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Lactogenic hormones stimulate expression of lipogenic genes but not glucose transporters in bovine mammary gland

Y. Shao[a], E.H. Wall[a], T.B. McFadden[b], Y. Misra[a], X. Qian[a], R. Blauwiekel[a], D. Kerr[a], F.-Q. Zhao[a,*]

[a] *Department of Animal Science, University of Vermont, Burlington, VT 05405, USA*

[b] *Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, Alberta T6G 2P5, Canada*

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Abstract

During the onset of lactation, there is a dramatic increase in the expression of glucose transporters (GLUT) and a group of enzymes involved in milk fat synthesis in the bovine mammary gland. The objective of this study was to investigate whether the lactogenic hormones mediate both of these increases. Bovine mammary explants were cultured for 48, 72, or 96 h with the following hormone treatments: no hormone (control), IGF-I, insulin (Ins), Ins + hydrocortisone + ovine prolactin (InsHPrl), or Ins + hydrocortisone + prolactin + 17β-estradiol (InsHPrlE). The relative expression of β-casein, α-lactalbumin, sterol regulatory element binding factor 1 (SREBF1), fatty acid synthase (FASN), acetyl-CoA carboxylase α (ACACA), stearyol-CoA desaturase (SCD), GLUT1, GLUT8, and GLUT12 were measured by real-time PCR. Exposure to the lactogenic hormone combinations InsHPrl and InsHPrlE for 96 h stimulated expression of β-casein and α-lactalbumin mRNA by several hundred-fold and also increased the expression of *SREBF1*, *FASN*, *ACACA*, and *SCD* genes in mammary explants ($P < 0.01$). However, those hormone combinations had no effect on GLUT1 or GLUT8 expression and inhibited GLUT12 expression by 50% after 72 h of treatment ($P < 0.05$). In separate experiments, the expression of GLUTs in the mouse mammary epithelial cell line HC11 or in bovine primary mammary epithelial cells was not increased by lactogenic hormone treatments. Moreover, treatment of dairy cows with bovine prolactin had no effect on GLUT expression in the mammary gland. In conclusion, lactogenic hormones clearly stimulate expression of milk protein and lipogenic genes, but they do not appear to mediate the marked up-regulation of GLUT expression in the mammary gland during the onset of lactation.

Keywords: Gene expression; Glucose transporters; Mammary gland; Lactogenic hormones

1. Introduction

Glucose is an important nutrient in general, but it is absolutely essential for milk production in the mammary gland of lactating animals because it is an energy source as well as a key substrate for synthesis of milk protein and lipids, and, especially, of lactose. The mammary gland does not synthesize glucose; thus, it needs to take up glucose from blood. Glucose uptake by mammary epithelial cells may play a rate-limiting role in milk production [1].

Glucose transport across the plasma membrane of mammary epithelial cells is mainly mediated by facilitative glucose transporters (GLUTs). The family of GLUTs consists of 13 isoforms, which are designated

* Corresponding author. Department of Animal Science, University of Vermont, 211 Terrill Hall, 570 Main Street, Burlington, VT 05405, USA. Tel.: +1 802 656 0786; fax: +1 802 656 8196.

E-mail address: fzhao@uvm.edu (F.-Q. Zhao).

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as GLUT1 to GLUT12 and H^+/myo-inositol cotransporter [2]. These transporters are structurally conserved and have different tissue distributions, transport kinetics, and regulatory properties. The lactating mammary gland mainly expresses GLUT1, GLUT8, and GLUT12 isoforms [3–5].

The demand for glucose by the mammary gland increases dramatically at the onset of lactation. The glucose transport activity increases 40-fold from the virgin state to the midlactating state [6,7]. Consequently, there is a coordinated increase in the expression of GLUTs in the mammary gland during the onset of lactation. From late pregnancy to early lactation, the mRNA abundance of GLUT1, GLUT8, and GLUT12 in bovine mammary gland increases markedly, by several-fold to several hundred-fold [1].

Expression of genes that code for enzymes and proteins required for de novo lipogenesis is also strikingly upregulated in the mammary gland during lactogenesis. For example, expression of sterol regulatory element binding factor 1 (SREBF1), the master regulator of lipid synthesis [8], increases approximately 2-fold in both mouse and cow mammary gland from late pregnancy to early lactation [9,10]. Expression of its known targets, fatty acid synthase (FASN) and acetyl-CoA carboxylase α (ACACA), also increases during the same period [9,10]. In addition, stearoyl-CoA desaturase (SCD) is upregulated more than 40-fold from late pregnancy to lactation [9].

Mammary development and milk synthesis are regulated both by systemic hormones and by local factors. It has been established that prolactin (Prl) and glucocorticoids (GCs) are the main mediators of secretory cell differentiation and lactogenesis [11,12]. In cows, concentrations of Prl, GCs, and GH in blood increase during late pregnancy and peak near parturition [11]. Concentrations of estrogens (Es) also increase gradually during late pregnancy, then surge several days before parturition. It has been shown that Prl-induced secretion of α-lactalbumin is markedly enhanced by adding 17β-estradiol to mammary explants [11]. Locally produced IGF-I is believed to mediate the effects of GH on the mammary gland, and the numbers of IGF-I receptors on mammary epithelial cells increase markedly during late gestation [11]. In addition, disruption of Prl signaling in the mammary gland decreased GLUT1 expression [13,14]. On the basis of these facts, we hypothesized that some combination of Prl, GCs, E, and/or IGF-I is responsible for the upregulation of GLUTs and lipogenic genes in the bovine mammary gland during onset of lactation. Our objective was to investigate the effects of those hormones on the expression of GLUTs and lipogenic genes in the mammary gland.

2. Materials and methods

2.1. Mammary tissue biopsy and explant culture

All animal use was approved by the University of Vermont Institutional Animal Care and Use Committee. Mammary tissues were obtained via biopsy from the rear quarters of 2 Holstein heifers and 2 multiparous Holstein dry cows approximately 37 d prepartum (37 ± 5 d). The biopsy procedures were performed as described previously [15].

Immediately after biopsy, a small piece (5 mg) of tissue was fixed in 4% paraformaldehyde for 4 h at 4°C. After fixation, the tissue was rinsed with 3 changes of cold PBS (2.67 mM potassium chloride, 1.47 mM potassium phosphate monobasic, 137.93 mM sodium chloride, and 8.06 mM sodium phosphate dibasic), immersed in 0.5 M sucrose in PBS overnight at 4°C, preserved in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA), and then frozen in liquid nitrogen chilled isopentane. Frozen tissues were stored at −80°C until use. The remaining tissue (~1 g) was cut into small pieces (~50 mg per piece). Two pieces of fresh tissue were immediately frozen in liquid nitrogen for RNA isolation. The remaining tissue was placed in 50-mL tubes containing basic medium [Medium 199 with Earle's salts and L-glutamine (Sigma, St. Louis, MO, USA), containing 26.2 mM sodium bicarbonate, 10 mM sodium acetate, 15 mM HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B; room temperature] and transported to the laboratory within 30 min. In a sterile, laminar flow hood, tissue was washed 3 times with PBS containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B and then diced into explants (~1 mm^3). Explants were washed 3 times with basic medium which was removed by use of a strainer after each wash. Explants were placed on siliconized lens paper (Whatman, Piscataway, NJ, USA) floating on culture media supplemented with various hormone treatments. Lens paper was siliconized as described previously [16]. There were 5 hormone treatments: NH (basic medium without hormone), IGF-I (basic medium + 200 ng/mL IGF-I), Ins (basic medium + 5 μg/mL insulin), InsHPrl (basic medium + 5 μg/mL Ins + 5 μg/mL ovine Prl + 1 μg/mL hydrocortisone), and InsHPrlE (basic medium + 5 μg/mL Ins + 5 μg/mL

ovine Prl + 1 μg/mL hydrocortisone + 500 ng/mL 17β-estradiol). Insulin-like growth factor I was purchased from PeproTech (Rocky Hill, NJ, USA), and all other hormones were products of Sigma. The IGF-I group was treated for 48 or 72 h. Other groups were treated for 48, 72, or 96 h. The doses of individual hormones used were based on previous studies [17–20]. There were 4 replicate cultures for each treatment, at each time point. After hormone treatment, a small piece (~5 mg) of explants was fixed and preserved for immunostaining as described above. The remaining explants were stored in liquid nitrogen for RNA isolation. The experiment was repeated with mammary tissues from 4 individual animals.

