The activity of transcription factor Stat5 responds to prolactin, growth hormone, and IGF-I in rat and bovine mammary explant culture

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ABSTRACT: Signal transducer and activator of transcription-5 (Stat5) is known to play a critical role in prolactin-induced β-casein gene transcription in rodents. In nonmammary cells, Stat5 is activated by multiple hormones and cytokines, including growth hormone. We hypothesized that Stat5 may serve as a common point in the signal transduction pathways of hormones that promote milk protein gene expression in bovine mammary cells, which are regulated by GH and IGF-I in addition to prolactin. Assays for Stat5 DNA binding activity and protein were validated in mammary explant culture. The Stat5 protein abundance was not changed by any of the short-term hormonal treatments used in our study, suggesting that short-term regulation of Stat5 is predominantly at the level of protein activation. Both rat and bovine explant culture showed a rapid stimulation of Stat5 DNA binding activity by prolactin, GH, and IGF-I at the high concentrations typically used in explant cultures as well as at levels within physiologic ranges. Growth hormone stimulated Stat5 activity at a lower concentration in bovine than in rat cultures, but in both species the presence of GH increased the response of Stat5 activity to prolactin. These results suggest that transcription factor Stat5 may represent part of a common route by which different extracellular signals converge and are transduced intracellularly to coordinately regulate cell function in the mammary gland.

Key Words: Bovidae, Insulin-Like Growth Factor, Mammary Glands, Prolactin, Signal Transduction, Somatotropin

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Introduction

Prolactin is a key factor in lactogenesis and maintenance of lactation. In ruminants, GH, and possibly IGF-I, are galactopoietic (Tucker, 1981; Bauman et al., 1985; Etherton and Bauman, 1998). Each of these factors has a specific cell surface receptor. Prolactin acts through its long-form receptor (Hennighausen et al., 1997), and mammary cells also express GH and IGF-I type I receptors (Rechner, 1985; Glimm et al., 1990; Ilkabahar et al., 1999). Separate signaling pathways originate from the respective receptors. However, because ultimately all three factors promote milk protein gene expression, these pathways must at some point interact or converge.

Mammary cells contain a latent transcription factor acting on casein gene promoter sequences, known as signal transducer and activator of transcription (Stat)-5 (Wakao et al., 1994). Stat5 is implicated in prolactin-induced β-casein gene transcription in rodent cells. The signaling pathway is proposed to involve activation of Janus Kinase 2 (Jak2) subsequent to receptor binding. Phosphorylation of Stat5 by Jak2 confers DNA binding activity (Gouilleux et al., 1994). Two isoforms of Stat5 (a and b) have been identified in mice. Stat5a-deficient transgenic mice fail to lactate after parturition, whereas Stat5b deficiency results in abnormal male-specific gene expression in the liver (Liu et al., 1997; Udy et al., 1997).

We hypothesized that Stat5 activation may be a common point in the signal transduction pathways of both prolactin and GH in mammary cells. In nonmammary cells, GH stimulates activation of Jak2 and Stat5 (Arendsinger et al., 1993; Cousin et al., 1999), so it seems likely that GH, like prolactin, may activate this pathway in mammary cells. We also thought it of interest to determine whether IGF-I interacts with the mammary Stat5 pathway; this is unknown for any tissue. We used mammary explants from lactating rats and cows in our
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studies to test the effects of prolactin, GH, and IGF-I on Stat5 activation and protein level.

Materials and Methods

Animals. Studies were carried out in compliance with the guidelines of the Canadian Council on Animal Care. Female Sprague-Dawley rats suckling 7 to 10 pups were from a colony maintained at the University of Alberta, and Holstein cows from the Dairy Research and Technology Center of the University were used. Animals of both species were in midlactation (rats, d 10 to 15 after parturition; cows, d 150 to 200 after parturition). Rats were killed by CO2 asphyxiation and the fourth mammary gland was dissected and removed aseptically. Mammary tissue was obtained by biopsy from cows that had not been housed in individual wire mesh cages in temperature- (24°C) and humidity-controlled (80%) rooms in a 12 h light–12 h dark cycle. Rats were fed laboratory chow containing 24% CP (Continental Grain, Chicago, IL). Cows were fed a total mixed ration containing 50% concentrate and 50% forage and housed in tie-stalls with 24 h light.

To reduce basal Stat5 activity, suckling (or milking) was suspended. Rats were separated from their pups for 24 h before tissue sampling. Rats were killed by CO2 asphyxiation and the fourth mammary gland was dissected and removed aseptically. Mammary tissue was obtained by biopsy from cows that had not been milked for the preceding 6 h or 22 h. The period of withdrawal from milking was later lengthened to 3 d because our first studies showed that bovine mammary Stat5 decreased more slowly than that in rats after withdrawal of milking.

