Tissue-specific Alterations of Glucose Transport and Molecular Mechanisms of Intertissue Communication in Obesity and Type 2 Diabetes

Abstract
Insulin resistance plays a major role in the pathogenesis of type 2 diabetes. Insulin regulates blood glucose levels primarily by promoting glucose uptake from the blood into multiple tissues and by suppressing glucose production from the liver. The glucose transporter, GLUT4, mediates insulin-stimulated glucose uptake in muscle and adipose tissue. Decreased GLUT4 expression in adipose tissue is a common feature of many insulin resistant states. GLUT4 expression is preserved in skeletal muscle in many insulin resistant states. However, functional defects in the intracellular trafficking and plasma membrane translocation of GLUT4 result in impaired insulin-stimulated glucose uptake in muscle. Tissue-specific genetic knockout of GLUT4 expression in adipose tissue or muscle of mice has provided new insights into the pathogenesis of insulin resistance. We recently determined that the expression of serum retinol binding protein (RBP4) is induced in adipose tissue as a consequence of decreased GLUT4 expression. We found that RBP4 is elevated in the serum of insulin resistant humans and mice. Furthermore, we found that increasing serum RBP4 levels by transgenic overexpression or by injection of purified RBP4 protein into normal mice causes insulin resistance. Therefore, RBP4 appears to play an important role in mediating adipose tissue communication with other insulin target tissues in insulin resistant states.

Introduction
A major goal of our laboratory has been to understand how alterations in the expression or function of GLUT4, the principal insulin-stimulated glucose transporter, influence whole body insulin-glucose homeostasis and contribute to the development of insulin resistant states such as obesity and type 2 diabetes. GLUT4 is expressed primarily in adipose tissue, skeletal muscle, and cardiac muscle with much lower levels in discrete areas of the brain and kidney [1]. GLUT4 mRNA and protein are downregulated in adipose tissue in the setting of obesity or type 2 diabetes in both humans and rodents [2]. In contrast, skeletal muscle GLUT4 expression remains intact in these conditions, but alterations in the distribution of GLUT4 between intracellular membranes and the plasma membrane and in insulin-stimulated translocation of GLUT4 to the plasma membrane results in impaired glucose transport [2]. Muscle is the major site for insulin-stimulated glucose uptake; in contrast, adipose tissue contributes minimally to whole body insulin-stimulated glucose disposal [2]. These observations raised questions regarding the relationship between downregulation of GLUT4 expression specifically in adipocytes and insulin resistance in obesity and type 2 diabetes.

Tissue specific alterations of GLUT4 expression
To better understand the role of GLUT4 in adipose tissue, we developed mouse models in which expression of GLUT4 was selectively altered in adipose tissue or muscle of mice. We recently determined that the expression of serum retinol binding protein (RBP4) is induced in adipose tissue as a consequence of decreased GLUT4 expression. We found that RBP4 is elevated in the serum of insulin resistant humans and mice. Furthermore, we found that increasing serum RBP4 levels by transgenic overexpression or by injection of purified RBP4 protein into normal mice causes insulin resistance. Therefore, RBP4 appears to play an important role in mediating adipose tissue communication with other insulin target tissues in insulin resistant states.
pose-GLUT4-Tg mice [4]). The mice exhibit 10–20-fold elevation of GLUT4 protein in white adipose tissue and brown adipose tissue [4]. Basal glucose transport in white adipocytes from adipose-GLUT4-Tg mice studied ex vivo is 20-fold increased and there is a further increase in glucose transport after insulin stimulation [4]. In vivo, at physiological glucose concentrations, insulin-stimulated glucose transport is increased ~2-fold in adipose-GLUT4-Tg mice [4].