2.2. Culture of HC11 cells and primary bovine mammary epithelial cells

HC11 (mouse mammary epithelial cell line) cells were grown in complete growth medium [RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 50 μg/mL gentamicin, 5 μg/mL Ins, and 10 ng/mL epidermal growth factor] and kept confluent for 3 d. Then, the cells were primed in RPMI 1640 medium supplemented with 10% charcoal-stripped horse serum (Cocalico Biologicals, Reamstown, PA, USA) and 5 μg/mL Ins for 2 d. For lactogenic hormone induction, ovine Prl (5 μg/mL) and dexamethasone (0.1 μM) were added to priming medium, and incubations were continued for an additional 48 h. Each experiment was repeated 3 times.

Bovine primary mammary epithelial cells (BMECs) were obtained as described [21]. Collagen was prepared from rat tail tendons, and cell culture dishes were coated with collagen gel according to the procedures described by Imagawa et al [22]. BMECs were seeded on collagen gels, grown in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum and 5 μg/mL Ins and allowed to grow until reaching confluence. The collagen gels were then released by rimming each gel with a sterile scalpel blade. For lactogenic hormone induction, ovine Prl (5 μg/mL) and hydrocortisone (1 μg/mL) were added to culture medium, and incubations continued for 48 h. Each experiment was repeated 3 times.

2.3. In vivo Prl administration

Five Holstein multiparous cows were injected intravenously with bovine Prl (provided by John Byatt, Monsanto Co., St. Louis, MO, USA; 1 μg/kg of body weight) twice daily during the first 3 wk of lactation, whereas 5 additional cows served as controls [23]. After 7 d of treatment, mammary tissue was obtained by biopsy from all 10 cows and was used for RNA isolation as described by Wall et al [23].

2.4. Quantitative real-time PCR

RNA was isolated from cells or mammary explants with the use of Trizol reagent (Invitrogen). The concentrations of isolated RNA were measured with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Reverse transcription (RT) was performed with 1 μg of total RNA and Moloney murine leukemia virus reverse transcriptase (USB, Cleveland, OH, USA) according to the manufacturer's protocol. TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA) were used to quantify mRNA for β-actin (Bt03279175), β-casein (Bt03217428), α-lactalbumin (Bt03213964), GLUT1 (Bt03215313), GLUT8 (Bt03217728), and GLUT12 (Bt03210346) with the use of a Bio-Rad (Hercules, CA, USA) CFX96 Real-Time PCR Detection System. Reactions were performed in duplicate in a 10-μL volume containing 5 μL of Universal PCR Master Mix (Applied Biosystems), 0.5 μL of TaqMan Assay mixture containing primers and probe, and 4.5 μL of diluted cDNA (corresponding to 50 ng of reverse-transcribed total RNA). The relative expression of target genes was normalized to that of β-actin and calculated by the $2^{-\Delta\Delta C}{}_T$ method [24]. Expression of lipogenic gene mRNA was measured by RT-PCR with the use of SYBR Green. Reactions were performed in duplicate in a 20-μL volume containing 10 μL of SsoFast EvaGreen Supermix (Bio-Rad), 1 μL of forward primer, 1 μL of reverse primer and 8 μL of diluted cDNA (corresponding to 25 ng of reverse-transcribed total RNA). All primers were designed to span the junction of 2 exons to avoid amplification of genomic DNA during quantitative PCR (primer sequences are shown in Table 1). The relative expression of target genes was normalized to β-actin and calculated by the $2^{-\Delta\Delta C}{}_T$ method.

2.5. Immunohistochemistry

Fixed and embedded mammary tissues were cut into 10-μm sections on a Cryostat HM 505 N (Microm, Walldorf, Germany) and mounted onto gelatin-coated slides. Tissue sections were immersed in goat serum (10% in PBS) for 1 h at room temperature to block nonspecific binding. After 3 PBS washes, tissue sections were incubated with a GLUT1 antibody (Millipore, Billerica, MA, USA) diluted 1:500 in PBS containing 1% bovine serum albumin for 1 h at room temperature, followed by 3 PBS washes for 5 min each

Table 1
Sequences of primers used in quantitative reverse transcription PCR for quantifying expression of lipogenic genes.

Gene	Forward	Reverse
ACTB	GATCTGGCACCACACCTTCT	CCAGAGGCATACAGGGACAG
SREBF1	ACCGCTCTTCCATCAATGAC	GCTGAAGGAAGCGGATGTAG
FASN	CTGAGTCGGAGAACCTGGAG	CGAAGAAGGAAGCGTCAAAC
ACACA	TGGTCTGGCCTTACACATGA	TGCTGGAGAGGCTACAGTGA
SCD	ACAATTCCCGACGTGGCTT	GGCATAACGGAATAAGGTGGC

Abbreviations: ACACA, acetyl-CoA carboxylase α; ACTB, β-actin; FASN, fatty acid synthase; SCD, stearoyl-CoA desaturase; SREBF1, sterol regulatory element binding factor 1

time. Sections were then incubated with goat anti-rabbit secondary antibody conjugated with Alexa Fluor 555 (Invitrogen) diluted 1:500 in PBS at room temperature in the dark. After 1-h incubation, sections were washed 3 times for 5 min each in PBS, stained with 4,6-diamidino-2-phenylindole (Invitrogen) diluted 1:10,000 in PBS for 5 min, and then washed twice in PBS for 15 min. Finally, slides were covered with a coverslip, and immunofluorescence was observed with a Zeiss (Thornwood, NY, USA) LSM 510 META Laser Scanning Microscope.

2.6. Statistical analysis

Statistical analyses were performed with JMP software (SAS Institute, Cary, NC, USA). The means of different hormone treatments were compared with ANOVA and the Tukey-Kramer honestly significant difference method was used for comparing individual means. For both studies (mammary explants and cell culture), hormone treatment was included in the model as fixed effect. For the explant study, the cow effect was used as block.

Before our in vivo experiment, we ran power calculations with the use of gene expression data from our previous, similar experiments. On the basis of those data, and using a 2-tailed *t*-test and $P < 0.05$, we calculated that we would have 80% to 90% power to detect a 1.5-fold difference in GLUT expression between treatment groups with 3 to 4 per group.

3. Results

3.1. Lactogenic hormones stimulate β-casein and α-lactalbumin gene expression in bovine mammary explants

Mammary explants were treated with or without Ins, IGF-I, InsHPrl, or InsHPrlE for 48, 72, and 96 h. Expression of β-casein mRNA in cultured explants, as well as in fresh tissue (FT), was measured by quantitative RT-PCR. Compared with FT, β-casein expression in explants exposed to the Ctrl, IGF-I, or Ins treatment decreased sharply ($P < 0.001$), whereas the InsHPrl and InsHPrlE treatments increased the expression of β-casein ($P < 0.05$) (Fig. 1A). In addition, β-casein expression did not differ between the InsHPrl and InsHPrlE treatments. We also measured α-lactalbumin expression in explants as an indicator of lactose synthesis. As shown in Fig. 1B, expression of α-lactalbumin in the InsHPrl and InsHPrlE treatments was 10-to several hundred-fold higher than that in the Ctrl, IGF-I, and Ins groups ($P < 0.01$). However, α-lactalbumin expression in explants was lower than that in FT ($P < 0.05$).

