

Decreased lactation capacity and altered milk composition in insulin receptor substrate null mice is associated with decreased maternal body mass and reduced insulin-dependent phosphorylation of mammary Akt

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Abstract

Expression of insulin receptor substrates (IRS)-1 and -2 within the mammary gland was found to be high at mid-lactation and dramatically decreased with mammary involution. This observation supports the hypothesis that these proteins are induced in the mammary gland with lactogenesis and involved in normal milk synthesis. To test this hypothesis, lactation capacity, along with indices of mammary secretory cell glucose metabolism and cell signaling were compared in normal mice and mice carrying targeted mutations in either the *Irs1* or *Irs2* genes. Mammary IRS-1 and IRS-2 protein levels were increased within 1 day of parturition and reached maximal levels by 5 days *post partum*. Dams carrying germline mutations of *Irs1* or *Irs2* displayed reduced lactation capacity as assessed by weight gain of pup litters. The reduction was more dramatic in *Irs1*^{-/-} versus *Irs2*^{-/-} dams. Maternal

body weight was also reduced in *Irs1*^{-/-} dams as well as in *Irs1*^{+/-}*Irs2*^{+/-} dams. The loss of IRS-1 had little impact on mammary gland expression of milk protein mRNAs, glucose transport, or on the abundance and subcellular localization of hexokinases I and II. The loss of IRS-1 was associated with a compensatory increase in insulin-induced IRS-2 phosphorylation; however, the loss of IRS-1 did also cause a reduction in insulin-dependent mammary gland-specific activation of Akt phosphorylation. These results support the conclusion that IRS-1 is important for insulin-dependent activation of Akt signaling within the lactating mammary gland, but that loss of this protein has only modest impact on normal milk synthesis, since related signaling proteins such as IRS-2 may act in compensatory fashion.

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Introduction

Establishment of a normal lactation is the result of a number of highly orchestrated processes occurring both during pregnancy and within the first several days of the *post partum* period. This process, referred to as lactogenesis, is a two-stage developmental program during which the mammary secretory cell prepares for the process of milk secretion. Lactogenesis I is characterized by the detectable induction of lactation-specific gene products within the mammary epithelium. In rodents, this process begins as early as day 10 of pregnancy and is associated with a variety of biochemical as well as morphological changes within the mammary epithelium (Bauman *et al.* 1974, Mellenberger & Bauman 1974b, Slaby & Brown 1974, Rosen & Barker 1976, Nakhasi & Qasba 1979, Schwertfeger *et al.* 2003). Lactogenesis II, also referred to as secretory activation, is

characterized by a switch that occurs within the secretory epithelium from the pre-lactating differentiated state described earlier, to a state of intense metabolic, biosynthetic, and secretory activity. It is most simply described as the onset of 'copious milk secretion' (Neville *et al.* 2001). From a mechanistic standpoint, secretory activation has best been characterized by the dramatic changes that occur both in milk volume and in milk composition (Neville & Morton 2001). Key events underlying these dramatic changes in milk composition and volume include closure of the epithelial cell tight junctions, upregulation of milk protein synthesis, upregulation of lactose and lipid synthesis, and increased oxidative metabolism (Mellenberger & Bauman 1974a,b, Rosen *et al.* 1978, Nguyen *et al.* 2001). Along with these, increases also occur in the expression and/or activation of enzymes associated with glucose metabolism such as hexokinase II, Glut 1, Glut 12, and

PFK2 (Sochor *et al.* 1984, Kaselonis *et al.* 1999, Nemeth *et al.* 2000, Macheda *et al.* 2003). Although the underlying trigger for these events is not completely understood, key events involved with lactogenesis include the drop in blood progesterone that coincides with parturition along with surges in blood prolactin and glucocorticoids (Neville & Morton 2001). Insulin is also believed to be important for supporting metabolic processes during lactation, despite the fact that its mammary-specific role has yet to be completely defined (Neville *et al.* 2002).

The mechanism through which insulin acts within cells and tissues has largely been defined in terms of effects mediated through the insulin receptor substrate proteins (IRS)-1 and -2 (Thirone *et al.* 2006). The IRS proteins serve as docking proteins to facilitate the interaction of a number of signal transducers (Yenush & White 1997). Most importantly, they link activation of the receptors for insulin and insulin-like growth factor-I (IGF-I) with a variety of biological responses in cells. Following activation, IRS proteins bind to effector molecules containing the signature src homology domain (SH2) within their amino acid sequence. Some of these effector molecules serve as docking proteins themselves, while others have intrinsic kinase or phosphatase activity. Among the best characterized of IRS-1 interactions is that which occurs with phosphatidylinositol 3'-kinase (Ruderman *et al.* 1990, Araki *et al.* 1994). This interaction is known to mediate the ability of receptors for insulin and IGF-I to activate the serine threonine kinase Akt (Datta *et al.* 1997). The activation of Akt in response to insulin or IGF-I stimulation mediates the ability of these two hormones to inhibit apoptosis (Datta *et al.* 1997). However, activation of Akt has also been demonstrated to mediate the effects of insulin on protein synthesis, carbohydrate metabolism, and lipid biosynthesis (Mendez *et al.* 1996, Gottlob *et al.* 2001, Schwertfeger *et al.* 2003).

Previous studies in our laboratory demonstrated that expression of IRS-1 and IRS-2 within the mammary gland is developmentally regulated and that both proteins are highly expressed during lactation (Lee *et al.* 2003b). The goal of the present study was to determine the importance of IRS-1 and IRS-2 to normal lactation and understand the potential mechanisms through which these two proteins might mediate the processes necessary to milk synthesis.

