Article

IRS1-Independent Defects Define Major Nodes of Insulin Resistance

Kyle L. Hoehn,[1] Cordula Hohnen-Behrens,[1] Anna Cederberg,[1] Lindsay E. Wu,[1] Nigel Turner,[1] Tomoyuki Yuasa,[2] Yousuke Ebina,[2] and David E. James[1],*

[1]Diabetes and Obesity Program, Garvan Institute of Medical Research, Darlinghurst, NSW 2010, Australia
[2]Division of Molecular Genetics, Institute for Enzyme Research, University of Tokushima, 2-24 Shinkura-cho, Tokushima 770-8501, Japan
*Correspondence: d.james@garvan.org.au
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SUMMARY

Insulin resistance is a common disorder caused by a wide variety of physiological insults, some of which include poor diet, inflammation, anti-inflammatory steroids, hyperinsulinemia, and dyslipidemia. The common link between these diverse insults and insulin resistance is widely considered to involve impaired insulin signaling, particularly at the level of the insulin receptor substrate (IRS). To test this model, we utilized a heterologous system involving the platelet-derived growth factor (PDGF) pathway that recapitulates many aspects of insulin action independently of IRS. We comprehensively analyzed six models of insulin resistance in three experimental systems and consistently observed defects in both insulin and PDGF action despite a range of insult-specific defects within the IRS-Akt nexus. These findings indicate that while insulin resistance is associated with multiple deficiencies, the most deleterious defects and the origin of insulin resistance occur independently of IRS.

INTRODUCTION

Insulin plays an essential role in metabolic homeostasis in mammals by accelerating the disposal of postprandial glucose into muscle and adipose tissue. This step is mediated via the insulin-regulated translocation of the glucose transporter GLUT4 from intracellular vesicles to the plasma membrane. This process is imperative, as mice lacking GLUT4 in muscle or fat are insulin resistant (Minokoshi et al., 2003), a condition defined as an impairment in insulin-stimulated glucose entry into these tissues. Insulin resistance and the associated glucose intolerance are important metabolic markers that predispose individuals to more severe disorders including type 2 diabetes. The origin of and the precise mechanism (or mechanisms) mediating insulin resistance are unknown. However, since glucose entry into the cell represents the earliest rate-limiting defect in insulin-resistant skeletal muscle (Petersen and Shulman, 2006), this suggests that the etiology of insulin resistance derives from a lesion in the pathway between the insulin receptor (IR) and GLUT4.

The precise path from the IR to GLUT4 is still unknown; however, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is essential for insulin-regulated glucose metabolism. The insulin-dependent phosphorylation of insulin receptor substrate (IRS) proteins is the initial step in the activation of PI3K that leads to the generation of phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which serves as a docking site for pleckstrin homology (PH) domain-containing proteins such as PDK1 and Akt. Akt is the pleiotropic kinase essential for most of the metabolic actions of insulin, and its activity is regulated by phosphorylation at Thr308 by PDK1 and Ser473 by the mTOR/Rictor complex. In many cases, Akt substrates that directly control metabolic steps have been identified; some of these include glycogen synthase kinase 3 (GSK3) (Cross et al., 1995), the transcription factor FOXO (Biggs et al., 1999; Paradis and Ruvkun, 1998), the Rheb GAP tuberous sclerosis protein 2 (TSC2) (Potter et al., 2002), the phosphodiesterase PDE3B (Kitamura et al., 1999), and the Rab GAP TBC1D4/AS160 (Kane et al., 2002). The last of these, TBC1D4, is the only Akt substrate that has been shown to play an important role in insulin-stimulated GLUT4 translocation (Kane et al., 2002). It should be noted that Akt-independent pathways have also been implicated in insulin action (Baumann et al., 2000; Farese et al., 2007); however, their role remains controversial (Huang and Czech, 2007). One possibility is that these pathways play a permissive rather than a regulatory role in insulin action. Regardless, their relevance to the development of insulin resistance in muscle and fat cells remains doubtful, particularly since some of these pathways appear to be cell type specific (e.g., c-Cbl).

While defects at any one of these loci between the IR and GLUT4 may contribute to insulin resistance, it is widely believed that defects at IRS1 represent a central feature of this disorder (Aguirre et al., 2000; Dresner et al., 1999; Gao et al., 2002, 2003; Gual et al., 2005; Hotamisligil et al., 1996; Morino et al., 2005; Ozcan et al., 2004; Rice et al., 1993; Turnbow et al., 1994; Ueki et al., 2004; White, 2002; Yu et al., 2002; Zick, 2005; Zierath et al., 1997). To date, considerable evidence has focused on IRS proteins as a major target of insulin resistance under a broad range of conditions. Several mechanisms have been reported to modify the activity of IRS, including protein/protein interactions, Ser/Thr hyperphosphorylation, tyrosine dephosphorylation, targeted degradation, and other posttranslational modifications such as O-linked glycosylation and acetylation (reviewed in Gual et al., 2005; Taniguchi et al., 2006).

There are several caveats to the model describing a major role for IRS proteins in insulin resistance. First, many studies of IRS function in insulin resistance focus on elements in the signal transduction cascade as the major readout of insulin resistance rather than terminal actions of insulin such as GLUT4 insertion into the
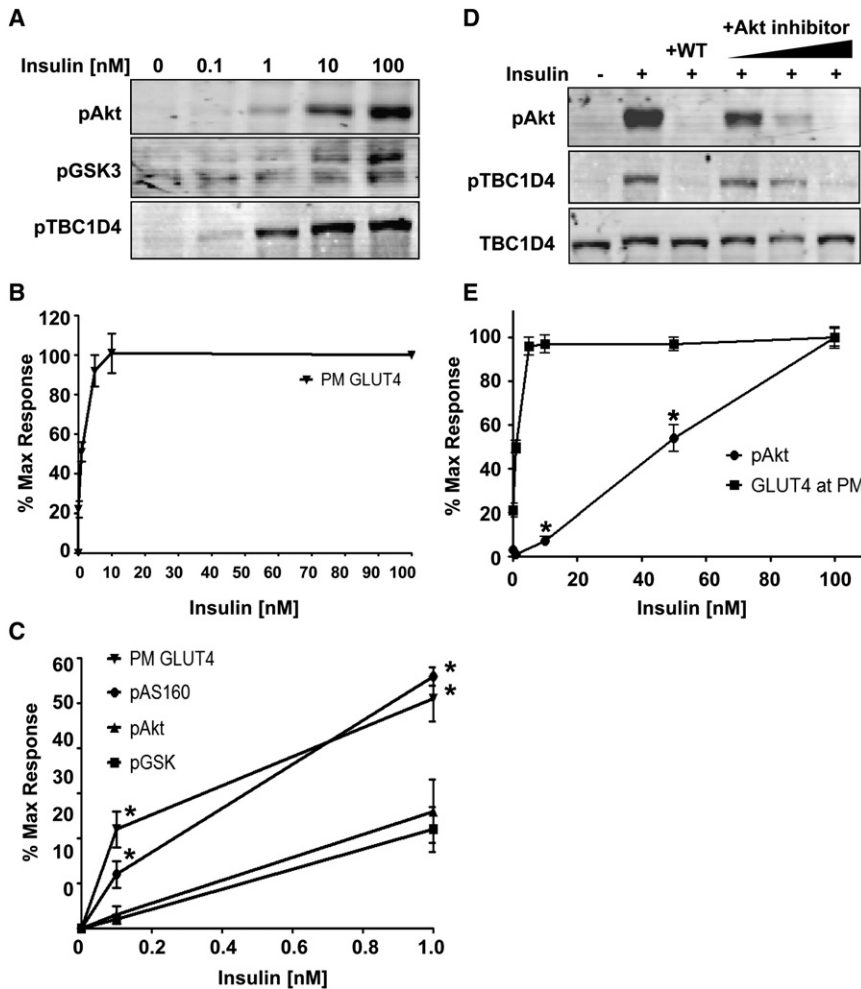
Figure 1. Insulin-Stimulated GLUT4 Translocation Is Not Proportional to Akt Phosphorylation

(A) 3T3-L1 adipocytes were treated with a dose response of insulin. At each dose, the phosphorylation status of Akt at Ser473, GSK3α/β at Ser9/21, and TBC1D4 at Thr642 were detected by western blot.