3.2. Lactogenic hormones regulate lipogenic gene expression in bovine mammary explants

In addition to milk protein and lactose synthesis, lactogenesis also involves induction of milk lipid synthesis, so we also investigated the regulation of SREBF1, FASN, ACACA, and SCD gene expression by lactogenic hormones in bovine mammary explants (Fig. 2). At 48 h, no difference was observed in SREBF1 mRNA expression among the Ctrl, Ins, and InsHPrl treatments. However, the InsHPrl treatment increased SREBF1 expression 2-fold ($P < 0.001$) at 96 h compared with Ctrl, whereas Ins had no effect (Fig. 2A). The expression of FASN in explants treated with InsHPrl was about 4 times higher than that of Ctrl explants throughout the experiment ($P < 0.001$). Regardless of treatment, expression of FASN in all cultured explants was lower than that in FT ($P < 0.001$; Fig. 2B). Incubation in InsHPrl increased the expression of ACACA relative to that of the Ctrl group throughout the experiment ($P < 0.001$), whereas Ins alone had no effect (Fig. 2C). However, Ins alone supported an increase of approximately 3.5-fold in SCD expression, relative to Ctrl ($P < 0.001$ at 48 and 72 h; $P < 0.05$ at 96 h; Fig. 2D). Incubation in the InsHPrl group further increased SCD expression by 5.7-fold (48 h), 7.4-fold (72 h), and 7.9-fold (96 h)

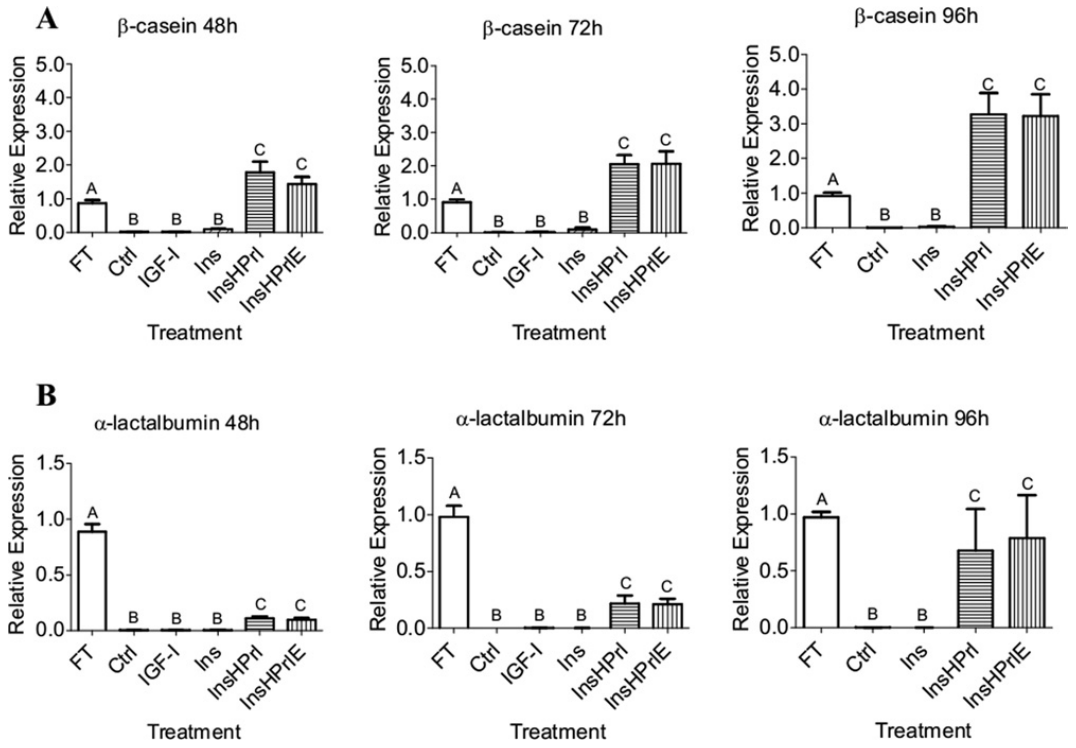

Fig. 1. Expression of mRNA for β-casein (panel A) and α-lactalbumin (panel B) in bovine mammary explants cultured in the presence of various hormones, including no hormone (Ctrl), IGF-I, insulin (Ins), insulin + hydrocortisone + prolactin (InsHPrl), and insulin + hydrocortisone + prolactin + 17β-estradiol (InsHPrlE) for 48, 72, or 96 h. Abundance of β-casein and α-lactalbumin mRNA transcripts was compared with that of fresh tissue (FT) and normalized to β-actin expression (n = 4). Different superscripts represent $P < 0.05$.

compared with Ctrl, at the same times ($P < 0.01$). Expression of SCD also differed between the Ins and InsHPrl treatments ($P < 0.01$).

3.3. Effects of lactogenic hormones on GLUT expression in bovine mammary explants

We next investigated the effect of lactogenic hormones on the expression of GLUT1, GLUT8, and GLUT12 in these explants (Fig. 3). At 48 and 96 h, GLUT1 mRNA expression was 50% to 80% higher in Ins alone relative to Ctrl ($P < 0.05$), but other treatments had no effect on GLUT1 expression (Fig. 3A). At 72 h, although all hormone treatments increased GLUT1 expression compared with Ctrl ($P < 0.05$), no difference was observed among the hormone treatments. None of the hormone treatments affected GLUT8 mRNA levels (Fig. 3B), nor did they affect GLUT12 expression at 48 h. But in explants incubated in InsHPrl or InsHPrlE, GLUT12 expression was 50% lower at 72 h and 96 h ($P < 0.05$; Fig. 3C). Compared with FT, explants from all treatments had higher GLUT1 expression and lower GLUT8 and GLUT12 expressions ($P < 0.05$). These results indicate that lactogenic hormones and IGF-I apparently do not stimulate expression of GLUT1, GLUT8, and GLUT12 in the bovine mammary gland.

3.4. Effect of lactogenic hormones on GLUT1 localization in bovine mammary explants

The subcellular localization of GLUT1 in the bovine mammary explants was examined by immunofluorescence. As shown in Fig. 4, GLUT1 was mostly localized to the plasma membrane of epithelial cells in all treatments, and no apparent difference was observed in subcellular localization of GLUT1. Explants did not exhibit fluorescence above background when normal (nonimmune) rabbit IgG replaced the first antibody nor when either the first or second antibody was omitted (data not shown).