Materials and Methods

Experimental animals

All animals were studied in accordance with procedures outlined in the NIH Guide to Care and Use of Experimental Animals. These experiments were approved by the Baylor College of Medicine Animal Care and Use Committee. For the analysis of IRS expression during secretory activation, mammary tissue samples were harvested from timed-pregnant CD-1 females (Charles River Laboratories) on 17 and 19 days of gestation and on 1, 2, 3, 4, and 5 days *post partum*. To

determine the importance of IRS-1 and IRS-2 to lactation, litter weight gain, milk composition, and mammary development were studied in mice that were either heterozygous or homozygous for targeted germline mutations. For both genes, litter weight gain was compared among wild-type (+/+), heterozygous (+/-), and null (-/-) females. Lactation performance was also compared among dams heterozygous for both IRS-1 and IRS-2. Mice carrying germline mutations in the *Irs1* or *Irs2* genes were previously described (Araki *et al.* 1994, Kubota *et al.* 2000). All animals were on a mixed genetic background consisting of FVB, SV129, and CBA. All genotype effects were tested for by experiments that used littermate comparisons. Cohorts of (totally eight) lactating females were obtained through the use of timed matings as previously described (Hadsell *et al.* 2003). On day 1 *post partum*, each dam received a cross-fostered litter of ten CD-1 pups. Litter weight was then recorded daily for 10 days. On day 10 *post partum*, milk and mammary tissue samples were collected and weighed from each dam as previously described (Hadsell *et al.* 2003). To determine the effects of *Irs1* mutation on insulin signaling capacity of the mammary gland, *Irs1*^{+/+} or *Irs1*^{-/-} lactating dams were given an i.v. injections of bovine insulin (Sigma-Aldrich) or long-R3 IGF-I (Sigma-Aldrich) as previously described (Lee *et al.* 2003a, Hadsell *et al.* 2005). On day 3 *post partum*, dams were fasted for 8 h and then separated from their litters for 2 h to bring endogenous concentrations of insulin and prolactin to a baseline. Mammary tissue samples were then collected 5 min following tail-vein injection of either insulin or long-R3 IGF-I (0.25 mg/kg). To compare the glucose transport capacity among *Irs1*^{+/+} and *Irs1*^{-/-} cells, primary mammary epithelial cell cultures were established from 16-day pregnant mice of each genotype as previously described (Aggeler *et al.* 1991).

Milk and tissue analysis

Milk samples were analyzed for lactose, nitrogen, and water as previously described (Hadsell *et al.* 2003). Milk samples were assayed for fat using the creatocrit assay (Mandel *et al.* 2005). Epithelial content of the mammary tissue in *Irs1*^{+/+} and *Irs1*^{-/-} dams was determined by segmentation analysis of images captured from hematoxylin- and eosin-stained mammary tissue as previously described (Hadsell *et al.* 2003).

Western and northern blotting

Total tissue protein extracts of mammary tissue were prepared from 50 mg tissue as previously described (Hadsell *et al.* 2001). Mammary tissue mitochondrial and post-mitochondrial fractions were prepared as previously described (Darley-Usmar *et al.* 1987). Total amounts of IRS-1, IRS-2, Akt, phospho-Akt, extracellular signal regulated kinases 1 and 2 (ERK1/2) and phospho-ERK1/2 were measured by western blotting as previously described (Hadsell *et al.* 2001). Phospho-Akt was measured using antibodies to phospho-Thr³⁰⁸ or

phospho-Ser⁴⁷³ (Cell Signaling Technology, Beverly, MA, USA). Phospho-ERK1/2 was measured using an antibody that detects dual phosphorylation of Thr²⁰² and Tyr²⁰⁴ (Cell Signaling Technology). Tyrosine phosphorylation of IRS-2 was measured by western blotting of IRS-2 immunoprecipitates (IPs). Each IP was prepared by incubating 0.5 mg mammary protein extract with a rabbit polyclonal antibody to IRS-2 (Upstate Biotechnology, Charlottesville, VA, USA) as previously described (Hadsell *et al.* 2001). Mitochondrial and post-mitochondrial fractions were blotted with antibodies to hexokinase I (Chemicon International, Temecula, CA, USA), hexokinase II (Chemicon International), cytochrome *c* oxidase subunit IV (COXIV; Abcam Inc., Cambridge, MA, USA), and tubulin (Abcam Inc). Western blotting for keratin 8 used the TROMA (trophoblastoma) I antibody (University of Iowa Developmental Studies Hybridoma Bank).

To check for equal loading, the samples were also run on gels which were subsequently stained with coomassie. Analysis of total mammary RNA for milk protein mRNA abundance was conducted by northern blotting as previously described (Hadsell *et al.* 2003). Densitometry data were collected using a molecular dynamics personal densitometer SI.

Glucose transport

To determine whether mammary cell glucose transport was impaired in response to loss of IRS-1, primary mammary cell cultures were prepared from *Irs1*^{+/+} and *Irs1*^{-/-} female mice at 16 days of pregnancy. Primary cells were prepared as previously described (Aggeler *et al.* 1991). All cell culture reagents were purchased from Sigma unless specified otherwise. Cells were suspended in 2×P media consisting of Dulbecco's minimum essential medium (DMEM) with antibiotics (100 U/ml penicillin, 100 U/ml streptomycin, and 50 µg/ml gentamicin), plus 2 mM glutamine, 2 µg/ml insulin, 20 ng/ml epidermal growth factor (EGF), 2 µM dexamethasone, and 25 mM glucose. Cells were then plated on six-well tissue plates that had been coated in 1 ml/well serum fetuin media for 24 h at 37 °C prior to plating (DMEM, 20% (v/v) heat-inactivated calf serum, 25 mM glucose, and 2 mg/ml fetuin). Cells were incubated for 24 h at 37 °C. After 24 h in culture, the media were changed to standard dexamethasone insulin prolactin (DIP) media, containing 25 mM glucose, 1 mg/l prolactin, 1 mg/l insulin, and 1 µM dexamethasone. The cells were maintained in this media for an additional 48 h. At 72 h after plating, a portion of the cells were harvested and DNA quantitated using the Hoechst assay. All other cells were incubated in glucose-free DIP media, plus or minus cytochalasin B (1 µg/ml) for 1 h at 37 °C. The media were then aspirated off and the cells were incubated for 15 min in glucose-free DIP, plus or minus cytochalasin B with 0.12 mCi (6.25 nmol/l) deoxy-D-glucose, 2-[³H(G)] (Perkin-Elmer, Waltham, MA, USA). Cells were washed thrice with ice-cold PBS and recovered using 500 µl of 5% TCA solution. Cells were placed in 5 ml Budget Solve Complete Counting Cocktail (Fisher Scientific, Pittsburg,

PA, USA) and counted on a Tri-Carb 2500 TR Liquid Scintillation Analyzer (Packard Bioscience).

