

Normal Akt/PKB with reduced PI3K activation in insulin-resistant mice

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Nadler, Samuel T., Jonathan P. Stoehr, Mary E. Rabaglia, Kathryn L. Schueler, Morris J. Birnbaum, and Alan D. Attie. Normal Akt/PKB with reduced PI3K activation in insulin-resistant mice. *Am J Physiol Endocrinol Metab* 281: E1249–E1254, 2001.—Insulin stimulates muscle and adipose tissue to absorb glucose through a signaling cascade that is incompletely understood. Insulin resistance, the inability of insulin to appropriately stimulate glucose uptake, is a hallmark of type 2 diabetes mellitus. The development of experimental systems that model human insulin resistance is important in elucidating the defects responsible for the development of type 2 diabetes. When two strains of mice, BTBR and C57BL/6J (B6), are crossed, the resultant male offspring (BtB6) demonstrate insulin resistance in muscle tissue. Here, we report an insulin resistance phenotype in adipose tissue from lean, nondiabetic BtB6 mice similar to that observed in human muscle. Adipocytes isolated from insulin-resistant male mice display 65% less insulin-stimulated glucose uptake compared with insulin-sensitive female mice. Similarly, adipocytes from insulin-resistant mice have diminished insulin-stimulated IRS-1 phosphorylation and phosphatidylinositol 3-kinase (PI3K) activation. However, normal activation of protein kinase B (Akt/PKB) by insulin is observed. Thus BtB6 mice demonstrate the dissociation of insulin-stimulated PI3K activity and Akt/PKB activation and represent a useful model to investigate the causes of insulin resistance in humans.

protein kinase B; phosphatidylinositol 3-kinase

INSULIN PROMOTES GLUCOSE UPTAKE in striated muscle and adipose tissue. The stimulation of glucose uptake is mediated by the translocation of glucose transporters (GLUT-4) from an intracellular pool to the plasma membrane (11, 12, 32). Upon binding to its receptor, insulin activates the receptor's intrinsic kinase, leading to autophosphorylation (20) and tyrosine phosphorylation of several substrates, including members of the insulin receptor substrate (IRS) family (27, 29, 45). Phosphorylation of IRSs recruits other signaling molecules, including phosphatidylinositol 3-kinase (PI3K) (4, 19, 43). Activation of PI3K has been shown to be absolutely required for insulin-stimulated glucose

transport (7, 30). However, the essential downstream targets of 3-phosphoinositides generated by PI3K are less clear.

One downstream target of 3-phosphoinositides is the serine/threonine kinase protein kinase B (Akt/PKB). The role of Akt/PKB activation in the regulation of glucose transport remains controversial (39). Expression of a constitutively active, membrane-targeted form of Akt/PKB increases glucose transport in 3T3-L1 adipocytes (25), isolated rat adipocytes (10, 42), and L6 muscle cells (15). However, expression of a dominant negative Akt/PKB mutant fails to suppress insulin-stimulated glucose uptake while simultaneously inhibiting insulin-stimulated protein synthesis (24) and phosphorylation of glycogen synthase kinase-3 (44). Most recently, normal insulin activation of PKB was demonstrated in obese and obese-diabetic patients despite decreased insulin-stimulated PI3K activity (22).

One of the hallmarks of non-insulin-dependent diabetes mellitus is insulin resistance, the inability of target tissues to adequately increase glucose transport in response to a physiological level of insulin. We have recently created a novel animal model of insulin resistance; the cross of BTBR and B6 mice (BtB6) leads to male offspring that are insulin resistant, whereas female offspring are insulin sensitive (34). When the *ob* allele is bred into the BTBR genetic background, the mice become overtly diabetic, in contrast to B6 *ob* mice, which are only transiently and moderately hyperglycemic (38). Furthermore, the difference between the phenotypes maps to two loci. One deleterious allele comes from the BTBR background, whereas the other is from the B6 strain. It is the combination of these two genetic backgrounds that leads to the disease phenotype in these mice.

Here, we show profound reductions in insulin-stimulated IRS-1 phosphorylation and anti-phosphotyrosine-associated PI3K activity as well as glucose uptake in isolated adipocytes from insulin-resistant mice; however, there is normal insulin activation of Akt/PKB. Thus BtB6 mice demonstrate the dissociation of

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insulin-stimulated glucose uptake and Akt/PKB activation and accurately model insulin resistance in humans.

