ABSTRACT: Differentiation of the mammary gland during pregnancy and the first several days following parturition is required for the establishment of a normal lactation. The expansion and development of the secretory compartment into lobuloalveolar structures that secrete milk is a complex process that is under the tight control of an array of hormones and growth factors that interact at multiple levels. Of the many factors that are required to orchestrate functional differentiation during pregnancy, insulin is often overlooked. This is likely related to the difficulty in manipulating insulin without disrupting its widespread systemic effects on metabolism. In addition, crosstalk with the IGF type 1 receptor (IGF-1R) and activation of similar downstream signaling pathways through the insulin receptor (IR) and IGF-1R often make it difficult to specifically link biological outcomes to insulin. The objective of this review is to summarize the available data that address a role for insulin in secretory differentiation of the mammary gland. Much of these data focus on a role for insulin in milk protein synthesis. However, data using conditional knockout of the IR in the mammary epithelium during mid pregnancy as well as transcriptomic approaches are discussed to present an expanded understanding of a role for insulin in functional differentiation that includes both transcriptional and post-transcriptional regulation of multiple genes involved in the process.

Key words: differentiation, insulin, lactation, mammary gland, pregnancy, review


INTRODUCTION

A role for insulin in mammary gland differentiation and subsequent lactation has been recognized for many years. Early studies showed that ablation of the endocrine pancreas with alloxan in lactating rats decreased growth of the pups, a measure of milk synthesis, which could be maintained with insulin replacement (Walters and McLean, 1968). Later studies linking streptozotocin-induced diabetes in rats with lactation deficiency (Lau et al., 1993) and insulin-dependent diabetes in women with delayed lactogenesis (Neubauer et al., 1993) also supported a role for insulin in mammary gland differentiation. However, the widespread systemic effects of insulin on general metabolism, as well as crosstalk between the insulin and IGF systems, have made it difficult to study the direct effects of insulin on the mammary gland.

Despite the presence of insulin receptors (IR) on mammary epithelial cells (MEC) that bind insulin at physiological concentrations, a role for insulin in general mammary gland development is typically not included in reviews on this topic. Differentiation is a broad term that is used to describe changes in the mammary gland that occur during pregnancy to prepare for lactation as well as the ability of the gland to initiate and maintain milk synthesis during established lactation. Therefore, the present review will focus on a range of studies that fall under the broad category of differentiation to cover the state of knowledge relative to the effects of insulin on this aspect of mammary gland function. Much of the work in this area has been done in rodents; however, applicable work in the cow will be discussed where available.

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GLUCOSE UPTAKE BY THE MAMMARY GLAND IS INSULIN INDEPENDENT

As a prelude to a review on a role for insulin in mammary gland differentiation, the lack of a role for insulin in glucose uptake in the mammary gland deserves mention. The lactating mammary gland requires large amounts of glucose to support milk production in addition to its role as a metabolic fuel. Glucose is the major precursor for lactose, the major sugar found in milk, and is also required for the synthesis of milk proteins (Qian and Zhao, 2014) and fat (Rudolph et al., 2007). Due to its lack of glucose-6-phosphatase, the mammary gland is unable to synthesize glucose from other precursors and is, therefore, dependent on uptake of glucose from the circulation to meet its glucose needs (Zhao, 2014). The mammary glands of all species require a large amount of glucose each day in support of milk production; in the lactating goat, this represents 60 to 85% of the glucose turnover in the body (Annison and Linzell, 1964). Because lactose controls milk volume via its effect on osmolality, glucose uptake by the mammary gland is considered a rate-limiting step in milk production (Rook, 1979).

