ABSTRACT: Signal transducer and activator of transcription (Stat)5 has been implicated in the signal transduction pathways of several factors that are lactogenic or galactopoietic in mammary cells, including prolactin, GH, and IGF-I. Data from cell or explant culture support the concept that Stat5 may represent part of a common route by which different extracellular signals converge and are transduced into the cell. There are few data on Stat5 activity and level in vivo, and we set out to determine whether physiological stimuli of milk synthesis, including GH, GH-releasing factor, and milking frequency, would be associated with alterations in Stat5 activity or protein. We measured Stat5 DNA binding activity using electrophoretic mobility shift assay and Stat5 protein by Western blot in bovine mammary tissue obtained by biopsy or slaughter. Stat5 activity was absent in nonlactating, nonpregnant cows and was present in late pregnancy and throughout lactation. Stat5 activity varied considerably among cows at similar stages of lactation. Mammary Stat5 activity and protein were determined in hormone-treated lactating cows and mammary quarters of cows milked at different frequencies. Infusion of GH and GH-releasing factor for 2 mo significantly raised levels of milk production and depressed mammary Stat5 activity without influencing Stat5 protein abundance. Mammary Stat5 was also influenced by milking frequency; once-daily milking reduced milk production, Stat5 activity, and protein abundance compared with twice-daily milking. Analysis of mammary Stat5 in relation to milk protein concentration in pooled data from lactating cows indicated that Stat5 activity was correlated \( r = 0.505, P < 0.05 \) with average milk protein concentration and not related to milk protein yield \( (P > 0.05) \). These results show that both Stat5 protein and Stat5 activity are modulated by different physiological signals in vivo and suggest that Stat5 lies within in the zone where signal transduction cascades from a variety of factors are convergent. Further work is required to clarify the role of Stat5 in relation to other factors in regulation of milk protein gene expression.

Key Words: Cows, Lactation, Milk, Protein, Somatotropin, Transcription Factors

Introduction

Milk synthesis in the bovine is regulated in part by hormones such as GH, which increases milk synthesis when infused or injected (Bauman and Vernon, 1993). There is also an element of hormone-independent local control. When mammary quarters are milked separately at different daily frequencies, there is a positive relationship between milk yield and milking frequency (Linzell and Peaker, 1971). The signaling pathways elicited by altered milking frequency are independent of circulating hormone levels. However, both hormonal and nonhormonal stimuli act ultimately to stimulate milk protein gene expression. It is not known how these physiological stimuli coordinately regulate milk synthesis in vivo, especially in the molecular connection of their respective signaling pathways to milk protein gene expression.

Signal transducer and activator of transcription (Stat)5 has been suggested to play a key role in regulation of milk protein gene expression, based on cell culture studies. Stat5 mutation results in loss of \( \beta \)-casein promoter activity and \( \beta \)-casein transcription in transfected cells (Schmitt-Ney et al., 1991; Standke et al., 1994). Two forms of Stat5 (a and b) were identified; Stat5a-deficient transgenic mice failed to lactate (Liu et al., 1995, 1997). In cultured cells, Stat5 is activated by GH and prolactin (Gouilleux et al., 1995; Ruff-Jami-
son et al., 1995; Wakao et al., 1995). We recently showed a rapid stimulation of Stat5 DNA binding activity by prolactin, GH, and IGF-I in bovine mammary explant culture (Yang et al., 2000). Results from these model systems suggest that Stat5 may represent part of a common route by which different extracellular signals converge and are transduced in mammary cells. There are few data on Stat5 DNA binding activity and protein level in vivo, and we set out to determine whether physiological stimuli of milk synthesis, including GH and milking frequency, would be associated with alterations in Stat5 activity or protein.

Materials and Methods

Animals. Studies were carried out in compliance with the guidelines of the Canadian Council on Animal Care. For studies of Stat5 activity in relation to stage of lactation, Holstein cows were used from the Dairy Research and Technology Centre of the University of Alberta. Cows were fed a total mixed ration containing 50% concentrate and 50% forage and housed in tie-stalls with 24 h of light and were milked twice daily at 0400 and 1600. Mammary tissue was obtained by surgical biopsy (Knight et al., 1992) at 1400. The biopsy site selected was in the basal (upper) portion of the left udder. Fat and large s.c. blood vessels were avoided wherever possible during incision. Fifteen Holstein cows were allocated for the lactation stages study, five animals in early lactation (d 30 to 53, nonpregnant), five animals in midlactation (d 90 to 110, not pregnant), and five animals in late lactation (d 218 and d 278, pregnant). Two further animals that were neither pregnant nor lactating were used.

