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SHORT COMMUNICATION

Insulin, a key regulator of hormone responsive milk protein synthesis during lactogenesis in murine mammary explants

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Abstract Murine milk protein gene expression requires insulin, hydrocortisone, and prolactin; however, the role of insulin is not well understood. This study, therefore, examined the requirement of insulin for milk protein synthesis. Mammary explants were cultured in various combinations of the lactogenic hormones and global changes in gene expression analysed using Affymetrix microarray. The expression of 164 genes was responsive to insulin, and 18 were involved in protein synthesis at the level of transcription and posttranscription, as well as amino acid uptake and metabolism. The folate receptor gene was increased by fivefold, highlighting a potentially important role for the hormone in folate metabolism, a process that is emerging to be central for protein synthesis. Interestingly, gene expression of two milk protein transcription factors, Stat5a and Elf5, previously identified as key components of prolactin signalling, both showed an essential requirement for insulin. Subsequent experiments in HCll cells confirmed that Stat5a and Elf5 gene expression could be induced in the absence of prolactin but in the presence of insulin. Whereas prolactin plays an essential role in phosphorylating and activating Stat5a, gene expression is only induced when insulin is present. This indicates insulin plays a crucial role in the transcription of the milk protein genes.

Keywords Elf5 · Stat5a · Insulin · Milk protein · Casein · Mammary gland

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Introduction

The lactating mammary gland is emerging as a specific target tissue of insulin action, in particular as a regulator of milk protein synthesis (Bolander et al. 1981; Choi et al. 2004; Chomczynski et al. 1984; Griinari et al. 1997; Kulski et al. 1983; Mackle et al. 1999, 2000; McGuire et al. 1995; Menzies et al. 2009b; Oka 1974). In preparation for lactation, the rodent mammary gland becomes sensitive to insulin at the onset of pregnancy and develops increased insulin sensitivity during late pregnancy due to an augmented kinase activity of the insulin receptor (Carrascosa et al. 1998).

Lactation involves the transition of the mammary gland to synthesise and secrete milk proteins and occurs in two distinguishable phases: secretory differentiation, followed by secretory activation. Secretory differentiation begins at midpregnancy, marked by expression of the milk protein genes (Neville et al. 2002). Activation of secretion occurs

around parturition and is characterised by further increase in milk protein gene expression and the formation of tight junctions between the alveoli cells to facilitate vectoral secretion of the milk constituents, including protein, into the alveoli lumen (Brisken and Rajaram 2006; Muller and Neville 2001; Neville et al. 2002). This transition is coordinated by the complex interplay of a milieu of hormones that includes insulin. The mammary explant culture model has been used to mimic the lactogenic process and examine the endocrine control of milk protein gene expression.

Mammary explant culture studies in the mouse and rat have shown there is an absolute requirement for insulin, hydrocortisone, and prolactin for the induction of casein gene expression (Bolander et al. 1981; Kulski et al. 1983; Nagaiah et al. 1981). Studies to date in the mouse have shown hydrocortisone and prolactin play an important role in both transcription of the casein gene and stabilisation of the messenger RNA (mRNA; Bolander et al. 1981; Choi et al. 2004; Kulski et al. 1983; Nagaiah et al. 1981). Historically, insulin was assumed to perform a permissive role of cell survival and maintenance in mammary culture models (Barnawell 1965; Rosen et al. 1980), however, mammary explant culture studies by Nicholas et al. (1983) showed insulin was essential for casein synthesis, and Chomczynski et al. (1984) showed the hormone to be essential for transcription of the β-casein gene, but not for stabilisation of the transcripts. These studies suggested that insulin clearly plays a role beyond cell survival and maintenance in the mammary explant culture model. More recent studies have shown that murine, bovine, and wallaby mammary explants can survive in the absence of insulin and any exogenous macromolecules (Brennan 2008; Brennan et al. 2008), supporting the concept that the primary role of insulin is regulating function of mammary epithelial cells.

