Original Article

Opposite Effect of JAK2 on Insulin-Dependent Activation of Mitogen-Activated Protein Kinases and Akt in Muscle Cells

Possible Target to Ameliorate Insulin Resistance

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Many cytokines increase their receptor affinity for Janus kinases (JAKs). Activated JAK binds to signal transducers and activators of transcription, insulin receptor substrates (IRSs), and Shc. Intriguingly, insulin acting through its own receptor kinase also activates JAK2. However, the impact of such activation on insulin action remains unknown. To determine the contribution of JAK2 to insulin signaling, we transfected L6 myotubes with siRNA against JAK2 (siJAK2), reducing JAK2 protein expression by 75%. Insulin-dependent phosphorylation of IRS1/2 and Shc was not affected by siJAK2, but insulin-induced phosphorylation of the mitogen-activated protein kinases (MAPKs) extracellular signal–related kinase, p38, and Jun NH_2-terminal kinase and their respective upstream kinases MKK1/2, MKK3/6, and MKK4/7 was significantly lowered when JAK2 was depleted, correlating with a significant drop in insulin-mediated cell proliferation. These effects were reproduced by the JAK2 inhibitor AG490. Conversely, insulin-stimulated Akt phosphorylation, glucose uptake, and GLUT4 translocation were not affected by siJAK2. Interestingly, in two insulin-resistant states, siJAK2 led to partial restoration of Akt phosphorylation and glucose uptake stimulation but not of the MAPK pathway. These results suggest that JAK2 may depress the Akt to glucose uptake signaling axis selectively in insulin-resistant states. Inhibition of JAK2 may be a useful strategy to relieve insulin resistance of metabolic outcomes. *Diabetes* 55: 942–951, 2006

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ERK, extracellular signal–related kinase; FBS, fetal bovine serum; IRS, insulin receptor substrate; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; PP2A, protein phosphatase-2A; siIRS-1, siRNA targeted against IRS-1; siJAK2, siRNA targeted against JAK2; siNR, siRNA targeted against nonrelevant control; SOCS, suppressors of cytokine signaling.

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Polypeptides such as erythropoietin, prolactin, leptin, angiotensin, growth hormone, most interleukins, and interferon-γ bind to receptors that lack intrinsic kinase activity, recruiting and activating cytoplasmic tyrosine kinases of the Janus family (JAK) consisting of JAK1, JAK2, JAK3, and Tyk2 (1–3). Activated JAK phosphorylates tyrosine residues within itself and the associated receptor forming high-affinity binding sites for a variety of signaling proteins containing Src homology 2 and other phosphotyrosine-binding domains, including signal transducers and activators of transcription, insulin receptor substrates (IRSs), and the adaptor protein Shc (1–4). Shc is responsible for the activation of the mixed-function tyrosine/serine/threonine kinases MEKs (mitogen-activated protein kinases [MAPKs] that in turn activate the extracellular signal–related kinanes [ERKs]) MAPKs, important for the mitogenic response (4). JAK is also the mediator of insulin-like effects of some polypeptides like growth hormone through phosphorylation of IRS and activation of their downstream effectors phosphatidylinositol (PI) 3-kinase and Akt, mainly required for metabolic functions (5–8). Moreover, JAK is the cytoplasmic kinase through which circulating factors such as tumor necrosis factor-α and pro-inflammatory cytokines negatively regulate insulin action through serine phosphorylation of IRS-1 or induction of suppressors of cytokine signaling (SOCS) expression (9).

Insulin increases the autophosphorylation and tyrosine kinase activity of its receptor and allows it to interact with intracellular substrates (10). The two major pathways thus activated are IRS→PI 3-kinase→Akt, leading to metabolic functions, and Shc→MAPKs, resulting in the insulin mitogenic effects (10–12). Interestingly, several studies have reported cross talk between the insulin receptor kinase and JAK signaling pathways. Insulin induces tyrosine phosphorylation of JAK1 and JAK2 and promotes their association with both insulin receptor and pp185 (IRS) in cells overexpressing insulin receptors (13–15). In vivo, upon administration of physiological concentrations of insulin to rats, JAK2 associates with the insulin receptor and is tyrosine phosphorylated in insulin-sensitive tissues (16). Insulin-stimulated JAK2 phosphorylation is variably regulated in animal models of insulin resistance, rising in liver of fasted rats (16) and dropping in heart of fasted (17)

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and aged rats (16). However, the implication of such changes on insulin action was not analyzed.

It is also unknown if and how insulin-mediated JAK2 activation impinges on insulin signaling in muscle cells, yet muscle tissue is the main site responsible for glucose uptake in response to insulin. The objective of this study was to determine the contribution of JAK2 to the metabolic and mitogenic responses of insulin, using siRNA-mediated specific gene silencing and a chemical inhibitor of JAK2. The transient nature of siRNA-mediated protein reduction minimizes the possibility of compensatory regulation of other proteins that may mask the biological consequences of eliminating JAK2. We used the L6GLUT4myc rat skeletal muscle cell line that stably expresses a myc-tagged GLUT4, where the expression of the GLUT4myc is under the control of a constitutive promoter (18). This allowed us to determine the importance of JAK2 in the acute regulation of glucose uptake and GLUT4 translocation (18–20).