Mammary tissue samples from 15 Holstein cows provided by H. A. Tucker (Michigan State Univ., East Lansing) were used to study Stat5 activity in relation to hormone treatments. These cows were in midlactation (d 118 to 181) and were not pregnant. Cows were allocated to receive three treatments (n = 5/treatment): 1) continuous i.v. infusion of recombinant bovine GH (29 mg/d, Somavute, Pharmacia & Upjohn, Peapack, NJ), 2) continuous i.v. infusion of recombinant Leu27, Hse45-bGHRF (1–45) lactone (12 mg/d, Pharmacia & Upjohn), or 3) no infusion (controls) for 63 d. Milk yield was recorded at each milking. Milk samples, combined from three consecutive milkings, were collected and analyzed for composition once a week for the 9 wk the cows were subjected to treatments. Data presented are average values for the entire 9-wk period. Analysis was by infrared analyzer (Multispec, Wheldrake, U.K.) at Michigan Dairy Herd Improvement Association (East Lansing). Cows were killed at the end of treatments approximately 8 h after the afternoon milking and mammary tissue was obtained within 20 min after killing (Binelli et al., 1995). Tissue was frozen and stored at −70°C.

Five cows in midlactation (d 115 to 130, nonpregnant) were used to study mammary Stat5 in response to milking frequency, which was implemented by milking left and right quarters of the mammary gland separately in each cow. Right quarters of the mammary gland of each cow were milked twice daily at 0400 and 1600, and the left quarters were milked once daily at 1600 for 2 wk. Milk yield was recorded at each milking and milk samples were collected separately from left and right quarters at d 1 to 3 and d 16 to 18. Milk protein concentration was determined by infrared analytic instrument (Robinson and Kennelly, 1989). Average milk and milk protein percentage were obtained from a pool of all milk collected on d 1 to 3 and d 16 to 18 of the milking schedule. At the end of the 18-d milking schedule, mammary tissue from front right and left quarters was separately biopsied at 1400. Mammary tissue was immediately placed in liquid nitrogen and stored at −80°C until nuclear extraction.

Preparation of Nuclear Extract. All the chemicals used in the analyses were purchased from Sigma (St. Louis, MO), unless stated otherwise. Mammary nuclear extracts were prepared as previously described (Yang et al., 2000).

Electrophoretic Mobility Shift Assay. The Stat5 binding site (5′-AGATTTCAGGAATTCATT-3′) based on the bovine β-casein promoter was used to design the probe for the electrophoretic mobility shift assay (EMSA) (Standke et al., 1994). Briefly, the double-stranded, labeled DNA probe was obtained by fill-in reaction with [α-32P]dATP. The DNA-binding reaction (20 μL) was carried out as described (Yang et al., 2000) at 25°C for 20 min. Poly (dI-dC) was added in proportion to the protein content of the nuclear extracts (1 μg/μg nuclear protein). Labeled DNA probe (2 μL) was added to the reaction. The Stat5-DNA complex was separated on a native 4% polyacrylamide gel. The gel was dried and used to expose an x-ray film. We have previously shown this assay to be specific in detection of bovine mammary Stat5, by “supershift” of the observed band for Stat5-DNA complex with an anti-Stat5 antibody. The assay is linear with protein content of the nuclear extracts (Yang et al., 2000).

Stat5 Protein Western Blotting. Nuclear protein electrophoresis and Western blotting were run as described (Wakao et al., 1995) with minor modifications (Yang et al., 2000). Nuclear protein was run on 7.5% SDS-polyacrylamide gels. Proteins from the gels were electrotransferred to NitroPure membranes (Micron Separations, Westborough, MA). Nonspecific binding was inhibited by incubation of membranes in phosphate-buffered saline with 10% nonfat dry milk and 0.1% Tween-20 for 1 h at 25°C. Membranes were incubated with 1 μg/mL anti-Stat5 antibody in PBS with 1% nonfat dry milk and 0.02% Tween-20 for 1 h. An antibody from Santa Cruz Biotechnology (Santa Cruz, CA) that recognizes both Stat5a and Stat5b was used in all Western blots. We have previously shown that the Western blotting detects both tyrosine-phosphorylated (active) and dephosphorylated (inactive) Stat5 and thus repre-
sents total Stat5 protein. The assay is linear with protein amount of nuclear extract (Yang et al., 2000).