Insulin has been shown to stimulate milk protein synthesis in murine mammary tissue in vitro (Wang and Amor 1971), and it is conceivable that insulin has a role in synthesis of the milk proteins and not simply expression of the milk protein genes. Choi and colleagues (2004) demonstrated that insulin and prolactin acted synergistically to enhance translation of the β-casein mRNA. Specifically, insulin enhances translation of β-casein by increasing initiation of translation and lengthening the poly(A) on mRNA by the cytoplasmic polyadenylation element binding protein (Choi et al. 2004). Recent studies in the cow have suggested a posttranscriptional role for insulin in milk protein synthesis via folate metabolism (Menzies et al. 2009b). It is plausible that insulin may also regulate folate in the murine mammary gland and thereby playing a posttranscriptional role in synthesis of the milk proteins.

The current study has exploited the mammary explant culture model to investigate the direct role/s of insulin in milk protein synthesis at the transcription level in the murine mammary gland. The use of Affymetrix microarray has allowed a global assessment of mammary gene in mammary explants and offers potential insight into the molecular mechanisms underlying the insulin-stimulated milk protein synthesis.

Materials and methods

Mice

Mice (CV40) were obtained from the Howard Flory Institute of The University of Melbourne, Parkville, Melbourne. Mice were maintained in the Zoology Department animal house, with feed and water provided ad libitum. Day 1 of pregnancy was identified as the first day a postcoital plug was observed. Mice were euthanased on day 12 of pregnancy, and their inguinal and abdominal mammary glands were excised under sterile conditions.

Tissue culture

Mammary gland explants from midpregnant mice were prepared and cultured in Medium 199 with Earle's salts (Gibco BRL Life technologies, Invitrogen, Melbourne, Australia) as described previously (Nicholas and Tyndale-Biscoe 1985), except that 10% foetal calf serum (FCS) was added, and bovine serum albumin was excluded from the media. Briefly, explants were incubated at 37°C and 5% CO_2 in 5 ml of media per well in six-well plates, and the media were changed every third day. Hormones were added at the following concentrations in the indicated combinations: bovine insulin (I; 100 ng/ml, Sigma, Sydney Australia), hydrocortisone (F; 50 ng/ml, Sigma), and ovine prolactin (P; 200 ng/ml, National Hormone and Pituitary Program USA). All explants were initially cultured in the absence of exogenous hormones (NH) for 8 days to allow effects of endogenous hormones and inflammatory response of the tissue to subside. At day 8, mammary explants were harvested for controls, and the remaining explants were cultured for 4 days in media containing FP or IFP. Mammary glands from a total of 12 mice were used in three separate culture experiments with mammary glands from four mice pooled for each culture experiment. In each experiment, three wells of explants were cultured for each hormone treatment and NH control. Explants were collected and stored at −80°C until RNA was extracted for microarray analysis.

Cell culture

HC11 cells, a mouse mammary epithelial cell line, were maintained in RPMI 1640 medium supplemented with 10%

FCS, 20 mM (HEPES) buffer solution, 6 mM L-glutamine, 5μg/ml I, 10 ng/ml epidermal growth factor (EGF, Sigma), 0.1125% $Na(CO_3)_2$, 50U/ml penicillin, and 50 g/ml streptomycin. For experiments involving P treatment, FCS was heat-inactivated by 30 min incubation at 50°C. All cell cultures were maintained in a 37°C, 5% CO_2 humidified incubator. HC11 cells were seeded in six-well plates at a density of 1×10^5 cells per well (day0) and allowed to grow to 70–80% confluence. On day3, the medium was changed and replaced with EGF free maintenance medium, and the concentration of I in the medium was adjusted (to 0, 0.05, 0.5, or 5 μg/ml). On day4, dexamethasone (dex; 0.5 μM, Sigma) and P (0 or 5 μg/ml) were added to cells to induce differentiation. The treatment was repeated daily until day8. Protein and total RNA extracts were isolated from the cells.

Microarray—hybridisation and analysis

Total RNA was extracted using an RNeasy Lipid Tissue Kit (Qiagen, Sydney, Australia) following manufacturers' instructions. The complementary DNA (cDNA) was synthesised from total RNA (20 μg) using Superscript II (Invitrogen). Synthesis of biotin-labelled complementary RNA was performed using BioArray High Yield RNA Transcript Labelling Kit (Enzo Diagnostics, USA). The cDNA probes were hybridised to Affymetrix MOE430 GeneChips overnight according to manufacturer's instructions. Three arrays were performed in total, each chip representing one treatment using pooled RNA from each of the three mammary explant culture experiments.