RESEARCH DESIGN AND METHODS

Phospho-specific antibodies to JAK2 (Tyr1007/1,008), Shc (Tyr317), IRS-1 (Ser307), Akt (Ser473), p38MAPK (Thr180/Tyr182), SAPK/Jun NH_2-terminal kinase (JNK) (Thr183/Tyr185), ERK (Thr202/Tyr204), MEK1/2 (Ser217/221), MKK3/MKK6 (Ser189/207), and SEK1/MKK4 (Thr261) were purchased from Cell Signaling (Beverly, MA). Monoclonal anti-phosphotyrosine, monoclonal anti-Rac, anti-JAK2, anti–IRS-1, and anti–IRS-2 (for immunoprecipitation and immunoblotting experiments) were from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti–SOCS-3 (H-103) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). WST-1/ECS cell proliferation assay kit was from Chemicon International (Temecula, CA). siRNAs targeted against JAK2 (siJAK2), IRS-1 (siIRS-1), and nonrelevant control (siNR) were purchased from Dharmacon (Lafayette, CO). The sequence of the JAK2 siRNA used was GGA AUG GCU UGC CUU ACA AdTdT; the IRS-1 was AAC AAG ACA GCU GGU ACC AGG; and the nonrelevant control X was AUU CUA UCA CUA GCG UGA CUU. The nonrelevant control siRNA sequence was screened against the EST Gene bank database and had no homology to the vertebrate genome. Tyrphostin B42 (AG490) and D-erythro-sphingosine, *N*-acetyl (ceramide) were from Calbiochem (San Diego, CA). Cytochalasin B, protease inhibitors, and O-phenylenediamine dihydrochloride (OPD reagent) were from Sigma (Oakville, Canada).

Tissue culture. L6 muscle cells stably expressing myc-tagged GLUT4 (L6-GLUT4myc cells) were described previously (20,21). GLUT4-myc myoblasts were differentiated into myotubes in α-minimum essential medium supplemented with 2% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution over 7 days. Cultures were deprived of serum for 5 h before stimulation with insulin.

Transfection of siRNA. L6 myoblasts were transfected with siJAK2, siIRS-1, or siNR using a calcium phosphate-based transfection reagent (CellPhect Transfection kit; Amersham Biosciences, Burlington, Canada) as previously described (22).

Measurement of cell proliferation in myoblasts. L6 myoblasts plated in a 96-well plate were transfected with 100 nmol/l siJAK2 on day 2, and the medium was changed 12–14 h later to α-minimum essential medium supplemented with 5% FBS with 1% antibiotic/antimycotic. On day 4, the cells were kept in 100 μl 0.1% FBS with or without insulin for 18 h. Ten microliters WST-1/ECS (cell proliferation assay kit) was added in each well, and after 30 min the absorbance was measured using a microplate reader at 420-nm wavelength.

Determination of 2-deoxyglucose uptake. 2-Deoxyglucose uptake measurements were carried out as previously described for 5 min in HEPES-buffered saline containing 10 μmol/l 2-[^{3}H] deoxyglucose (0.5 μCi/ml) in the absence of insulin (18,23).

Immunoprecipitation of IRS-1 and IRS-2. IRS-1 and IRS-2 were immunoprecipitated from L6 myotubes as previously described (22). Briefly, 500 μg or 1 mg protein of whole-cell lysates in TX-100 were incubated with IRS-1– or IRS-2–specific antibodies at 4°C overnight under constant rotation and then pulled down with protein A/G Sepharose beads. The pelleted protein was resolved by 7.5% SDS-PAGE, and tyrosine phosphorylation of IRS-1 or IRS-2 was detected by immunoblotting with monoclonal anti-phosphotyrosine antibody.

Immunoprecipitation of Akt1 and Akt2. Immunoprecipitation of Akt1 and Akt2 was performed as previously described (22,23). The antibody-antigen complex was pulled down by protein A/G Sepharose beads and resolved by 10% SDS-PAGE. Serine phosphorylation of Akt1 or Akt2 was detected by immunoblotting using anti–phospho-Akt-Ser473 antibody.

Measurement of GLUT4myc translocation in L6 myotubes. Cell surface myc-tagged GLUT4 density in L6 myotubes was quantified by an antibody-coupled colorimetric assay as validated previously (19).

Detection of cellular protein expression and protein phosphorylation. Whole-cell lysates were made from myotubes after incubation with insulin (0–100 nmol/l) for 10 min, the time when stimulation yields maximal phosphorylation of Akt and MAPKs (22,24). Forty micrograms of lysates was resolved by SDS-PAGE and transferred onto polyvinylidine fluoride filters, which were incubated with primary antibodies overnight at 4°C under constant agitation (dilutions: polyclonal anti–IRS-1 or anti–IRS-2, 1:1,000; anti-Akt, anti-ERK, anti-JNK, and anti-p38MAPK, 1:500 to 1:1,000; anti–phospho-Akt-Ser473, anti–phospho-ERK, anti–phospho-p38MAPK, anti–phospho-JNK, anti–phospho-MKK1/2, anti–phospho-MKK3/6, and anti–phospho-MKK4/7, 1:500), followed by 1 h of incubation with horseradish peroxidase–conjugated secondary antibody (1:15,000 goat anti-rabbit antibody; 1:7,500 sheep anti-mouse antibody). Protein bands were visualized by enhanced chemiluminescence and Kodak X-Omat-blue film and scanned within the linear range using ImageJ software.

Rac activation by insulin. Activated Rac was pulled down from cell lysates using a glutathione S-transferase–fusion protein of the CRIB domain of p21 kinase conjugated to glutathione beads as described previously (25). Pulled down proteins were resolved by 10–12% SDS-PAGE gels, followed by immunoblotting using anti-Rac antibody.