3.5. Effects of lactogenic hormones on GLUT expression in bovine primary mammary cells and mouse mammary epithelial cell line HC11 cells

To verify the results obtained from explants, we also cultured BMECs on collagen gel with various hormone treatments (Fig. 5). Relative to Ins, expression of β-ca-

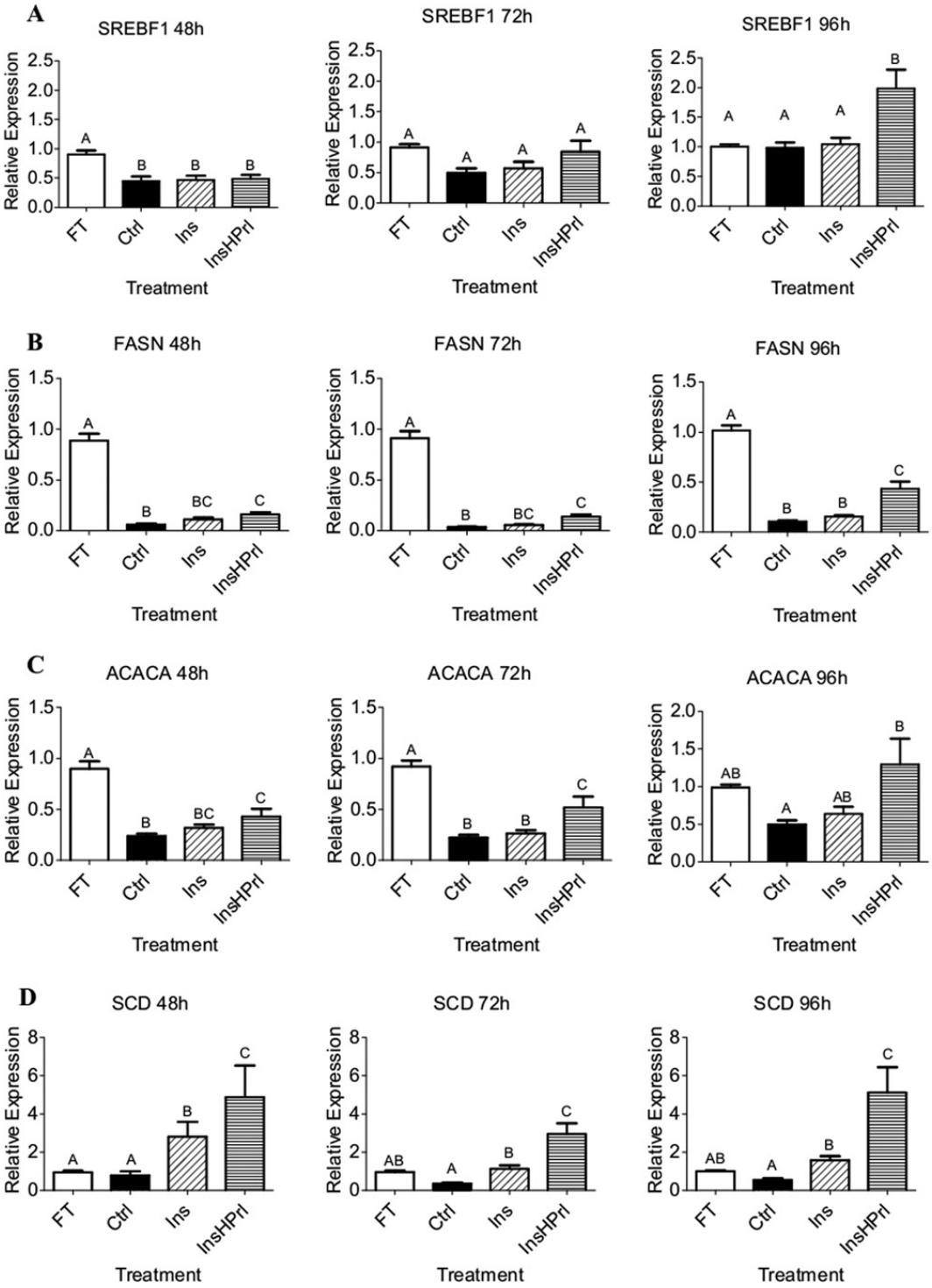

Fig. 2. Expression of mRNA for sterol regulatory element binding protein 1 (SREBF1; panel A), fatty acid synthase (FASN; panel B), acetyl-CoA carboxylase α (ACACA; panel C), and stearoyl-CoA desaturase (SCD; panel D) in bovine mammary explants cultured in the presence of no hormone (Ctrl), insulin (Ins), or insulin + hydrocortisone + prolactin (InsHPrl) for 48, 72, or 96 h. The mRNA abundance of target genes was compared with that of fresh tissue (FT and normalized to β-actin expression (n = 4). Different superscripts represent $P < 0.05$.

sein mRNA in these cells was increased 20-fold by the InsHPrl treatment for 48 h ($P < 0.01$); however, no effect was observed of InsHPrl on expression of GLUT1, GLUT8, or GLUT12 mRNA.

We further examined the effects of these hormones on the HC11 mouse mammary epithelial cell line (Fig. 6). Similar to the explants and the primary mammary epithelial cells, the lactogenic complex, InsHPrl, in-

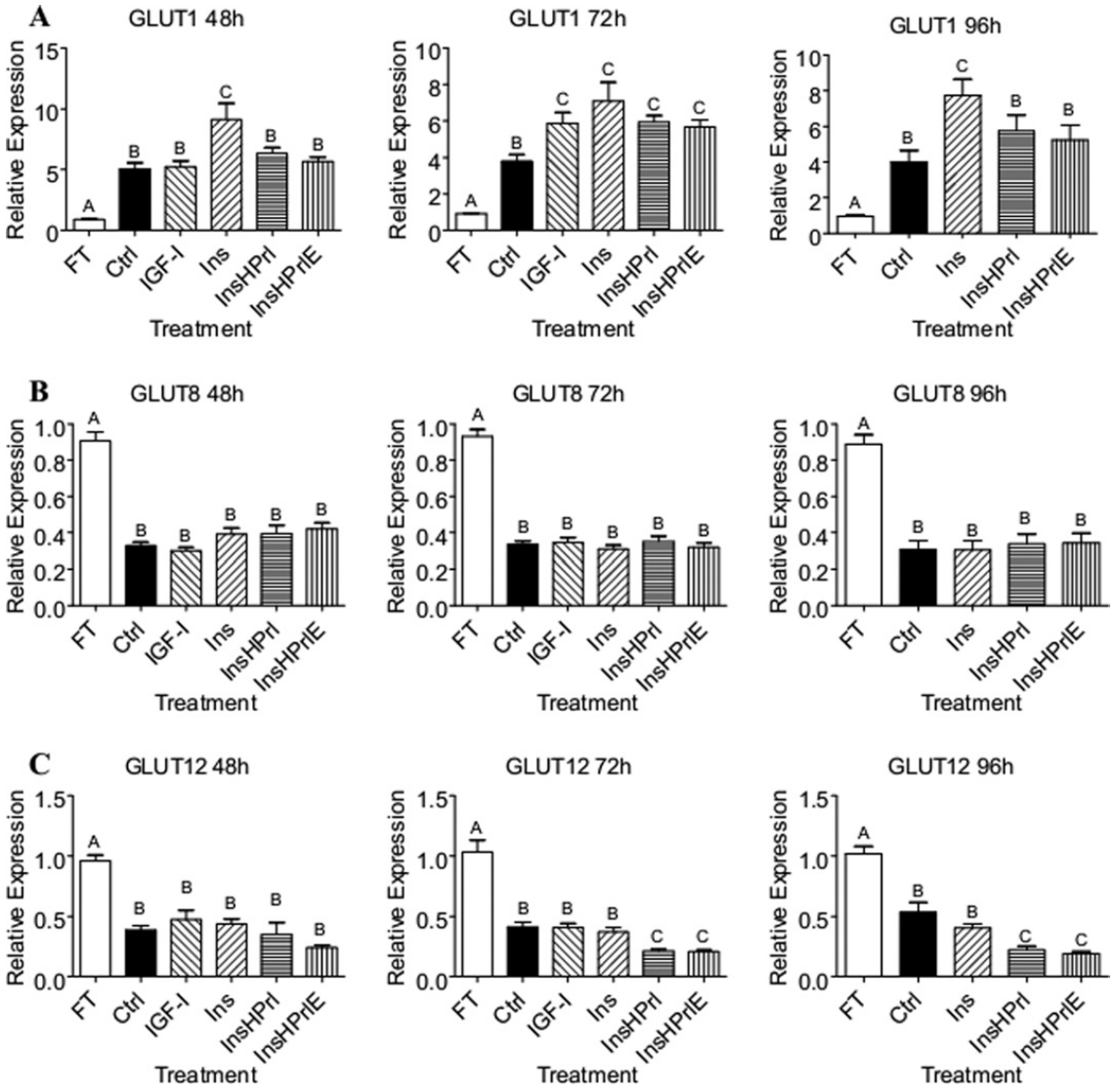

Fig. 3. Expression of mRNA for GLUT1 (panel A), GLUT8 (panel B), and GLUT12 (panel C) in bovine mammary explants cultured in the presence of different hormones, including no hormone (Ctrl), IGF-I, insulin (Ins), insulin + hydrocortisone +prolactin (InsHPrl), and insulin + hydrocortisone + prolactin + 17β-estradiol (InsHPrlE) for 48, 72, or 96 h. Abundance of GLUT mRNAs was compared with that of fresh tissue (FT) and was normalized to β-actin expression (n = 4). Different superscripts represent $P < 0.05$.

duced β-casein mRNA more than 100-fold ($P < 0.01$) but had no effect on expression of GLUT1, GLUT8, or GLUT12 mRNA (Fig. 6).