Initial analysis was performed using the Affymetrix GeneChip® Operating Software to assess array quality. Signal intensities of each gene were obtained using the robust multiarray average function of the Affy package in bioconductor (http://www.bioconductor.org). The cutoff threshold for gene expression intensity was 200, and differential gene expression was assessed by fold change. IFP-responsive genes were determined by identifying genes differentially expressed by at least twofold in explants cultured in IFP compared to explants cultured in NH. Insulin-responsive genes were determined by identifying genes that were either up- or downregulated at least twofold in mammary explants cultured in IFP compared to FP. The biological functions of each gene within the datasets was manually researched in the literature and PubMed database (http://www.ncbi.nlm.nih.gov) and then classified by cellular function.

Quantitative polymerase chain reaction

Total RNA was extracted from HC11 cells using TRIZOL Reagent (Invitrogen) according to the manufacturer's instructions and purified using Qiagen RNeasy Mini Spin columns. Triplicate RNA samples were assessed for quality and quantity using the 2100 Bioanalyzer (Agilent, Forrest Hill, Australia). Synthesis of cDNA from HC11 cells was performed using SuperscriptTM II Reverse Transcriptase (Invitrogen). Quantitative polymerase chain reaction (PCR) reactions were performed in triplicate using the TaqMan probe-based system (Elf5 Mm00468732_m1, Stat5a Mm00839861_ml, β-casein Mm00839664_m1, and Wap Mm00839913_m1) on the ABI 7900HT, and relative quantification of the product was performed by comparison to an internal control (mHPRT Mm00446968_m1). Significant changes in gene expression were assessed by unpaired, two-tailed *t* test. Gene expression data is presented as the mean± standard error of the mean (SEM).

Western blot

Protein lysates were prepared from HC11 cells in lysis buffer (20 mM Tris pH7.2, 0.15 M NaCl, 10 mM NaF, 1 mM $NaVO_4$, 1% Triton X-100, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 2.5 mM $Na_4P_2O_7$, 1 mM glycerolphosphate, 0.1% sodium docecyl sulphate, and 1% sodium deoxycholate) containing protease inhibitor cocktail (Roche). Lysates were sonicated briefly before protein determination was performed using bicinchoninic acid assay reagents (Thermo Scientific). Lysate samples were prepared and resolved using Invitrogen's NuPAGE reagents, and gel system before proteins were transferred onto polyvinylidene fluoride membranes. Blocked membranes were incubated with α-milk antibody (Accurate Chemical & Scientific) overnight followed by a horseradish peroxidase-linked secondary antibody. Protein bands were visualised by chemiluminescence using electrochemiluminescence reagent (Perkin Elmer, Sydney, Australia).

Results

Microarray analysis of cultured mammary explants

Microarray analysis confirmed that the mammary explant culture model is suitable for studying secretory differentiation and milk protein synthesis. Maximal induction of both the major casein (three- to fivefold) and whey (tenfold) milk protein genes required I in the presence of FP (Table 1). A total of 354 genes were differentially expressed by at least twofold in mammary explants cultured in IFP compared to explants cultured in NH. Fifty-two genes in the IFP dataset, 46 upregulated and six downregulated, were identified as key regulatory and marker genes of lactogenesis within mammary alveoli in the mouse (supplementary data). These key genes fall into the general categories of milk

Table 1 I-responsive genes involved in protein synthesis in cultured murine mammary explants

Gene description	Affy ID	Gene symbol	Fold change FP→IFP
Milk proteins			
Lactalbumin, alpha-	1418363_at	Lalba	10
Lactoferrin	1450009_at	Ltf	10
Whey acidic protein	1448386_a_at	Wap	10
Casein alpha S1	1420627_a_at	Csn1s1	5
Casein kappa	1419735_at	Csn3	3
Casein beta	1420370_s_at	Csn2	3
Casein alpha S2-like A	1420633_a_at	Csn1s2a	3
Regulation of transcription			
E74-like factor 5 (ets domain transcription factor)	1419555_at	Elf5	5
Signal transducer and activator of transcription 5A	1421469_a_at	Stat5a	2
Posttranscriptional regulation—one-carbon pool			
Aldehyde dehydrogenase 1 family, member L1	1424400_a_at	Aldh1l1	3
Folate receptor 1 (adult)	1450995_at	Folr1	5
Nucleotide supply			
Solute carrier family 28 (sodium-coupled nucleoside transporter), member 3	1419571_at	Slc28a3	10
Ectonucleotide pyrophosphatase/phosphodiesterase 2	1448136_at	Enpp2	2
Amino acid supply			
Aldehyde dehydrogenase family 1, subfamily A7	1418601_at	Aldh1a7	3
Aldehyde dehydrogenase 1 family, member L1	1424400_a_at	Aldh1a1	3
Glutamic-pyruvate transaminase (alanine aminotransferase)	1426502_s_at	Gpt	2
Sulphotransferase family, cytosolic, 1A, phenol-preferring, member 1	1427345_a_at	Sult1a1	2
Solute carrier family 7 (cationic amino acid transporter, Y+ system), member 5	1418326_at	Slc7a5	2