Ceramide treatment. Serum-starved (3 h) L6 GLUT4myc myotubes were treated with or without 100 μmol/l ceramide or vehicle for the last 2 h. After treatment, myotubes were treated with or without 100 nmol/l insulin at 37°C for 20 min for glucose uptake or for 5 min for Akt phosphorylation.

RESULTS

JAK2 silencing using siRNA and insulin-induced JAK2 phosphorylation. To determine the contribution of JAK2 to insulin action, we induced gene-specific silencing using siJAK2 in L6GLUT4myc myotubes. A 48-h treatment with SiJAK2 caused a 75% drop in JAK2 protein expression (Fig. 1*A*). The reduction in JAK2 is expressed as percentage of the amount of protein present in cells treated with siNR. To examine the insulin-mediated tyrosine phosphorylation of JAK2, siRNA-treated L6GLUT4myc myotubes were incubated without or with 100 nmol/l insulin for 5 min, and whole-cell lysates were immunoblotted with anti–phospho-JAK2. Insulin-stimulated phosphorylation of JAK2 was reduced to 50 ± 9% in cells treated with siJAK2 compared with cells treated with siNR (Fig. 1*B*). Potentially, the 25% remaining JAK2 is either insufficient or unavailable for phosphorylation, or the sensitivity of the phosphorylation assay may have missed detecting a small increase in JAK2 phosphorylation. Regardless, this result suggests that siJAK2 is effective in blocking insulin signaling via JAK2.

JAK2 silencing does not affect insulin-mediated IRS1/2 and Shc phosphorylation. The first step in insulin signaling involves phosphorylation of IRSs and Shc. To examine the effect of JAK2 kinase elimination on IRS-1, IRS-2, and Shc, siJAK2-treated L6GLUT4myc myotubes were incubated without or with 100 nmol/l insulin, and immunoprecipitates of IRS-1 or IRS-2 were immunoblotted with anti–phospho-tyrosine antibodies. Depletion of JAK2 did not affect insulin-stimulated IRS-1 (Fig. 2*A*) or IRS-2 (Fig. 2*B*) phosphorylation. Similar results were obtained for insulin-mediated phosphorylation of the interacting protein Shc (Fig. 2*C*). siRNA-mediated reduction of JAK2 also had no impact on IRS-1, IRS-2, or Shc protein expression (data not shown).

JAK2 participates in insulin-stimulated phosphorylation of ERK, p38, and JNK and cell proliferation. From the previous experiments, we concluded that JAK2 is not required for insulin-stimulated phosphorylation of

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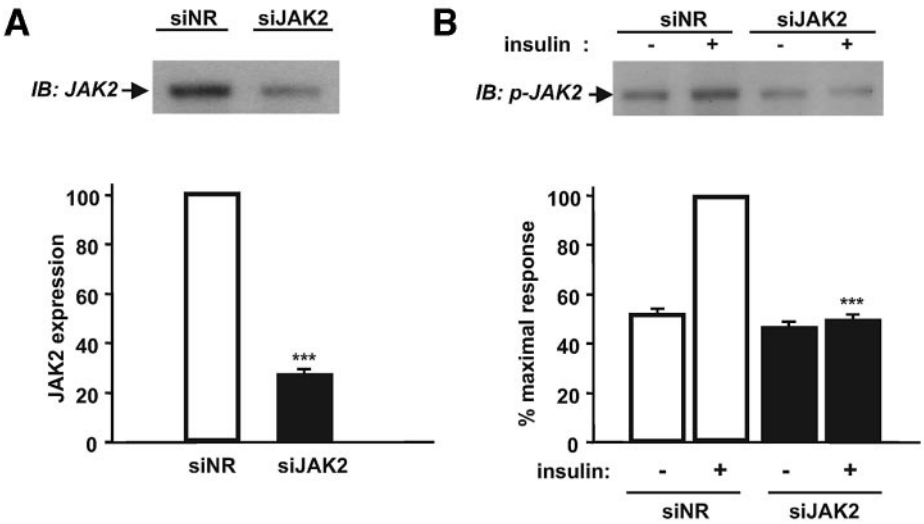

FIG. 1. JAK2 silencing using siRNA and effect on insulin-induced JAK2 phosphorylation in L6 muscle cells. *A*: Cell lysates from myotubes transfected with siJAK2 or siNR were prepared and immunoblotted (IB) for JAK2. A representative blot is shown, and the results represent the means ± SE of four independent experiments. ***$P < 0.001$, siNR vs. siJAK2. *B*: Total cell lysates were immunoblotted for phospho-JAK2. A representative blot and the results represent the means ± SE of four independent experiments. ***$P < 0.001$, insulin siNR vs. insulin siJAK2.

the early steps in insulin signal transduction. To investigate whether JAK2 impinges on downstream insulin signals, we first focused on MAPK signaling. In cells treated with siJAK2, insulin-stimulated phosphorylation of ERK dropped to 30 ± 3% of the value on cells treated with siNR (Fig. 3*A*). Likewise, p38 MAPK and JNK phosphorylation were reduced in cells depleted from JAK2 to 49 ± 9 and 51 ± 8%, respectively (Fig. 3*B* and *C*). SiJAK2 transfection had no effect on ERK, p38 MAPK, or JNK protein expression (data not shown). Consistent with the impairment in MAPK activation, the insulin-mediated increase in myoblast proliferation was reduced by siJAK2 (Fig. 3*D*).