EXPERIMENTAL PROCEDURES

Adipocyte isolation and glucose uptake. Ten- to twelve-week-old male or female mice were killed at 1200 after a 4-h fast, and their epididymal fat pads were promptly removed. Adipocytes were isolated by a protocol modified from Rodbell (35). Briefly, fat pads were minced in Krebs-Ringer phosphate HEPES (KRPH) buffer (recipe) with 2% BSA (Intergen, lot no. R9607) and 0.5 mg/ml collagenase (Worthington Biochemical, lot no. 47D1076) and incubated at 37°C for 40 min with shaking at ~100 rpm. After digestion, the cell suspension was passed through a 900- μ m filter, and the cells were washed five times with fresh KRPH plus BSA. After the final wash, cells were diluted to a 20% suspension and aliquotted into reaction vials. Cells were treated with basal (no insulin), submaximal (150 μ U/ml), or maximal (10,000 μ U/ml) insulin (Humulin, Novolin) for 15 min before addition of D-[U-¹⁴C]glucose. Glucose uptake was stopped by spinning an aliquot over oil and discarding the infranatant. Glucose uptake into the cells was measured by counting cell pellets solubilized in Biosafe II liquid scintillation analysis cocktail.

Immunoblotting. After treatment with or without insulin, isolated adipocytes were lysed in 10 mM Tris buffer (pH 7.4) containing 1% NP-40, 137 mM NaCl, 10% glycerol, 1 mM each EDTA, sodium orthovanadate, and phenylmethylsulfonyl fluoride, 10 mM NaF, 10 μ g/ml leupeptin, and 2 μ g/ml aprotinin. Lysates were spun at 13,200 rpm for 15 min at 4°C, and the fat cake was removed. Laemmli SDS-PAGE sample buffer was added, and samples were heated to 95°C for 10 min. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. All blots were blocked in Tris-buffered saline plus Tween 20 with 1% BSA. Primary antibodies used in Western analysis were anti-IRS-1 (1 μ g/ml; Upstate Biotechnology), anti-phosphotyrosine (1 μ g/ml; Upstate Biotechnology, clone 4G10), and anti-actin (2 μ g/ml; Santa Cruz Biotechnology). After incubation with appropriate secondary antibody (Sigma) conjugated to alkaline phosphatase, proteins were detected using AttoPhos substrate (JBL Scientific) and quantitated with a fluorescence imager (Molecular Dynamics).

PI3K assay. Isolated adipocytes were exposed to insulin for 10 min. The medium was removed, and cells were resuspended in lysis buffer as previously described (33). Lysates were immunoprecipitated by 5 μ g of anti-phosphotyrosine antibody (Transduction Labs). PI3K activity was measured as described.

Akt/PKB assay. Immunoprecipitation was performed by the method of Summers et al. (40). Briefly, lysates were immunoprecipitated with anti-Akt2 antibody and protein A agarose beads. Beads were washed three times with lysis buffer and three times with kinase buffer. Enzymatic activity was assayed by addition of reaction cocktail [20 mM HEPES, pH 7.2, 5 mM MgCl₂, 1 mM dithiothreitol, 0.2 mM EGTA, 2 μ g of the peptide inhibitor of cAMP-dependent protein kinase, 25 μ g histone H2B, 10 μ M ATP (5 μ Ci/reaction)], and incubation at 30°C for 30 min. Reactions were stopped by boiling for 2 min, and the supernatant was loaded on a 12.5% SDS-PAGE gel. Bands were quantified by phosphorimager analysis using ImageQuant software.

RESULTS

Adipocytes from male BtB6 mice have a blunted insulin response. We have previously shown (34) that, compared with insulin-sensitive BtB6 mice, insulin-resistant mice have impaired oral glucose tolerance and blunted insulin-stimulated glucose uptake in isolated muscle. To determine whether fat tissue similarly demonstrated altered insulin responsiveness, we performed insulin-stimulated glucose uptake experiments in isolated adipocytes from insulin-resistant and insulin-sensitive BtB6 mice.

Adipocytes isolated from insulin-resistant BtB6 mice show a significant blunting of insulin-stimulated glucose uptake (Fig. 1). Maximal doses of insulin (10,000 μ U/ml) lead to a 13.5-fold stimulation of glucose uptake in adipocytes from insulin-sensitive BtB6 females. In contrast, adipocytes from insulin-resistant BtB6 males show only a 4.7-fold increase. Thus adipocytes from insulin-resistant BtB6 mice demonstrate a pronounced attenuation of the effects of insulin on glucose uptake.