3.6. Effect of Prl administration in vivo on GLUT expression in bovine mammary gland

To examine possible effects of Prl on GLUT expression in the mammary gland, in vivo, 5 cows in early lactation were treated with Prl, and mRNA levels of GLUT1, GLUT8, and GLUT12 in the mammary gland were measured. Although Prl injections resulted in increased milk yield and a 2-fold increase in α-lactalbumin mRNA expression in the mammary gland [23], no difference was observed in expression of GLUT mRNAs between Prl-treated and nontreated cows (Fig. 7).

4. Discussion

Lactogenesis in the mammary gland is stimulated by the lactogenic hormones, mainly Prl [12]. Bovine explant culture has been used extensively for in vitro studies of functional differentiation of the mammary gland and hormonal induction of milk synthesis because there are few, if any, bovine mammary epithelial cell lines that are capable of undergoing hormonal induction of milk gene expression in vitro [25,26]. In those studies, it was clearly established that expression

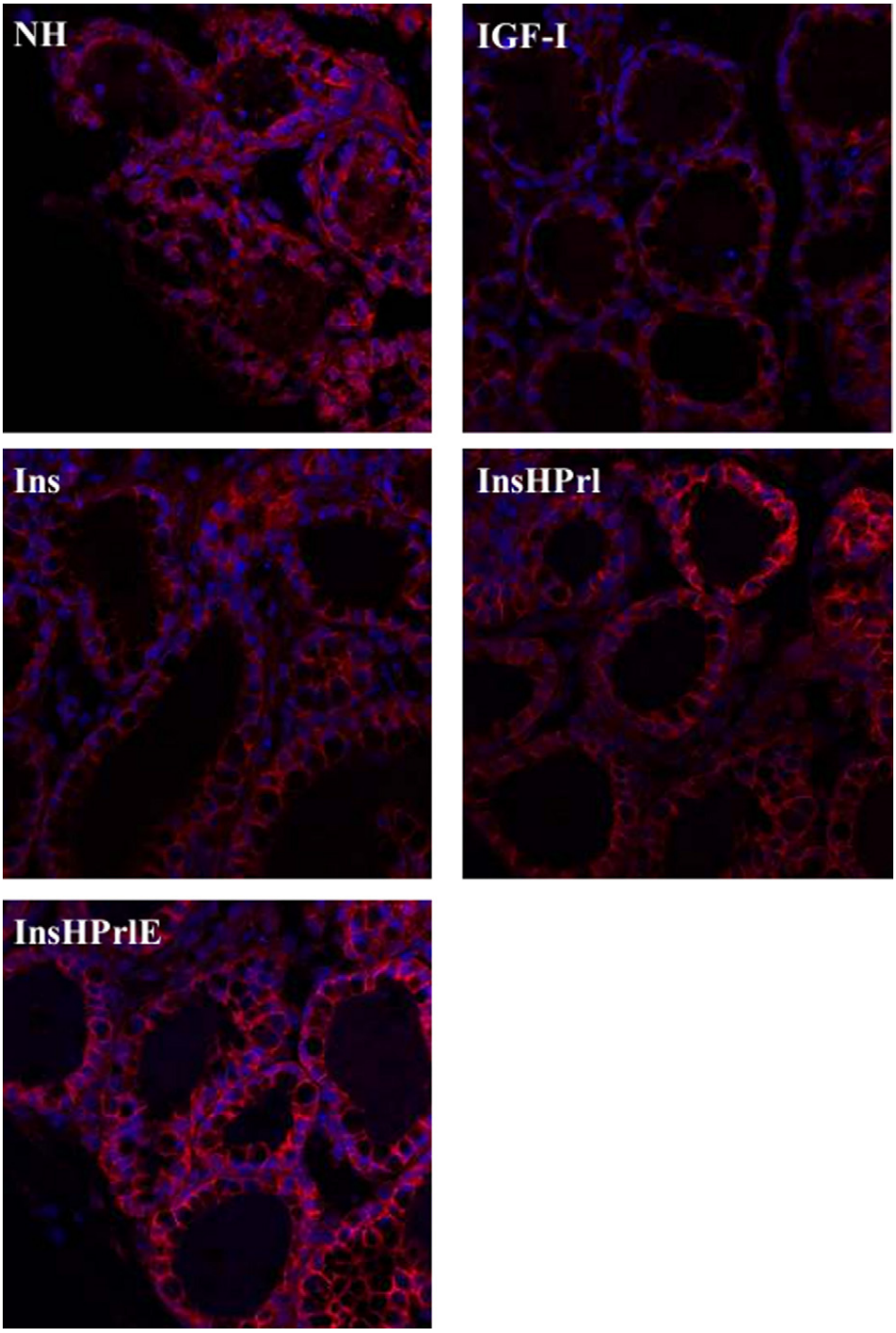

Fig. 4. Immunofluorescent staining (red) of GLUT1 subcellular localization in bovine mammary explants cultured in the presence of different hormones, including no hormone (Ctrl), IGF-I, insulin (Ins), insulin + hydrocortisone + prolactin (InsHPrl), and insulin + hydrocortisone + prolactin + 17β-estradiol (InsHPrlE) for 48 h. Nuclei of epithelial cells were stained with 4,6-diamidino-2-phenylindole (blue).

of β-casein and α-lactalbumin mRNA can be induced by the lactogenic complex of Ins, Prl, and GCs [25,26] and that Prl-induced secretion of α-lactalbumin can be markedly enhanced by addition of Es [11]. These results were verified in our explant cultures, except that 17β-estradiol did not enhance the induction of milk protein gene expression stimulated by the lactogenic complex in our study. Reasons for this inconsistency are unknown, but Barrington et al [27] reported similar findings.