In addition to the opposite alterations of GLUT4 expression in adipose tissue, adipose-GLUT4-KO mice and adipose-GLUT4-Tg mice exhibit reciprocal metabolic phenotypes. Adipose-GLUT4-KO mice have normal body weight and exhibit insulin resistance and glucose intolerance [3]. Under euglycemic hyperinsulinemic clamp conditions, adipose-GLUT4-KO mice have defects in insulin-stimulated glucose uptake in skeletal muscle and insulin-mediated suppression of glucose production in liver [3]. Signaling studies confirmed that insulin-stimulated phosphatidylinositol 3-kinase activity is impaired in both muscle and liver of adipose-GLUT4-KO mice [3]. In contrast, adipose-GLUT4-Tg mice have increased adiposity, due to increased numbers of adipocytes, but are more insulin sensitive and develop hypoglycemia when food is restricted [4,5]. When normal mice are treated with the beta cell toxin, streptozotocin, they develop diabetes due to insulin deficiency. They also become insulin resistant secondary to hyperglycemia and other metabolic derangements. However, when adipose-GLUT4-Tg mice are treated with streptozotocin, they are partially protected from hyperglycemia-associated insulin resistance compared to nontransgenic mice treated with streptozotocin [6]. Therefore, the level of GLUT4 expression in adipose tissue can exert dominant effects to alter whole body insulin-glucose homeostasis by altering insulin action in other tissues.

We have also generated mice with Cre/lox-mediated muscle specific knockout of GLUT4 expression (muscle-GLUT4-KO mice [7]). These mice develop insulin resistance and glucose intolerance at an early age [7]. Muscle-GLUT4-KO mice secondarily develop insulin resistance in adipose tissue and liver, in part due to chronic effects of hyperglycemia [7]. We bred muscle-GLUT4-KO mice with adipose-GLUT4-KO mice to generate adipose/muscle-GLUT4 double knockout mice (adipose/muscle-GLUT4-KO mice [8]). Surprisingly, we found that the degree of fasting hyperglycemia and glucose intolerance of adipose/muscle-GLUT4-KO mice is comparable to that observed in mice with absence of GLUT4 only in muscle or adipose tissue (i.e., in either the adipose-GLUT4-KO or the muscle-GLUT4-KO mice [3, 7, 8]). The adipose/muscle-GLUT4-KO mice exhibit hyperinsulinemia, but only modestly elevated blood glucose on a normal chow diet [8]. So, the combined effects of adipose tissue and muscle GLUT4 knockout are neither additive nor synergistic.

To better understand how the adipose/muscle-GLUT4-KO mice maintain glucose homeostasis without developing frank diabetes, we performed an in depth analysis of their fuel utilization characteristics. Adipose/muscle-GLUT4-KO mice cleared an oral lipid load more rapidly. This, combined with a reduced respiratory quotient during both the light and dark phases, indicated that these animals have adapted to utilize lipid substrates more efficiently [8]. This switch in fuel utilization was accommodated through quite profound adaptations of the liver. Glucokinase expression and glucose uptake were increased in liver [8]. Acetyl CoA carboxylase expression was increased 1.6-fold and hepatic de novo fatty acid synthesis and glucose incorporation into fatty acids were increased (2-fold and 3-fold, respectively [8]). And finally, hepatic triglyceride synthesis and release were increased [8]. These changes may reflect activation of a hepatic lipogenic gene program mediated by the transcription factor, SREBP1c, which was increased by ~2-fold in the liver of the adipose/muscle-GLUT4-KO mice [8]. Therefore, specifically-defined genetic defects in adipose tissue and muscle created changes in programs of gene expression and metabolism in the liver that allowed for glucose – which could not be transported into adipose tissue or muscle – to be transformed into lipid fuels for muscle [8].

In summary, it appears that no matter which tissue harbors the primary genetic defect of GLUT4 expression, the other insulin target tissues are ultimately affected. So, muscle and liver are affected by adipose-GLUT4 knockout, adipose tissue and liver are affected by muscle-GLUT4 knockout, and liver is affected by the combined adipose tissue- and muscle-GLUT4 knockout.