For biopsy, deep sedation of the cow was achieved by slow i.v. injection of xylazine. Cows were tipped and held recumbent on their right side. Local anesthesia was by a line block with s.c. injection of 5 to 10 mL of 2% lignocaine HCl. A 5 to 10-g portion of secretory tissue was surgically removed (Knight et al., 1992). Hemostasis was achieved with suturing. Milk yield of tissue was measured as the amount of enzyme that will hydrolyze 1.0 nmol of p-nitrophenyl phosphate per minute at 30°C, pH 7.0.

Preparation of Nuclear Extract. Nuclear extracts were prepared as described (Standke et al., 1994) with modifications. Chemicals used were purchased from Sigma unless otherwise noted. Tissue was pulverized in liquid nitrogen using a prechilled (−70°C) mortar and pestle. The frozen, pulverized tissue was immediately transferred to a 14-mL Falcon tube and minced in 2 mL of lysis buffer (10 mM HEPES, pH 7.6, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 2 μg/mL pepstatin) in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Then, 3 to 5 mL of the same buffer was added and the dispersed tissue was homogenized in a 7-mL glass Dounce tissue grinder (Wheaton Scientific, Millville, NJ). The homogenates were centrifuged on a cushion of the lysis buffer at 100,000 × g (Sorvall Ultra Centrifuge, Du Pont, Wilmington, DE) for 20 min using a Sorvall TH 641 rotor. Nuclei were recovered at the bottom of the centrifugation tube and the supernatant was discarded. The DNA yield (8 to 10 mg/g tissue) from the nuclear purification was determined using the method of Labarca and Paigen (1980) and was not different among experimental treatments. The pellets were resuspended in hypertonic buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 20% glycerol, 1 mM dithiothreitol (DTT), 2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 2 μg/mL pepstatin for 20 min on ice. The extracts were then centrifuged at 15,800 × g for 5 min at 4°C. Nuclear extracts were frozen in liquid nitrogen and stored at −70°C. The protein contents in the nuclear extracts were measured (Bio-Rad Laboratories, Hercules, CA) and used as a basis for sample loading in the assay of Stat5 activity and Western blots (see below). For the study of dephosphorylation of Stat5, nuclear extracts were isolated without using sodium orthovanadate in the extract buffer, and protein tyrosine phosphatase (human recombinant, Calbiochem, San Diego, CA) was used at the concentration of 1 unit/μg nuclear protein to dephosphorylate nuclear extracts. One unit of protein tyrosine phosphatase is defined as the amount of enzyme that will hydrolize 1.0 nmol of p-nitrophenyl phosphate per minute at 30°C, pH 7.0.

Electrophoretic Mobility Shift Assay. Stat5 DNA binding activity was detected using electrophoresis mobility shift assay (EMSA), in which a labeled double-stranded DNA sequence was used as a DNA probe to bind active Stat5 protein in nuclear extracts. The Stat5-DNA complex and the free DNA were separated using polyacrylamide gel electrophoresis and visualized by autoradiography. The Stat5 binding site (5′-AGATTCTAGGAAATTCGAATTCCAT-3′) of the bovine β-casein promoter was used to design the probe. This oligonucleotide was hybridized with its complimentary oligonucleotide (5′-GATTGGAAATTCGAATT-3′) at 55°C for 10 min. The double-stranded labeled DNA probe was obtained by fill-in reaction with [α-32P]dATP (Amersham) using DNA polymerase I.

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Klenow fragment (Pharmacia Biotech, Piscataway, NJ) at 37°C for 1 h. The unincorporated radioactive dATP was removed by Sephadex G-50 (DNA grade, Pharmacia Biotech) spin column chromatography. Specific activities of 1.5 to 3 × 10^6 cpm/ng were obtained. The DNA binding reaction was carried out as described at 25°C for 20 min (Standke et al., 1994). Nuclear extracts were incubated in a 20-μL reaction containing 10 mM HEPES (pH 7.8), 50 mM KCl, 5 mM MgCl2, 10% (vol/vol) glycerol, 5 mM DTT. Poly (dl-dC) was added in proportion to the protein content of the nuclear extracts (1 μg/μg nuclear protein), where the same amount of nuclear protein from each sample was added for a run of EMSA. Labeled DNA probe (2 μL) was added to the reaction solution at the final step. After completion of the binding reaction, 2 μL of loading buffer containing bromphenol blue was added, and the mixture was loaded immediately on a native 4% polyacrylamide gel. Electrophoresis was run in 0.25× TBE (0.1× Tris-borate, 0.5 mM EDTA, pH 8.0) at 10 V/cm and 25°C with cooling water circulation between the plates. In all of the figures shown, the unbound free DNA probe had run out of the gel, so that only Stat5-DNA complex is evident. The gels were used to expose x-ray film, with different times of exposure to ensure that the detected bands were within the saturation limits of the film.