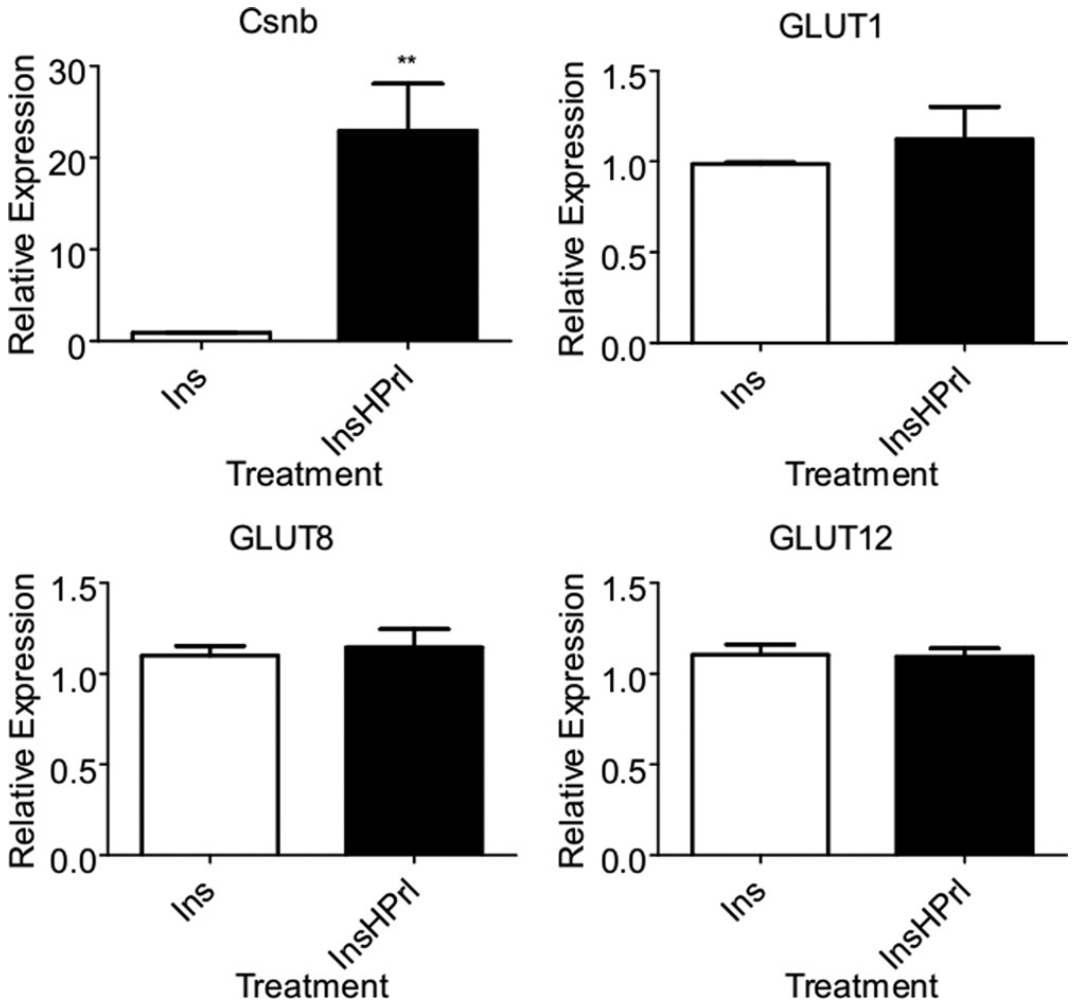

Fig. 5. Expression of mRNA for β-casein and glucose transporters in bovine primary mammary epithelial cells. Cells were treated with insulin (Ins) or insulin + hydrocortisone + prolactin (InsHPrl) for 48 h. Gene expression of the InsHPrl group was compared with that of the Ins treatment and was normalized to β-actin expression (n = 3). $^{**}P < 0.01$.

In addition to the milk protein genes, a set of genes encoding enzymes involved in milk lipid synthesis pathways are also under tight regulation in the mammary gland. The mRNA levels of several key enzymes in fatty acid synthesis were increased at secretory activation in both mouse [28] and cow [9]. We hypothesized that upregulation of lipogenic genes during this time is also mediated by lactogenic hormones. Indeed, SREBF1, FASN, ACACA, and SCD were upregulated by lactogenic hormones in our explant cultures, supporting our hypothesis. In hepatocytes and adipocytes, Ins promotes lipid synthesis by upregulating SREBF1, FASN, and ACACA as one of the mechanisms for maintaining blood glucose homeostasis [29]. However, in our mammary explant cultures, Ins alone had no effect on expression of these 3 genes. This may indicate tissue specificity in response to Ins. However, Ins alone stimulated SCD expression in our explant cultures, and the addition of Prl and hydrocortisone further increased its expression, indicating that SCD expression is regulated by mechanisms that differ from those controlling expression of SREBF1, FASN, and ACACA. In addition, among the lipogenic genes assayed in our explants, SCD had the strongest response to lactogenic hormones. This is consistent with the findings of Bionaz and Loor [9] who examined the expression of 45 genes associated with lipid synthesis and secretion in the bovine mammary gland from late pregnancy to lactation and reported that SCD was one of the most abundantly expressed genes during lactation and was upregulated more than 40-fold from late pregnancy to peak lactation.

During the transition from late pregnancy to early lactation the mammary gland undergoes dramatic functional differentiation, leading to the onset of lactation. This is accompanied by a markedly increased demand for glucose by the mammary gland. This demand is met mainly by increased expression of GLUTs. The mRNA

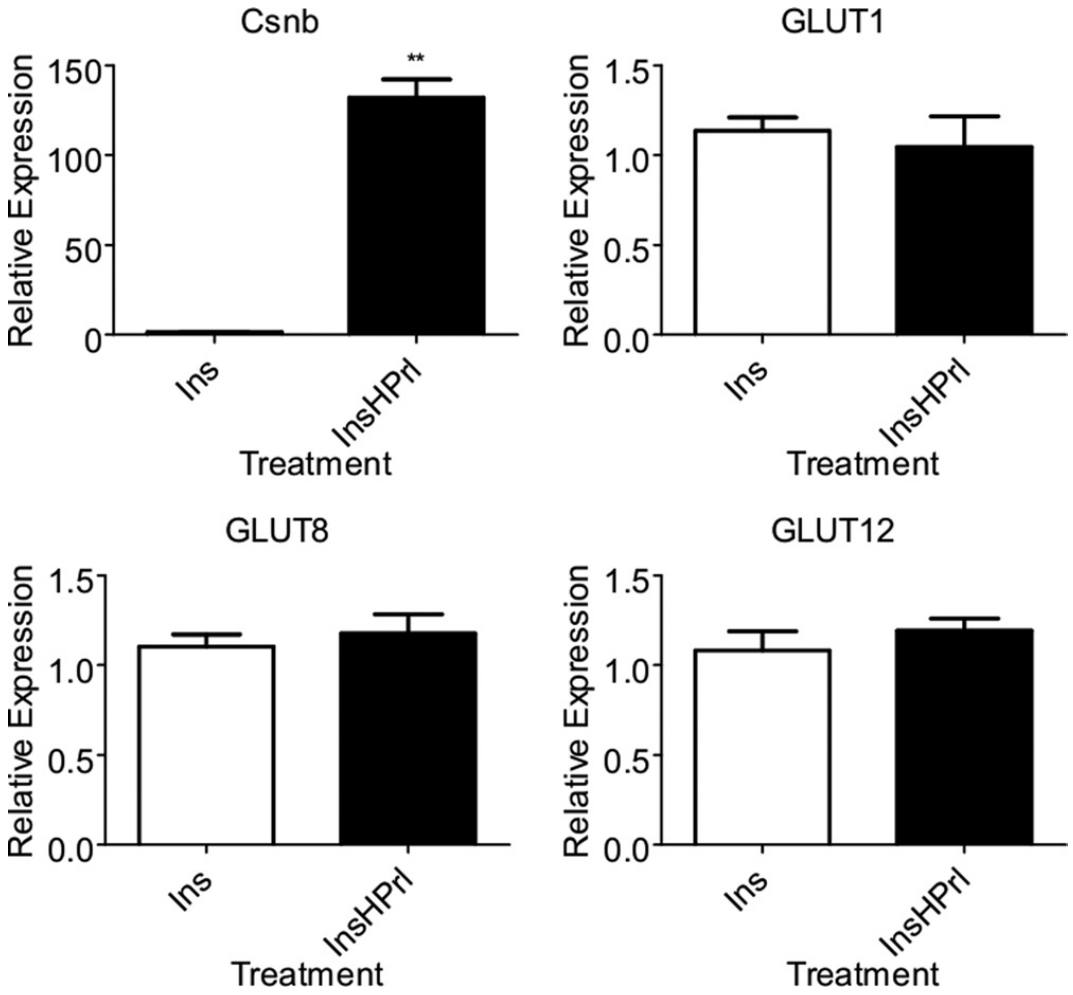

Fig. 6. Expression of mRNA for β-casein and glucose transporters in cells of the HC11 mouse mammary epithelial cell line. Cells were treated with insulin (Ins) or insulin + hydrocortisone + prolactin (InsHPrl) for 48 h. Gene expression of the InsHPrl group was compared with that of the Ins treatment and was normalized to β-actin expression (n = 3). $^{**}P < 0.01$.