**Statistical Analysis.** The bands on EMSA and Western blots were analyzed by Imaging Densitometry (Bio-Rad Laboratories, Hercules, CA) and data are expressed in units of adjusted volume (optical density $\times$ mm $\times$ mm). The least squares means and standard errors of the means were obtained from the general linear model (GLM) procedure of SAS (SAS Inst. Inc., Cary, NC). In the study of effects of lactational stage on Stat5 activity, the main effect of treatment in this study was intended as stage of lactation on Stat5 activity or protein. However, the loss of several samples precluded further statistical analysis of the treatment effect. In the hormone treatment and milking frequency experiments, the main effects considered were hormone treatment and different milking frequency. For analysis of the relationship of Stat5 activity to average milk protein concentration, within each treatment (control, GH, and GHRF), animals were classified into either a group with high milk protein concentration or a group with low milk protein concentration. Data from 15 animals (five animals for each treatment) were used in the GLM of SAS. Significant differences between treatment or group means were taken from the matrix of student’s $t$-test within each set of least squares means. Significance was determined at $P < 0.05$.

**Results**

**Stat5 DNA-Binding Activity and Protein Abundance in Bovine Mammary Gland.** Stat5 DNA binding activity was detected in the mammary gland of cows in midlactation, but no Stat5 activity was observed in nonlactating, nonpregnant cows (Figure 1). We had originally allocated five animals to biopsy in early, middle, and late lactation, but several samples were lost due to problems with either the biopsied material or the biopsy procedure. In the end, we did not get enough samples from all of the stages of lactation to do the planned comparisons, so we present the individual data to illustrate the degree of variation that we encountered in the nine cows from which we obtained usable material (Figure 2). Three cows in early lactation (30 to 53 d) had a similar mammary Stat5 activity and protein abundance. However, mammary Stat5 activity showed considerable variations in the five cows between 90 and 110 d of lactation. One cow in late lactation (218 d in lactation) had Stat5 activity similar to that of cows in earlier stages of lactation. The nuclear extracts were also used to detect Stat5 protein by Western blotting. The density of the bands varied among cows at similar stages of lactation but showed less variably than that of Stat5 DNA binding activity.

**Stat5 DNA Binding Activity and Protein Abundance in Mammary Gland of Control, GH-, and GH Releasing Factor-Treated Cows.** Mammary Stat5 was analyzed in GH- and GH releasing factor-treated animals, in which chronic infusion of GH and GH releasing factor signifi-

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**Figure 1.** Stat5 activity was detected in mammary gland from lactating cows but not in that from nonlactating, nonpregnant cows. Nuclear extracts were prepared from mammary tissue samples of two lactating cows (lanes 1 and 2) and two nonlactating, nonpregnant cows (lanes 3 and 4). Electrophoretic mobility shift assay was performed using 4 $\mu$g of nuclear protein.

**Figure 2.** Stat5 activity and protein in the mammary gland of cows at different stages of lactation. Nuclear extracts were prepared from mammary tissue of lactating cows. Days in lactation for individual cows are shown above each lane. Electrophoretic mobility shift assay was performed using 4 $\mu$g of nuclear protein from each sample, and 15 $\mu$g of nuclear protein was used for Western blotting to detect Stat5 protein. The density of the band for Stat5 activity and protein was quantified and is charted in the graph.
Mammary Stat5 in vivo variations

Figure 3. Stat5 activity in the mammary gland of control, GH-, and GH releasing factor-treated cows. Mammary tissue was obtained from 15 cows: five cows were used as controls, five cows received GH treatment, and five cows received GH releasing factor treatment for 63 d. Nuclear extracts were isolated from the mammary tissue and 4 μg of nuclear protein from each cow was used in the Stat5 electrophoretic mobility shift assay. The density of the Stat5-DNA complex bands was scanned and quantified. The least squares means and standard error of the densities from each treatment (n = 5) are shown. Bars identified by a different letter differ at P < 0.05.