(B) Under the same insulin treatment conditions, GLUT4 translocation to the plasma membrane (PM) was detected using the GLUT4 translocation assay described in Experimental Procedures. Results are presented as means ± SEM of 3–4 experiments.

(C) To directly compare the insulin-stimulated response of each parameter in (A) and (B), the western blots and translocation data were quantified and normalized to 0 and 100 nM insulin, representing minimal and maximal stimulation. An evaluation of submaximal stimulation reveals a distinct nonlinearity between Akt phosphorylation and GLUT4 translocation to the plasma membrane. However, phosphorylation of TBC1D4 at submaximal insulin levels is more consistent with GLUT4 translocation than Akt, indicating an increased activity toward this substrate over others like GSK3. Results are presented as means ± SEM of 3–4 experiments. *$p < 0.05$ relative to Akt phosphorylation at the given dose of insulin by Student's t test.

(D) The PI3K inhibitor wortmannin (WT, 100 nM for 10 min prior to insulin) and an Akt inhibitor (at concentrations of 0.1, 1, and 10 μM) inhibit Akt phosphorylation and subsequent phosphorylation of TBC1D4 in 3T3-L1 adipocytes treated with 100 nM insulin for 12 min, indicating that Akt is the sole insulin-regulated kinase acting on this site. Representative western blots of three separate experiments are shown.

(E) Insulin (20 min) was added to L6 myotubes at the concentrations indicated, and the Akt phosphorylation and GLUT4 translocation responses were analyzed as a percent of maximal stimulation. Results are presented as means ± SEM, n = 3. *$p < 0.05$ by Student's t test.

plasma membrane. Second, there is evidence that insulin resistance is not always associated with defects in Akt activity (Kim et al., 1999; Nadler et al., 2001). Third, as originally shown by Kono and colleagues (Kono and Barham, 1971) for the IR, there is often considerable "spareness" in various elements of the insulin signaling cascade, and the same applies to IRS1 (Rice et al., 1992). This means that incomplete inhibition of IRS may not have a major impact on metabolism. Consistent with this, mice heterozygous for IRS1 do not show defects in insulin action under normal conditions (Shirakami et al., 2002), and partial knockdown of IRS1 in skeletal muscle has no effect on insulin action (Cleasby et al., 2007). Thus, it remains unclear whether reduced insulin signaling through IRS is a primary cause of insulin resistance or simply a secondary consequence of an alternate primary disorder.

RESULTS

Lack of Linearity between Akt and GLUT4

Akt has been shown to play an essential role in many actions of insulin, including GLUT4 translocation (Kohn et al., 1996). How-

ever, as shown in Figure 1, the dynamic range of Akt activity with respect to substrate phosphorylation and GLUT4 translocation is considerably lower than its maximal capacity. This is consistent with previous reports showing a similar nonlinearity between insulin-stimulated Akt activation and glucose uptake (Whitehead et al., 2001). Strikingly, the dose-response relationship of Akt substrate phosphorylation was discordant with that of insulin-stimulated Akt phosphorylation, and a further discordance was observed between distinct Akt substrates (Figure 1C). In adipocytes incubated with 0.1–1 nM insulin, a concentration within the high physiological range, GLUT4 translocation and TBC1D4 phosphorylation increased to ∼50% of their maximum values while Akt and GSK3 phosphorylation reached only 20% of their maximum output. The discordance between insulin-activated TBC1D4 phosphorylation is not likely due to an additive contribution of other insulin-stimulated upstream kinases on TBC1D4, because its phosphorylation was inhibited using a specific Akt inhibitor (Figure 1D). Furthermore, insulin had a maximal effect on GLUT4 translocation in L6 myotubes at concentrations where only 5% of the total Akt pool was phosphorylated (Figure 1E).

Hence, these data indicate several important aspects of insulin signaling and insulin action not previously emphasized. First, there is considerable spareness in the activity of Akt in vivo with respect to insulin action as defined by GLUT4 translocation. Second, there is discordance in the efficiency of Akt substrate phosphorylation such that certain substrates such as TBC1D4 are more efficiently phosphorylated than others such as GSK3. One ramification of these data is that a modest defect in upstream elements of the insulin signaling cascade per se, as is often described in insulin resistance, may not translate into downstream defects due to spareness in the pathway. To test this hypothesis, we conducted an extensive analysis of the insulin action pathway in a variety of models of insulin resistance.

PDGF Mimics Insulin Action Independently of IRS

To dissect the specific role of IRS in insulin resistance, we established an experimental system utilizing the platelet-derived growth factor (PDGF) pathway to bypass the requirement for IRS while preserving other elements of the insulin action cascade. Muscle and fat cells do not possess sufficient levels of the PDGF receptor to trigger a PDGF response in these cells (Whiteman et al., 2003; unpublished data). Therefore, the human PDGF β receptor (PDGFR) was ectopically expressed in mouse 3T3-L1 preadipocytes and rat L6 myoblasts using recombinant retrovirus (Figure 2A; Whiteman et al., 2003). Upon differentiation into adipocytes or myotubes, we evaluated insulin and PDGF action to define the optimal doses and incubation times with ligand to achieve the most consistent amplification of signal transduction and GLUT4 translocation (data not shown). In both cell culture models, a maximum dose of insulin (100 nM) and PDGF (20 ng/ml) was utilized, as this produced a consistent and similar response facilitating a direct comparison between both agonists (see Figure 2B). Both hormones activated PI3K without detectable crosstalk at the level of IRS1 as indicated by the absence of PDGF-stimulated IRS1 tyrosine phosphorylation at the PI3K binding site (Tyr612), compared to the robust phosphorylation observed with insulin. Conversely, whereas PDGF stimulated tyrosine phosphorylation of the PDGF receptor in both myotubes and adipocytes, this was not observed in response to insulin stimulation (Figure 2B). Importantly, both insulin and PDGF stimulated Akt and TBC1D4 phosphorylation and GLUT4 translocation with similar amplitude (Figures 2B and 2C) and kinetics (data not shown) in both cell types. Consistent with previous studies (Whiteman et al., 2003), these data show that IRS proteins are not necessary for PDGF-induced GLUT4 translocation but are rather a specific feature necessary for other aspects of insulin action. Hence, this offers an ideal experimental system with which to assess the role of IRS proteins in insulin resistance.