protein synthesis, lipid synthesis and secretion, carbohydrate synthesis, defence factors, tight junction formation, extracellular matrix, molecular transporters, and signal transduction.

A total of 164 genes were responsive to insulin in cultured mammary explants, and 18 were identified to be involved in protein synthesis (Table 1). These genes included induction of two major milk protein gene transcription factors signal transducer and activator of transcription 5A (Stat5a; twofold) and E74-like factor 5 (ets domain transcription factor; Elf5; fivefold), as well as the folate receptor alpha (Folr1; fivefold; Fig. 1a).

Confirmation of I-regulated Stat5a and Elf5 gene expression in HC11 model

HC11 mouse mammary cells are capable of producing milk proteins when cultured in the presence of I, dex, and P. In order confirm the key results obtained from explant studies, we treated HC11 cells with and without I in the presence of P and dex. Cells cultured in I, P, and dex for 5 days showed a significant increase in β-casein and Wap mRNA expression of more than 15- and 200- fold, respectively (both $P< 0.05$; Fig. 1b). In contrast, there was little change in mRNA levels for these genes when either I or P was absent, indicating that I as well as P is essential for milk protein expression in these cells. There was no clear dose effect of I on milk protein mRNA levels ($P> 0.05$), however Western blot analysis of HC11 cell lysates showed a clear dose effect of I on synthesis in of the β-casein protein (Fig. 1c).

The effect of P on HC11 milk protein gene expression was independent of changes in Elf5 mRNA levels, however, cells cultured in the absence of I showed greatly reduced Elf5 mRNA expression ($P< 0.05$ Fig. 1b). This result suggests that I mediates its lactogenic effect via Elf5 and is consistent with the mammary explant experiments. Similarly, Stat5a levels were reduced in cells cultured in the absence of I ($P<0.05$).

Discussion

This study demonstrates that insulin is essential for milk protein synthesis at multiple levels in the murine mammary gland. A central role for insulin was highlighted by the requirement of insulin for two milk protein transcription factors, Stat5a and Elf5, which have previously been