IRS-1 phosphorylation is altered in insulin-resistant vs. insulin-sensitive adipocytes. Insulin induces the activation and autophosphorylation of the insulin receptor as well as recruitment to the plasma membrane and subsequent tyrosine phosphorylation of signaling molecules such as IRS-1. To determine whether proximal elements of the insulin-signaling cascade were responsible for the blunted response in insulin-resistant adipocytes, we examined the relative abundance and degree of tyrosine phosphorylation IRS-1.

Incubation with insulin of adipocytes isolated from insulin-sensitive mice causes a significant increase in tyrosine phosphorylation of IRS-1, as determined by Western blot analysis (Fig. 2). Both the abundance of IRS-1 and the degree of tyrosine phosphorylation are

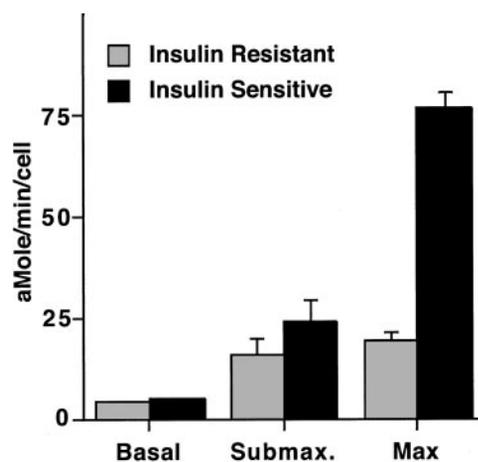


Fig. 1. Glucose uptake into isolated adipocytes. Adipocytes were isolated from epididymal fat pads of 10- to 12-wk-old mice as described in EXPERIMENTAL PROCEDURES and incubated in the absence or presence of submaximal (Submax. 150 μ U/ml) or maximal (Max. 10,000 μ U/ml) insulin for 15 min. Glucose uptake was assessed by adding trace D-[U-¹⁴C]glucose. Assays were stopped by spinning cells over oil and counting the cell pellet. Uptake is representative of 3 separate experiments, each with 4–6 mice.

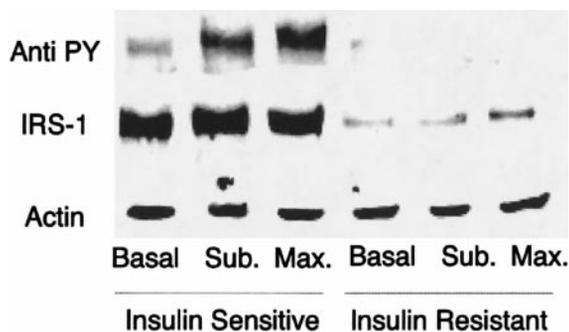


Fig. 2. Insulin receptor substrate-1 (IRS-1) expression and phosphorylation in response to insulin. Adipocytes were stimulated with or without insulin for 10 min. Cell lysates were separated on a 6% (IRS-1) or 12% (actin) SDS-PAGE gel and transferred to nitrocellulose membranes. Blots were probed with anti-phosphotyrosine (Anti PY), anti-IRS-1, or anti-actin antibodies. Results are representative of 3 independent experiments, each with 4–6 mice.

markedly reduced in adipocytes isolated from insulin-resistant mice compared with adipocytes from insulin-sensitive littermates. An actin immunoblot shows that equivalent amounts of cell lysates were used for this analysis.

PI3K activity is decreased in insulin-resistant vs. insulin-sensitive adipocytes. PI3K is an important mediator of insulin signaling downstream of the IRSs. Insulin-stimulated phosphorylation of tyrosines facilitates the association of PI3K with signaling molecules such as IRS-1. We measured anti-phosphotyrosine-associated PI3K activity in isolated adipocytes from insulin-resistant and insulin-sensitive BtB6 mice. In agreement with previous observations (14), submaxi-