**RBP4, a molecular mediator of intertissue communication**

A remarkable feature of the adipose-GLUT4-KO mouse model is that the insulin resistance in muscle is no longer evident when individual hind limb muscles are dissected and studied ex vivo [3]. This led us to hypothesize that the impairment of insulin action in muscle in vivo might be mediated by a circulating factor that is eliminated when the muscle is isolated and studied ex vivo. We further hypothesized that such a circulating factor might be a secreted protein or lipid produced by adipose tissue from adipose-GLUT4-KO mice as a consequence of decreased GLUT4 expression and/or the impairment of insulin-stimulated glucose transport. We found that the serum levels of potential lipid mediators of insulin resistance, including free fatty acids and triglycerides, were normal in the adipose-GLUT4-KO mouse [3]. Furthermore, triglyceride content of muscle and liver of the adipose-GLUT4-KO mouse is not altered [3]. The serum levels of several previously identified adipose tissue-secreted proteins implicated in the regulation of insulin-glucose homeostasis (i.e., adipokines such as leptin) were found to be normal ([3] and unpublished data). Therefore, it appeared that a potentially novel mediator of insulin resistance might be present in the circulation of adipose-GLUT4-KO mice. As a proof of principal, we incubated differentiated 3T3L1 adipocytes overnight with serum from adipose-GLUT4-KO mice. Treatment of the cultured adipocytes with serum from adipose-GLUT4-KO mice inhibited insulin-stimulated glucose transport in a dose-dependent manner, whereas treatment with serum from wild type littermate mice did not (unpublished data). Attempts to purify the factor present in adipose-GLUT4-KO serum mediating this effect were hampered by lack of sufficient serum to perform serial purifications on the basis of the activity.

As an alternative approach, we performed global gene expression analysis in white adipose tissue of adipose-GLUT4-KO mice and adipose-GLUT4-Tg mice. Since the two lines of mice have opposite alterations of GLUT4 expression and inverse metabolic phenotypes, we focused our efforts on identifying genes with inverse patterns of expression in the two models. By looking at only mRNAs that were inversely regulated (i.e., increased in the adipose-GLUT4-KO mouse but decreased in the adipose-GLUT4-Tg mouse, or vice-versa), we could narrow down the number of candidate genes. Furthermore, we looked for mRNAs encoding proteins either known to be secreted or proteins containing a traditional secretory sequence tag. We then began a process of investigating the potential for the proteins encoded by each of the genes we found to mediate systemic insulin resistance. One of these proteins was the serum retinol binding protein (RBP4, [9]). RBP4 is a 21 kiloDalton serum protein expressed mainly in liver and adipose tissue [10]. It is a member of the lipocalin family, a
class of proteins that bind and transport small lipophilic or amphipathic molecules such as fatty acids, steroids, bilins, and retinoids [11]. RBP4 is the sole specific serum transport protein for retinol (vitamin A). Retinol delivered to tissues is metabolized into retinoic acid which regulates the expression of at least 300 different genes. RBP4 circulates in a 78 kiloDalton complex with transthyretin (also known as pre-albumin), and formation of this complex stabilizes RBP4 in serum by preventing glomerular filtration of the relatively smaller RBP4 [12].

Quantitative real-time PCR confirmed that RBP4 mRNA is increased 2- to 3-fold in adipose tissue of adipose-GLUT4-KO mice [9]. Liver has traditionally been considered the main source of circulating RBP4; however, we found that RBP4 mRNA expression is unaltered in liver of adipose-GLUT4-KO mice [9]. We found that serum levels of RBP4 are elevated in multiple different mouse models of insulin resistance, including mice on a high fat diet and mice with monogenic forms of obesity [9]. Ob/ob mice, which lack leptin [13], exhibited the greatest elevation of serum RBP4, approximately 10-fold over lean ob/+ control mice [9]. Even wild type mice on a high fat diet exhibited a ~3-fold elevation of serum RBP4 in comparison to mice on a normal chow diet [9]. Therefore, elevation of serum RBP4 appears to be a feature common to many different models of insulin resistance with both genetic and dietary etiologies.

We hypothesized that if the elevation of RBP4 is related to insulin resistance, then insulin sensitizers should reverse it. To test this, we treated adipose-GLUT4-KO knockout mice with the insulin-sensitizing drug, rosiglitazone, by gavage for 3 weeks. Rosiglitazone treatment lowered serum RBP4 levels in the adipose-GLUT4-KO mice to the levels of the untreated wild type littermate control mice [9]. However, rosiglitazone treatment did not alter serum RBP4 levels in the non-insulin resistant wild type littermate control mice [9]. RBP4 mRNA in adipose tissue was increased about 3 fold in adipose-GLUT4-KO mice compared to controls before rosiglitazone treatment and markedly improved after rosiglitazone treatment [9]. There was however no change in RBP4 mRNA in the liver [9], so it appears that RBP4 expression is altered specifically in adipose tissue in this insulin resistant state.