Figure 4. Stat5 protein abundance in the mammary gland of control, GH-, and GH releasing factor-treated cows. Mammary tissue was obtained from 15 cows: five cows were used as controls, five cows received GH treatment, and five cows received GH releasing factor treatment for 63 d. Nuclear extracts were isolated from the mammary tissue and 4 μg of nuclear protein from each cow was used in the Stat5 electrophoretic mobility shift assay. The density of the Stat5-DNA complex bands was scanned and quantified. The least squares means and standard error of the densities from each treatment (n = 5) are shown. Bars identified by a different letter differ at P < 0.05.

Significantly increased circulating blood GH levels and milk yield (Dahl et al., 1993). Serum GH concentrations were elevated approximately sixfold in GH- and GH releasing factor-treated cows compared with levels seen in control cows (3 ng/mL). Milk yield of the GH- and GH releasing factor-treated animals was about 33 to 34 kg/ d on average and significantly higher than the control of 29 kg/d (Binelli et al., 1995). Mammary Stat5 activity was significantly lower in GH- (~50%) and GH releasing factor- (~60%) treated cows than in control cows (n = 5; P < 0.05, Figure 3). Treatment with GH and GH releasing factor had a tendency to suppress Stat5 protein abundance (P < 0.1) compared to control cows (Figure 4).

Mammary Stat5 Response to Different Levels of Milking Frequency. Milk and milk protein yields were influenced by the milking schedule (Table 1). Once-daily milking significantly reduced milk yield and milk protein yield in comparison with twice-daily milking. Milk protein concentration increased during d 1 to 3 (P < 0.01) after the transition to once-daily milking, but it was not significantly different between once- and twice-daily milking by d 16 to 18 (P > 0.05). Stat5 DNA binding activity was depressed (~19%) by once-daily milking in comparison with twice-daily milking (P < 0.01, Figure 5).

Mammary quarters milked once daily had lower level of Stat5 protein (~36%) than the quarters milked twice daily (P < 0.01, Figure 6).

Stat5 Activity in Relation to Average Milk Protein Concentration. Because Stat5 is a transcription factor involved in regulation of milk protein gene expression, and levels of milk protein production show considerable variability among cows at similar lactational stages, the physiological variability in Stat5 may represent dif-
Table 1. Influence of milking frequency on milk and milk protein yielda

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Frequency of daily milking</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Once</td>
<td>Twice</td>
</tr>
<tr>
<td>Days 1 to 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk yield, kg/d</td>
<td>8.35 ± 0.46</td>
<td>14.26 ± 1.92</td>
</tr>
<tr>
<td>Milk protein, %</td>
<td>3.22 ± 0.03</td>
<td>3.08 ± 0.04</td>
</tr>
<tr>
<td>Milk protein yield, kg/d</td>
<td>0.27 ± 0.02</td>
<td>0.44 ± 0.06</td>
</tr>
<tr>
<td>Days 16 to 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk yield</td>
<td>9.15 ± 0.84</td>
<td>16.6 ± 1.21</td>
</tr>
<tr>
<td>Milk protein, %</td>
<td>3.29 ± 0.05</td>
<td>3.26 ± 0.02</td>
</tr>
<tr>
<td>Milk protein yield, kg/d</td>
<td>0.30 ± 0.03</td>
<td>0.55 ± 0.04</td>
</tr>
<tr>
<td>Days 1 to 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk yield, kg/d</td>
<td>8.6 ± 0.75</td>
<td>15.5 ± 1.42</td>
</tr>
</tbody>
</table>

a^n = 5.

Figures 5 and 6: Mammary Stat5 activity and protein responses to once- and twice-daily milking. Stat5 activity and protein were measured in mammary tissue biopsied at the end of the milking schedule from both quarters. Nuclear extracts were isolated and analyzed using the Stat5 electrophoretic mobility shift assay (Figure 5) and Western blotting (Figure 6). The density of the bands was scanned and quantified. The least squares means are shown. Bars identified by different letters differ at P < 0.01.

Influence of milking frequency on milk and milk protein yield. We analyzed Stat5 activity in relation to milk protein concentration (Figure 7). Within each treatment (control, GH, GH releasing factor), animals were classified into either a low or a high average milk protein concentration. When control and GH releasing factor-treated cows were divided into groups based on their milk protein concentration, there was significantly higher Stat5 activity in the mammary tissue of animals producing higher protein concentrations (Figure 7A,B). In this experiment, the GH-treated cows showed a relatively small range of milk protein concentrations (Figure 7C), and there was no relation with Stat5 activity in this group. To evaluate the overall relationship of mammary Stat5 and milk protein concentration, we carried out regression analysis on the pooled data of control, GH-, and GH releasing factor-treated cows based on their relative Stat5 activity.
ity. This analysis revealed that mammary Stat5 was correlated to average milk protein concentration (n = 15, r = 0.505, P < 0.05, Figure 8) but not significantly related to milk protein yield (data not shown, P > 0.05). Using anti-Stat5 antibody, the protein abundance of Stat5 was analyzed in the same tissue samples. There were no significant differences between animals producing high and low protein concentrations in control and GH releasing factor-treated cows (Figure 9). The nuclear extracts of the mammary tissue from GH-treated cows were not available for Stat5 protein analysis.