levels of GLUT1, GLUT8, and GLUT12 increase by more than 100-fold, 10-fold, and 10-fold, respectively, in bovine mammary gland from 40 d before lactation to 7 d of lactation [1]. We hypothesized that this increased GLUT expression was stimulated by lactogenic hormones because expression of GLUT genes has similar patterns to the expression of milk protein genes. In addition, in CIT3 mouse mammary epithelial cells, treatment with Prl, hydrocortisone, and Ins caused a 15-fold induction of GLUT1 [30]. Three mouse models

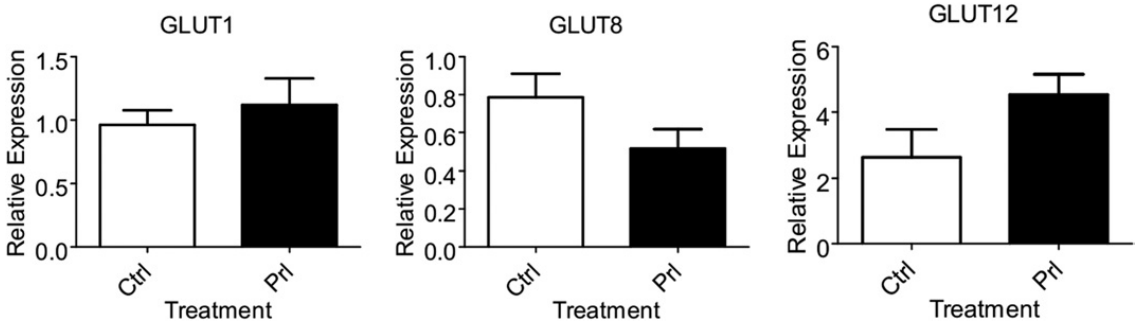

Fig. 7. Expression of mRNA for glucose transporters in early lactating cows injected intravenously with bovine prolactin twice daily for 7 d. Gene expression of prolactin-treated cows was compared with that of control cows and was normalized to β-actin expression (n = 5).

with disrupted Prl signaling pathways exhibited a failure of secretory activation and decreased expression of GLUT1 [13]. In a similar vein, use of bromocriptine to inhibit Prl secretion in lactating rats caused a 37% decrease in GLUT1 expression. A more profound inhibition (up to 90% decrease) was observed in response to treatment with bromocriptine plus a growth hormone-neutralizing antibody [14]. Contrary to these previous findings and our hypothesis, our study showed that lactogenic hormones did not stimulate the expression of GLUT1, GLUT8, or GLUT12 in bovine mammary explants, BMECs, or in the mouse HC11 cell line. Furthermore, GLUT expression was not affected in early lactating cows that were treated with Prl intravenously, even though expression of the milk protein gene, α-lactalbumin, was induced [23]. Thus, our data suggest that the lactogenic complex does not mediate the marked upregulation in expression of GLUTs in the bovine mammary gland during the onset of lactation. Reasons for the discrepancy between our results and those of previous studies in rodents [13,14,30] are not clear. It may reflect species differences between cattle and rodents, but expression of GLUTs was also not affected by the lactogenic hormones in the mouse mammary epithelial cell line HC11 in this study. It is possible that the lactogenic hormones do not regulate GLUT1 expression directly. For example, Prl is essential for mammary secretory activation and lactogenesis, and, if Prl secretion is disrupted, the mammary gland may display a lower than normal metabolic rate, which could, in turn, reduce GLUT1 expression indirectly. Along these lines, one of the main challenges in studying regulation of lactogenesis and milk secretion has been the universal inability to elicit lactose synthesis by in vitro mammary model systems, despite clear induction of the A and B components of the lactose synthase enzyme. Thus, it may be that the failure to induce lactose synthesis uncouples the expected demand for glucose and so increased GLUT expression is not recruited. Our data also present a novel possible explanation for the failure to induce lactose synthesis in vitro; perhaps the inability to recruit heightened GLUT expression deprives the biosynthetic machinery of the sole substrate required for lactose synthesis. In addition, we cannot rule out the possibility that the lactogenic hormones may induce GLUT1 expression in the mammary gland specifically in lactogenesis stage 2 because the main increase of GLUT expression in bovine mammary gland occurs around parturition [1], and the explant tissues used in our study were taken from pregnant animals about 1 mo before parturition.

Transcriptional regulation of GLUT1 gene expression has been studied in some detail because of its role in cancer development and the response of cells to stress. Increased GLUT1 expression is elicited by a variety of factors and stressful stimuli, including hypoxia, glucose deprivation, hyperosmolarity, growth factors, and transformation and inhibition of oxidative phosphorylation [31–36]. Of all of these factors, hypoxia is of most interest. It is well established that GLUT1 expression is increased in many cell types in response to hypoxia [37]. Mammary consumption of oxygen increases steadily during late pregnancy and peaks during lactation [38]. This may well result in a chronic local hypoxia. In addition, it has been shown that mice in which the hypoxia-inducible factor 1α has been deleted from the mammary gland exhibit impaired mammary differentiation and lipid secretion, striking changes in milk composition, and failure to lactate [39]. Thus, hypoxia may also be an important regulator of GLUT1 expression in the mammary gland.

Progesterone is another hormone that may regulate GLUT expression in the mammary gland during the transition period. Concentrations of progesterone in blood remain high throughout pregnancy but drop dramatically shortly before parturition [11]. High concentrations of progesterone may inhibit expression of GLUTs, and progesterone withdrawal prepartum may be permissive for induction of GLUT expression in the mammary gland. However, others have reported that progesterone upregulates GLUT1 expression in Ishikawa endometrial cancer cells [40] and primary murine and human endometrial stromal cells [41]. Therefore, the role of progesterone in regulating GLUT expression in the mammary gland needs to be investigated.

Little research has been done on the regulation of GLUT8 and GLUT12 expression, and, to our knowledge, this is the first report on the regulation of GLUT8 and GLUT12 expression by lactogenic hormones. Each GLUT isoform serves specific physiological functions, and each is regulated by distinct mechanisms. It is not surprising that different factors may be responsible for upregulating expression of individual GLUTs in the mammary gland. This is supported by our observations that, although Ins alone increased GLUT1 expression in bovine mammary explants, it had no effect on GLUT8 or GLUT12 expression. Likewise, exposure to the lactogenic complex of hormones reduced expression of GLUT12, but not GLUT1 or GLUT8, in bovine mammary explants.

We conclude that IGF-I, 17β-estradiol, and lactogenic hormones do not elicit the upregulation of GLUTs in bovine mammary epithelial cells during the transition period from late pregnancy to early lactation. Hypoxia, or other factors, may play a role, but need further investigation.

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References

[1] Zhao F-Q, Keating AF. Expression and regulation of glucose transporters in the bovine mammary gland. J Dairy Sci 2007; 90(suppl 1):E76–E86.

[2] Zhao F-Q, Keating AF. Functional properties and genomics of glucose transporters. Curr Genomics 2007;8:113–28.

[3] Miller PJ, Finucane KA, Hughes M, Zhao F-Q. Cloning and expression of bovine glucose transporter GLUT12. Mamm Genome 2005;16:873–83.