Data analysis

Litter weight gain and dam weight were analyzed using the repeated measures procedure of SPSS (version 12.01 for Windows, SPSS Inc., Chicago, IL, USA) with genotype as the fixed variable and day *post partum* as a repeated measure within each dam. Northern and western blotting data, milk composition data, litter size, and glucose transport data were all analyzed as one-way ANOVA designs using genotype as independent variables. Conception rate data among the genotypes were compared using the Kruskal–Wallis test in SPSS. All data are presented as $\text{lsmeans} \pm \text{s.e.m.}$ Differences were considered statistically significant at $\alpha = 0.05$.

Results

Our previous work showed that IRS-1 and IRS-2 levels were developmentally regulated in the mammary gland (Lee *et al.* 2003b). Interestingly, the expression profile of IRSs was suggested to be similar to that of milk proteins such as β -casein and whey acidic protein (Rosen *et al.* 1999), with increased expression during late pregnancy, dramatically elevated levels during lactation, and rapid loss of protein expression during involution. However, this initial study had only analyzed samples in mid-lactation and could provide no indication of how rapidly the expression of IRS-1 and IRS-2 increases during lactation. To determine whether increased IRS-1 and IRS-2 protein expression was linked to the process of secretory activation, we measured the abundance of these two proteins by immunoblotting of mammary tissue extracts prepared from mice during the immediate peri-parturient period (Fig. 1). These blots demonstrated a twofold increase ($P < 0.05$) in the abundance of both IRS-1 and IRS-2 by 2 days *post partum* compared with -1 day *post partum* (Fig. 1A). Densitometric analysis demonstrated that both proteins appeared to reach a maximum level at 4 days *post partum*, while abundance of the epithelial marker keratin 8 did not change appreciably (Fig. 1B). These results support the conclusion that the expression of both IRS-1 and IRS-2 is induced in the mammary gland during secretory activation.

The observation that IRS-1 and IRS-2 expression was induced during the immediate *post partum* period, suggested that these proteins might be required for normal lactation. To test this hypothesis, lactation capacity was compared between wild-type dams and dams which were either heterozygous or null for *Irs1* and/or *Irs2* (Fig. 2). Lactation was established in these mice through the injection of pregnant mare serum gonadotropin (PMS) and human chorionic gonadotropin (HCG) coupled with timed mating. This approach allowed for the establishment of lactation not only in the wild-type mice, but also in females that were heterozygous for either or both of the *Irs* mutations or null for either *Irs1* or *Irs2*. However, although fertility was similar

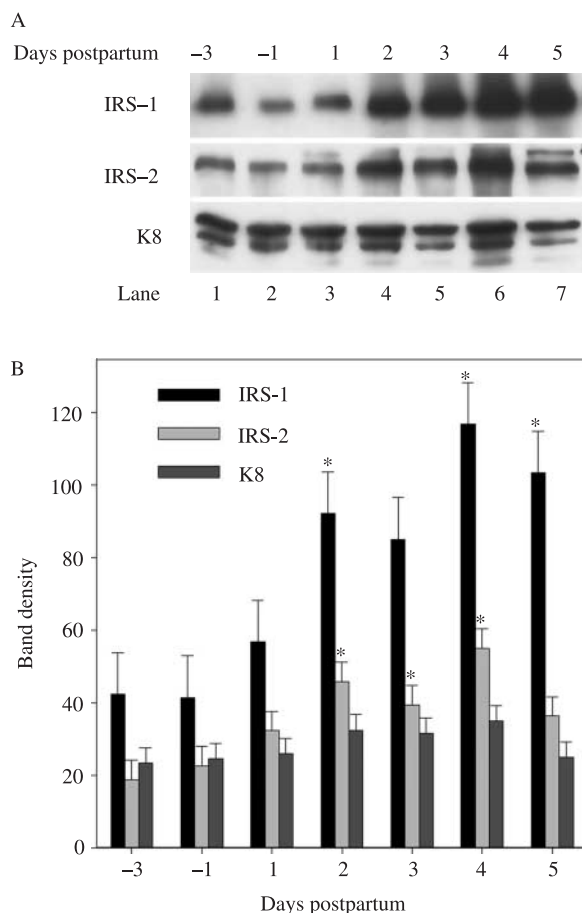


Figure 1 IRS-1 and IRS-2 protein levels are rapidly increased during secretory activation. Protein abundance of IRS-1 and IRS-2 was measured by immunoblotting (A) of mammary tissue extracts prepared from mice on days 17 (–3) and 19 (–1) of pregnancy and on days 1, 2, 3, 4, and 5 *post partum*. Immunoblotting of lysates for cytokeratin 8 (K8) was used to control for loading. Densitometry data collected from the immunoblots were used to quantify changes in abundance with time (B). Each bar represents the $\text{Ism}_{\text{means}} \pm \text{S.E.M.}$ for three animals. *represents $P < 0.05$.

among $Irs^{+/+}$ females and females that were either $Irs1^{+/-}$, $Irs2^{+/-}$, or $Irs2^{-/-}$, fertility was significantly reduced in $Irs1^{-/-}$ females. This reduction was evident not only in conception rates, but also in litter sizes. In comparison with $Irs^{+/+}$ dams, $Irs1^{-/-}$ dams had lower ($P < 0.05$) conception rates (52 vs 14% respectively) and smaller litter sizes (10 ± 1 vs 7 ± 1 respectively). This decreased fertility, though relatively minor for the first three cohorts studied, became a significant impediment to further studies of lactation in $Irs1^{-/-}$ dams. Consequently, lactation data from $Irs1^{-/-}$ dams were obtained from only the first three of the eight cohorts that were studied.