Discussion

Sources of Variation of Stat5 Activity In Vivo

The general pattern of Stat5 activity in bovine mammary gland was similar to that reported in rodents.

A. Control Animals

<table>
<thead>
<tr>
<th>Milk protein concentration (%)</th>
<th>3.04 ± 0.007</th>
<th>3.22 ± 0.030</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stat5-DNA complex Density</td>
<td>32.1 ± 8.0</td>
<td>54.9 ± 9.5</td>
</tr>
</tbody>
</table>

B. GHRF – treated animals

<table>
<thead>
<tr>
<th>Milk protein concentration (%)</th>
<th>3.00 ± 0.05</th>
<th>3.24 ± 0.074</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stat5-DNA complex Density</td>
<td>37.1 ± 4.5</td>
<td>53.1 ± 3.2</td>
</tr>
</tbody>
</table>

C. GH-treated animals

<table>
<thead>
<tr>
<th>Milk protein concentration (%)</th>
<th>2.97 ± 0.046</th>
<th>3.17 ± 0.078</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stat5-DNA complex Density</td>
<td>70.4 ± 10.6</td>
<td>71.2 ± 15.4</td>
</tr>
</tbody>
</table>

Figure 7. Mammary Stat5 activity in relation to milk protein concentration in control, GH-, and GH releasing factor-treated cows. Stat5 activity was analyzed in control (Figure 7A), GH releasing factor- (Figure 7B), and GH-treated animals (Figure 7C). Animals within each group were ranked according to their average milk protein concentration. The Stat5-DNA binding reaction was carried out using 4 μg from each sample in the electrophoretic mobility shift assay. The density of each band was scanned and quantified. The least squares means and standard error of mean are shown below the autoradiography. *P < 0.05; n.s., not significant.

A. Control Animals

<table>
<thead>
<tr>
<th>Milk protein concentration (%)</th>
<th>3.04 ± 0.007</th>
<th>3.22 ± 0.030</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stat5 protein Density</td>
<td>10 ± 1.7</td>
<td>11 ± 2.2</td>
</tr>
</tbody>
</table>

B. GHRF-treated Animals

<table>
<thead>
<tr>
<th>Milk protein concentration (%)</th>
<th>3.00 ± 0.051</th>
<th>3.24 ± 0.074</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stat5 protein Density</td>
<td>6.1 ± 0.6</td>
<td>5.3 ± 1.0</td>
</tr>
</tbody>
</table>

Figure 8. Correlation of mammary Stat5 activity and milk protein concentration. The density of the Stat5-DNA complex from control, GH-, GH releasing factor-treated animals in Figure 7 was plotted against milk protein concentration for each animal.

Figure 9. Stat5 protein abundance in relation to milk protein concentration. Western blot for the same samples illustrated in Figures 7A and B. Mammary tissue samples were from control and GH releasing factor-treated cows. Fifteen micrograms of nuclear protein from each sample was used. The bands were scanned and quantified and the least squares means and standard error of mean are shown.
Stat5 activity was expressed just prior to parturition and throughout lactation but was absent in nonlactating, nonpregnant cows. In mice, Stat5 activity was detected at day 15 of pregnancy, remained strong during lactation, and ceased at the end of lactation (Schmitt-Ney et al., 1999). The presence of Stat5 activity in late pregnancy prior to the onset of lactation may be related to a role for this factor in proliferation or differentiation of mammary cells.

There is only one prior published report of mammary Stat5 in the bovine (Wheeler et al., 1997). In that study, bovine tissue showed Stat5 mRNA as well as Stat5 protein in Western blotting. However, either no signal, or a very weak signal, was detected for Stat5 DNA binding activity in the electrophoretic mobility shift assay. The reasons for the difference in Stat5 activity between the results of Wheeler et al. (1997) and the strong activity in our study are unclear. However, it seems likely that Stat5 was present but inactive for some reason in their tissue samples.