Although insulin plays a major role in facilitated transport of glucose in tissues such as adipose and skeletal muscle, glucose uptake by the lactating mammary gland is insulin independent (reviewed in Zhao, 2014). In the cow, this is supported by in vivo studies that show that milk production does not increase when insulin levels are elevated 5-fold whereas glucose levels are maintained within 10% of baseline values (i.e., hyperinsulinemic and euglycemic clamp studies; McGuire et al., 1995). The major insulin-responsive glucose transporter (GLUT), GLUT4, is lacking in the lactating and nonlactating bovine mammary gland (Zhao et al., 1995; Komatsu et al., 2005; Finucane et al., 2008), which likely explains the insensitivity of the mammary gland to insulin-stimulated glucose uptake. Mammary glands of cows, rats, and mice express other facilitative glucose transporters such as GLUT1, GLUT8, and GLUT12 as well as the Na⁺–glucose co-transporter (SGLT) 1 and SGLT2. Whereas the mRNA levels of GLUT1, GLUT8, and GLUT12 increase with the onset of lactation (Zhao, 2014), studies using bovine mammary explants, primary bovine MEC, or the mouse HC11 MEC line indicate that insulin and other lactogenic hormones do not increase their expression, indicating that other factors must control their regulation in the lactating mammary gland (Shao et al., 2013).

THE INSULIN–IGF SYSTEM

Insulin belongs to the family of ligands that make up the insulin–IGF system. The system consists of 3 peptides, insulin, IGF-I, and IGF-II, which exhibit significant structural homology with identical disulfide bonding and similar tertiary structures (LeRoith et al., 1995). The receptors for these ligands are also structurally similar (Fig. 1). Although the receptors differ in ligand binding kinetics, crosstalk between these ligands and receptors can occur (LeRoith, 2000). Insulin is a small polypeptide of 51 AA that is synthesized by the pancreas. Although the AA sequence of insulin varies across species, its 3-dimensional conformation is highly conserved. In contrast to insulin, IGF are made in most tissues of the body (Cohick and Clemmons, 1993). The mature circulating forms of IGF-I and IGF-II are slightly larger than insulin at 70 and 67 AA, respectively, due to differences in processing of the proteins. Circulating concentrations of IGF-I are approximately 100-fold greater than those of insulin in lactating dairy cows. Levels of both hormones generally reflect the energy status of the animal and, therefore, decline with parturition when the demands of milk synthesis drive the animal into negative energy balance (Vicini et al., 1991). In dairy cows, insulin levels average approximately 1.5 ng/mL 28 d before parturition and 0.4 ng/mL 7 d post-partum (Rhoads et al., 2004), whereas in rodents, insulin levels of 5 and 2 ng/mL are reported for pregnancy and lactation, respectively (Moffett et al., 2013).

The insulin–IGF ligands mediate their effects through binding to heterodimeric transmembrane receptors. The IR and IGF type 1 receptor (IGF-1R) each consist of 2 extracellular α-subunits and 2 transmembrane β-subunits held together by disulfide bonds (Fig. 1). At physiological concentrations, insulin mediates its effects through the IR whereas IGF-I signals through the IGF-1R (Siddle et al., 2001). There are 2 IR isoforms, IR-A and IR-B, which differ by a 16-AA splicing event. Insulin receptor-B has a high affinity for insulin and binds IGF ligands only at supraphysiological concentrations. It plays a primary role in metabolic regulation in the adult. The major isoform of IR in the fetus is thought to be IR-A, although IR-A mRNA is detectable in many adult tissues and it is upregulated in many cancers (Belfiore and Frasca, 2008; Belfiore et al., 2009). Insulin receptor-A binds insulin and IGF-II with similar affinity but does not bind IGF-I at physiological concentrations; therefore, IR-A may represent a physiological receptor for IGF-II (Frasca et al., 1999). In addition to IR-A, IR-B, and IGF-1R, hybrid receptors exist that consist of 1 dimer of the IGF-1R and another dimer of either IR-A or IR-B (Fig. 1). These hybrid receptors have differing affinities for all 3 li-
gands (Belfiore et al., 2009). Therefore, the sensitivity of any given tissue to the 3 ligands may depend on the relative expression of each of the receptor isoforms in a specific physiological state. The presence of hybrid receptors with differing affinity for IGF-I and IGF-II may help explain why these 2 peptides have differing effects on mammary gland development (Brisken et al., 2002; Rowzee et al., 2008).