To assess lactation capacity, average pup weight during the first 10 days of lactation was measured in litters of ten pups each that were cross-fostered onto $Irs^{+/+}$ dams, or dams that were heterozygous or null for either $Irs1$ (Fig. 2A) or $Irs2$ (Fig. 2B), or

dams that were double heterozygotes ($Irs1^{+/-}Irs2^{+/-}$; Fig. 2C). Pup's weight gain was decreased in litters nursed by either $Irs1^{-/-}$ or $Irs2^{-/-}$ dams. However, pup weight was unaffected in litters nursed on $Irs1^{+/-}$, $Irs2^{+/-}$, or $Irs1^{+/-}Irs2^{+/-}$ dams. These data support the conclusion that the loss of IRS-1 or IRS-2 diminishes lactation capacity.

Comparison of the body weights among dams of the different genotypes demonstrated that $Irs1^{-/-}$ dams were significantly smaller than their wild-type littermates (Fig. 2D). Dams that were $Irs2^{-/-}$ had no discernable difference in body weight from $Irs2^{+/+}$ dams at the beginning of lactation, but failed to undergo the same weight gain during early lactation as the $Irs2^{+/+}$ and $Irs2^{+/-}$ dams (Fig. 2E). Dams that were $Irs1^{+/-}Irs2^{+/-}$ were also significantly smaller than their $Irs^{+/+}$ dams (Fig. 2F), yet they had exactly the same lactation capacity, indicating that differences in observed lactation capacity may not have been simply related to decreased body size.

In order to further understand the basis for the reduced pup litter weight gain on the Irs null dams, milk samples and mammary tissue were collected on day 10 *post partum*. Both the amount of milk recovered after oxytocin injection, and the wet weights of the number 4 mammary glands were significantly lower ($P < 0.05$) in $Irs1^{-/-}$ mice than their wild-type siblings (Fig. 3A). However, these differences were proportional to the lowered body weight and were similar among both genotypes when expressed on a body weight basis (data not shown). For $Irs2^{-/-}$ dams, mammary gland and milk weight were unchanged (Fig. 3B). In addition, comparison of hematoxylin–eosin-stained mammary tissue sections among $Irs^{+/+}$ or $Irs^{-/-}$ dams, and $Irs2^{+/+}$ or $Irs2^{-/-}$ dams revealed no alterations due to genotype (data not shown).

To determine whether the reduced lactational performance in the $Irs1^{-/-}$ dams was associated with alterations in milk composition, we measured the fat, protein, lactose, and water content of samples collected on day 10 *post partum* (Fig. 4). This analysis revealed that milk from $Irs1^{-/-}$ dams had significant, though modest, decreases ($P < 0.05$) in both water and lactose content. Fat content on the other hand tended to be higher ($P < 0.1$) in milk from $Irs1^{-/-}$ dams than that from their wild-type siblings. This result suggests that loss of IRS-1 has a modest impact on milk synthesis.

To determine whether the diminished milk synthesis capacity and altered milk lactose in $Irs1^{-/-}$ mice might be linked with alterations in milk protein gene expression or in mammary secretory cell carbohydrate metabolism, we used northern blotting to measure the mammary tissue abundance of the β -casein and α -lactalbumin mRNAs, we compared 2-deoxy-glucose (2DOG) uptake in primary mammary epithelial cells, and we measured the abundance of hexokinase I and II isoforms in mitochondrial and post-mitochondrial tissue fractions isolated from $Irs1^{+/+}$ or $Irs1^{-/-}$ mice. The loss of $Irs1$ had no impact on the mRNA abundance of β -casein and α -lactalbumin (data not shown). Uptake of

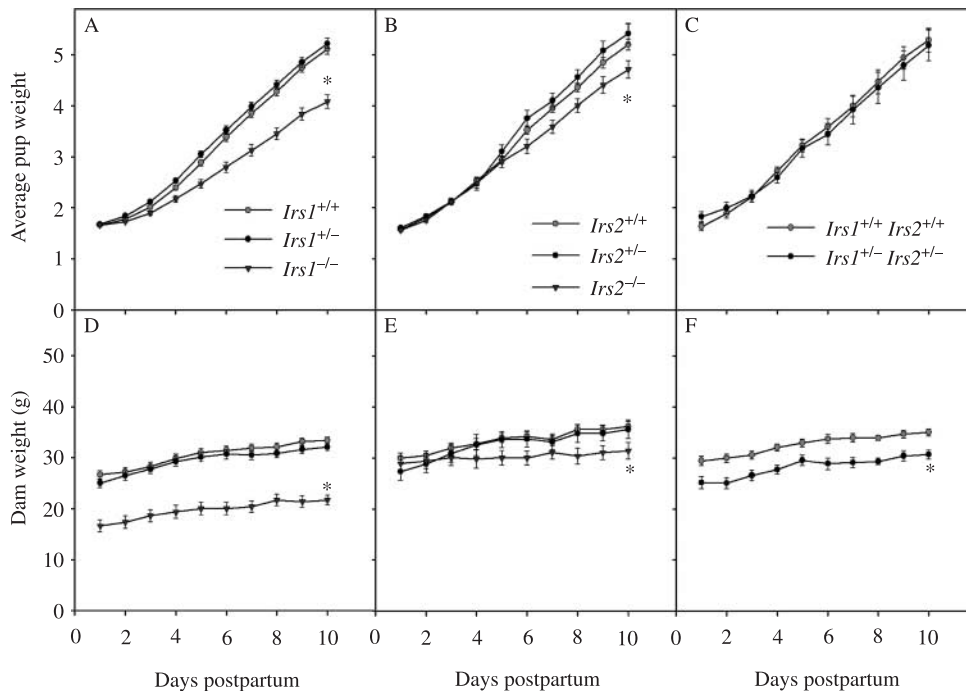


Figure 2 Lactation performance is reduced in *Irs1*^{-/-} and *Irs2*^{-/-} dams, but not in *Irs1*^{+/-} *Irs2*^{+/-} dams. Litter weight gain was measured for CD-1 cross-fostered litters (ten pups) that were nursed by dams that were wild-type (+/+), heterozygous (+/-) or null (-/-) for *Irs1* (A) or *Irs2* (B), or heterozygous for both *Irs1* and *Irs2* (C). Body weight was also compared among dams that were wildtype, heterozygous or null for *Irs1* (D), *Irs2* (E), or heterozygous for both *Irs1* and *Irs2* (F). Each symbol represents the $\text{I mean} \pm \text{S.E.M.}$ for 4–13 animals. Asterisks indicate either a significant ($P < 0.05$) main effect of genotype (D and F), or a significant day-by-genotype interaction (A, B, and E).

