; Calbiochem-Novabiochem, La Jolla, CA) for 2 h. LF mRNA expression was measured by real-time quantitative RT-PCR using the TaqMan universal master mix (Applied Biosystems, Foster City, CA). The thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min and 40 cycles at 95 °C for 15 s, and at 60 °C for 1 min. Normalization was performed using β-actin mRNA levels as controls. The primers used for LF (L08604) analysis were as follows: forward, 5'-GGTGAAGAAGGACTGTT-3', and reverse, 5'-CAAAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic probe, 5'-FAM-AGCAAGGCGCAGGAAATTTGGAA-BHQ-1-3'; 5'-ACTGAGCAGAGCTGGACTCAGAT-3'; TaqMan fluorogenic
probe, 5′-FAM-TTCCGCTGCCCTGAGGCTCTCTTC-BHQ-1-3′. The data were analyzed by ANOVA, followed by Bonferroni’s method of multiple comparisons between pairs.

Initially we established primary cultured bMECs, as described above. Although these cells spontaneously secreted LF into the culture supernatant, PRL enhanced the secretion of LF in a time-dependent manner, and maximum secretion was observed at 48 h after stimulation (Fig. 1A). Primary cultured bMECs are a heterogeneous population with respect to their potential to synthesize LF. To minimize this heterogeneity and exclude the possibility that other types of cells contaminated the primary bMECs cultures, we next performed a limiting dilution of primary bMECs cultures and established cloned bMECs (CL1). This clonal line formed a cobblestone-like monolayer and expressed cytokeratin, a marker of epithelial cells (data not shown). The cells were then stimulated with 8 μg/ml of PRL (closed circle) or the vehicle (open circle) for the indicated periods. The reaction was terminated by collecting the conditioned medium. Each value represents the mean ± SE of triplicate determinations. *P < 0.05 as compared with the value of vehicle.

![Fig. 1. Effects of PRL on LF Secretion in Primary Cultured bMECs (A) and Cloned bMEC Line (CL1) (B).](image)

Subconfluent bMECs were cultured in RPMI 1640 medium containing 5% FCS, 5 μg/ml of insulin, and 5 μg/ml of dexamethasone for 48 h, and further cultured in the medium alone for 48 h. The cells were then stimulated with 8 μg/ml of PRL (closed circle) or the vehicle (open circle) for the indicated periods. The reaction was terminated by collecting the conditioned medium. Each value represents the mean ± SE of triplicate determinations. *P < 0.05 as compared with the value of vehicle.

Next we investigated the effect of PRL on the mRNA expression of LF in CL1 cells. As shown in Fig. 2, PRL stimulated mRNA expression of LF in a time-dependent manner for up to 72 h in these cells. mRNA expression of LF was significant after 24 h. A similar observation was reported using a mouse tissue culture system, indicating that LF responds to PRL in mammary explants,11) but, there are contradictory reports that LF secretion is not dependent on PRL in primary bMECs and the cloned bMEC line.12,13) In our experiments, LF secretion from cells without serum starvation was not dependent on the presence of PRL, but mRNA expression of LF was enhanced by PRL (data not shown). It has been reported that the amount of secreted LF is regulated by FCS and the extracellular matrix in bMECs.7) This discrepancy between previous studies and the present one can be attributed to differences in cell culture conditions, such as serum starvation time and the cell culture substratum. Furthermore, mRNA expression of LF is independent of PRL in the CID-9 mouse mammary epithelial cell line.14) The latter finding is contradictory to our result, which suggests that the expression of bovine LF mRNA is stimulated by PRL. Since the previous study utilized cultured CID-9 cells on an Engelbreth-Holm-Swarm tumor matrix, the discrepancy in the effect of PRL on mRNA expression of LF might also be due to the difference in cell culture substratum. The amount of secreted LF was approximately 10-fold lower than that previously reported,12) in which primary bMECs plated
on type I collagen gels were used. Although our culture conditions using plastic dishes in the absence of FCS were not ideal for maximal induction of LF synthesis in bMECs, we can exclude the possibility that other factors affect PRL-stimulated LF synthesis in bMECs.

It is known that the PRL receptor is associated with Janus kinase (JAK)2 and that it activates JAK2 rapidly upon exposure to PRL. In order to throw light on the involvement of JAK2-mediated signaling in the PRL-induced LF synthesis in bMECs, we next examined the effect of AG-490, a specific inhibitor of JAK2, on LF mRNA expression in CL1 cells. As shown in Fig. 3, AG-490 reduced PRL-induced LF mRNA expression in a dose-dependent manner. This result suggests that the upregulation of LF transcripts by PRL is mediated by JAK2 in CL1 cells. It has been reported that the promoter region of the bovine LF gene contains a putative binding site for STAT5, a downstream mediator of JAK2 activation in mammary epithelial cells. Taken together, these observations support the suggestion that mRNA expression of LF is stimulated by PRL in bMECs.

It is known that the plasma concentration of PRL reaches maximum levels near parturition in dairy cows. PRL concentrations in bovine mammary secretions increase throughout the prepartum period, and are much higher than plasma PRL in prepartum cows. Because the concentrations of LF in bovine mammary secretions are much higher during development and in colostrum than in milk during lactation, we speculate that PRL might be involved in the regulation of LF synthesis in the bovine mammary gland, especially in the prepartum period. The present results provide an initial means to study the mechanisms of PRL-stimulated LF synthesis in bovine mammary glands. Further investigation is necessary to clarify the mechanism underlying LF synthesis.

In conclusion, within the limitations of our in vitro experimental model under serum-free conditions, our findings suggest that PRL stimulates LF synthesis in bMECs and that this effect is mediated by JAK2.

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References