The upstream kinases responsible for ERK, p38, and JNK activation are MKK1/2, MKK3/6, and MKK4/7, respectively. Insulin-mediated MKK1/2 phosphorylation significantly dropped to 57 ± 9% in siJAK2-transfected cells (Fig. 4*A*). Concomitant reductions were observed for MKK3/6 (to 62 ± 6%; Fig. 4*B*) and MKK4/7 (to 58 ± 4%; Fig. 4*C*). Taken together, these results indicate that the JAK2 input into mitogenic pathways stimulated by insulin occurs at the level of the MKKs, downstream of Shc or IRS activation.

The insulin-mediated metabolic pathway does not require JAK2. Upon tyrosine phosphorylation, IRSs bind and activate PI 3-kinase, and a downstream effector of this enzyme is Akt, which regulates mainly the insulin metabolic functions. In contrast to what was observed for the MAPKs, reduction of JAK2 expression did not affect phosphorylation of T308 and S473 on Akt (mediated by the upstream kinases PDK1 and PDK2) (Fig. 5*A* and *B*). The Akt phosphorylation shown in Fig. 5*A* and *B* reflects the net behavior of all Akt isoforms. To examine the contribution of JAK2 to the activation of major Akt isoforms 1 and 2, these were individually immunoprecipitated from siJAK2-transfected L6 myotubes incubated without or with 100 nmol/l insulin for 10 min, followed by immunoblotting with anti–phospho-Akt-Ser473 antibody. Figure 5*C* and *D* shows that reduction of JAK2 expression did not affect insulin-stimulated serine phosphorylation of either Akt isoform (nor protein expression; data not shown). One immediate metabolic response downstream of insulin-stimulated Akt is GLUT4 translocation and subsequent stimulation of glucose uptake. Consistent with the lack of input by JAK2 into Akt activation by insulin, siJAK2

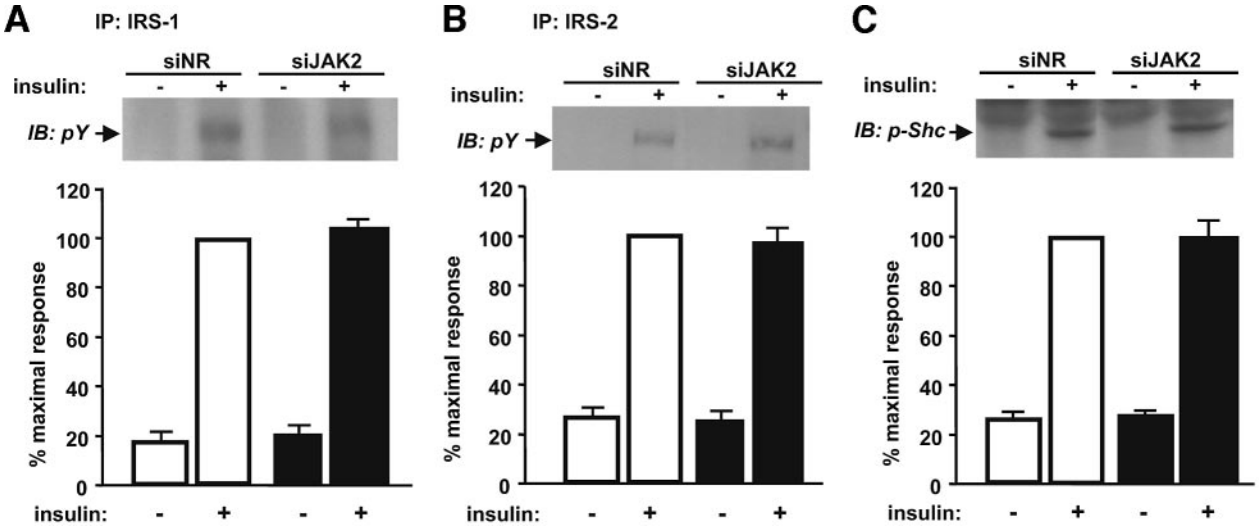

FIG. 2. JAK2 silencing does not affect insulin-mediated IRS1/2 and Shc phosphorylation. Cells transfected with siNR or siJAK2 were selectively immunoprecipitated (IP) with anti–IRS-1 (*A*) or anti–IRS-2 (*B*) antibody, and tyrosine phosphorylation was determined by immunoblotting (IB). *C*: Total cell lysates were immunoblotted for phospho-Shc. Representative blots are shown, and the results represent the means ± SE of five independent experiments.