2DOG was similar among *Irs1*^{+/+} and *Irs1*^{-/-} cells (Fig. 5A). Specificity of 2DOG uptake was measured by comparing uptake in the presence and absence of cytochalasin B. Non-specific uptake accounted for about 30% of total 2DOG uptake. As an independent method to test for possible alterations in glucose metabolism in response to loss of IRS-1, the abundance of the two enzymes hexokinases I and II was compared among mitochondrial and cytosolic fractions prepared from mammary tissue of *Irs1*^{+/+} and *Irs1*^{-/-} dams. Western blotting was conducted for hexokinases I and II, COXIV, and cytokeratin 8 (Fig. 5B). Densitometric analysis of these blots (Fig. 5C and D) demonstrated that both the overall abundance of these enzymes and their distribution between mitochondrial and post-mitochondrial tissue fractions were similar among *Irs1*^{+/+} and *Irs1*^{-/-} mice. This result supports the suggestion that loss of IRS-1 probably had little impact on the expression of milk protein genes, glucose transport, or on the hexokinase activity and subsequent glucose phosphorylation.

To determine whether the loss of IRS-1 had any impact on insulin- or IGF-I-dependent signaling within the lactating mammary gland, we measured the phosphorylation of Akt and ERK1/2 in mammary tissue collected from 3-day lactating, insulin- or IGF-I-stimulated, dams (Fig. 6). Immunoblotting of mammary tissue extracts with

phospho-specific antibodies demonstrated increased Akt phosphorylation on both Thr³⁰⁸ and Ser⁴⁷³ in response to insulin and IGF-I (Fig. 6A and B). This induction was greatest with insulin. However, we noted significantly lower induction of Akt phosphorylation in *Irs1*^{-/-} mammary tissue compared with *Irs1*^{+/+} mammary glands. Insulin, but not IGF-I caused a modest increase in the phosphorylation of ERK1/2 on Tyr²⁰⁴ and Thr²⁰². However, this induction was similar among *Irs1*^{-/-} and *Irs1*^{+/+} mammary glands (Fig. 6B). These results suggest that insulin-dependent activation of Akt was diminished in mammary tissue from *Irs1*^{-/-} dams.

To determine whether the loss of IRS-1 impacted the ability of signaling pathways to activate IRS-2, we compared the phosphorylation and p85-binding activity of IRS-2 in *Irs1*^{+/+} and *Irs1*^{-/-} mammary glands (Fig. 7A). Immunoprecipitation of IRS-2 was followed by anti-phosphotyrosine immunoblotting. Densitometry on these blots revealed 20-fold higher IRS-2 tyrosine phosphorylation in mammary tissue from *Irs1*^{-/-} dams than that in *Irs1*^{+/+} dams (Fig. 7B). This data therefore show that the loss of IRS-1 attenuates insulin-induced Akt phosphorylation, but that there is a compensatory increase in insulin-stimulated IRS-2 phosphorylation in IRS-1 null mammary glands.

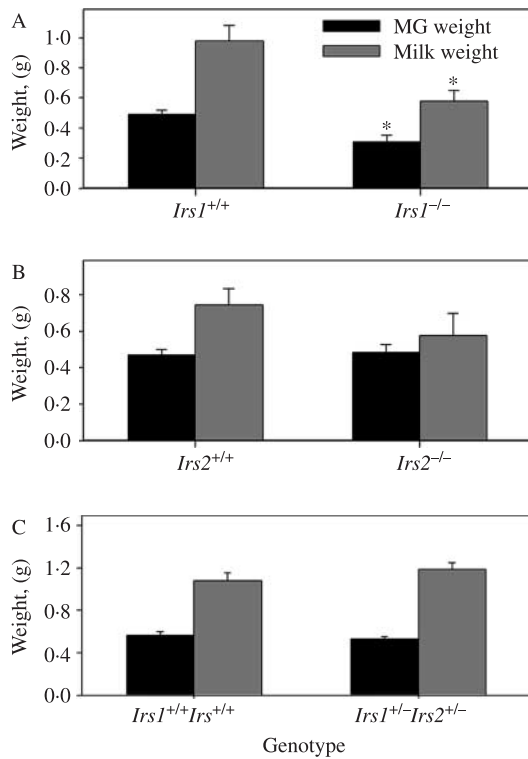


Figure 3 Mammary gland wet weight, and milk volume recovered following oxytocin administration, is decreased in *Irs1*^{-/-} dams. Wet weight of the number 4 mammary gland and weight of milk recovered from all ten glands following oxytocin treatment were compared among *Irs1*^{+/+} and *Irs1*^{-/-} dams (A), *Irs2*^{+/+} and *Irs2*^{-/-} dams (B), and *Irs1*^{+/+}*Irs2*^{+/+} and *Irs1*^{+/+}*Irs2*^{+/-} dams (C). Each bar represents the mean \pm S.E.M. for 4–13 mice. Asterisks indicate a significant decrease ($P < 0.05$) in comparison with wildtype.