The β-subunits of the IR and IGF-1R possess intrinsic tyrosine kinase activity and are activated by ligand binding to the extracellular α-subunit. Following autophosphorylation, the phosphotyrosines serve as binding sites for signaling molecules, which subsequently provide docking sites for multiple effectors that ultimately activate 2 major signaling pathways, Ras/Raf/mitogen-activated protein kinase (MAPK) or phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR; Fig. 2). The protein mTOR belongs to the large mTOR complex 1 that functions to enhance translation initiation by phosphorylating 2 major targets, the eukaryotic translation initiation factor (eIF) 4E binding proteins (4E-BP) and ribosomal protein S6 kinase (S6K) that cooperate to regulate translation initiation rates (Petroulakis et al., 2006; Ma and Blenis, 2009). Interestingly, both insulin and IGF activate similar signaling cascades through their receptors, indicating that the availability of the individual receptors may control the biological action of the different ligands across different physiological stages in the mammary gland. Moreover, IGF circulate bound to IGF binding proteins, so their accessibility to cell surface receptors is reduced. In contrast, insulin does not bind to this family of proteins. Therefore, IGF binding proteins also likely modulate different biological outcomes between the 2 ligands (Cohick, 1998; Clemmons, 2006).

Insulin binds to lactating rat mammary tissue and activates IR signaling concentrations of around 10 ng/mL, indicating that physiological levels of insulin bind IR in the lactating gland (Flint et al., 1984; Burnol et al., 1990). Both IR-A and IR-B mRNA are present in the mammary gland of pregnant and lactating rats, with IR-B exhibiting a marked upregulation on d 1 of lactation (Berlato and Doppler, 2009). Similar changes are observed in the murine MEC line HC11 when these cells are induced to differentiate (Berlato and Doppler, 2009). Total IR mRNA also increases across lactation
Figure 2. Mammalian target of rapamycin (mTOR) signaling regulates translation initiation by integrating several different inputs. Insulin, hormones, and growth factors activate the phosphatidylinositol-3-kinase (PI3K)/Akt signaling cascade. Akt receives inputs from phosphoinositide dependent kinase 1 (PDK1) and the rictor/mTOR complex. Energy status (the adenosine monophosphate [AMP]:ATP ratio) modulates AMP kinase (AMPK) activity. These pathways affect tuberous sclerosis (TSC) 1/2 to regulate mTOR activity via Rheb. The raptor/GβL/mTOR complex mediates the phosphorylation of 4E binding proteins (4E-BP) and S6 kinase (S6K). Pharmacological inhibition of PI3K (wortmannin or LY294002) and mTOR (rapamycin or its analogs) or activation of AMPK (by increased AMP/ATP, metformin, or 5-aminoimidazole-4-carboxamide ribonucleotide [AICAR]) are indicated. Reprinted from Petroulakis et al. (2006) with permission from Macmillan Publishers Ltd. on behalf of Cancer Research UK (the British Journal of Cancer). eIF = eukaryotic translation initiation factor; GTP = guanosine-5'-triphosphate; IRS-1 = insulin receptor substrate-1; rpS6 = ribosomal protein S6.
in the mammary gland of cows relative to d 15 prep-
tum, as does insulin receptor substrate-1 (IRS-1) mRNA
(Bionaz et al., 2012). Determining if changes in IR-B
relative to IR-A occur at the protein level is not possible
at this time due to the lack of antibodies that distinguish
between IR-A and IR-B, given that these proteins differ
by only 16 AA. Rowzee et al. (2009) developed a specif-
ic quantitative PCR assay to compare IR-A, IR-B, and
IGF-1R in the same RNA sample. Using this approach,
both IR isoform mRNA are 3- to 16-fold greater than
IGF-1R mRNA expression in primary MEC isolated
from glands of virgin mice as well as early-, mid-, and
late-pregnant mice whereas IR protein is 3- to 10-fold
greater than IGFR protein. However, despite the greater
prevalence of IR mRNA and protein, IGF ligands are
more effective than insulin in stimulating the IRS-1/
PI3K/Akt pathway in MEC isolated from mammary
glands of virgin animals. Whether this will hold true dur-
ning pregnancy and lactation is unknown. Interestingly,
the amount of hybrid receptor in the mammary glands of
virgin mice was shown to be developmentally regulated
in this study (Rowzee et al., 2009). Information on IR-A,
IR-B, and hybrid receptors in domestic species is lack-
ing at this time and a complete understanding of the role
of hybrid receptors in mammary gland development and
differentiation across all species requires further study.