Bypassing IRS Does Not Override Insulin Resistance

To study the role of IRS in insulin resistance, we next generated a range of metabolic disorders in L6 myotubes or 3T3-L1 adipocytes overexpressing PDGFR using insults that recapitulate common causes of insulin resistance in mammals, including hyperinsulinemia (chronic insulin, CI), inflammation (tumor necrosis factor α, TNF), Cushing's syndrome/anti-inflammatory steroids (dexamethasone, DEX), and hyperlipidemia/dyslipidemia (palmitate, PALM). Oxidative stress, which has also been linked to insulin resistance (Houstis et al., 2006; Kaneto et al., 2005), was
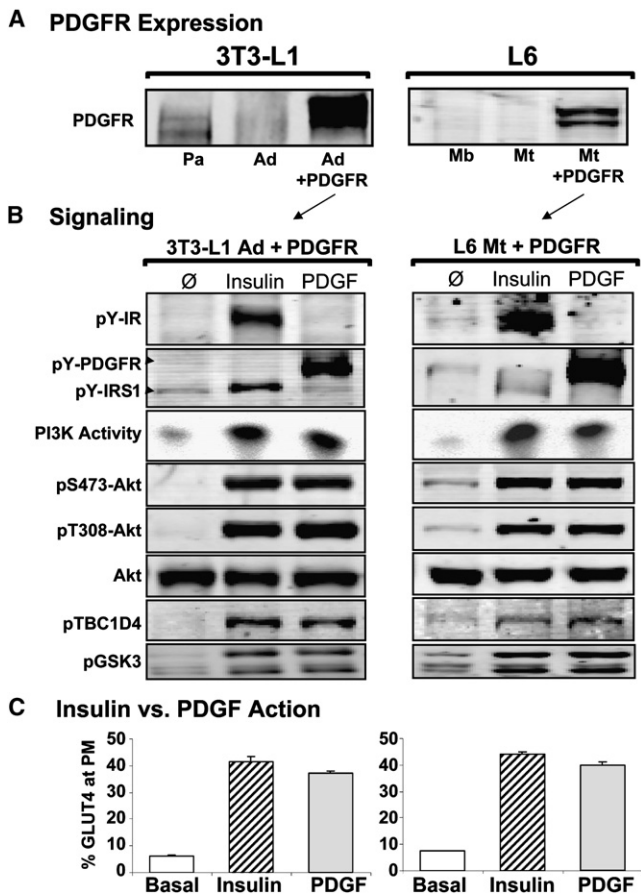

**Figure 2. PDGF Mimics Insulin Action While Circumventing the Insulin Receptor and IRS**

(A) The human PDGF receptor (PDGFR) was ectopically overexpressed in mouse 3T3-L1 adipocytes and rat L6 myotubes. Representative western blots of PDGFR expression are shown. Pa, preadipocytes; Ad, adipocytes; Mb, myoblasts; Mt, myotubes.

(B) PDGFR-expressing adipocytes (left panel) and myotubes (right panel) were serum starved for 90 min prior to acute stimulation with either 100 nM insulin or 20 ng/ml PDGF for 20 min. Representative western blots of cell and tissue extracts show similar amplification of insulin- and PDGF-stimulated signaling intermediates.

(C) Insulin- and PDGF-stimulated GLUT4 translocation to the plasma membrane as a percentage of total HA-GLUT4 in adipocytes (left panel) and myotubes (right panel). In both cell types, insulin was slightly more potent than PDGF (89% ± 2% and 90% ± 3% of maximal insulin for adipocytes and myotubes, respectively). Results are presented as means ± SEM of 5 experiments of >3 measurements each.

generated by incubating cells with glucose oxidase (GO). To avoid toxic or nonspecific effects, the treatment dose and/or duration for all insults was optimized to define minimal conditions required to elicit maximal insulin resistance (data not shown). GO, CI, TNF, DEX, and PALM inhibited insulin-stimulated GLUT4 translocation by 45%–60% in both 3T3-L1 and L6 cells, indicating marked insulin resistance with each model (Figure 3). However, PDGF was unable to overcome these defects, indicating that IRS1 per se is not a major contributor to insulin resistance (Figure 3). Notably, however, we did observe a consistent restoration of GLUT4 translocation with PDGF in myotubes, and to

A 3T3-L1 Adipocytes
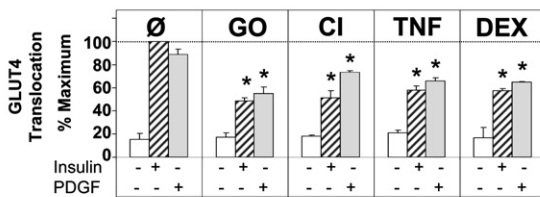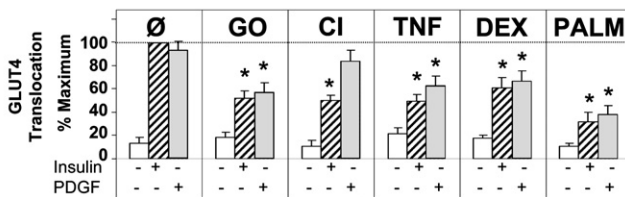

B L6 Myotubes

Figure 3. Multiple Insults Antagonize Insulin and PDGF Action

(A) GLUT4 translocation to the plasma membrane was measured after glucose oxidase (GO, 50 mU/ml for 2 hr), chronic insulin (CI, 10 nM for 24 hr), TNFα (TNF, 2 ng/ml for 4 days), or dexamethasone (DEX, 20 nM for 8 days) treatment in 3T3-L1 adipocytes stimulated with insulin or PDGF.

(B) GLUT4 translocation to the plasma membrane was analyzed in L6 myotubes treated with the above conditions or palmitate (PALM, 150 μM for 18 hr). All data were normalized to maximum stimulation with 100 nM insulin for each cell type.

Results are displayed as means ± SEM of 3–5 experiments for each data set. *p < 0.05 versus respective insulin- or PDGF-stimulated control (Ø) by Student's t test.

a lesser extent in adipocytes, treated with CI (Figure 3). This emphasizes the utility of this system, clearly indicating that in the case of the CI model, the major defect must occur at components unique to the insulin pathway involving either the IR or IRS1.

Upstream Signal Transduction in Insulin Resistance

We next examined IRS1 expression and phosphorylation since defects in both of these parameters have been reported in many insulin-resistance models. A significant reduction in IRS1 protein levels was observed in adipocytes treated with CI, TNF, and DEX, consistent with previous studies (Figure 4A). However, this did not consistently correlate with changes in insulin-stimulated IRS1 tyrosine phosphorylation as exemplified in cells treated with DEX. Moreover, we observed uncoupling between IRS1 tyrosine phosphorylation and downstream signaling in adipocytes treated with CI. In this instance, we observed persistent tyrosine phosphorylation of IRS1 following chronic insulin treatment despite an extensive insulin washout period. The insulin washout was clearly effective because Akt phosphorylation had returned to basal levels (see CI, Figure 4A). Hence, this suggests that additional aspects of IRS1 function, such as subcellular localization (Clark et al., 1998; Inoue et al., 1998), may play an important role in its coupling to downstream signaling elements. Finally, GO treatment had no effect on IRS1 tyrosine phosphorylation or abundance despite significant insulin resistance (Figure 3A), indicating that this insult likely functions downstream of IRS as suggested recently (JeBailey et al., 2007).

We next focused on Akt to determine whether the changes in IRS function described above were transmitted distally throughout the insulin signaling cascade. Phosphorylation of Akt at both Thr308 and Ser473 was measured as an index of its activation by upstream regulators. To determine the in vivo activity state of Akt, the phosphorylation of TBC1D4, a key target of Akt involved in GLUT4 translocation (Kane et al., 2002; Larance et al., 2005), was measured. We and others have shown that Thr642 is one of the key Akt substrate sites in TBC1D4 that plays an essential role in its function (Kane et al., 2002; Ramm et al., 2006; Sano et al., 2003). Therefore, a phosphorylation-specific antibody targeting this site was produced and used to probe cell lysates.