[4] Zhao F-Q, Glimm DR, Kennelly JJ. Distribution of mammalian facilitative glucose transporter messenger RNA in bovine tissues. Int J Biochem 1993;25:1897–903.

[5] Zhao F-Q, Miller PJ, Wall EH, Zheng YC, Dong B, Neville MC, et al. Bovine glucose transporter GLUT8: cloning, expression, and developmental regulation in mammary gland. Biochim Biophys Acta 2004;1680:103–13.

[6] Prosser CG, Topper YJ. Changes in the rate of carrier-mediated glucose transport by mouse mammary epithelial cells during ontogeny: hormone dependence delineated *in vitro*. Endocrinology 1986;119:91–6.

[7] Threadgold LC, Kuhn NJ. Monosaccharide transport in the mammary gland of the intact lactating rat. Biochem J 1984;218: 213–9.

[8] Eberlé D, Hegarty B, Bossard P, Ferré P, Foufelle F. SREBP transcription factors: master regulators of lipid homeostasis. Biochimie 2004;86:839–48.

[9] Bionaz M, Loor JJ. Gene networks driving bovine milk fat synthesis during the lactation cycle. BMC Genomics 2008;9: 366.

[10] Rudolph MC, Monks J, Burns V, Phistry M, Marians R, Foote MR, et al. Sterol regulatory element binding protein and dietary lipid regulation of fatty acid synthesis in the mammary epithelium. Am J Physiol Endocrinol Metab 2010;299:E918–27.

[11] Akers RM. Lactation and the Mammary Gland Ames, IA: Iowa State Press; 2002.

[12] Neville MC, McFadden TB, Forsyth I. Hormonal regulation of mammary differentiation and milk secretion. J Mammary Gland Biol Neoplasia 2002;7:49–66.

[13] Naylor MJ, Oakes SR, Gardiner-Garden M, et al. Transcriptional changes underlying the secretory activation phase of mammary gland development. Mol Endocrinol 2005;19: 1868–83.

[14] Fawcett HA, Baldwin SA, Flint DJ. Hormonal regulation of the glucose transporter GLUTI in the lactating rat mammary gland. Biochem Soc Trans 1992;20:17S.

[15] Wall EH, Auchtung TL, Dahl GE, Ellis SE, McFadden TB. Exposure to short day photoperiod during the dry period enhances mammary growth in dairy cows. J Dairy Sci 2005;88: 1994–2003.

[16] Ginsburg E, Vonderhaar BK. Whole organ culture of the mouse mammary gland. In: Ip MM, Asch BB, editors. Methods in Mammary Gland Biology and Breast Cancer Research. New York: Kluwer Academic/Plenum Press; 2000, p. 147–54.

[17] Cohick WS, Wang B, Verma P, Boisclair YR. Insulin-like growth factor I (IGF-I) and cyclic adenosine 3',5'-monophosphate regulate IGF-binding protein-3 gene expression by transcriptional and posttranscriptional mechanisms in mammary epithelial cells. Endocrinology 2000;141:4583–91.

[18] Djiane J, Delouis C, Denamur R. Lactogenesis in organ cultures of heifer mammary tissue. J Endocrinol 1975;65:453–4.

[19] Collier RJ, Bauman DE, Hays RL. Lactogenesis in explant cultures of mammary tissue from pregnant cows. Endocrinology 1977;100:1192–200.

[20] Nickerson SC, Heald CW, Bibb TL, McGilliard ML. Cytological effects of hormones and plasma on bovine mammary tissue in vitro. J Endocrinol 1978;79:363–8.

[21] Wellnitz O, Kerr DE. Cryopreserved bovine mammary cells to model epithelial response to infection. Vet Immunol Immunopathol 2004;101:191–202.

[22] Imagawa W, Yang J, Guzman RC, Nandi S. Collagen gel method for the primary culture of mouse mammary epithelium. In: Ip MM, Asch BB, editors. Methods in Mammary Gland Biology and Breast Cancer Research. New York: Kluwer Academic/Plenum Press; 2000, p. 111–23.

[23] Wall EH, Crawford HM, Ellis SE, Dahl GE, McFadden TB. Mammary response to exogenous prolactin or frequent milking during early lactation in dairy cows. J Dairy Sci 2006;89: 4640–8.

[24] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta delta C(T)) method. Methods 2001;25:402–8.

[25] Gertler A, Weil A, Cohen N. Hormonal control of casein synthesis in organ culture of the bovine lactating mammary gland. J Dairy Res 1982;49:387–98.

[26] Goodman GT, Akers RM, Friderici KH, Tucker HA. Hormonal regulation of alpha-lactalbumin secretion from bovine mammary tissue cultured in vitro. Endocrinology 1983;112:1324–30.

[27] Barrington GM, Besser TE, Gay CC, Davis WC, Reeves JJ, McFadden TB. Effect of prolactin on *in vitro* expression of the bovine mammary immunoglobulin G1 receptor. J Dairy Sci 1997;80:94–100.

[28] Anderson SM, Rudolph MC, McManaman JL, Neville MC. Key stages in mammary gland development. Secretory activation in the mammary gland: it's not just about milk protein synthesis! Breast Cancer Res 2007;9:204.
[29] Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 2001;414:799–806.
[30] Haney PM. Localization of the GLUT1 glucose transporter to brefeldin A-sensitive vesicles of differentiated CIT3 mouse mammary epithelial cells. Cell Biol Int 2001;25:277–88.
[31] Behrooz A, Ismail-Beigi F. Dual control of GLUT1 glucose transporter gene expression by hypoxia and by inhibition of oxidative phosphorylation. J Biol Chem 1997;272:5555–62.
[32] Boado RJ, Pardridge WM. Glucose deprivation and hypoxia increase the expression of the GLUT1 glucose transporter via a specific mRNA cis-acting regulatory element. J Neurochem 2002;80:552–4.
[33] Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of GLUT1 mRNA by hypoxia-inducible factor-1. Interaction between h-RAS and hypoxia. J Biol Chem 2001;276:9519–25.
[34] Hwang DY, Ismail-Beigi F. Stimulation of GLUT-1 glucose transporter expression in response to hyperosmolarity. Am J Physiol Cell Physiol 2001;281:C1365–72.
[35] Murakami T, Nishiyama T, Shirotani T, Shinohara Y, Kan M, Ishii K, et al. Identification of two enhancer elements in the gene encoding the type 1 glucose transporter from the mouse which are responsive to serum, growth factor, and oncogenes. J Biol Chem 1992;267:9300–6.
[36] Sánchez-Feutrie M, Viñals F, Palacín M, Zorzano A. Identification of a novel proliferation-dependent c-rich element that mediates inhibition of the rat GLUT1 promoter. Gene 2003; 322:47–55.
[37] Cassavaugh J, Lounsbury KM. Hypoxia-mediated biological control. J Cell Biochem 2011;112:735–44.
[38] Reynolds M. Mammary respiration in lactating goats. Am J Physiol 1967;212:707–10.
[39] Seagroves TN, Hadsell D, McManaman J, Palmer C, Liao D, McNulty W, et al. HIF1alpha is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. Development 2003;130:1713–24.
[40] Medina RA, Meneses AM, Vera JC, Gúzman C, Nualart F, Rodriguez F, et al. Differential regulation of glucose transporter expression by estrogen and progesterone in Ishikawa endometrial cancer cells. J Endocrinol 2004;182:467–78.
[41] Frolova A, Flessner L, Chi M, Kim ST, Foyouzi-Yousefi N, Moley KH. Facilitative glucose transporter type 1 is differentially regulated by progesterone and estrogen in murine and human endometrial stromal cells. Endocrinology 2009;150: 1512–20.