In our studies, all tested lactating animals showed a robust Stat5 DNA-binding activity. There were substantial interindividual variations in this activity among animals at similar stages of lactation. This variation has implications for the study of Stat5. In physiological experimentation on dairy cattle, mammary tissue is available from animals killed at the end of study, which is costly, or from biopsy, which is both costly and invasive. These factors tend to limit sample size in studies of dairy cattle and it is thus important to identify and control for sources of variation. Diurnal variation in milking stimulation could be one source. In the accompanying paper we obtained evidence for a decline in Stat5 activity over time since the last milking (Yang et al., 2000), although this was relatively slow compared with the laboratory rat. It would be difficult to study diurnal variation in Stat5 activity in dairy cattle because the study design would entail multiple biopsies from each animal on the same day. In the absence of such data, it would seem appropriate to sample at a constant interval after the last milking. When this approach was taken (i.e., Figure 5) and controlled comparison made within animals, significant differences of the order of 20% in Stat5 activity could be detected with a sample size of five. Some other factors that may make a contribution to the interindividual variations in Stat5 activity among animals in the same stage of lactation may include variation in the biopsy site and seasonal changes in various reproductive and lactational hormones in studies of extended duration.

Lactation is initiated by a period during which mammary cell proliferation and differentiation exceed the rate of cell death. Lactation ends with a period of involution in which apoptosis predominates. Stat5 may be involved in regulation of these cellular changes, in a manner that is separate from a role in transcriptional regulation of milk protein synthesis during lactation. There are dramatic changes in cell differentiation and the secretory machinery during late gestation, and these changes are largely induced by prolactin (Akers et al., 1981). It has been suggested that the signal transduction pathway leading to mammary gland development involves Stat5 (Hennighausen, 1997; Liu et al., 1997). We also observed activity of Stat5 during late pregnancy, and it seems likely that Stat5 activity in lactating animals may be dependent on their pregnancy status.

From in vitro studies it is clear that Stat5 activity is highly sensitive to the concentration of hormones such as prolactin. Stat5 activity fell to zero within 60 min of incubation of bovine mammary explants if prolactin, GH, or IGF-I was not present to maintain activity. Hormonal status of the animal is thus a likely contributor to level of Stat5 activity. Stat5 DNA-binding activity in bovine mammary explants seems highly sensitive to prolactin concentrations within the physiologic range, and we suggested that Stat5 could be regulated in vivo over a broad range of activation by prolactin alone (Yang et al., 2000). In explants, GH alone at a physiologic concentration of 5 ng/mL did not by itself stimulate Stat5 activity. At 50 ng/mL, a concentration achieved in some infusion trials, GH stimulated Stat5 activity and potentiated the response to prolactin.

Our results obtained in vivo support the idea that the GH status of individual animals is a source of variation in Stat5 activity. Stat5 activity was strongly reduced by long-term elevation of circulating GH levels, in contrast to the activation of Stat5 by GH in explant culture. One possible explanation is that there is a down-regulation in the overall train of signal transduction in response to elevated GH levels. In rat liver, a dramatic down-regulation of the Stat5 activation pathway has been shown to result from chronic plasma GH stimulation (Choi and Waxman, 1999). Alternatively, it may be that GH infusion alters the circulating profile of other factors that also influence Stat5 activity, such as prolactin (Borromeo et al., 1995; Cecim et al., 1995).