Consistent with changes in IRS function, we observed a significant reduction in Akt phosphorylation in cells treated with CI, TNF, and DEX, but not in cells treated with either GO or PALM (Figure 4B). These data are consistent with previous reports showing upstream signaling defects upon treatment with CI, TNF, and DEX (Aguirre et al., 2000; Ball et al., 2006; Hotamisligil, 1999; Houstis et al., 2006; Ishizuka et al., 2007; Paz et al., 1997; Saad et al., 1993; Sun et al., 1999; Turnbow et al., 1994). Interestingly, PDGF treatment overcame the defect in Akt activation in cells treated with CI and TNF, but not in cells treated with DEX. To determine whether these changes in Akt phosphorylation resulted in defective Akt signaling, we next examined TBC1D4 phosphorylation (Figure 4C). Surprisingly, we observed a complete discordance between these parameters. For example, there was no effect of GO on either insulin- or PDGF-stimulated Akt phosphorylation (Figure 4B), whereas the specific activity of TBC1D4 phosphorylation was significantly diminished (Figure 4C). Conversely, with CI, despite reduced insulin-stimulated Akt phosphorylation, TBC1D4 phosphorylation was unimpaired (Figure 4C). This highlights a degree of spareness at the level of Akt as described in Figure 1. TNF-treated cells exhibited a distinct phenotype at the level of TBC1D4 compared with either GO- or CI-treated cells. Here we observed a dramatic reduction in TBC1D4 protein levels. Despite this, the specific activity of TBC1D4 phosphorylation at Thr642 was unchanged in response to either insulin or PDGF. Again this emphasizes the spareness in these signaling intermediates because TBC1D4 is believed to be a negative regulator of GLUT4 translocation under basal conditions and phosphorylation overcomes this negative regulation (Kane et al., 2002; Larance et al., 2005; Ramm et al., 2006). Hence, it is notable that cells were able to tolerate this substantial reduction in TBC1D4 levels with only a mild increase in basal cell surface levels of GLUT4 (Figure 3A). Intriguingly, DEX yielded changes at the level of TBC1D4 that were diametrically opposed to TNF. TBC1D4 total protein levels and Thr642 phosphorylation in response to insulin and PDGF were significantly enhanced in these cells. Considering that both TNF and DEX caused significant insulin resistance at the level of GLUT4 translocation, these data suggest that intermediates such as TBC1D4 may operate as regulated switches with an intrinsic threshold such that modest defects upstream of this molecule may not directly translate downstream.

Effects of Palmitate on Insulin Action

The most striking discrepancy between Akt activation and GLUT4 translocation was observed in L6 myotubes incubated with palmitate. In the presence of 150 μM palmitate, insulin- and
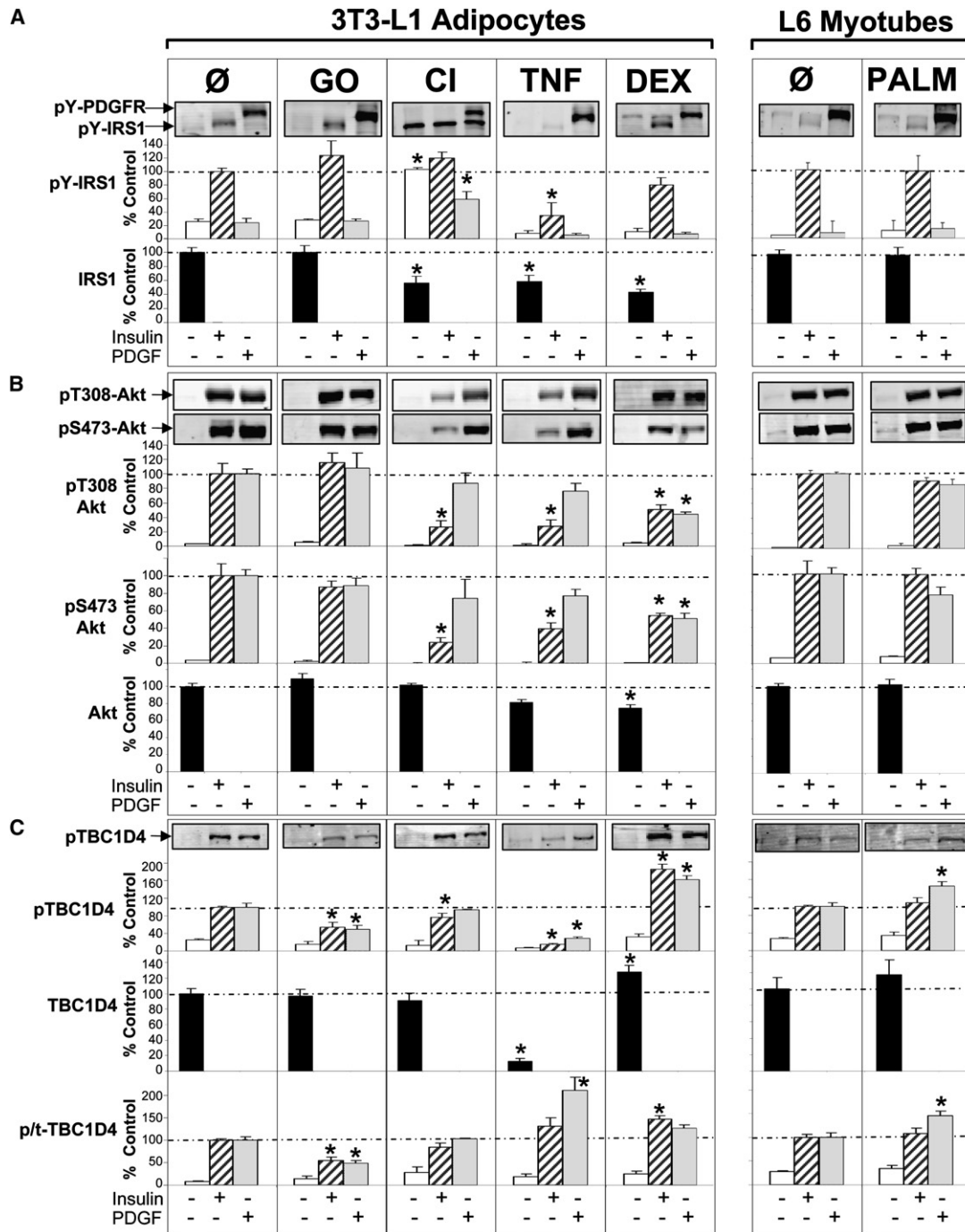
Figure 4. Signal Transduction in 3T3-L1 Adipocytes and L6 Myotubes Treated with Multiple Models of Insulin Resistance

Cells were left in unstimulated basal conditions or stimulated with insulin or PDGF.

(A) Representative western blots and quantitative bar graphs are shown for the detection of PDGFR and IRS1 tyrosine phosphorylation and total IRS1 protein levels. The phospho-Tyr612 IRS1 (pY-IRS1) antibody detects phosphorylation of the PI3K binding site in both molecules.

(B) Representative western blots and quantification of Akt Ser473 and Thr308 phosphorylation and total Akt levels.

(C) Cell lysates were probed for TBC1D4 phosphorylation at Thr642 and total TBC1D4 expression. The bottom panel represents a quantification of phosphorylated to total TBC1D4 (p/t-TBC1D4).