**Stat5 Protein Western Blotting.** Nuclear extracts were electrophoresed through 7.5% SDS-polyacrylamide gels run at a constant voltage of 100 V. Gels were electrotransferred to NitroPure membranes (Micron Separations, Westborough, MA). A Ponceau S stain of the membrane was used to confirm equal sample loading. Nonspecific binding was inhibited by incubation of membranes in blocking buffer (10% nonfat dry milk, 0.1% Tween-20 in phosphate-buffered saline) for 1 h at 25°C with shaking, followed by washing in three changes of 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween-20 (TBST) for 5 min each. Membranes were incubated with the first antibody (1 μg/mL) in phosphate-buffered saline containing 1% nonfat dry milk and 0.02% Tween-20 for 1 h at 25°C or overnight at 4°C and washed with TBST three times for 5 min each. The primary antibody was rabbit polyclonal anti-mouse Stat5a+b antibody and anti-mouse Stat5a (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-Stat5a+b is shown to recognize both isoforms of Stat5a and Stat5b, and anti-Stat5a is shown to be Stat5a-specific (Gebert et al., 1997). Horseradish peroxidase-conjugated anti-rabbit IgG was then incubated with the membranes at 0.1 μg/mL for 1 h at 25°C and then washed. Detection reagent for ECL and Hyperfilm ECL (Amersham, Arlington Heights, IL) were used to detect antibodies bound to the Stat5 protein.

**Study Design.** A series of experiments was conducted to validate the measures used and thereafter to test the response of Stat5 activity and protein to prolactin, GH, and IGF-I. Each experiment was conducted on tissues collected from animals on the same day. Harvested tissues were either immediately frozen (i.e., not incubated) or subjected to explant culture and then frozen, on that day. All samples from each experiment were then studied within a single assay for Stat5 DNA binding activity or protein.

In Exp. 1, we tested bovine mammary nuclear extract in the Stat5 electrophoretic mobility shift assay and investigated its DNA binding activity in relation to the amount of nuclear protein added to the binding reaction. In Exp. 2, the specific antibody to Stat5 a-b was included in the reaction mixture of the Stat5 DNA binding assay. In this approach, the resulting Stat5-DNA antibody complex is “supershifted” (i.e., migrates more slowly in the electrophoresis than the Stat5-DNA complex alone). This shift in the mobility of the band by a specific antibody confirms its identity as Stat5.

The effects of the hormone treatments were thereafter studied. Within each set of cultures, the control treatment consisted of Medium 199 containing 1 μg/mL insulin, 1 μg/mL hydrocortisone, and 1 mg/mL bovine serum albumin (Sigma) (hereafter referred to as basic culture medium). Ovine prolactin was from Sigma and IGF-I (human recombinant) was from Gibco BRL. Rat and bovine GH were a gift from A. F. Parlow through the National Hormones and Pituitary Program, National Institute of Health (Bethesda, MD). Exp. 3 was conducted to confirm that the incubated mammary explants were responsive to added hormone. We determined whether prolactin was able to induce Stat5 activation in rat mammary explant culture to a similar extent and over a similar time course as seen in vivo in response to suckling. Stat5 activity was determined in mammary tissue of rats that had been separated from their pups for 24 h, and then after replacement of pups for 30 and 60 min. Mammary tissue was also obtained from rats 24 h after withdrawal of pups and immediately used to make explants and cultured in the presence or absence of prolactin (1 μg/mL) for 10, 20, 30, and 60 min. Experiment 3 was repeated twice in explant cultures obtained from different rats.