What about RBP4 in human diabetes? In studies of micronutrient status in people with type 2 diabetes, there are several reports of elevated serum RBP4 levels in human type 2 diabetics [14, 15]. The relationship between insulin resistance and RBP4 was not studied. Further supporting a potential role for RBP4 in human disease, the human RBP4 gene locus on 10q23 is located within a known “hotspot” for type 2 diabetes established by two separate linkage analyses, one study among Mexican Americans [16] and another study among American Caucasians in the Framingham Offspring Study [17].

We measured serum RBP4 in lean subjects, obese nondiabetic subjects, and obese subjects with diabetes (in collaboration with Drs. Theodore P. Ciaramaldi and Robert R. Henry, VA-SD and University of California San Diego). In this cohort, every insulin resistant person was found to have elevated serum RBP4 [9]. Serum RBP4 levels were increased on average by ~80% in both obese nondiabetic and obese diabetic subjects [9, 18], indicating that obesity-related insulin resistance and not diabetes, per se, is associated with elevations of serum RBP4. We sought to relate serum RBP4 levels to clinical components of the metabolic syndrome in these subjects. We found a high degree of correlation of RBP4 with BMI and fasting insulin levels, and an inverse correlation of RBP4 with glucose disposal rate during euglycemic hyperinsulinemic clamp, indicating that more insulin resistant people have higher RBP4 levels [18]. Serum triglycerides and hemoglobin A1c also correlated with serum RBP4 levels [18]. To determine whether lowering serum RBP4 improves insulin sensitivity, we studied mice with a targeted genetic deletion of RBP4 (RBP4 KO mice [21]). RBP4 KO mice had previously been generated by Drs. William Blaner and Max Gottesman (Columbia University) for the study of retinol transport. RBP4 KO mice have no detectable serum RBP4 and are hyperglycemic [21]. The homozygous RBP4 KO mice display ~50% reduced levels of serum RBP4 as compared with wild type littermates [21]. The homozygous RBP4 KO mice exhibit normal body weight, as compared to wild type control mice [9, 20]. However, RBP4 KO mice have elevated fasting plasma glucose and insulin resistance by insulin tolerance testing [9]. Retinol and its retinoic acid metabolites are very important in development, and therefore we sought to rule out the possibility that serum RBP4 causes insulin resistance solely through developmental effects of the RBP4 transgene. Therefore, we purified recombinant RBP4 and injected it into wild type adult mice 3 times a day for 3 weeks. Injection of RBP4 resulted in approximately 3-fold elevation of RBP4 over baseline levels and caused insulin resistance and glucose intolerance [9]. Together, these findings confirm that elevation of serum RBP4 does in fact cause insulin resistance and hyperglycemia in mice.

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play lower levels of free fatty acids, but fed-state glucose, insulin, leptin, adiponectin, and resistin levels are normal [9].

To obviate potential developmental effects of the RBP4 gene knockout, we treated wild type adult mice with the drug fenretinide [N-(4-hyroxylphenyl)retinamide]. Fenretinide has a structure that is similar to retinol but contains a bulky hydrophobic group [22]. Fenretinide binds to the retinol binding pocket of RBP4 with high affinity, and the bulky hydrophobic group displaces RBP4 from its serum binding partner transthyretin [22]. This results in rapid glomerular filtration and renal excretion of RBP4 [23]. We fed wild type mice a high fat diet either with or without fenretinide. Admixture of fenretinide did not affect food intake, or weight gain on the high fat diet up to 16 weeks of treatment [9]. Mice on the high fat diet without fenretinide, developed 2–3-fold elevation of serum RBP4 [9]. Fenretinide treatment prevented the elevation of serum RBP4 levels and did not affect weight gain on the high fat diet [9]. Fenretinide-treated mice on high fat diet displayed normal glucose tolerance and improved insulin sensitivity [9]. Therefore, targeted pharmacological lowering of serum RBP4 in adult mice which are obese due to a high fat diet improves insulin action. These findings also suggest that disruption of the serum RBP4-transthyretin complex may be a useful strategy for treating insulin resistance. \( \text{Fig. 1} \)