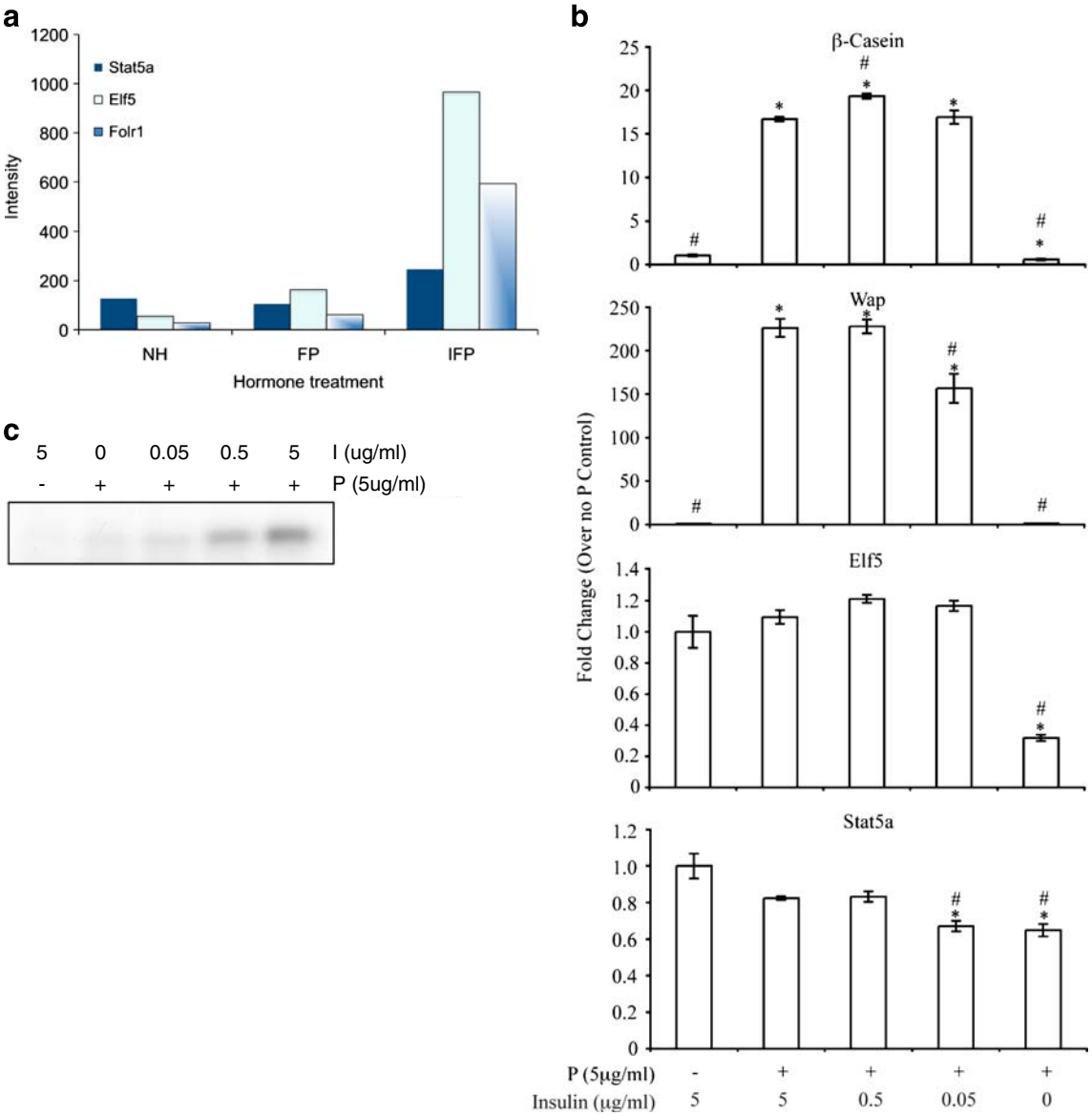

Fig. 1 Insulin stimulated milk protein synthesis **a** Microarray analysis showed maximal expression of signal transducer and activator of transcription 5A (Stat5a), E74-like factor (Elf5), and folate receptor 1 (Folr1) genes required insulin (I) in the presence of hydrocortisone (F) and prolactin (P) in mammary explants. Mammary explants from 12 midpregnant mice were cultured with no hormone (NH) for 8 days and then with FP or IFP for a further 3 days. Cultures were performed in triplicate using mammary explants from four mice for each culture. Mammary explant RNA of the same hormone treatment was pooled. **b** Maximal expression of β-Casein, Wap, Elf5, and Stat5a required the presence of insulin in HC11 cells that were cultured for 4 days in dexamethasone and various concentrations of I in the presence or absence or P. There was no clear dose effect of I on milk protein gene expression. RNA extracts of cultured HC11 cells were analysed by real-time PCR for gene expression, and data were presented as means ±SEM. **c** The synthesis of β-casein protein in cultured HC11 cells was dose-responsive to the amount of insulin included in the culture media. Cells were cultured for 4 days in dexamethasone and various concentrations of I and P, protein extracts prepared, and Western blot analysis of β-Casein performed using an antimilk antibody. *$P<0.05$ (indicates significant fold change in gene expression compared to no P control), #$P<0.05$ (indicates significant fold change in gene expression compared to P+5 ug/ml insulin)

considered prolactin-responsive. The concentration of insulin used in the current study is significantly lower than is commonly used in culture experiments, but is still sufficient to induce milk protein gene expression, as observed in earlier work by Bolander et al. (1981). This concentration of insulin (100 ng/ml media) is below the insulin receptor saturation rate (Akers 2002), and therefore, the lactogenic response observed in the cultured explants is independent of IGF-1 receptor signalling, contrary to prior suggestion by Akers (2002) and Neville et al. (2002).