Prolactin at 1 to 5 μg/mL is commonly used to induce and maintain differentiation of explant cultures (Topper et al., 1975). The physiological concentration range of prolactin in rodents is 10 to 800 ng/mL. To investigate whether Stat5 responded to physiological concentration of prolactin, rat mammary explants were cultured in 200 ng/mL prolactin for 1 h in Exp. 4. In Exp. 5 stimulation of Stat5 activity by prolactin and GH was determined for bovine mammary, initially using high levels of hormones. Tissue was obtained at 6 h after milking, and explants were cultured in basic culture medium alone or supplemented with prolactin (1 μg/mL) or GH (1 μg/mL) for 30 and 60 min. To test for response in a physiologic hormone concentration range, Exp. 6 was then conducted. Tissue was obtained from two lactating cows that had not been milked for 3 d. Two hormone concentrations (50 and 200 ng/mL for prolactin and 5 and 50 ng/mL for GH) were compared with basic culture medium, and the explants were incubated for 1 h. The
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The combined effects of prolactin and GH in both species were studied in Exp. 7. Mammary tissue was obtained from four lactating rats that had been separated from pups for 20 to 24 h and from three lactating cows that had not been milked for 3 d. Explants were cultured in basic culture medium alone or supplemented with 200 ng/mL prolactin, 50 ng/mL GH, or both, for 1 h. The explant culture was done in triplicate for each of the described treatments.

The effect of IGF-I on Stat5 activation was studied using mammary tissue from lactating rats (without suckling for 22 h) and cows at 22 h after milking in Exp. 8. Prolactin and IGF-I were added at 1 μg/mL and 50 ng/mL, respectively, and the explants were incubated for 1 h. This experiment was repeated in different lactating animals. In Exp. 9, the effect of cycloheximide (0.5 mM) on Stat5 activity in response to prolactin and GH was studied using tissue from three lactating cows that had not been milked for 3 d. The explant culture was done in triplicate for each of the described treatments. Explants were incubated with 200 ng/mL prolactin or 50 ng/mL GH in the presence or absence of 0.5 mM cycloheximide for 1 h.

Quantification and Statistical Analysis. Bands representing Stat5-DNA complex in EMSA and Stat5 Western blots were scanned and quantified by Imaging Densitometry (Bio-Rad Laboratories). The density volume for each Stat5-DNA complex or band on Western blot was deducted from its own lane background. The data are expressed as means ± SEM of the density unit of adjusted volume (OD × mm × mm). Statistical analyses were conducted using the general linear models procedure of SAS (SAS Inst. Inc., Cary, NC). In the study of suckling and prolactin effects on Stat5 activity (Figure 3), the main effects in the SAS model included treatment (suckling or prolactin addition), time, and time × treatment. In the analysis responses to prolactin and GH (Figures 6, 7, and 9), the statistical model included treatment and sampled rats or cows within treatment. The main effects of treatment were tested using the sampled rats/cows with treatment mean square as the error term. In Figures 12 and 13B, comparisons between treatment and control were performed using Fisher’s two-tailed least significant difference test. Differences with probability less than 0.05 were considered significant.

Results

Stat5 EMSA and Supershift Assay. Stat5 activity was detected in bovine mammary nuclear extract and was proportional to nuclear protein content in the binding reaction. Stat5 activity was almost undetectable at 0.01 μg of nuclear protein but increased linearly with amount of nuclear protein from 1.0 to 4.0 μg (Figure 1). When antibody to Stat5 was included, the resulting Stat5-DNA-antibody complex supershifted (migrated

![Graph](Figure 1. Stat5 activity and protein are proportional to nuclear protein levels. Nuclear extracts were prepared from mammary tissue of lactating cows. (A) Electrophoretic mobility shift assay was carried out using the bovine β-casein gene promoter-based DNA probe and 0.01 to 4 μg nuclear protein. (B) Detection of Stat5 protein was by Western blotting. Varying amount of nuclear protein (8 to 16 μg) were separated on 7.5% SDS polyacrylamide gels and electrophoretically transferred to membrane and immunoblotted with anti-Stat5a-b antibody. Results are expressed as densitometric volume and plotted against amount of nuclear protein.)
Figure 2. Stat5 electrophoretic mobility shift and supershift assay. Nuclear extracts were isolated from mammary gland of a lactating cow. Nuclear protein (2 μg/lane) and varying amounts of antibody to Stat5 from 2 to 0.01 μg were added to the binding reaction with radioactively labeled DNA probe. An antibody to tubulin was used as a control antibody and added to the binding reaction (lane 2).

more slowly in the electrophoresis) (Figure 2). This shift in the mobility of the band by a specific antibody confirmed its identity as Stat5. With a decrease in antibody from 2 to 0.1 μg, the density of the supershifted band decreased and the density of the band corresponding to the Stat5-DNA complex increased accordingly.