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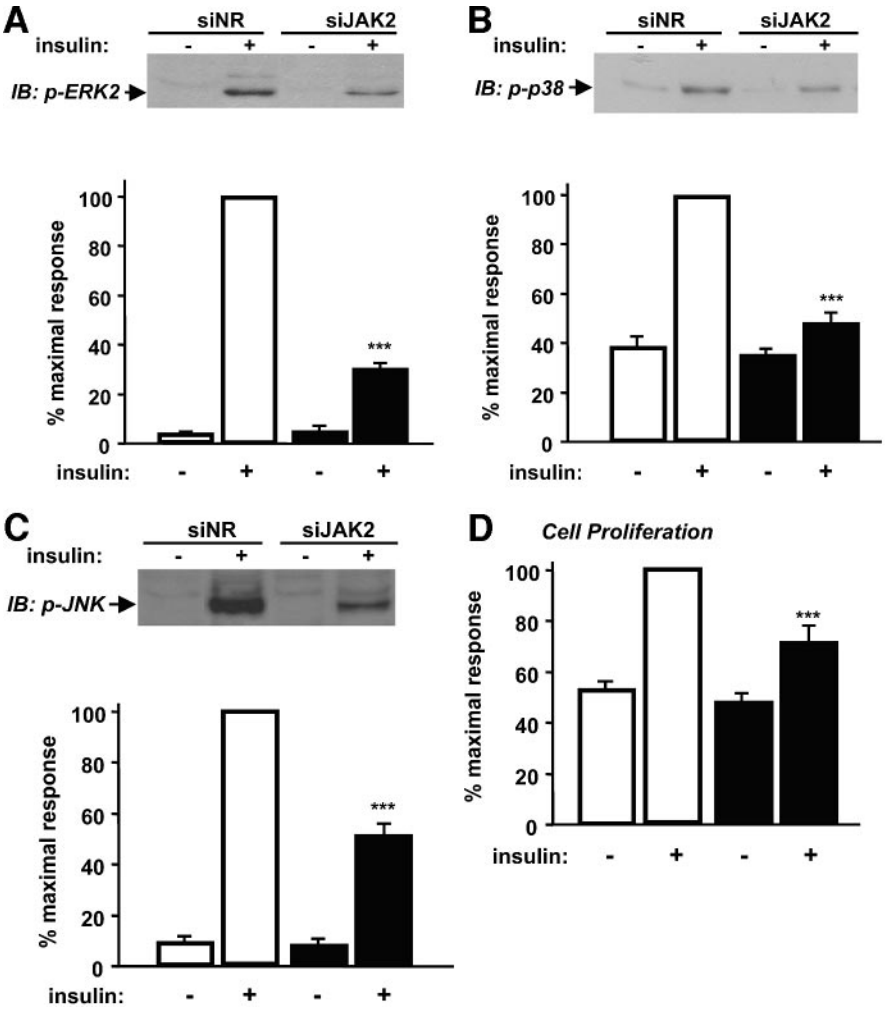

FIG. 3. JAK2 participates in insulin-stimulated phosphorylation of ERK, p38, and JNK and cell proliferation. Total cell lysates were prepared from myotubes transfected with siNR or siJAK2 and immunoblotted (IB) for phospho-ERK (*A*), phospho-p38 MAPK (*B*), and phospho-JNK (*C*). Representative blots are shown, and the results are means ± SE of five independent experiments. *D*: L6 myoblasts were transfected with siRNAs, and cell proliferation was measured. The results represent the means ± SE of four independent experiments. ***$P < 0.001$, insulin siNR vs. insulin siJAK2.

depletion affected neither glucose uptake (Fig. 5*D*) nor GLUT4 translocation (Fig. 5*E*).

AG490 mimics the effects of siRNA JAK2 and reveals Rac participation upstream of MAPKs. L6 myotubes were depleted of serum for 5 h and incubated without or with 10 μmol/l AG490, a chemical inhibitor of JAK2, for the same period (26,27). The cells were then incubated with 100 nmol/l insulin for 5 min before analysis of kinase activation or for 20 min before measurement of GLUT4 translocation and glucose uptake. It is noteworthy that AG490 reproduced the results described above for siJAK2 (Table 1).

Several Rho-family GTPases can activate the MKKs, and notably, Rac mediates ERK, p38, and JNK phosphorylation

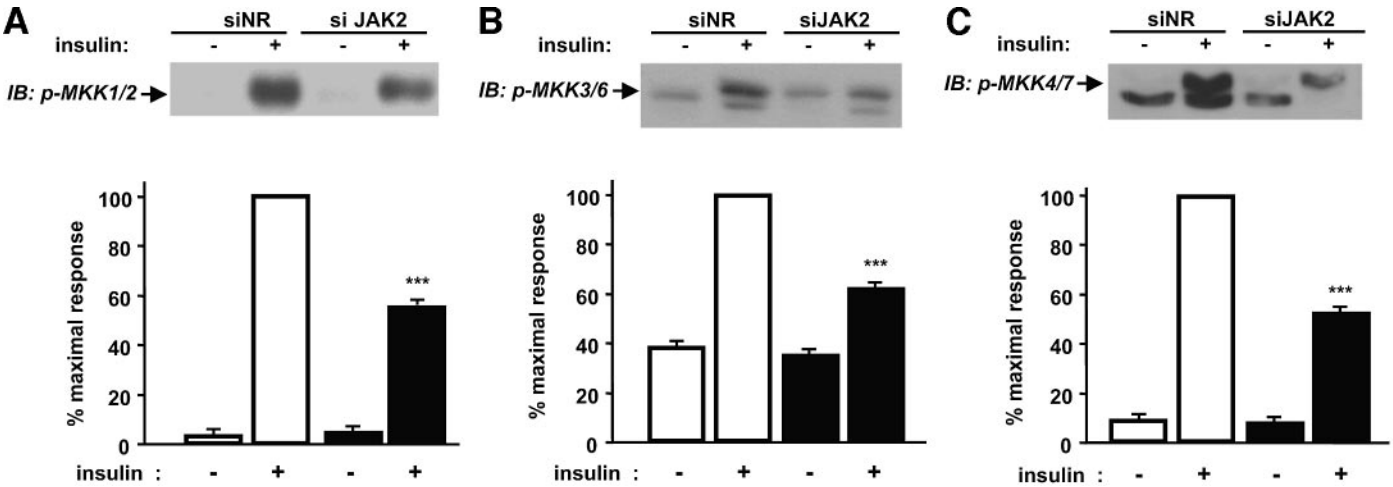

FIG. 4. JAK2 participates in insulin-stimulated phosphorylation of MAPK kinase. Total cell lysates from myotubes transfected with siJAK2 or siNR were immunoblotted (IB) for phospho-MKK1/2 (*A*), phospho-MKK3/6 (*B*), and phospho-MKK4/7 (*C*). Representative blots are shown, and the results represent the means ± SE of five independent experiments. ***$P < 0.001$, insulin siNR vs. insulin siJAK2.