GENERAL OVERVIEW OF HORMONAL
REGULATION OF DIFFERENTIATION

The hormonal regulation of mammary gland differ-
entiation is a major area of interest in mammary gland
biology. This topic has obvious relevance for milk pro-
duction in domestic animals as well as humans. It is
also important in the biology of breast cancer because
pregnancy and lactation drive differentiation and both
decrease breast cancer risk in humans and rodents
(Sinha et al., 1988; Medina, 2004; Misra et al., 2012).

The mammary gland is one of the few organs that
undergoes a majority of its growth postnatally. The
gland cycles through repeated rounds of growth, dif-
fentiation, and involution with pregnancy and lac-
tation. The different developmental stages are under
complex hormonal control with GH, estrogen, epider-
mal growth factor, and IGF-I in general regulating lon-
gitudinal growth and branching of the ductal structures
from birth through puberty, whereas progesterone and
prolactin (PRL) contribute to enhanced side-branching
and alveolar bud formation after puberty (Akers, 2006;
McNally and Martin, 2011; Lee and Ormandy, 2012).
Functional differentiation of the mammary gland in
preparation for lactation occurs in several phases
(Andereson et al., 2007). During early pregnancy, a pro-
liferative phase occurs that involves the formation of
the alveolar structures containing the milk-producing
cells in the gland. The epithelial compartment of the
murine gland expands from less than 10% of the total
volume to 90% by parturition, with the greatest pro-
liferation occurring before d 12 of pregnancy (parturi-
tion occurs between d 19 and 21; Richert et al., 2000).
Studies using radiolabeled thymidine incorporation
show that 25% of MEC are proliferating on d 5 of
pregnancy in the mouse (Traurig, 1967). Progesterone
and PRL from the ovaries and the pituitary gland, re-
spectively, regulate this proliferative phase, which de-
creases by mid pregnancy (Oakes et al., 2006; Obr and
Edwards, 2012; Briskin and Ataca, 2015). Secretory
differentiation (also referred to as lactogenesis stage
I) marks the next phase of differentiation, which is
characterized by major biochemical changes that lead
to a prelactational state. These changes include cyto-
plasmic lipid droplet formation, casein accumulation,
and lumina expansion (Russell et al., 2007). The actual
secretion of milk is blocked by the high plasma con-
centration of progesterone during pregnancy. A decline
in plasma progesterone at parturition removes the inhi-
bition on milk secretion, leading to secretory activation
(Andereson et al., 2007). A further increase in milk pro-
tein gene expression occurs during this phase. In addi-
tion, tight junctions form between MEC that compose
the alveoli to facilitate directional secretion of milk
constituents into the alveolar lumen. Lactation marks
the physiological state where the mammary gland is
fully differentiated and continuous milk production oc-
curs (Neville et al., 2002).