Stat5 Activity in Response to Suckling and Prolactin in Rat Mammary Tissue. Prolactin secretion in rodents is strongly influenced by the suckling of pups (Grosvenor et al., 1979). We investigated Stat5 activity in response to suckling to determine the physiological time course of Stat5 activation by prolactin in vivo. Mammary Stat5 DNA binding activity in lactating rats was very low at 24 h after removal of the pups; however, Stat5 activity was evident at 30 min after restoration of the pups and increased linearly with time of suckling up to 60 min (Figure 3). For mammary tissue stimulated with prolactin in vitro, Stat5 activity was detected at 10 min and increased linearly for 60 min thereafter (Figure 3). These data indicate prolactin is able to induce Stat5 activation in explant culture to a similar extent and over a similar time course as seen in vivo in response to resumption of suckling.

The physiological concentration range of prolactin in the rat is from 10 to 800 ng/mL (Arbogast and Voogt, 1996). This is much lower than the hormone concentration used (1 μg/mL) in many reported studies of mammary explant culture in which prolactin was added to provoke differentiation. Stat5 DNA-binding activity was undetectable in tissue that was frozen but not incubated, and activation of Stat5 was not inducible in explant culture by insulin and hydrocortisone alone (Figure 4). Stat5 DNA-binding activity was present in explants supplemented with prolactin (200 ng/mL), suggesting that prolactin at physiologic concentrations is also able to induce Stat5 activation in rat mammary explant culture.

Stat5 Response to Prolactin and GH in Bovine Mammary Explant Culture. Stat5 activity was detected in
the bovine mammary tissue collected at 6 h after the last milking (Figure 5). Stat5 activity was also present in explants cultured with insulin and hydrocortisone for 30 min. However, by 60 min of incubation, Stat5 activity became undetectable in the presence of only insulin and hydrocortisone. Stat5 activity in the mammary explants was maintained during 60 min of incubation in medium supplemented with either prolactin or GH at 1 μg/mL. This experiment yielded similar results when repeated with tissue from different lactating cows.

In Exp. 6, Stat5 activity was undetectable in the bovine mammary tissue (data not shown) and when explants of that tissue were cultured in the medium that was supplemented with only insulin and hydrocortisone (Figure 6). During 1 h of incubation, Stat5 activity in the explants was induced by 50 and 200 ng/mL prolactin and 50 ng/mL GH, but not by 5 ng/mL GH. The relative Stat5 activity induced by 200 ng/mL prolactin was about twice that induced by 50 ng/mL prolactin (P < 0.01), and the Stat5 activity induced by 50 ng/mL GH was also higher than that induced by 50 ng/mL prolactin (P < 0.05). Stat5 activity was sensitive to prolactin and GH at 50 ng/mL, but a low level of GH (5 ng/mL) was unable to independently induce Stat5 activation in the bovine mammary explants.

Combined Action of Prolactin and GH in Stat5 Activation. The separate and combined effects of prolactin and GH on Stat5 DNA binding activity are shown for rat and bovine mammary explant cultures in Figure 7. Stat5 activity was undetectable in the rat explants incubated with 50 ng/mL GH. However the response to prolactin was higher by 40% in the presence of 50 ng/mL GH (P < 0.001). In the bovine, Stat5 activity was detected in prolactin-, GH-, and prolactin + GH-treated mammary explants. The relative signal of Stat5 activity was different among the three treatments (P < 0.01). Stat5 activity induced by prolactin and GH was higher by 40% than that induced by prolactin alone.

Stat5 Activation Responds to IGF-I in Rat and Bovine Mammary Explants. In experiments to investigate Stat5 activity response to IGF-I, prolactin induction of Stat5 activity was used as a positive control, whereas the incubation with only insulin and hydrocortisone was used as negative control (Figure 8A). Stat5 activity was detected in prolactin and IGF-I treated explants but was not induced by insulin and hydrocortisone alone in rat mammary explant culture (Figure 8A). In bovine mammary explant culture, in which Stat5 activity was presented in a low level in the tissue that was not incubated (Figure 8B), prolactin and IGF-I supplementation during 30- and 60-min incubation increased Stat5 activity to a similar degree.

Cycloheximide Does Not Influence the Response of Stat5 Activity to Prolactin and GH. It is possible that GH activation of Stat5 might be through increasing mammary IGF-I secretion. The addition of cyclohexi-
Figure 7. The additive effects of prolactin and GH on Stat5 DNA binding activity. Mammary tissue was taken from four lactating rats that had been separated from their pups for 20 to 24 h. Mammary tissue was obtained from three lactating cows that had not been milked for 3 d. Mammary explants were cultured in basic culture medium alone and with prolactin (200 ng/mL, three lanes), GH (50 ng/mL, three lanes), and prolactin + GH (three lanes). The incubation was carried out for 1 h and explants were frozen until nuclear isolation. The Stat5 electrophoretic mobility shift assay was carried out using 4 μg of nuclear protein. Data in the graphs represent means ± SEM for rats (n = 4) and cattle (n = 3). A representative electrophoretic mobility shift assay is shown. Treatments with different alphabetical superscripts within each species are different (P < 0.05).