Western blots are representative and statistical values are displayed as means ± SEM of 3–5 experiments. *p < 0.05 versus respective control (Ø) by Student's t test.
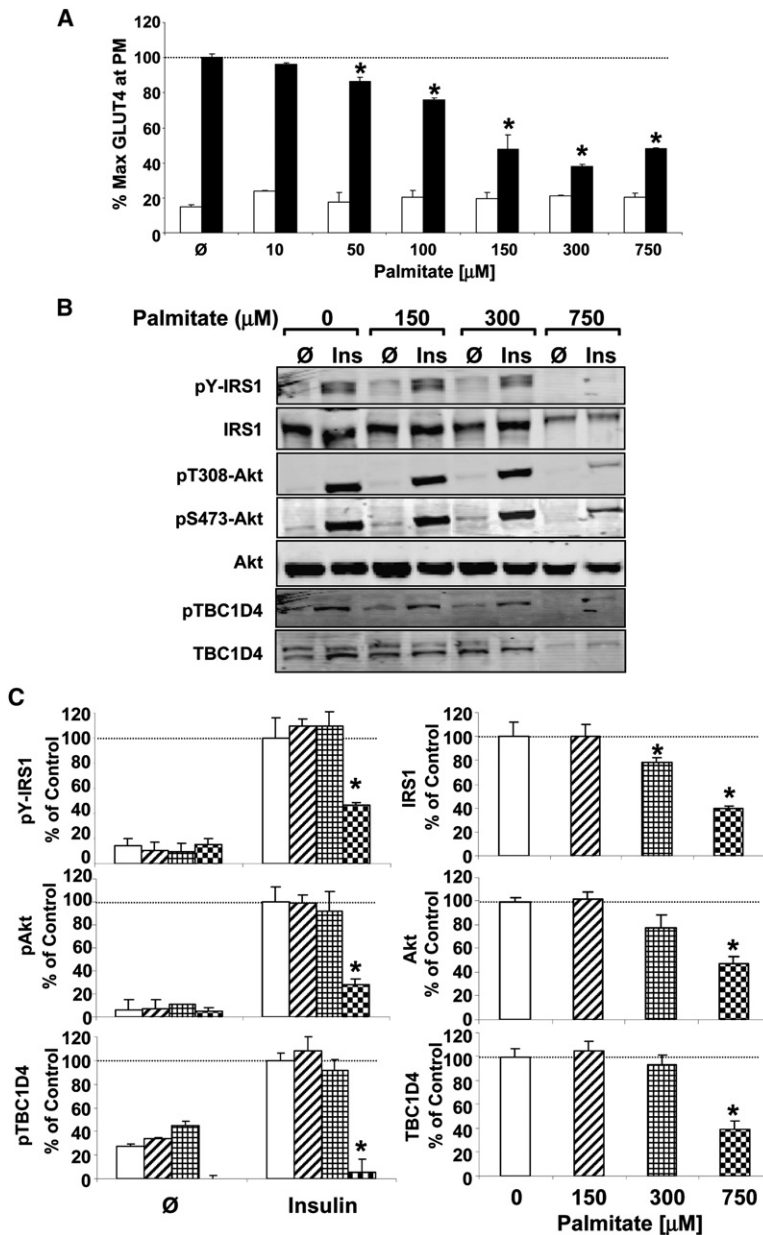
Figure 5. Palmitate Antagonizes Insulin Action at GLUT4 without Disrupting Signal Transduction through TBC1D4 in L6 myotubes

(A) Basal (white bars) and insulin-stimulated (black bars) GLUT4 translocation were measured in L6 myotubes incubated with a dose response of palmitate for 18 hr. Results are presented as means ± SEM of 3 independent experiments of >3 measurements each. *p < 0.05 between palmitate and carrier control by Student's t test.

(B) Representative western blots of phosphorylated and total IRS1, Akt, and TBC1D4 in palmitate-treated myotubes.

(C) No significant difference was observed in the phosphorylation of IRS1, Akt, or TBC1D4 between control myotubes and those treated with up to 300 μM palmitate. However, higher concentrations severely antagonized insulin action at these molecules as shown. Results are presented as means ± SEM. *p < 0.05 between palmitate and control by Student's t test.

No defects in either IRS1 expression or insulin-stimulated IRS1 tyrosine phosphorylation, Akt phosphorylation, or TBC1D4 phosphorylation were observed using palmitate concentrations between 0 and 150 μM (Figures 5B and 5C), whereas at concentrations exceeding 300 μM, substantial defects in each of these signaling parameters were observed. Again, this model of insulin resistance points to a major node of insulin resistance distal to IRS/PI3K/Akt and suggests that signaling defects may arise either as a consequence of insulin resistance per se (i.e., reduced GLUT4 translocation) or independently of insulin resistance, possibly due to toxic "off-target" effects.

The Role of IRS/Akt in High-Fat-Fed Mice

The above studies indicate that while a range of signaling defects can be observed in some (GO, CI, TNF, and DEX), but not all (PALM), models of insulin resistance, it is apparent that there is sufficient spareness in many of these elements such that they may represent neither the sole nor the primary defect. While such cell systems have been invaluable in dissecting the molecular basis of insulin resistance, it is important to determine whether such findings can be translated to a more physiological setting. Hence, we next performed a series of studies in transgenic mice overexpressing PDGFR in muscle. PDGF stimulated signal transduction and glucose transport to an extent similar to insulin in isolated soleus muscle from these mice (Figures 6A–6C). Thus, these animals provide a unique system with which to assess the role of IRS and downstream signaling elements in insulin resistance in vivo.

We created insulin resistance in this model by dietary modification to reflect the physiological impact of poor diet. The diets consisted of either standard lab chow (8% of calories from fat, 21% of calories from protein, 71% of calories from carbohydrates; 2.6 kcal/g) or a high-saturated-fat diet (HFD; 45% of calories from fat, of which 87% was lard and 13% safflower oil by weight, 20% of calories from protein, and 35% of calories from

PDGF-stimulated GLUT4 translocation were inhibited by >50% (Figure 3B) without any observed signaling defects at IRS1, Akt, or TBC1D4 (Figure 4). This was surprising, as other investigators have described defects in insulin signaling following incubation of either adipocytes or myotubes with palmitate (Powell et al., 2004; Sinha et al., 2004). However, many of these studies utilized much higher doses of palmitate to achieve these effects than those used in the present study. In our preliminary investigations, we observed that high (>300 μM) palmitate doses were toxic to cells, resulting in morphological changes and even detachment from the substratum. Hence, we further characterized the dose-response relationship between palmitate and insulin action. We observed a significant reduction in insulin-stimulated GLUT4 translocation with palmitate doses as low as 50 μM, reaching a maximum diminution at 150 μM (Figure 5A).
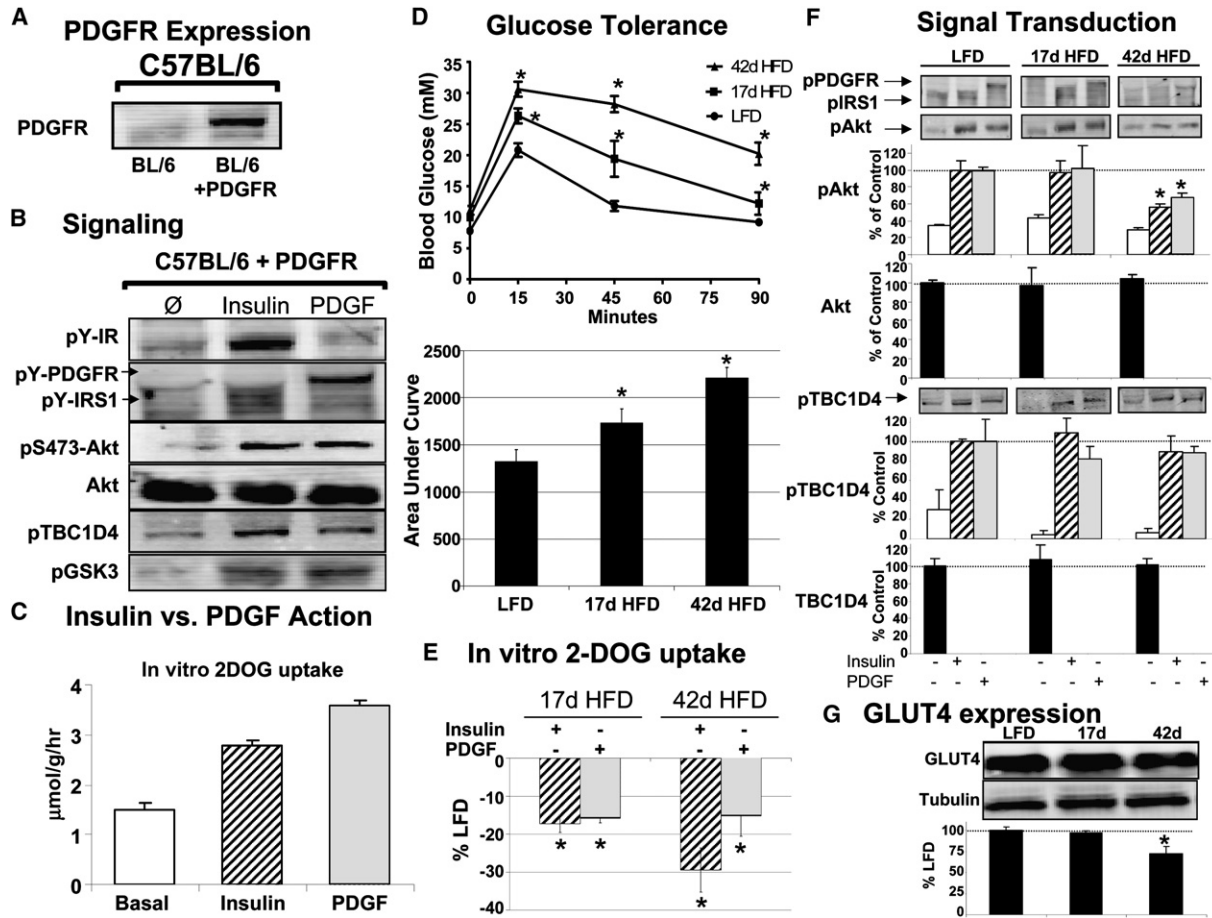
Figure 6. Modulation of Insulin and PDGF Action in High-Fat-Fed PDGFR Transgenic Mice

(A) Intact soleus muscles from transgenic mice were blotted for expression of PDGFR.