The secretory differentiation stage of lactogenesis begins around midpregnancy and involves induction of the milk

protein genes (Hartmann 1973; Neville et al. 2002). Maximal induction of the major milk protein genes required the coordinate involvement of insulin, glucocorticoid, and prolactin, which is in agreement with previous studies (Bolander et al. 1981; Kulski et al. 1983; Nagaiah et al. 1981). The second phase of lactogenesis, secretory activation, occurs around parturition when the mammary epithelial cells begin to synthesise and secrete protein, lipid, lactose, and other milk constituents such as defence factors into the alveoli lumen (Brisken and Rajaram 2006; Muller and Neville 2001; Neville et al. 2002). In accordance with this process occurring in the cultured mammary explants, there was an increase in expression of genes involved the synthesis of these major milk constituents in response to insulin, glucocorticoid, and prolactin. The differential regulation of genes involved in the concomitant synthesis of extracellular matrix, signal transduction, and the closure of tight junctions between mammary epithelial cells, was also evident, the latter necessary for cells to maintain polarity around a central lumen and prevent paracellular communication such that milk constituents may be secreted into the alveoli lumen (Blanchard et al. 2006; Itoh and Bissell 2003; Linzell and Mepham 1974; Morgan and Wooding 1982; Nguyen and Neville 1998). Furthermore, 13 signalling and transporter genes, as well as one lipid synthesis gene, identified in the current study are common to the datasets arising from three studies that have utilised functional genomics to identify key regulatory genes involved in secretory activation in the murine mammary gland (Naylor et al. 2005; Ramanathan et al. 2007; Rudolph et al. 2003). This indicates that the mammary explants were a useful model to examine the role of insulin in induction of milk protein synthesis that occurs at lactogenesis.

Insulin and milk protein gene transcription

The current study showed that the major casein and whey protein genes are insulin-responsive and confirms earlier studies on the hormonal regulation of casein gene expression (Bolander et al. 1981; Goodman et al. 1983; Kulski et al. 1983; Nagaiah et al. 1981). Consistent with this induction of the milk protein genes is a concomitant increase in gene expression of two key milk protein gene transcription factors, Stat5a and Elf5 (Gass et al. 2003; Harris et al. 2006; Wakao et al. 1995, 1992), in response to insulin. A major role for insulin in Elf5 and Stat5a gene expression was confirmed in HC11 cells, which showed only reduced expression of these genes in the absence of insulin. In contrast, prolactin was not required for maximal expression of Elf5 and Stat5a. Interestingly, both Stat5a and Elf5 have been identified as major regulators of the prolactin signalling pathway to induce milk protein gene expression and mammary development (Delcommenne and Streuli 1995; Gass et al. 2003; Gouilleux et al. 1994; Harris et al. 2006; Naylor et al. 2005; Neville et al. 2002; Philp et al. 1996; Zhou et al. 2005).

Insulin has previously been identified to play a role in Stat5 signalling by Chen et al. (1997) who showed the major insulin receptor substrate, IRS-1 activates Stat5 by phosphorylation. Phosphorylation of the Stat5 proteins, Stat5a and Stat5b, is important for their dimerisation and binding to GAS (γ-interferon activation sequence) elements in the promoter region of milk protein genes (Gouilleux et al. 1994; Groner et al. 1994; Litterst et al. 2003; Wartmann et al. 1996). Although the heterodimer of Stat5a and Stat5b proteins, as opposed to homodimers, is essential for its recognised role in milk protein gene expression (Calvert and Shennan 1996), the level of expression of the Stat5a gene is greater in mammary epithelial cells, and this protein is regarded the main regulator in mammary gland functions (Teglund et al. 1998). Prolactin (Burdon et al. 1994; Philp et al. 1996) and insulin (Chen et al. 1997), to a lesser extent, have been recognised a key role in phosphorylation of Stat5a, but the current study shows Stat5a gene transcription is insulin-responsive.