Discussion

The requirement of insulin for normal lactation has been illustrated in animal models in a variety of ways (Walters & McLean 1968a,b, Martin & Baldwin 1971, Kunjara *et al.* 1986, Lau *et al.* 1993). Thus, a reasonable hypothesis is that key mediators of insulin signaling might be necessary to establish or maintain a normal lactation. To date, there have been only limited attempts at establishing the functional importance of specific insulin signaling molecules to the process of lactogenesis (Li *et al.* 2002, Schwertfeger *et al.* 2003). Our studies make several novel observations concerning the importance of IRS proteins to lactation. First, mammary expression of both IRS-1 and IRS-2 was doubled by 48 h after the onset of lactation. Secondly, the loss of both IRS-1 and IRS-2 caused decreased lactation capacity. Thirdly, the loss of IRS-1 caused a modest decrease in both milk water content and lactose concentration. Fourthly, the loss of IRS-1 decreases insulin-dependent induction of Akt phosphorylation. Fifthly, the loss of IRS-1 resulted in an increase in the phosphorylation of IRS-2 and increased association of IRS-2 with the p85 regulatory subunit of PI3 kinase. Finally, these

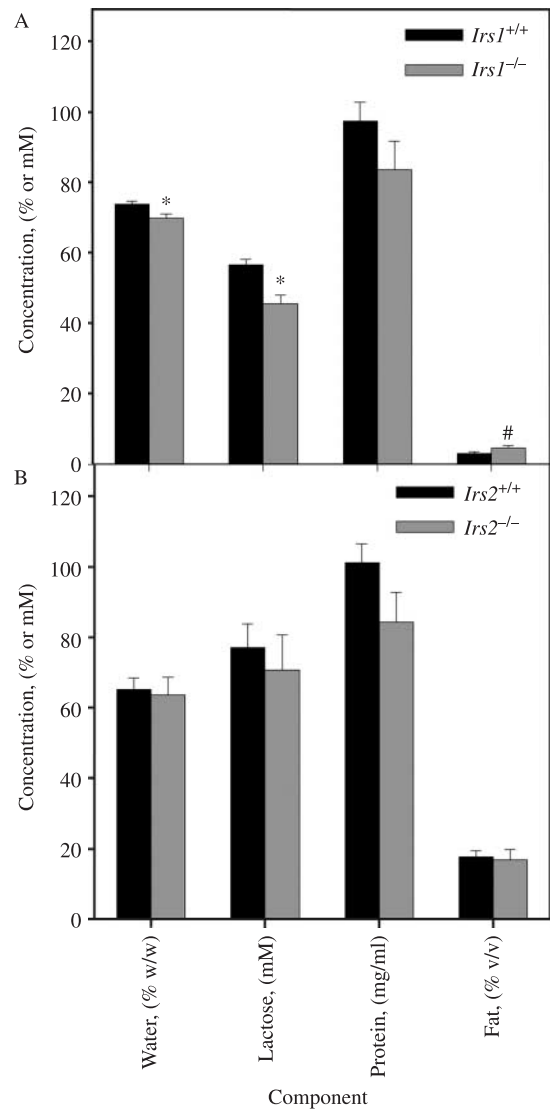


Figure 4 Water and lactose are modestly reduced in milk from *Irs1*^{-/-} dams. Water, fat, lactose, and protein were measured in milk samples collected from *Irs1*^{+/+} and *Irs1*^{-/-} dams (A), and from *Irs2*^{+/+} and *Irs2*^{-/-} dams (B) on day 10 *post partum* (A). Each bar represents the mean \pm S.E.M. for 5 and 11 *Irs1*^{-/-} and *Irs1*^{+/+} dams, and 5 and 11 *Irs2*^{+/+} and *Irs2*^{-/-} dams respectively. Asterisks indicate a statistically significant ($P < 0.05$) decrease.

changes in lactation capacity, milk composition, and mammary cell signaling were associated with little or no change in milk protein gene expression, mammary glucose transport, or in the abundance and subcellular localization of hexokinases I and II. Taken together, the results support our hypothesis that IRS-1 and IRS-2 are important for normal lactation, but they also suggest that compensatory mechanisms within the secretory epithelium may prevent the appearance of a more dramatic phenotype in animals with a single deletion of either IRS-1 or IRS-2.

The induction of IRS protein expression during the first few days of lactation is consistent with previous studies published in our laboratory which demonstrated higher levels of IRS protein expression during mid-lactation compared with late pregnancy (Lee *et al.* 2003b). The immunoblot results in the current study extend that observation by demonstrating that this upregulation of IRS-1 and IRS-2 is

an early event that is temporally linked with the process of secretory activation. The published results on other components of the insulin signaling pathway have also demonstrated increased expression of both the insulin receptor and Akt with the onset of lactation, suggesting that the coordinated upregulation of multiple insulin signaling pathway components may occur during the early *post partum* period (Flint 1982, Schwertfeger *et al.* 2003).

The fact that the loss of IRS-1 or IRS-2 resulted in decreased lactation capacity, as measured by the ability of the dams to support the growth of cross-fostered litters, also supports the suggestion that these two proteins are important to lactation. However, neither one alone was absolutely necessary to lactation since null animals could still lactate albeit at reduced capacity. This observation coupled with the observation that tyrosine phosphorylation of IRS-2 was increased in the mammary tissue of IRS-1 knockout dams supports the idea that compensatory interactions exist between IRS-1 and IRS-2 within the mammary gland. Although this phenomenon of compensatory interaction between IRS-1 and IRS-2 has been demonstrated in earlier studies, it appears to be tissue specific (Araki *et al.* 1994, Yamauchi *et al.* 1996). In other studies on compensatory interactions between IRS-1 and IRS-2, the phenomenon was clearly indicated by genetic crosses in which both genes were targeted in the same animal (Withers *et al.* 1999, Kido *et al.* 2000, Miki *et al.* 2001). Although similar crosses were made for the purpose of testing the extent of compensatory interactions between IRS-1 and IRS-2 in the mammary gland, only double heterozygous progeny were fertile.