ROLE OF INSULIN IN MORPHOLOGICAL
DIFFERENTIATION OF THE MAMMARY GLAND

As previously mentioned, insulin is typically not
included as a hormone required for the morphological
differentiation that occurs during pregnancy. However,
data of Neville et al. (2013) indicate that in the rodent,
insulin may play a role in these processes. These au-
thors used a Cre-lox transgenic system to specifically
knock out IR in the mammary epithelium of mice.
Using growth rate of the litters as a measure of milk se-
cretion, they found that pups born to dams lacking the
IR in MEC exhibited severely compromised growth,
indicating that MEC IR is required for normal lacta-
tion. Histological analysis of the glands indicated a
severe developmental defect at pregnancy d 13.5. The
area occupied by alveoli and the number of alveoli per
field was reduced by 50% in mammary gland sections
of the dams that lacked IR in their MEC (Neville et
al., 2013). In addition, MEC were isolated from preg-
nant mice on d 13.5 and gene expression was evalu-
ated using Affymetrix gene arrays (Affymetrix Inc.,
Santa Clara, CA). Genes associated with differentiated function were downregulated in MEC from dams that lacked IR in MEC, whereas genes associated with the cell cycle and extracellular matrix and the cytoskeleton were upregulated. The authors concluded that insulin and IR may serve as a major regulator of the switch from proliferation to differentiation in the second half of pregnancy (Neville et al., 2013).

**ROLE OF INSULIN IN MILK PROTEIN SYNTHESIS DURING PREGNANCY AND LACTATION**

Although few in vivo studies have examined a role for insulin in regulating morphological differentiation of the gland during pregnancy, a role for insulin in differentiation as it relates to milk protein synthesis has been studied for many years using in vitro methods. Early studies used mammary gland explant or organ culture to mimic the lactogenic process and examine the endocrine control of milk protein gene expression. These methods are advantageous because the normal histology of the gland is preserved, including contributions from both stromal and epithelial components. Also, the first 24 h in culture are generally representative of the in vivo situation. A drawback is that cultures are typically functional for less than 96 h (Andersen and Larson, 1970; Topper et al., 1975; Brennan et al., 2008). Historically, although the combination of insulin, hydrocortisone (HC), and PRL were all shown to be required for maximal induction of casein synthesis, insulin was assumed to perform a permissive role in cell survival and maintenance in rodent or redundant mammary explant models. In addition, many of these early studies were complicated by the use of high levels of insulin (1 to 5 μg/mL) that activate the IGF-1R (Andersen and Larson, 1970; Topper et al., 1975; Collier et al., 1977). However, studies in the early 1980s showed that insulin at physiological concentrations (i.e., 1 to 10 ng/mL) was essential for casein synthesis in the rodent gland and that IGF-I could not replace insulin for casein synthesis (Bolander et al., 1981; Kulski et al., 1983). Our current understanding is that insulin, glucocorticoids, and PRL each contribute to the regulation of milk protein synthesis at multiple levels including transcriptional regulation, stabilization of milk protein mRNA, and translation of milk protein mRNA. In addition, there is considerable synergy among the actions of these hormones in terms of regulating milk protein synthesis (Rhoads and Grudzien-Nogalska, 2007; Menzies et al., 2009, 2010). Other factors, such as extracellular matrix components and cell–cell interactions, also contribute to the complexity of milk protein synthesis (Bionaz et al., 2012).

The finding that insulin regulates protein synthesis in the mammary gland through translational control (Rhoads and Grudzien-Nogalska, 2007) supports much of the older literature using mammary gland explant culture that showed a greater accumulation of β-casein mRNA or milk proteins in the media relative to new protein synthesis in the presence of lactogenic hormones. In general, insulin regulates protein synthesis in the mammary gland as it does in adipose and skeletal muscle by activating the IR and the PI3K/Akt/mTOR downstream signaling cascade, leading to regulation of the translation initiation machinery (Ma and Blenis, 2009). However, the effect of insulin on protein translation can occur through multiple mechanisms and in synergy with other lactogenic hormones. In the differentiating mouse MEC line CID 9, insulin alone was shown to stimulate synthesis of milk and nonmilk proteins whereas PRL alone had no effect. However, insulin plus PRL selectively stimulated synthesis of milk proteins more than insulin alone (Choi et al., 2004). Inhibitors of the PI3K, MAPK, and mTOR pathways block the effect of insulin but not the synergistic effect of the 2 hormones together, whereas the polyadenylation inhibitor cordycepin effectively blocks this effect. Additional experiments led to the conclusion that insulin and PRL act synergistically to enhance translation of β-casein by increasing phosphorylation of the cytoplasmic polyadenylation element binding protein, resulting in lengthening of the mRNA poly(A) tail (Choi et al., 2004).