Cycloheximide to bovine mammary explant culture at concentrations that block protein synthesis completely inhibits IGF-I secretion (Campbell et al., 1991). Cycloheximide did not alter Stat5 activity induced by either prolactin or GH (Figure 9), suggesting that neither GH nor prolactin action on Stat5 activation is via IGF-I production and does not require new protein synthesis.

Stat5 Protein Response to Prolactin and GH in Rat and Bovine Mammary Explant. Two antibodies to Stat5 were used. The anti-Stat5a+b antibody was developed based on the common sequence of both Stat5a and b isoforms and reacts with both. Anti-Stat5a was based on a Stat5a-specific sequence and only reacts with Stat5a. We used both antibodies to detect Stat5 protein in the nuclear extracts from rat and bovine mammary tissue that had been obtained in the various experiments described above. Stat5 immunoblots showed a signal at the expected molecular weight of 97 kDa (Wakao et al., 1994) (Figure 10). A doublet Stat5 band was detected in rat tissue, but two bands were not always clearly resolved in the bovine tissue using anti-Stat5a+b antibody. One clear band was detected in tissue of both species using anti-Stat5a antibody. The upper band obtained using the antibody that reacts to both isoforms corresponds to the molecular size of the single band seen in the Stat5a blots. Thus, the upper bands in the rat tissue are Stat5a and the lower bands are Stat5b protein in the anti-Stat5a+b antibody-based Western blotting. Two bands were not always clearly distinguishable in mammary tissue from bovines, and this may be due to poorer electrophoretic resolution or possibly to differential specificity of the antibodies toward bovine Stat5 isoforms. The density of Stat5 protein was linearly related to the amount of nuclear protein loaded on the gel (Figure 1).

To determine whether the antibody against both Stat5 isoforms reacted with phosphorylated (active) and dephosphorylated (inactive) forms of the protein, we treated nuclear extracts with protein tyrosine phosphatase (Figure 11). Protein tyrosine phosphatase treatments for 1 to 4 h abolished Stat5 DNA binding activity in EMSA but did not influence Stat5 protein abundance as determined by Western blot. Addition of vanadate (an inhibitor for protein phosphatase) to the treatments could maintain the DNA binding activity in the nuclear extracts. These results indicate that the anti-Stat5a+b antibody reacted equally well with the
Figure 9. Cycloheximide has no effect on Stat5 activity in response to prolactin and GH in bovine mammary explant. Mammary tissue was obtained from lactating cows that had not been milked for three d. The explants were cultured in basic culture medium or 200 ng/mL prolactin and 50 ng/mL GH, in the presence and absence of 0.5 mM cycloheximide as indicated. Mammary tissue was incubated for 1 h and then frozen until nuclear isolation. Stat5 electrophoretic mobility shift assay was carried out using 4 μg nuclear protein. The graph represents the average data (means ± SEM) from the three animals, and only one representative Stat5 electrophoretic mobility shift assay is shown. Treatments with different alphabetical superscripts are different (P < 0.05).

phosphorylated and dephosphorylated Stat5 protein and thus reflected total Stat5.

Figure 10. Detection of Stat5 (a and b) protein in bovine and rat mammary tissue. Nuclear extracts were isolated from the mammary gland of two lactating rats and two lactating cows. Fifteen micrograms of nuclear protein from each sample was used for detecting Stat5 protein by Western blotting. The membrane was incubated with anti-Stat5a+b then the membrane was stripped and incubated with anti-Stat5a antibody.

We analyzed Stat5 protein in the nuclear extracts previously isolated for determination of Stat5 DNA binding activity. Stat5 protein abundance was determined for Exp. 7. Whereas hormone treatments had induced Stat5 activity (Figure 7), the density of Stat5 immunoblots was not different among the control, prolactin, GH, and prolactin + GH treatments (P > 0.05) in the rat mammary explants (Figure 12). Further analysis of bovine mammary nuclear extracts also revealed that prolactin and GH treatment during explant culture

Figure 11. Treatment of nuclear extracts with protein tyrosine phosphatase. Nuclear extracts were isolated from rat mammary tissue as described except without adding sodium orthovanadate in the extraction buffer. The nuclear extracts were treated with protein tyrosine phosphatase for the times indicated. Sodium orthovanadate (2 mM final concentration) was added to the samples in lanes 3, 5, and 7. Stat5 DNA binding activity was detected with 4 μg nuclear extract by electrophoretic mobility shift assay, and the protein abundance of Stat5 was determined using 10 μg of nuclear protein in the Western blot.