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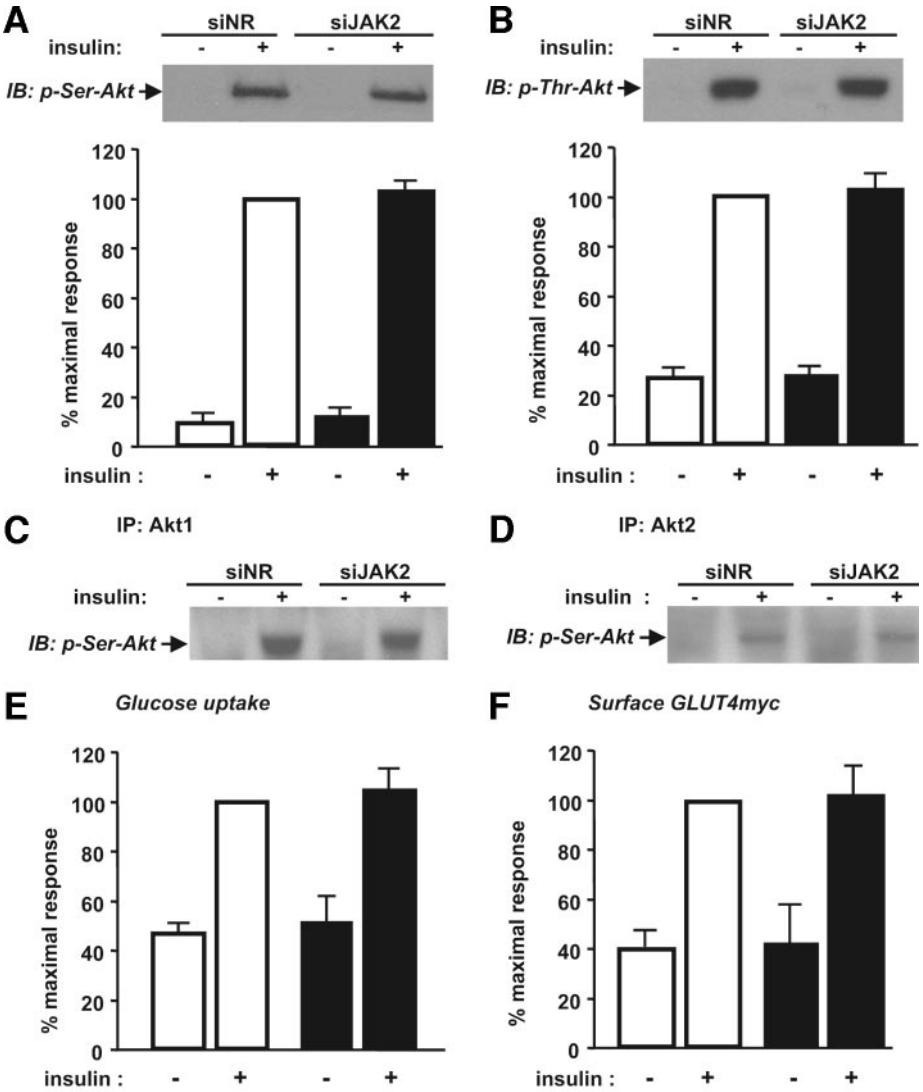

FIG. 5. Insulin-mediated stimulation of Akt, GLUT4 translocation, and glucose uptake do not require JAK2. Lysates from myotubes transfected with siJAK2 or siNR were immunoblotted (IB) for phospho-Akt Ser473 (*A*) and phospho-Akt Thr 308 (*B*). Akt1 (*C*) or Akt2 (*D*) was selectively immunoprecipitated, and serine phosphorylation was determined by immunoblotting with phospho-Akt Ser473. 2-Deoxyglucose uptake (*E*) and cell surface GLUT4myc (*F*) were measured in myotubes transfected with siJAK2 or siNR. The results represent the means ± SE of six to eight independent experiments.

in different cell models (28–30). AG490 treatment provoked a 50% reduction on insulin-mediated Rac activation (Table 1), suggesting the following signaling sequence: JAK2→Rac→MKKs→MAPKs. Preliminary studies in our laboratory have also revealed that siRac leads to a significant drop in insulin-mediated MAPK phosphorylation (data not shown), supporting this possible flow model.

TABLE 1
Reductions in signaling molecule activities in response to AG490 treatment

	AG490
Phospho–IRS-1 (%)	99 ± 7
Phospho–IRS-2 (%)	96 ± 3
Phospho-Shc (%)	98 ± 10
Rac activation (%)	52 ± 10*
Phospho-ERK (%)	58 ± 6†
Phospho-p38MAPK (%)	60 ± 3†
Phospho-JNK (%)	52 ± 10†
Phospho-MKK1/2 (%)	59 ± 10†
Phospho-MKK3/6 (%)	61 ± 9†
Phospho-MKK4/7 (%)	54 ± 9†
Phospho-Akt Ser 473 (%)	99 ± 8
Phospho-Akt Thr 308 (%)	101 ± 7
Glucose uptake (%)	102 ± 9
GLUT4 translocation (%)	105 ± 10

Data are means ± SE of the insulin-stimulated response in AG490-treated cells in comparison with vehicle-treated cells. *$P < 0.01$, †$P < 0.001$, insulin plus AG490 vs. insulin plus vehicle.