Stat5 DNA Binding Activity and Protein Response to Milking Frequency

In the bovine there are four separate mammary glands. The left and right quarters are physically separated by a medial suspensory ligament. By altering milking frequency in left and right quarters, we were able to study local effects in a model in which all four quarters could be presumed to receive the same hormonal stimuli through the common blood supply. The DNA-binding activity and protein level of Stat5 in the mammary gland of lactating cows were both depressed by once-daily milking in comparison with twice-daily milking. Modulation of Stat5 in response to milking frequency adds support to the idea that Stat5 serves as a common point of the signaling pathways of different physiologic stimuli in vivo. Stat5 may be not only involved in signaling pathways of multiple hormones, but also may convey signals into the cell from nonhormonal stimuli. It is not known what specific signals could in-
fluence Stat5 protein and activation in response to milking frequency. We speculate that there may be some regulatory signals that are candidates for local activation of Stat5. First, it has been reported that milk synthesis is regulated by autocrine control through a milk protein, such as feedback inhibitor of lactation and some peptides generated from milk protein (Wilde et al., 1995; Aslam and Hurley, 1998). Increased milking frequency may remove those feedback inhibitors and thus reduce their inhibitory effects on milk synthesis. Second, decreasing milking frequency results in an increased physical pressure, which may reduce secretory cell volume or cellular hydration states in the mammary gland. Cellular hydration states are regulated by cell swelling and shrinking. Cell swelling activates membrane transport pathways, which leads to the net loss of osmolytes and osmotically obligated water. Conversely, cell shrinking activates a membrane transport system, which acts to increase the amount of solutes, and hence water, entering the cell. In mammary cells, cellular hydration is an important cellular signal and is capable of mimicking the effects of some hormones, and protein synthesis is acutely regulated by cellular hydration (Haussinger, 1996; Millar et al., 1997). The transduction pathways for both lactation feedback inhibitors and cellular hydration in the mammary gland are not known at present, but these may interact with a signaling pathway involving Stat5.

Another factor that must be considered is the possibility that reduction of milking to once daily may have induced partial involution and that part of the change in Stat5 activity or protein may be attributable to a shift in cell populations from active proliferation and differentiation toward apoptosis. It is known that mammary involution in dairy cows occurs slowly by decreased milking frequency, and lactation can be reinitiated by milking (Capuco and Akers, 1999).

Milk protein concentration represents the net balance of milk protein gene expression and protein synthesis and catabolism of milk proteins in the mammary epithelial cells. Transcriptional regulation is thus only one of multiple elements that influence milk protein production. In transfected cells there is a close coupling between Stat5 activity and β-casein transcription in response to prolactin stimulation. The data presented here, however, indicate no clear relationship between the DNA-binding activity of the transcription factor Stat5 and milk protein production. When milk protein synthesis was increased by increased frequency of milking, Stat5 activity was elevated; however, when milk synthesis was increased by hormonal injection, Stat5 activity fell. These results show that both Stat5 protein and Stat5 activity are modulated by different physiological signals in vivo and suggest that Stat5 lies within the zone where different signal transduction cascades are convergent. Further work is required to clarify the role of Stat5 in relation to other factors in regulation of milk protein gene expression.

It remains unclear whether Stat5 activity is related to milk protein concentration. The 15 lactating cows from the hormone infusion trial had milk protein concentrations ranging from 2.97 to 3.24%, and Stat5 activity was correlated with average milk protein concentration. There was no relationship between Stat5 activity and milk protein yield. In the study of milking frequency, the Stat5 activity as well as milk protein yield were significantly increased by twice-daily milking in comparison with once-daily milking. However, milk protein concentration was not different between once- and twice-daily milking during the final 3 d before biopsy (3.26 + 0.02 vs 3.29 + 0.05%, respectively).

A limitation of this work is that the available methods do not allow separate determination of the amounts and activities of Stat5a and Stat5b. A number of recent studies in knockout mice have revealed clear distinctions between the actions of Stat5a and b in mediating hormone actions. For example, the lipolytic action of GH is lost after Stat5a gene disruption (Fain et al., 1999), and Stat5b gene disruption leads to loss of GH-dependent body growth and male-specific liver gene expression (Park et al., 1999). Further study is required to determine the relative amounts of Stat5 a and b in mammary and their respective degree of activation in response to different stimuli.

In conclusion, Stat5 DNA-binding activity and protein were present in bovine mammary gland throughout lactation. Stat5 activity and protein in mammary tissue seem to be related to hormonal status and milking frequency.

Implications

In comparison with the understanding of lactation in rodents, knowledge of bovine lactation is limited, especially in the area of molecular regulation. Stat5 is a recently identified regulator in the signaling pathway of several lactogenic and galactopoietic hormones in rodents and cell culture. The specific roles of Stat5 in bovine mammary gland have not been well studied. This work reports evidence that in vivo mammary Stat5 is modulated by infusion of GH and local changes inside the mammary gland caused by different milking frequencies, indicating that it is involved in some manner in the signal transduction of hormonal as well as non-hormonal stimuli. However, an uncoupled relationship between Stat5 DNA-binding activity and levels of milk protein production under different physiological states were also observed, implying roles of factors other than Stat5 in controlling milk protein synthesis in the mammary gland.

Literature Cited