Figure 12. Stat5 protein level is insensitive to prolactin and GH in rat mammary explants. Lactating rats were separated from their pups for 20 to 24 h and killed to obtain mammary tissue. Mammary explants were prepared and cultured in basic culture medium with the addition of prolactin (200 ng/mL) and GH (50 ng/mL) as described in the legend to Figure 7. Nuclear extracts (15 μg) were used to detect Stat5 protein by Western blotting. The densities of the Stat5 bands were quantified and the mean ± SEM of each treatment is shown. No significant differences were noted amongst treatments. Stat5 DNA binding activity for this experiment is shown in Figure 7.
did not influence Stat5 protein abundance, nor did treatment with cycloheximide (Figure 13).

**Discussion**

Several methodologic considerations underlie interpretation of this work. Prolactin, synergistically working with insulin and hydrocortisone, induces casein synthesis in mammary explants (Juergens et al., 1965). Mammary explant seems to be a suitable system to explore the actions of prolactin and other hormones on Stat5 DNA binding activity and protein. In lactating rats whose pups were removed for approximately 1 d, mammary tissue Stat5 activity decreased to an undetectable level. Restoration of suckling rapidly re-induced Stat5 activation and in vitro stimulation of mammary tissue with prolactin-induced Stat5 activity over a similar time scale, suggesting that the incubated tissue was physiologically normal in this response. In freshly isolated mammary tissue from cows milked twice daily, a robust Stat5 DNA binding activity was detected in the electrophoretic mobility shift assay. This apparent Stat5 DNA binding activity was verified by supershift with a specific anti-Stat5 antibody. In the bovine, mammary tissue Stat5 DNA binding activity was reduced at 24 h after cessation of milking and fell to zero by 72 h after the last milking. Although it was necessary to stop milking for a relatively long time in the bovine to lower Stat5 activity in the tissue, both bovine and rat explant cultures showed a rapid stimulation of Stat5 DNA binding activity by prolactin, GH, and IGF-I. These hormones induced Stat5 activity at the high concentrations typically used in explant cultures as well as at levels within physiological ranges.

Antibodies that were commercially available at the time this study was done were used to detect Stat5 protein. In the rat, two Stat5 isoforms were clearly detected in Western blots using an antibody specific for Stat5a and b, and the larger isoform was selectively detected using anti-Stat5a. In the bovine two bands were not clearly resolved in all blots, suggesting poorer electrophoretic resolution or differential specificity of the antibodies toward bovine Stat5 isoforms. The experiment with protein tyrosine phosphatase establishes that Stat5 antibodies react equally with phosphorylated and dephosphorylated Stat5 and therefore reflect total Stat5 protein in the nuclear extract. This variable was not changed by up to 60 min of treatment with prolactin, GH, or IGF-I in our study, suggesting that the short-term regulation of Stat5 is most likely at the level of protein activation. 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 these reports and the strong activity in our study are unclear. However, it seems likely that Stat5 was present but inactive for some reasons in their tissue samples.