The fact that body weight was significantly decreased in both the IRS-1 and IRS-2 null dams suggests that at least some of the lactation phenotype may be attributable to the fact that the dams are smaller. In this regard, there is clearly a link between mature body weight and lactation capacity not only in mice but also within other species. More specifically, selection for milk production capacity produces larger animals in both mice and cows (Nagai & Sarkar 1978, Hansen 2000). However, the difference in body weight and lactation capacity in the *Irs1*^{-/-} and *Irs2*^{-/-} mice is somewhat mitigated by the fact that

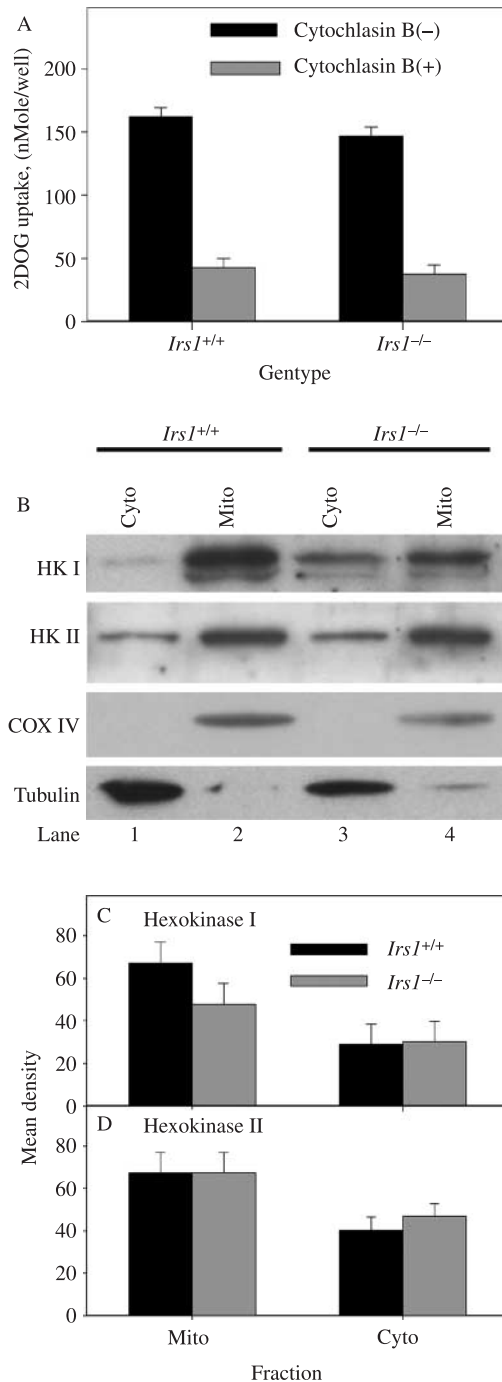


Figure 5 Mammary cell glucose uptake and mammary abundance and localization of hexokinases (HKs) I and II are unaffected by loss of the *Irs1* gene. Mammary epithelial cells were isolated from mice at 16 days of pregnancy and then plated in serum fetuin-coated plastic six-well plates. Total glucose uptake was measured by incubating the cells with 2DOG for 15 min (A). Cytochalasin B was included in some of the wells to provide a measurement of non-specific uptake. Western blotting (B) of mammary mitochondrial and cytoplasmic fractions was used to determine whether the loss of IRS-1 had any impact on HK localization. Purity of the fractions was demonstrated by probing the blots for cytochrome *c* oxidase subunit IV (COXIV) and tubulin. Densitometry was used to quantify the mitochondrial (mito) and cytoplasmic (cyto) abundance of HKI (C) and HKII (D) in fractions prepared from *Irs1*^{+/+} and *Irs1*^{-/-} mammary tissue. In (A), each bar represents the mean \pm S.E.M. for three wells. In (C) and (D), each bar represents the mean \pm S.E.M. for five mice.

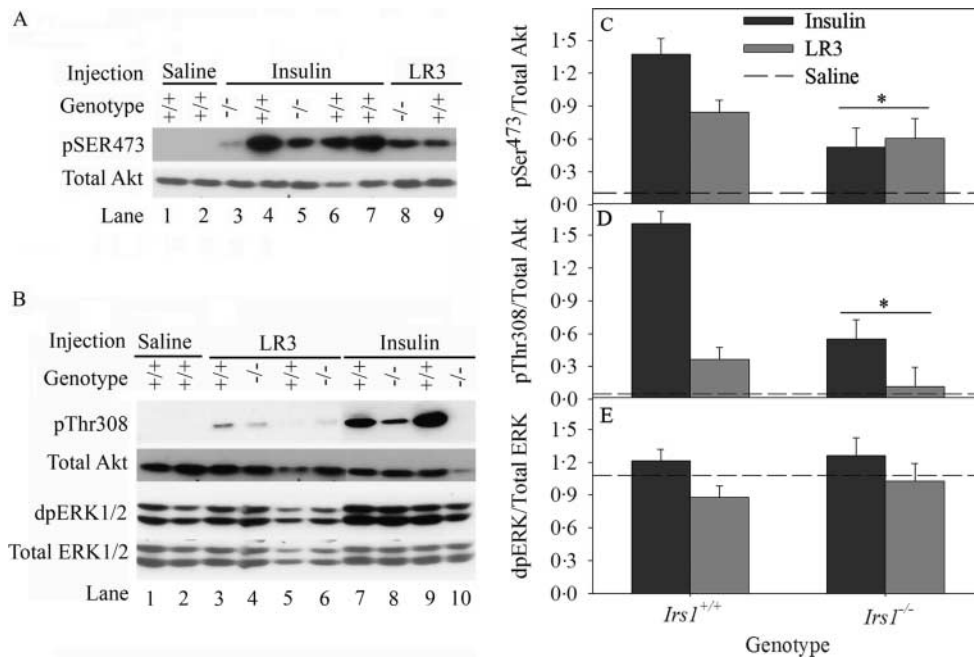


Figure 6 Insulin- and LR3-IGF-I-dependent phosphorylation of Akt is decreased in mammary tissue from *Irs1*^{-/-} dams. Protein phosphorylation was measured by western blotting of mammary tissue extracts prepared from 3-day lactating *Irs1*^{+/+} (+/+) or *Irs1*^{-/-} (-/-) mice. On day 3 *post partum*, the mice were fasted for 8 h, separated from their litters for 2 h, and then given a tail-vein injection of either saline, insulin, or LR3-IGF-I (LR3). Tissue was harvested at 5 min following injection. Phosphorylation of Akt was measured with antibodies specific for Ser⁴⁷³ (A) and Thr³⁰⁸ (B). Phosphorylation of MAPK4/3, ERK 1/2 (B) was measured with an antibody that detects dual phosphorylation at Thr²⁰² and Tyr²⁰⁴. Densitometry (C, D, and E) was used to quantitate the signal intensities in each of the blots. Each bar represents the mean \pm S.E.M. for two to four mice. * indicate a statistically significant ($P < 0.05$) genotype effect.