Modulation of IRS-1 serine phosphorylation and SOCS3 expression in JAK2-silenced cells. Circulating factors, such as tumor necrosis factor-α, that engage JAK decrease insulin signaling by impairing insulin-stimulated tyrosine phosphorylation of IRS molecules via phosphorylation of IRS-1 Ser307 and induction of SOCS proteins (9,31,32). JNK causes phosphorylation on Ser307 on IRS-1, preventing further tyrosine phosphorylation of IRS-1 (33). The siJAK2-induced drop in JNK phosphorylation (Fig. 3*C*) correlated with a loss of Ser307 phosphorylation of IRS-1 (Fig. 6*A*) and a drop in SOCS-3 expression (Fig. 6*B*). Despite such reductions, insulin-stimulated tyrosine phosphorylation of IRS-1 or IRS-2 was not enhanced (Fig. 2*A*). This likely reflects the robust action of insulin signal transduction at maximal insulin levels and the limited impact of insulin-dependent JAK2 activity on IRS phosphorylation.

JAK2 contributes to Akt downregulation in insulin-resistant states. The results so far reveal that the modest level of insulin-mediated JAK2 activation is insufficient to modulate insulin metabolic signaling via Akt. However,

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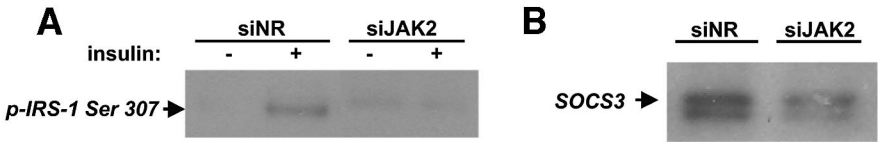

FIG. 6. Modulation of IRS-1 serine phosphorylation and SOCS3 expression in JAK2-silenced cells. Lysates from myotubes transfected with siRNAs were immunoblotted for phospho-IRS-1 (Ser307) (*A*) and SOCS3 (*B*), and representative blots are illustrated, the results of four to five independent experiments.

JAK2 activation by agents that induce insulin resistance is more potent. Therefore, we next investigated whether in insulin-resistant states the heightened activation of JAK2 could override the resilience of the metabolic pathway. We chose to challenge the cells with ceramide, a sphingomyelin-derived lipid molecule that rises in cells exposed to fatty acids, causing JAK2 activation (34,35). In L6 myotubes, a 2-h ceramide treatment led to an increase JAK2 phosphorylation of 1.7-fold (results not shown). Importantly, ceramide exerts a profound inhibitory effect on insulin-induced activation of Akt in muscle and fat cells but does not affect early insulin signals such as IRS-1 tyrosine phosphorylation and association with PI 3-kinase in both 3T3-L1 adipocytes (36,37) and L6 muscle cells (38) (L. JeBailey and A. Klip, unpublished observations). Hence, the curbing effect of ceramide on Akt phosphorylation is likely independent of upstream effects on IRS-1. This is further highlighted by the fact that ceramide treatment actually prevented insulin-stimulated phosphorylation of JNK and of Ser307 on IRS-1 (Fig. 7*A*), signals that, if elevated, would have potentially reduced IRS-1 tyrosine phosphorylation.

The above results establish ceramide as an agent activating JAK2 and causing insulin resistance at the level of Akt. We therefore sought to determine whether, under these conditions, reducing JAK2 via siRNA would rescue Akt phosphorylation. As shown in Fig. 7*B*, ceramide treatment caused a loss in insulin-stimulated Akt phosphorylation in siNR-transfected cells (down to 19 ± 2% of the response in the absence of ceramide). As hypothesized, siJAK2 partially rescued the insulin-mediated Akt phosphorylation reduced by ceramide treatment (up to 35 ± 5% of the value in untreated, siNR-transfected cells). An even more impressive restoration was achieved for glucose uptake (Fig. 7*C*) and for Akt phosphorylation at Thr308 (Fig. 7*D*, *top panel*). Conversely, the decreased ERK phosphorylation elicited by ceramide treatment was not affected by siJAK2 transfection (Fig. 7*D*, *bottom panel*). Similarly, reducing JAK2 levels via siJAK2 did not recover the dampened insulin-induced phosphorylation of JNK or p38 MAPK caused by ceramide (data not shown). These results suggest that JAK2 contributes to the down-regulation of Akt and its metabolic effect on glucose uptake provoked by ceramide but has no effect on the ceramide-induced suppression on MAPKs.