The signaling molecule mTOR represents an important control point in the MEC that integrates extracellular signals from hormones and growth factors with AA availability and intracellular energy status to control translation rates of milk proteins (Fig. 2; Toerien and Cant, 2007; Hayashi et al., 2009; Bionaz et al., 2012). Burgos et al. (2010) conducted a study using cultures of mammary acini isolated from lactating bovine mammary glands to examine the effects of all 3 factors (i.e., hormones, AA availability, and energy status) on milk protein synthesis and mTOR signaling. They found that the lactogenic hormones HC, insulin, and PRL alone had no effect on protein synthesis, whereas AA alone increased protein synthesis by 50% (Burgos et al., 2010). This effect was enhanced with HC, insulin and PRL, although it should be noted that insulin was added at 1 μg/mL in that study, which does not rule out effects of insulin acting through the IGFR. This enhancement by the lactogenic hormones was associated with increased phosphorylation of the mTOR substrates p70S6K1 and 4E-BP1 as well as dissociation of 4E-BP1 from elf4E (Burgos et al., 2010). This finding supports in vivo studies with insulineemic, euglycemic clamp approaches in lactating dairy cows, which show that
insulin can increase milk protein production when AA substrate availability is increased (Mackle et al., 1999).

A transcriptomics approach has been used to more broadly address a role for insulin in milk protein synthesis (Menzies et al., 2009). Mammary gland explants derived from mammary biopsies from late pregnant cows were cultured with or without lactogenic hormones. In this study, insulin was used at a concentration of 100 ng/mL, which should not activate the IGFR. The validity of the system as a model for lactation was determined by comparing gene profiles regulated by insulin, HC, and PRL with murine transcriptome studies that have profiled gene changes across pregnancy and lactation (Naylor et al., 2005; Ramanathan et al., 2007; Rudolph et al., 2007). To derive an insulin-responsive set of genes, Menzies et al. (2009) compared genes regulated by HC and PRL with those regulated by HC, PRL, and insulin. They found that 264 genes covering a range of cellular processes were determined to be insulin responsive, with 125 of these upregulated and 139 downregulated. Of these genes, 29 were related to the validity of the system as a model for lactation was determined by comparing gene profiles regulated by insulin, HC, and PRL with murine transcriptome studies that have profiled gene changes across pregnancy and lactation (Naylor et al., 2005; Ramanathan et al., 2007; Rudolph et al., 2007). To derive an insulin-responsive set of genes, Menzies et al. (2009) compared genes regulated by HC and PRL with those regulated by HC, PRL, and insulin. They found that 264 genes covering a range of cellular processes were determined to be insulin responsive, with 125 of these upregulated and 139 downregulated. Of these genes, 29 were related to processes involved in protein synthesis, including E74-like factor 5, a transcription factor associated with milk protein gene expression, and genes coding for proteins involved in protein synthesis at post-transcriptional and translational levels as well as protein packaging, genes that regulate folate metabolism, and enzymes involved in catabolism of essential AA and biosynthesis of non-essential AA. These studies indicate a global role for insulin in transcriptional regulation of secretory differentiation of the bovine mammary gland. However, the specific mechanisms by which insulin acts to regulate these processes remains to be elucidated.

SUMMARY AND CONCLUSIONS

Significant evidence now exists to indicate that insulin plays an important role in regulating multiple processes that occur in the mammary gland in preparation for lactation. Although insulin clearly regulates milk protein synthesis at both the transcriptional and post-transcriptional levels, transcriptomic studies indicate a more general role in regulating overall protein synthesis in support of the global remodeling and secretory activation required for lactation. An open question is whether insulin affects alveolar development in cows and other domestic animals to the extent it does in rodents based on the phenotype of the conditional IR knockout mouse. Also, the specific mechanisms by which insulin synergizes with PRL and HC as well as with other hormones and growth factors to regulate differentiation of MEC remain to be elucidated.

LITERATURE CITED


Insulin and mammary gland differentiation

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