Irs1^{+/-}*Irs2*^{+/-} mice were significantly smaller than their *Irs1*^{+/+}*Irs2*^{+/+} littermates, but both sets of mice had identical lactation capacity. This result would indicate that either there is a threshold for the effect of body weight on lactation capacity or that at least part of the lactation defect in the *Irs1*^{-/-} dams was intrinsic to the mammary gland. Subsequent analysis of the mammary tissue from these mice was intended to determine whether the lactation defect could be at least partially attributable to mammary-specific effects. Although not all of these measurements supported the presence of a mammary-specific defect in the *Irs1*^{-/-} mice, one of them did. The phosphorylation of mammary Akt in response to an insulin or IGF-I challenge was decreased in *Irs1*^{-/-} mice.

The fact that IRS-1 dams displayed reduced lactation capacity along with decreased concentrations of milk lactose suggested that the mammary cells isolated from these mice could have had decreased glucose transport capacity or altered glucose metabolism. In mammary secretory cells, insulin does not acutely stimulate glucose transport, but is required for the maintenance of transport capacity (Prosser *et al.* 1987, Nemeth *et al.* 2000). In addition, the expression of hexokinase II increases dramatically during secretory activation and treatment of lactating rats with neutralizing

antisera to insulin has been shown to cause an alteration in the subcellular distribution of mammary hexokinase activity in conjunction with a reduction in glycolytic activity (Walters & McLean 1968a,b). Consequently, we were somewhat surprised to find that the loss of IRS-1 had no perceptible impact on mammary on glucose uptake or mitochondrial hexokinase abundance. This surprise was even greater in light of the fact that one of the mammary-specific endpoints which was affected by the loss of IRS-1 was insulin-dependent induction of Akt phosphorylation.

The *Irs2* gene was originally identified because of a residual substrate protein that was phosphorylated in response to insulin in *Irs1*^{-/-} mice (Kadowaki *et al.* 1996). Subsequent studies have demonstrated numerous instances of compensation between IRS-1 and IRS-2, however, the proteins clearly have both unique and conserved function. We found a compensatory increase in IRS-2 activity in insulin-stimulated IRS-1 null mammary glands. However, despite this induction of IRS-2 phosphorylation, the loss of IRS-1 was clearly associated with decreased insulin-dependent phosphorylation of Akt at both Thr³⁰⁸ and Ser⁴⁷³. The loss of insulin signaling in *Irs1*^{-/-} mice has previously been reported for a number of tissues (Valverde *et al.* 1999, Ueki *et al.* 2000). However, some tissues such as

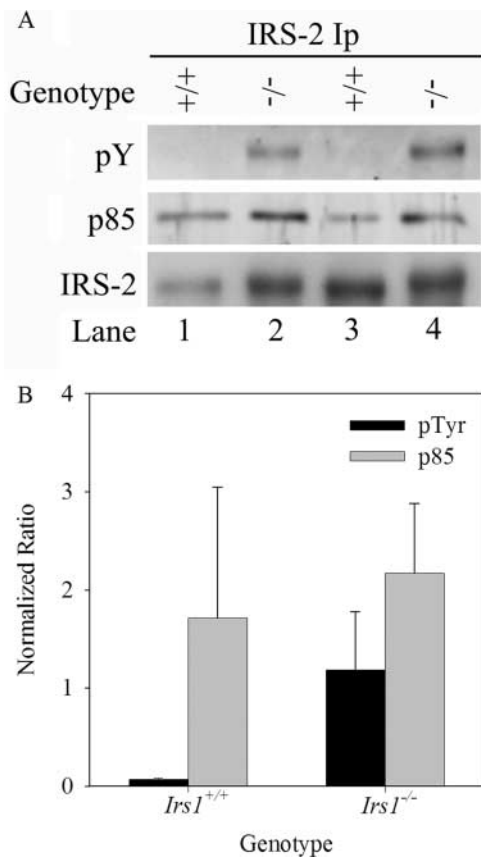


Figure 7 Increased tyrosine phosphorylation and p85 binding of IRS-1 in mammary tissue from lactating *Irs1*^{-/-} females. Tyrosine phosphorylation (pY) of IRS-2 and association of IRS-2 with p85 were measured by western blotting of IRS-2 immunoprecipitates prepared from insulin-injected *Irs1*^{+/+} or *Irs1*^{-/-} mice (A). Each lane is loaded with an extract from one animal. Densitometry analysis (B) demonstrated that tyrosine phosphorylation, but not p85 association, was increased for IRS-2 in the *Irs1*^{-/-} mammary tissue. The normalized ratio is the ratio of integrated optical density for phosphotyrosine to that of total IRS-2. This ratio was 20-fold greater in *Irs1*^{-/-} mammary tissue than in *Irs1*^{+/+} mammary tissue. Each bar represents the mean \pm s.d. for two mice.

white adipose tissue or brown preadipocytes are still capable of exhibiting insulin-stimulated Akt phosphorylation even with the loss of IRS-1. Although the basis for this tissue specificity is not clear, it appears that the mammary cells depend on IRS-1 for insulin-dependent Akt phosphorylation.

In summary, we have shown that both *Irs1*^{-/-} and *Irs2*^{-/-} mice show reduced lactation capacity. The loss of IRS-1 is associated with minor alterations in milk composition but no change in milk protein mRNAs, glucose transport, or the abundance and subcellular localization of hexokinases I and II. The minor lactation phenotype may in part be explained by an apparent compensatory increase in insulin-induced IRS-2 phosphorylation. Further studies are required to determine the impact of loss of both IRS-1 and IRS-2 on lactation capacity.

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