**Potential mechanisms of RBP4-induced insulin resistance**

How does RBP4 cause insulin resistance? We had previously reported that the adipose-GLUT4 KO mice display a post-receptor impairment of insulin signaling in muscle and liver at the level of insulin-stimulated PI 3-kinase activity ([3] and unpublished data). We therefore sought to determine whether insulin signaling was similarly impaired in the muscle or liver of mice with increased or decreased serum RBP4. In RBP4 Tg mice and in mice injected with purified RBP4, we found reduced insulin-stimulated PI 3-kinase activity in muscle but not in liver. We also found in muscle reduced insulin-stimulated tyrosine phosphorylation of IRS-1 at amino acid residue 612, an important docking site for the p85 subunit of PI 3-kinase [9]. Conversely, RBP4 KO mice displayed increased insulin-stimulated PI 3-kinase activity and increased IRS-1 tyrosine 612 phosphorylation [9]. Phosphorylation of the insulin receptor was not altered in these models [9]. Therefore, specific genetic or pharmacological manipulations to increase or decrease RBP4 appear to regulate insulin signaling in skeletal muscle at the level of PI 3-kinase activation, and this is consistent with the defect we observed in the adipose-GLUT4 KO mice.

However, in contrast to the adipose-GLUT4 KO mice, we found that mice with increased levels of serum RBP4 (i.e., RBP4 Tg mice or RBP4-injected mice) do not display impairment of insulin stimulated PI 3-kinase activity or IRS-1 tyrosine 612 phosphorylation in liver [9]. We therefore hypothesized that RBP4 might alter insulin action through a different mechanism in the liver. It has been reported that dietary retinol and its metabolite retinoic acid can regulate gluconeogenic enzymes in the liver [9]. We found increased expression of PEPCK mRNA in mice injected with RBP4 [9]. To determine whether RBP4 may directly influence glucose production we treated cultured H4IIe hepatocytes with purified RBP4 in vitro. RBP4 treatment induced expression of PEPCK protein, increased basal glucose production, and reduced the ability of insulin to suppress glucose production in these cells [9]. These findings indicate that RBP4 may contribute to glucose intolerance and insulin resistance through direct effects on hepatic glucose production.

**Further exploration into mechanisms of RBP4 induced insulin resistance**

We are presently approaching the question of how RBP4 causes insulin resistance by considering the possibility that RBP4 may...
act through both retinol-dependent and retinol-independent mechanisms. Retinol dependent mechanisms might involve retinoic acid receptor action [11]. In support of a retinol-dependent mechanism, we found elevation of mRNA of several retinoic acid-responsive genes, including PEPCk in liver and retinoic acid receptor β1, acetyl coA carboxylase β1, and stearoyl coA desaturase-1 in skeletal muscle [9]. Retinol independent mechanisms might conceivably involve binding of RBP4 to a specific cell surface receptor [24,25] – which has not yet been identified – modulation of biological activities of RBP4’s binding partner, transthyretin, or transport of ligands other than retinol.

Conclusions

Insulin-stimulated glucose transport mediated by GLUT4 in adipose tissue and muscle is a critical regulator of whole body insulin-glucose homeostasis. Impaired expression or function of GLUT4 in adipose tissue, muscle, or both tissues can induce insulin resistance and other metabolic adaptations in other target tissues of insulin action, including liver. Alterations of GLUT4 expression may result in aberrant glucose flux and/or release of endocrine mediators that regulate insulin action and glycemia systemically. RBP4, a novel adipokine produced as a consequence of decreased GLUT4 expression in adipose tissue, is increased in the serum of insulin resistant humans and rodents and mediates insulin resistance in muscle and increased glucose production in liver. Further studies are needed to define the molecular basis of RBP4 action and how it contributes to the pathogenesis of insulin resistance and type 2 diabetes.

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Note Added in Proof:
Since submission of the manuscript, several important advances have occurred. 1) We reported that RBP4 mRNA is increased in adipocytes of insulin resistant human subjects and expressed in inverse proportion to GLUT4 in visceral adipose tissue (Klöting N, et al. Cell Metab 2007; 6: 79-87). This is consistent with our findings in perigonadal fat of adipose-GLUT4 KO mice [9]. 2) In addition, polymorphisms in the human RBP4 gene were reported to be associated with elevated serum RBP4, insulin resistance, and/or type 2 diabetes (Craig RL, et al., Mol Genet Metab 2007; 90: 338-344; Munkhtulga L, et al., Hum Genet 2007; 120: 879-88; and Kovacs P, et al., Diabetes 2007; Epub in press). 3) A putative membrane receptor for RBP4 was identified (Kawaguchi R, et al. Science 2007; 315: 820-825).

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