(B) Isolated soleus muscles were incubated in vitro and stimulated with 2 mU/ml insulin or 500 ng/ml PDGF for 30 min, and western blots were performed on whole-cell lysates. Representative blots are shown. Phosphorylation of Akt at Thr308 was undetectable.

(C) PDGF had a more potent effect on 2-[2-6-^{3}H]deoxyglucose (2-DOG) uptake into soleus muscle than insulin in transgenic mice (3.59 ± 0.09 versus 2.78 ± 0.12 μmol 2-DOG/g/hr), consistent with previous studies (Yuasa et al., 2004). Results are presented as means ± SEM, n = 5–7 soleus muscles per group.

(D) Glucose tolerance tests were performed on mice in the 4 hr fasted state to predict insulin resistance. Diet durations of both 17 and 42 days produced significant reductions in glucose tolerance as indicated by differences in blood glucose levels of mice fed a high-fat diet (HFD) versus the group fed a low-fat diet (LFD). Individual points are displayed as means ± SEM, n = 7–9 mice per curve. *p < 0.05 between LFD and HFD groups by Student's t test.

(E) Reduction in insulin-stimulated (hashed bars) and PDGF-stimulated (gray bars) 2-DOG uptake in isolated soleus muscles from mice fed HFD for 17 days (−17% ± 6% and −16% ± 5% of control insulin- and PDGF-stimulated uptake, respectively) or 42 days (−29% ± 5% and −15% ± 2% of control insulin- and PDGF-stimulated uptake, respectively). Results are displayed as means ± SEM, n = 3–7 soleus muscles per data set. *p < 0.05 between LFD and HFD groups by Student's t test.

(F) Representative western blots and bar graphs for soleus muscles stimulated with insulin (hashed bars) or PDGF (gray bars) for 30 min. Total protein levels are shown in white or black bars. Results are displayed as means ± SEM, n = 3–5 soleus muscles per experiment. *p < 0.05 between LFD and HFD groups by Student's t test.

(G) Representative western blots and quantification of GLUT4 expression in mice fed LFD or HFD for 17 and 42 days. Results are displayed as means ± SEM, n = 4 per group. *p < 0.05 between LFD and HFD groups by Student's t test.

carbohydrates; 4.7 kcal/g). Consistent with other reports (Bonnard et al., 2008), we observed glucose intolerance within weeks of HFD feeding (Figure 6D). To study the mechanism of this effect, we examined the capacity of insulin and PDGF to increase glucose uptake and activate signal transduction in this model (Figures 6E and 6F). HFD feeding for as little as 17 days resulted in a significant reduction in both insulin- and PDGF-stimulated 2-[2-6-^{3}H]deoxyglucose (2-DOG) uptake, without any detectable decrease in GLUT4 levels (Figure 6G) or Akt or TBC1D4 phosphorylation. As was the case with the cell models described

above, impaired insulin- and PDGF-stimulated Akt phosphorylation was apparent only with longer-term dietary treatment (42 days HFD; Figure 6F). It should also be noted that we observed a significant 30% reduction in total muscle GLUT4 levels in the 42 days HFD animals. Intriguingly, this was accompanied by an additional decrease in insulin-stimulated, but not PDGF-stimulated, 2-DOG uptake compared to that observed in 17 days HFD animals. This suggests that IRS-dependent defects might contribute to a worsening or maintenance of the condition. These data are consistent with the in vitro PALM data in

Table 1. Summary of Defects

	IRS1 Defect		Phospho-Akt		Phospho-TBC1D4		GLUT4/2-DOG		
	Phospho-Tyr	Expression	Insulin	PDGF	Insulin	PDGF	Insulin	PDGF	Primary Defect
Glucose oxidase	−	−	−	−	↓↓	↓↓	↓↓	↓↓	IRS-independent
Chronic insulin	−	↓↓	↓↓	−	−	−	↓↓	↓	IRS-dependent
TNFα	↓↓	↓↓	↓↓	−	−	−	↓↓	↓↓	both
Dexamethasone	−	↓↓	↓↓	↓↓	−	−	↓↓	↓↓	both
Palmitate	−	−	−	−	−	−	↓↓	↓↓	IRS-independent
17 days HFD	−	−	−	−	−	−	↓↓	↓↓	IRS-independent

Summary of defects in insulin- and PDGF-stimulated signal transduction and GLUT4 translocation (for cell culture models) and 2-DOG uptake (for animal model). Arrows indicate the severity of the defect for each variable, and minus sign (−) represents the absence of a defect. The last column indicates the existence of IRS-dependent and/or -independent defects.

myotubes indicating that early defects leading to insulin resistance are not due to impaired IRS/Akt function and that while defects in Akt phosphorylation are observed with prolonged HFD, there is sufficient spareness in the system such that these defects are not necessarily transmitted further down the pathway since phosphorylation of TBC1D4 remained intact.

DISCUSSION

Defining the mechanism (or mechanisms) of insulin resistance is challenging. Numerous insults can cause insulin resistance, yet it has been difficult to establish whether there is a common link between these disparate triggers. A common dogma in the field is that IRS proteins represent a major node of insulin resistance for most of these models (Aguirre et al., 2000; Dresner et al., 1999; Gao et al., 2002, 2003; Gual et al., 2005; Hotamisligil et al., 1996; Morino et al., 2005; Ozcan et al., 2004; Rice et al., 1993; Turnbow et al., 1994; Ueki et al., 2004; White, 2002; Yu et al., 2002; Zick, 2005; Zierath et al., 1997). In the present study, we provide evidence that while defects in upstream elements of the insulin cascade may occur in various insulin-resistance models, it is unlikely that this is a major cause of impaired glucose metabolism, the defining feature of insulin resistance.

We have utilized a system enabling us to test the role of IRS proteins in insulin resistance by comparing the effects of insulin and PDGF on signaling and metabolism. For example, with chronic insulin, a significant reduction in insulin-stimulated GLUT4 translocation was observed whereas PDGF-stimulated GLUT4 translocation was similar to that observed in control cells. This indicates that with chronic insulin, the major impairment likely involves a target specific to the IR, most likely IRS1. This is consistent with previous literature and acts as an important control for the present model system. In contrast, in the other models, which more closely parallel physiologic states of insulin resistance, we observed a similar impairment in GLUT4 translocation with both ligands. The most extreme discordance between upstream signaling and GLUT4 translocation was observed in palmitate-treated L6 myotubes and HFD-fed mice. Here, using either low doses of palmitate or relatively short exposure to HFD, we observed impaired insulin- and PDGF-stimulated GLUT4 translocation and 2-DOG uptake, respectively, without any detectable defect within the IRS/PI3K/Akt nexus. This raises the possibility that upstream signaling defects are a consequence of insulin resistance rather than a cause of the

condition. In this scenario, lesions in signal transduction possibly exacerbate the insulin-resistant state to support a more chronic and progressive dysfunction resulting in metabolic diseases like type 2 diabetes. Collectively, these studies suggest that while defects in IRS/PI3K/Akt may occur in insulin resistance, it is unlikely that such defects contribute to its early development. Alternatively, these signaling defects may be completely independent of the metabolic milieu and may possibly involve cytotoxicity, a potential consequence of chronic exposure to cytokines, lipids, and other metabolic insults that in many cases lead to cellular stress (Bloch-Damti and Bashan, 2005; Evans et al., 2005; Kaneto et al., 2005).