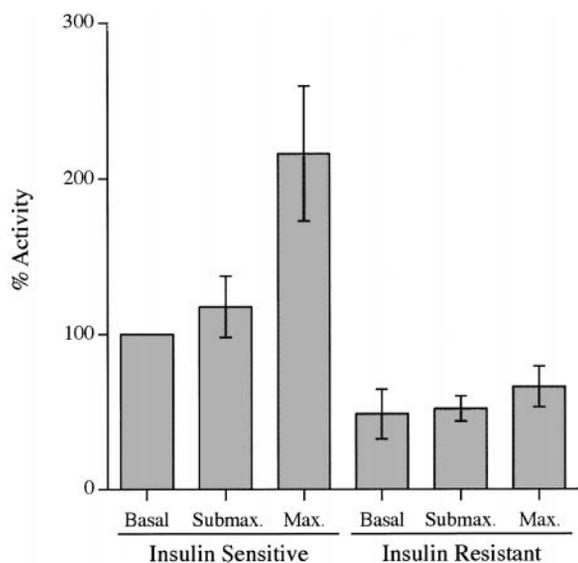


Fig. 3. Phosphatidylinositol 3-kinase (PI3K) activation by insulin. Lysates from adipocytes stimulated with various insulin concentrations were immunoprecipitated by anti-phosphotyrosine antibody. Immunocomplexes were washed 3 times and before kinase assay. Lipid products were separated by TLC and quantitated by phosphorimager. Basal activity in insulin-sensitive adipocytes was normalized to 100%. Results represent 3 independent experiments, each with 4–6 mice.

mal insulin levels caused a slight increase in PI3K activity, whereas maximal insulin levels led to a full stimulation (Fig. 3). In contrast, insulin-resistant adipocytes demonstrate a blunted stimulation of PI3K at both submaximal and maximal insulin levels. Thus PI3K activation is impaired in insulin-resistant adipocytes. Additionally, absolute PI3K activity in adipocytes from insulin-resistant mice was decreased relative to insulin-sensitive mice; at maximal stimulation, PI3K activity in insulin-resistant adipocytes was only 24% of the activity observed in the insulin-sensitive adipocytes.

Akt/PKB activation is normal in insulin-resistant and insulin-sensitive adipocytes. In response to insulin, Akt/PKB is recruited to the plasma membrane by 3-phosphoinositides generated by activated PI3K (13). There, Akt/PKB is phosphorylated at two distinct sites and becomes fully activated. We predicted that Akt/PKB activation would be significantly reduced in insulin-resistant mice as the upstream signaling molecule PI3K showed a blunted response to insulin. Surprisingly, stimulation by insulin led to an eight- and sixfold activation in insulin-sensitive and insulin-resistant adipocytes, respectively (Fig. 4). In striking contrast to insulin-stimulated glucose transport, there was no significant difference in insulin-stimulated Akt/PKB activity between the insulin-sensitive and insulin-resistant adipocytes.

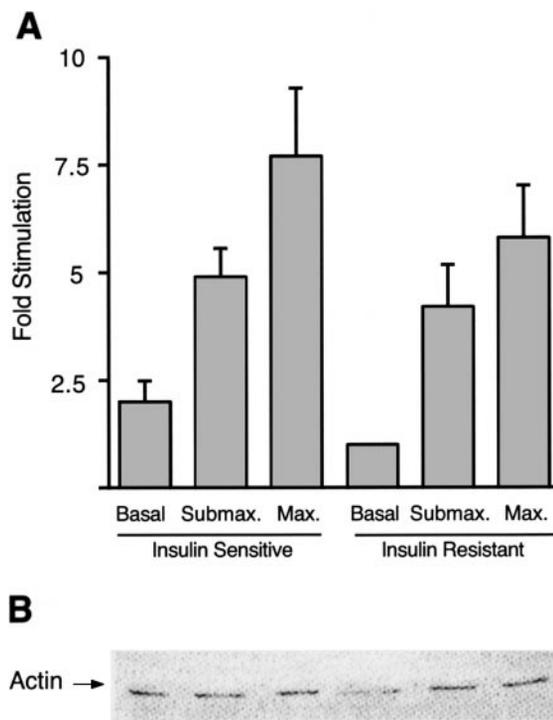


Fig. 4. Protein kinase B (Akt/PKB) activation by insulin. Isolated adipocytes were incubated with the listed insulin doses for 10 min. Lysates were prepared and immunoprecipitated as described in EXPERIMENTAL PROCEDURES. PKB activity against histone H2B was measured. Results are the average of 4 independent experiments, each with 4–6 mice.