Stat5 seems to be a common point in multiple signal transduction pathways in the mammary gland. In COS cell culture, prolactin, GH, and some cytokines have been independently demonstrated to stimulate the DNA binding activity of Stat5, by co-transfection of vectors encoding Stat5 cDNA and respective hormone receptors (Gouilleux et al., 1995; Wakao et al., 1995). We demonstrated here that Stat5 activity was induced by prolactin, GH, and IGF-I in mammary explant culture. In the accompanying article (Yang et al., 2000), we show that hormonal infusion and local changes elicited by different frequencies of milking in vivo also could influence Stat5 activity in the mammary gland. These results support our hypothesis that Stat5 serves as a common point in the signaling pathways of various extracellular stimuli after they are transduced intracellularly. Although GH and IGF bind their respective receptors and activate their specific pathways, they also interact with the prolactin signaling pathway by activating Stat5. We observed additive effects of prolactin and GH action on Stat5 activation in bovine mammary explant culture and enhanced prolactin action in Stat5 activation by GH in rat explants. These data indicate that Stat5 activation could be additive when two stimuli for Stat5 are present simultaneously. Stat5 is also involved in prolactin signaling interaction with extracellular matrix in mammary cell culture (Streuli et al., 1995), and it has functional interactions with glucocorticoid and progesterone receptors in transfected COS cells (Stocklin et al., 1996; Richer et al., 1998). Stat5 may represent part of a common route by which different extracellular signals are transduced in mammary cells.
Both rat and bovine mammary explants showed Stat5 activation at prolactin concentrations from within the physiologic levels induced by suckling (10 to 800 ng/mL in rats and 10 to 200 ng/mL in the cow; Arbegast and Voogt, 1996; Tucker, 1971). Although the small amounts of tissue available from biopsy precluded the study of a full range of hormone concentrations, Stat5 activity at 50 and 200 ng/mL (Figure 6) suggests a steep dose response and potentially a high degree of sensitivity to prolactin concentrations. We thus speculate that Stat5 activity could be regulated over a broad range of activation by prolactin alone in both species. Growth hormone is more likely to modulate the prolactin response. In rat mammary explants, GH only induced Stat5 activity at a pharmacological concentration of 1 µg/mL, and GH at 50 ng/mL (about two- to fivefold the physiological concentration) was ineffective by itself in stimulating Stat5 activity. However, in the presence of 50 ng/mL GH (an otherwise ineffective dose), the response of Stat5 to 200 ng/mL prolactin was increased in the rat compared with prolactin addition alone. Because GH concentrations do not change in response to suckling or milking (Tucker, 1971; Escalada et al., 1997), it may be that it acts as a secondary factor modulating the prolactin response or setting a basal level of activation. This type of relationship has been demonstrated in the human interleukin-3-dependent erythroleukemia cell line F-36P, in which IGF-I augmented erythropoietin-induced Stat5 activity but alone failed to induce Stat5 activation (Okajima et al., 1998). Growth hormone at 50 ng/mL, which was ineffective in the rat, induced Stat5 activity by itself in bovine mammary explant culture, and this response was slightly greater than that seen with 50 ng/mL prolactin. This may reflect a lower threshold for activation of Stat5 by GH in the bovine. Although 50 ng/mL is above the circulating concentrations in lactating cows, it is a level achieved in GH infusion trials. Although the increased milk production by GH infusion involves various aspects of GH action in lactating cows (Etherton and Bauman, 1998), its inability to stimulate rat lactation (Flint, 1995) may be related to a higher threshold for activation of Stat5 by GH in rats.

Bovine and rat mammary tissues in explant culture have the capacity to respond to GH through a signal transduction cascade involving Stat5. Direct and specific induction of Stat5 activity by GH has been shown in COS cells transfected with the GH receptor (Argersinger et al., 1993) and in pancreatic β-cells (Cousin et al., 1999). However, GH action in the mammary gland is thought to be effected by a direct action of GH upon its receptor and also by an indirect action mediated by IGF-I. There is evidence that both GH and IGF-I receptors are present in bovine mammary cells (Rechler, 1985; Glimm et al., 1990). Growth hormone-induced activation of Stat5 was rapid and was not inhibited by cycloheximide, an agent that inhibits the production of IGF-I in mammary cells (Campbell et al., 1991). It seems likely that there is a GH-receptor-mediated action on Stat5 activity and an additional effect of IGF-I per se. Here we present the first observation that IGF-I stimulation is associated with activation of Stat5. This response must entail activation of a kinase. There is an IGF-I receptor-associated kinase, and a number of kinases are known to be involved in IGF-I signal transduction, including insulin receptor substrate-I and phosphatidylinositol-3-kinase (Jones and Clemmons, 1995; Melmed et al., 1996). It remains to be established whether IGF-I activated Stat5 via Janus kinase 2 or one of these other enzymes.

In conclusion, our work provides evidence that prolactin and GH independently induce Stat5 activation in bovine mammary explant culture. In both rat and bovine explants, prolactin and GH showed additive effects in Stat5 activation. We also demonstrated that IGF-I signal transduction pathway may involve Stat5 in rat and bovine mammary cells. These results from in vitro explant culture indicate that Stat5 serves as a common point of signal transduction pathways of several lactogenic and galactopoietic hormones in the mammary gland.
Implications

Multiple hormones regulated mammary function through their respective signaling pathways. It has not been well established how these pathways integrate to coordinate their actions in the mammary gland. This study begins to investigate one particular regulatory molecule signal transducer and activator of transcription (Stat)-5, which is known to play a critical role in rodent lactation. In both rat and bovine explant culture, we showed a rapid stimulation of Stat5 DNA binding activity by prolactin, GH, and IGF-I. Protein abundance of Stat5 was not changed by hormone treatments for up to 60 min in our study, suggesting that short-term regulation of Stat5 is predominantly at the level of protein activation. Prolactin and GH in mammary explants showed additive effects in their action on Stat5 activation. The results indicate that Stat5 is a potential common point in the signaling pathways of various hormones in the mammary gland.

Literature Cited