A major problem with many studies that have implicated an important role for upstream signaling elements in insulin resistance is that they assume that such pathways operate stoichiometrically such that a modest defect in one component will be linearly transmitted all the way down the pathway. However, our analysis of insulin action under control and insulin-resistant conditions indicates that these pathways are governed more by a stochastic operating principle. This can be illustrated by the observation that only a finite amount of Akt is required to be phosphorylated in order to orchestrate a maximal biological response (Figure 1). Moreover, once activated, there is discordance in the ability of Akt to activate discrete downstream substrates such as TBC1D4 and GSK3. The nature of this differential transmission downstream of Akt may involve a role for discrete Akt isoforms, the unique localization of certain Akt substrates, or the assembly of unique signaling subcomplexes involving scaffolding proteins, and this certainly requires further investigation. Careful analysis of our insulin-resistance models as summarized in Table 1 reveals a somewhat fragmented response pattern in upstream elements of the pathway, which again is consistent with stochastic regulation involving many negative feedback loops. However, a unifying feature that emerges from this analysis is that despite this fragmented pattern upstream, both insulin- and PDGF-stimulated TBC1D4 phosphorylation responded quite normally in the face of insulin resistance at the level of GLUT4 translocation.

How do insulin-sensitive cells achieve graded outputs in response to different concentrations of the relevant agonist given that the active range of Akt with respect to glucose transport appears to be extremely finite? One possibility is that signaling through Akt is a digital rather than an analog response. A similar mechanism has been described for Ras signaling (Tian et al., 2007). In addition, we have recently presented a model whereby

Akt may control GLUT4 translocation via a quantal release mechanism again consistent with a digital or threshold response (Larance et al., 2008). Such a mechanism likely denotes signaling subcompartments that are not resolved by the types of analyses employed in this and many other studies. For example, it has been suggested that there are critical pools of IRS1 that may be localized close to the plasma membrane (Clark et al., 1998), in which case one can envisage that defects in these compartments may disrupt signaling through the pathway in an apparently nonlinear fashion. While we cannot dismiss these possibilities, it seems likely that such adaptations would merely account for the nonlinearity in the system, thus emphasizing the need to exercise caution when interpreting analyses involving simple changes in total levels or the phosphorylation status of such intermediates. With this in mind, it is therefore important to consider the output from signaling nodes such as Akt substrate phosphorylation or GLUT4 translocation, which in both cases was discordant with the flux through these upstream elements.

How can the present data be interpreted in light of numerous studies suggesting that IRS1 is the major node of insulin resistance? First, it is noteworthy that many studies have focused on signaling rather than metabolic endpoints as their index of insulin resistance. Here we utilized GLUT4 translocation or glucose transport as our index of insulin resistance. This is important because not only is this process rate limiting for many of the metabolic actions of insulin, but defects in glucose transport appear to represent some of the earliest and most significant defects observed in insulin resistance (Petersen and Shulman, 2006). Moreover, our analysis of GLUT4 translocation was normalized to the total expression of GLUT4 in the cell, thus overcoming the contribution of altered GLUT4 expression in response to insulin-resistance treatments (Hajduch et al., 1992, 1995; Kaestner et al., 1991; Lundgren et al., 2004; Stephens et al., 1997; Stephens and Pekala, 1992). We also suggest that this assay is superior to the measurement of cellular glucose transport or glycogen synthesis, as this is easily influenced by changes in the expression of alternate transporter isoforms such as GLUT1, a common feature of in vitro cell culture models of insulin resistance (Hosaka et al., 1999; Mei et al., 2003; Sakoda et al., 2000) but not in vivo insulin resistance (Kahn et al., 1991). Second, many studies have also overlooked the temporal and/or dose-response characteristics of the metabolic insult with respect to the onset of insulin resistance. In the present study, we carefully characterized minimal conditions required to achieve maximal insulin resistance as defined by inhibition of GLUT4 translocation to the plasma membrane in cell culture or glucose uptake in mice. This was essential, as in at least two models (150 μM palmitate in L6 myotubes and 17 days HFD in mice) we observed marked insulin resistance without any significant change in signaling, whereas under more extreme conditions signaling defects became apparent. Finally, many studies implicating IRS proteins in insulin resistance rely on correlative changes between metabolism and IRS Ser/Thr phosphorylation in response to various reversal strategies. For instance, inhibition of mTOR with rapamycin or JNK with peptide and small-molecule drugs has been used in this way, yet these agents likely impact upon many cellular processes due to their effect on protein synthesis and gene expression, respectively. Thus, if insulin resistance is independent of the signaling defects as suggested here, then any manipulation that corrects insulin resistance might also potentially override defects in signaling that derive from the same insult.

A major question arising from these studies is, what are the molecular targets that contribute to insulin resistance? Based on the present studies, we conclude that these defects likely occur downstream of IRS, and in some cases possibly downstream of Akt. However, in relation to the latter it is important to emphasize that the regulation of Akt is proving to be quite complex, as indicated by recent studies showing a role for the Akt-binding protein APPL1 in insulin-stimulated GLUT4 translocation (Saito et al., 2007). Hence, this may invoke some type of nanoregulation that will be challenging to dissect. This could involve the assembly of signaling modules close to or at the plasma membrane. In this regard, it is interesting that reduced levels of PIP$_2$ at the cell surface may disrupt the ability to recruit GLUT4 vesicles to this site, possibly due to disruptions in the actin cytoskeleton (McCarthy et al., 2006; Strawbridge and Elmendorf, 2005). This poses yet another important question: Does insulin resistance represent a uniform disruption in all of the metabolic actions of insulin, or is there a temporal progression of defects whereby an initial defect in glucose transport, for example, could subsequently lead to defects in other metabolic parameters such as glycogen and lipid synthesis? Consistent with such a hypothesis is the observation that deletion of GLUT4 in adipose tissue results in insulin resistance in other tissues in the intact animal (Abel et al., 2001). Hence, this potentially places GLUT4 translocation and glucose transport at the center of this disorder.

In conclusion, these data reveal that upstream elements of the insulin signaling cascade are not a central feature of the origin of insulin resistance as commonly thought. While defects in these upstream signaling elements may be frequently observed in insulin resistance in view of the stochastic nature of the pathway, such defects may or may not be transmitted further downstream. Moreover, such defects may occur as a consequence of an alternate initial defect, possibly to sustain the insulin-resistant state. The nature of this initial defect (or defects) remains unknown at the present time. It is likely that a search for additional Akt substrates and other downstream regulators of this pathway will be revealing. The identification of such targets will be of enormous utility in the design of targeted therapies for prevention of insulin resistance. This study provides molecular insight into the mechanisms and origins of insulin resistance and should facilitate a renewed focus on the pursuit of new molecular targets downstream of IRS.

EXPERIMENTAL PROCEDURES

Models of Insulin Resistance

3T3-L1 preadipocytes or L6 myoblasts were infected with replication-incompetent retroviruses expressing HA-GLUT4 and human PDGF β receptor and selected with puromycin and Geneticin. Cells were differentiated into adipocytes or myotubes as described previously (Lee et al., 2006).