DISCUSSION

Many animal model systems have involved the use of pharmacological agents or gene knockouts to generate insulin resistance (18). Targeted disruption of the insulin receptor in mice is lethal within 1 wk of birth (2, 17). Muscle-specific disruption of the insulin receptor in mice causes whole animal insulin resistance but does not dramatically affect blood glucose or insulin levels (5). This is likely due to a redistribution of substrate flux to adipose tissue (21). Mice lacking IRS-1 are insulin resistant and have reduced insulin-stimulated PI3K activity in both muscle and liver (3, 41). Muscle-specific elimination of GLUT-4 in mice leads to profound insulin resistance (46). Mice with adipose tissue-specific GLUT-4 knockouts are also insulin resistant and have impaired insulin action in liver and muscle (1). However, very few single-gene disorders leading to insulin resistance have been identified in humans.

Other insulin resistance models demonstrate similar changes to those observed in BtB6 mice. However, obesity or hyperglycemia can coexist with insulin resistance. Thus it is unclear whether tissue defects are a consequence of obesity or can exist in the absence of obesity. Obese Zucker rats demonstrate decreased insulin-stimulated phosphorylation of IRS-1 and -2 as well as diminished insulin-stimulated PI3K and PKB in adipose tissue (6, 23). Diabetic Goto-Kakizaki rats similarly show evidence of insulin resistance as well as impaired insulin-stimulated PKB activation in muscle (26, 37). Obese diabetic mice have impaired GLUT-4 translocation as well as reduced PKB phosphorylation and activation in response to insulin in adipose tissue (36). However, in muscle from humans with type 2 diabetes mellitus, normal insulin stimulation of PKB is observed despite decreased PI3K activation (22). Our observations in lean BtB6 mice demonstrated that defects in PI3K activation are present in the prediabetic, nonobese state, whereas PKB activation by insulin remains normal. These defects preceded the onset of hyperglycemia and hyperinsulinemia and were present in the absence of obesity. Therefore, these alterations in insulin signal transduction are specific to the insulin-resistant state and are not results of hyperglycemia or obesity.

In humans, insulin resistance and type 2 diabetes mellitus are polygenic disorders. The combination of the BTBR and B6 backgrounds led to insulin resistance in BtB6 mice (34). Furthermore, genetic obesity in these hybrid mice leads to the development of diabetes (38). Although the genetics of human diabetes have yet to be fully understood, the genetic contributions leading to diabetes in BtB6 mice are better understood. Three loci have been identified that control the susceptibility of these mice to developing hyperglycemia (38). It is the combination of alleles from each strain that synergistically leads to an increased susceptibility to the development of diabetes. Thus this murine model will facilitate both a genetic and a biochemical ap-

proach to studying the development of insulin resistance.

Glucosamine treatment of cultured adipocytes and 3T3-L1 cells leads to insulin resistance (16, 28). Similar to adipocytes from BtB6 male mice, glucosamine-treated 3T3-L1 cells display decreased insulin-stimulated insulin receptor autophosphorylation and IRS-1 phosphorylation, and GLUT-4 translocation is observed (16). Furthermore, glucosamine infusion of rats leads to insulin resistance in muscle and postreceptor defects similar to those observed in BtB6 mice (31). However, normal insulin-stimulated Akt/PKB activity is observed, even with reduced IRS-1-associated PI3K activity. Both glucosamine-treated 3T3-L1 cells and adipocytes from BtB6 male mice demonstrate normal PKB activation in response to insulin (Fig. 4) despite insulin resistance and prominent postreceptor signaling defects. Thus our results with the use of BtB6 mice in the absence of glucosamine demonstrate a mechanism of insulin resistance similar to that in glucosamine-treated 3T3-L1 cells.

Parallel pathways exist in the activation of PKB and glucose transport. In contrast to glucosamine-induced insulin resistance, osmotic shock promotes glucose uptake while inhibiting PKB activation (8). Hyperosmotic treatment of 3T3-L1 cells promotes GLUT-4 translocation, although not to the same degree as insulin treatment. Further stimulation of glucose transport by insulin is prevented by maintaining PKB in a dephosphorylated, inactive state (9). Conversely, β 1-integrin treatment of adipocytes leads to elevated PI3K and PKB activity but no increase in glucose uptake. Thus there is not a direct relationship between PKB activation and increased glucose uptake into cells.

In conclusion, we have reported that in a lean, non-diabetic mouse model of insulin resistance, IRS-1 phosphorylation, anti-phosphotyrosine-associated PI3K, and glucose transport are markedly reduced. However, similar to the findings in humans, activation of Akt/PKB by insulin remains normal (22). This was observed in the absence of overt hyperglycemia or obesity. From these studies, we conclude that there are several routes to Akt/PKB activation and that Akt/PKB activation alone is neither necessary nor sufficient to promote glucose transport.

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