Elf5 is an Ets transcription factor that regulates secretory alveolar epithelium differentiation during mammary morphogenesis, lobuloalveolar development in the mammary gland, and transcription of the milk protein genes (Harris et al. 2006; Li et al. 2005; Oakes et al. 2008; Zhou et al. 2005, 1998). Elf5 directly activates a GGAA site in the Wap gene promoter (Thomas et al. 2000), but the mechanism by which Elf5 regulates transcription of the casein genes remains to be elucidated. It has been suggested Elf5 has a negative regulatory domain that inhibits DNA binding (Oettgen et al. 1999), but it is not known if this is related to casein gene transcription. Fibroblast growth factor (Fgf) signalling is essential for Elf5 expression in the lung epithelium, and explant culture studies suggest this regulation of Elf5 by Fgf is by means of the PI3-kinase/Akt pathway (Metzger et al. 2007). Insulin can signal by the PI3-kinase/Akt pathway (Saltiel and Pessin 2002), and this signalling pathway has been identified a central role in lactation (Lemay et al. 2007). While insulin has also been shown to regulate Elf5 in bovine mammary tissue (Menzies et al. 2009b), whether insulin regulates Elf5 in the mammary gland via PI3-kinase/Akt signalling mechanisms or not remains to be resolved.

A posttranscriptional role for insulin

The synthesis of β-casein protein, but not transcription of the β-casein, or Wap, genes in cultured HC11 cells was dose-responsive to the amount of insulin included in the culture media. This provides evidence of a posttranscriptional role for

insulin in milk protein synthesis and is consistent with the microarray data that revealed a number of the genes responsive to insulin were involved in protein synthesis at the posttranscriptional level. An increase in expression of several genes involved in the uptake and metabolism of nonessential amino acids (Alnouti and Klaassen 2008a, b; Davis and Mepham 1976; Jakoby and Ziegler 1990; Kansal et al. 2000; Mepham 1982; Roh et al. 1994; Sharma and Kansal 1999; Welch 1972) is also consistent with recent data from the hormone's action in cultured bovine mammary explants (Menzies et al. 2009b). The increased gene expression of the Y+ cationic transporter gene (Slc7a5) provides a molecular mechanism for the requirement of insulin to stimulate cationic amino acid transport into the mammary gland (Kansal et al. 2000; Menzies et al. 2009b; Roh et al. 1994; Sharma and Kansal 1999).

Insulin and folate metabolism

Recent comparative genomics studies that combined bioinformatics and animal models with a high (and increased) milk protein production during an established lactation have indicated a key role for folate receptor alpha (Folr1) in the mammary gland for milk protein production (Menzies et al. 2009a). A specific role for folate in milk protein synthesis is also supported by folate supplementation studies in lactating cows, which resulted in a positive response to milk production and milk protein yields (Girard and Matte 1998, 2005; Graulet et al. 2007). The current study indicated that two genes that are central to the metabolic pathway of folate were responsive to insulin in the cultured mammary explants. Folr1 was upregulated fivefold, and expression of the gene encoding the folate reducing enzyme, formyltetrahydrofolate dehydrogenase (Aldh1l1) gene, was also increased (Shane 1989). A role for insulin in stimulating mammary genes involved in folate uptake and metabolism is consistent with a recent report that these genes were also insulin responsive in bovine mammary tissue (Menzies et al. 2009b).

Folate metabolism plays an important role in protein synthesis due to the single, important biochemical function of folate in mammals to accept and release one-carbon units, otherwise known as the one-carbon pool (Choi and Mason 2000). This role is essential for the synthesis of purines and pyrimidines, generation of methionine, and the de novo synthesis of methyl groups for formation of the primary methylating agent, *S*-adenosylmethionine (Bailey and Gregory 1999). The importance of Folr1 for cellular uptake of folate was established by the analysis of renal folate handling in mice with targeted gene knockouts of folate binding proteins 1- and 2 (also known as Folr1 and Folr2) (Birn 2006). Once in the cell, Aldh1l1 plays a central role in reducing folate to its active forms of tetrahydrofolates (Barlowe and Appling 1988; Cook et al. 1991; Shane 1989).

The current study demonstrates that insulin plays an important role in milk protein synthesis at multiple levels in the murine mammary gland. The new data presented demonstrates that the requirement of insulin for milk protein gene expression may primarily be facilitated by the major milk protein transcription factors, Elf5 and Stat5a. Whereas prolactin plays an essential role in phosphorylating and activating Stat5a, insulin plays an integral role in the gene expression of this protein. This indicates that insulin plays a crucial role in the prolactin-induced expression of the milk protein genes and confirms that the primary role of insulin, together with hydrocortisone, equally as important as prolactin in milk protein synthesis. Therefore, this study has begun to elucidate the molecular mechanisms of insulin underpinning the coordinate induction of milk protein synthesis.

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