Oxidative Stress

Oxidative stress (GO) was induced in 3T3-L1 adipocytes by washing cells three times in phosphate-buffered saline (PBS) and incubating with serum-free DMEM supplemented with 0.1% BSA (stepdown medium) containing 50 mU/ml glucose oxidase or carrier (0.16 μM sodium acetate [pH 7.4]) for 2 hr. This generated a final H$_2$O$_2$ concentration of 25.8 ± 0.1 μM, while total glucose levels (measured with an Accu-Chek II glucometer [Roche]) decreased slightly from 24.7 ± 1.6 mM to 23.3 ± 1.9 mM.

Chronic Low-Dose Inflammation
Chronic low-dose inflammation (TNF) was mimicked in 3T3-L1 adipocytes by incubation with 2 ng/ml TNFα (Calbiochem) for 4 days. Medium was changed every 24 hr. On the fourth day, cells were washed three times with PBS and incubated in stepdown medium in the absence of TNFα for 90 min prior to acute insulin or PDGF stimulation.

Hyperinsulinemia
Hyperinsulinemia (CI) was created by addition of 10 nM insulin to adipocytes at 1200, 1600, and 2000 hr on day 1 and 0800 hr on the following day. At 1200 hr on the second day, cells were washed 4–5 times with PBS and cultured in stepdown medium for 90 min before acute insulin or PDGF stimulation.

Glucocorticoid
Glucocorticoid-induced insulin resistance (DEX) was created with 20 nM dexamethasone (0.01% ethanol carrier as control) changed every other day for 8 days. On day 8, the cells were washed three times with PBS and incubated for 90 min in stepdown medium before acute stimulation.

Palmitate
Palmitate (PALM) was conjugated to 20% BSA in culture medium at 5-fold of the final concentration. BSA/palmitate or BSA/ethanol (control) was heated to 50°C for 20 min, cooled to 37°C, and diluted five times with culture medium before filter sterilizing. The flowthrough was delivered to cells at the specified doses with a final BSA concentration of 4% in all treatments. The complete treatment lasted 18 hr and consisted of two additions of palmitate. The first treatment was performed in the presence of 2% fetal calf serum for 16.5 hr, after which the myotubes were washed three times with PBS, and the second incubation was identical to the first except that it was performed without serum for 90 min prior to acute stimulation with insulin or PDGF.

All insulin-resistance treatment regimens were initiated in 3T3-L1 adipocytes between 7 and 12 days postdifferentiation and in L6 myotubes between 6 and 9 days postdifferentiation. After the insulin-resistance treatment, 100 nM insulin or 20 ng/ml PDGF was added to the cells for 20 min before harvest or analysis of GLUT4 translocation. Total GLUT4 protein levels were measured by western blot following each treatment condition. While we did not observe a significant change in GLUT4 levels following GO, CI, or PALM treatments, GLUT4 levels were increased by 22% and 94% after treatment with TNF and DEX, respectively (data not shown). Therefore, all measures of GLUT4 translocation were normalized to total GLUT4 and are represented as the percent of GLUT4 at the plasma membrane.

Animals
Male C57BL/6 mice overexpressing PDGFR in muscle were obtained from Yuasa et al. (2004). Homozygous transgenic mice (clone #2) were fed a chow diet until 8 weeks of age, when they were split into two groups. One group was maintained on standard lab chow (8% of calories from fat, 21% of calories from protein, 71% of calories from carbohydrates; 2.6 kcal/g), and the other was switched to a high-saturated-fat diet (45% of calories from fat, of which 87% was lard and 13% safflower oil by weight, 20% of calories from protein, and 35% of calories from carbohydrates; 4.7 kcal/g; based on Rodent Diet #D12451 [Research Diets, Inc.]) for a period of 17 days or 6 weeks. Mice were housed in a temperature-controlled room (22°C ± 1°C) on a 12 hr light/dark (0700/1900) cycle.

Materials
Rat recombinant PDGF-BB, puromycin, dexamethasone, biotin, isobutylmethylxanthine, pyruvate, mannitol, thin-layer chromatography plates, potassium oxalate, and glucose oxidase were from Sigma. Geneticin, 100× penicillin/streptomycin/glutamine solution, NuPAGE precast gels, DMEM, and α-MEM were from Invitrogen. [1-^{14}C]mannitol, 2-[2-6-^{3}H]deoxyglucose, and [γ-^{32}P]ATP were from GE Health Care. Fetal calf serum was from Thermo (Melbourne, Australia). Standard rodent chow was from Gordon's Specialty Stock Feeds. The Akt1/2 inhibitor was obtained from Symansis; details on its characterization can be found elsewhere (DeFeo-Jones et al., 2005).

Antibodies
Phospho-Ser473 Akt, phospho-Thr308 Akt, total Akt, and phospho-GSK3 were from Cell Signaling Technology. Antibodies against IR, PDGFR, IRS1, and phospho-Tyr99 (used in PI3K assays) were from Santa Cruz Biotechnology. Phospho-Tyr IR and phospho-Tyr IRS antibodies were from Millipore

(formerly Upstate Biotechnology). The phospho-Thr642 TBC1D4 antibody was created for us by Symansis.

Animal Experiments
Intraperitoneal (i.p.) glucose tolerance tests (GTTs) were performed on mice on the morning of sacrifice. Mice were fasted for 4 hr (0730–1130) before i.p. injection of 25% glucose solution at a dose of 2g/kg. Blood glucose was measured by sampling blood from the tail tip with an Accu-Chek II glucometer. After the GTT, mice were killed by cervical dislocation, soleus muscles were rapidly dissected tendon to tendon, and in vitro 2-DOG uptake was measured as described previously (Turner et al., 2007).

Soleus muscles (∼10 mg) were processed for immunoblotting by homogenizing at 4°C with 250 μl of 1× PBS (pH 7.4) containing 1 mM EDTA, 30 mM sodium pyrophosphate, 10 mM NaF, 150 mM NaCl, 2 mM Na$_3$VO$_4$, 1% NP-40, 10% glycerol, and 1× protease inhibitor cocktail (Roche) using a Polytron instrument (Kinematica). The lysates were rotated at 4°C for 1 hr before being centrifuged at 16,000 × g for 15 min at 4°C. Supernatant protein concentration was determined by the bicinchoninic acid (BCA) method (Pierce).

Immunoblots
3T3-L1 and L6 cultures were washed twice with cold PBS before harvesting with HES buffer (pH 7.4) containing 2% SDS. Lysates were sonicated for 15 s and centrifuged at 15,000 × g for 20 min at 15°C. Supernatants were then assayed for protein content by BCA assay. Homogenates from soleus muscle were prepared as described above. Clarified lysates were then diluted with 4× Laemmli buffer and heated to 65°C for 3 min. Equal amounts of protein were electrophoresed through self-made 8% polyacrylamide gels or NuPAGE precast gradient gels, transferred to PVDF membranes, and blocked with 5% BSA in Tris-buffered saline (TBS) for 1 hr. Blocked membranes were incubated overnight at 4°C in primary antibodies diluted in TBS-0.1% Tween 20 (TBST) containing 5% BSA and 0.05% sodium azide. The following morning, membranes were incubated for 0–2 hr more at room temperature and washed for 30 min in TBST. Membranes were then incubated with their respective fluorescently labeled secondary antibodies diluted in TBST + 5% BSA + 0.1% SDS in the dark at room temperature for 1 hr and washed three times for >30 min in the dark. Membranes were then developed and quantified using a LI-COR Odyssey infrared imaging instrument and software. 14-3-3 expression and Ponceau staining were utilized as loading controls for these experiments.

PI3K Activity Assays
These assays were performed as described previously (Wang and Summers, 2003).

GLUT4 Translocation Assays
These assays were performed as described previously (Govers et al., 2004).

Statistical Analyses
Data are expressed as means ± SEM. For GLUT4 translocation analysis, unless otherwise specified, each experiment was normalized to 100% of maximal insulin before comparison across experiments to decrease interassay variation and allow direct comparison across all insults of the same cell type. p values were calculated by two-tailed Student's t test using Microsoft Excel or GraphPad Prism. Statistical significance was set at $p < 0.05$.

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