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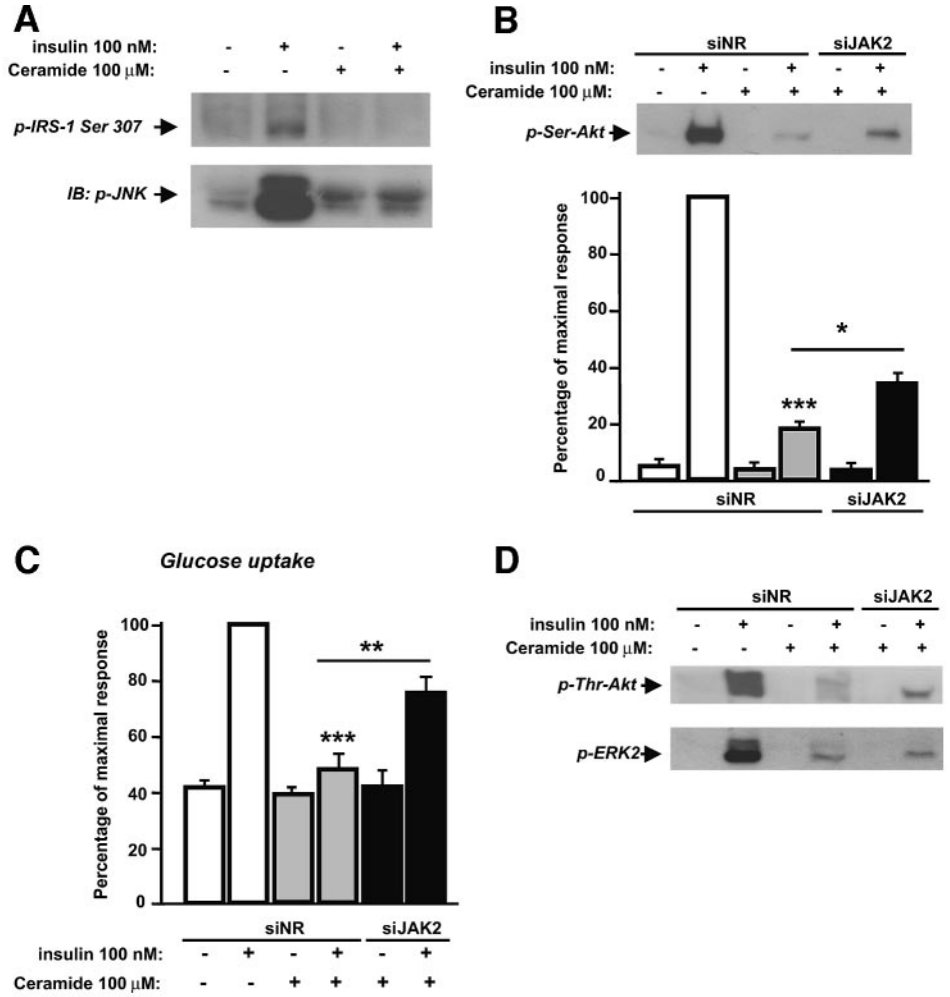

FIG. 7. Contribution of JAK2 to Akt down-regulation in ceramide-treated cells. Myotubes were depleted of serum for 3 h followed by 2 h of incubation with 100 mmol/l ceramide followed by insulin stimulation (100 nmol/l, 10 min). *A*: Total cell lysates were prepared and immunoblotted (IB) for phospho–IRS-1 Ser307 and phospho-JNK. *B–D*: Myotubes transfected with siJAK2 or siNR were treated as in *A*. In *B* and *D*, lysates were immunoblotted for phospho-Akt Ser473, phospho-Akt Thr 308, and phospho-ERK; in *C*, 2-deoxyglucose uptake was measured. Representative blots are shown, and the results represent the means ± SE of four to five independent experiments. ***$P < 0.001$, insulin siNR vs. insulin siNR plus ceramide; *$P < 0.05$, **$P < 0.01$, insulin siNR plus ceramide vs. insulin siJAK2 plus ceramide.

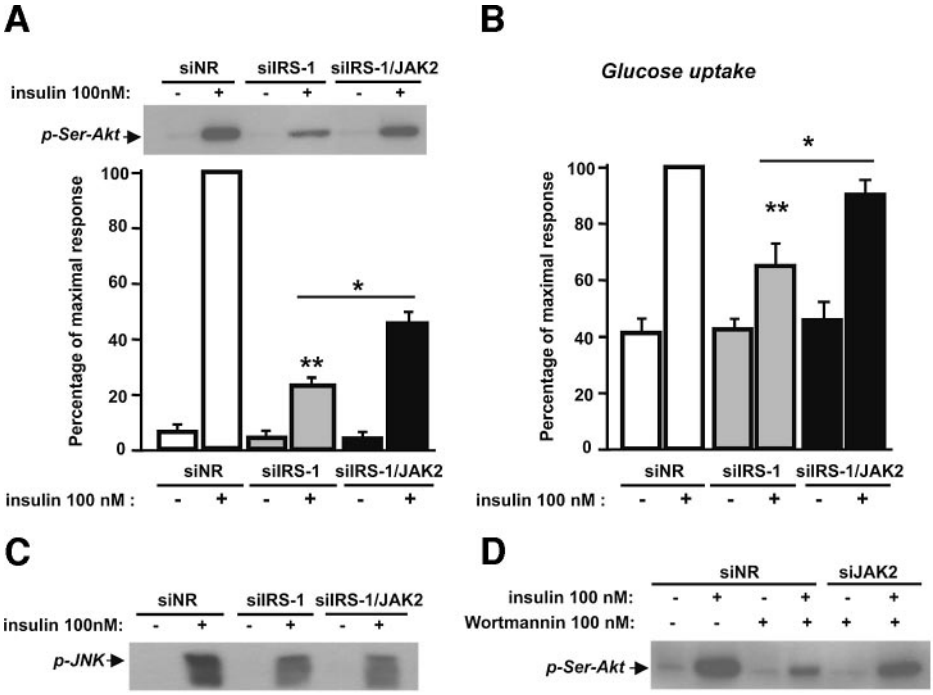

FIG. 8. JAK2 contribution to Akt downregulation in insulin resistance does not occur at the level of IRS-1 and is PI 3-kinase independent. L6 myotubes were transfected with siNR, siIRS-1, or combined IRS-1– and JAK2-specific siRNAs. *A* and *C*: Total cell lysates were prepared and immunoblotted for phospho-Akt Ser473 and phospho-JNK. In *B*, 2-deoxyglucose uptake was performed. Representative blots and the means ± SE are shown (n = 4–6). **P < 0.001, insulin siNR vs. insulin siIRS-1; *P < 0.05, insulin siIRS-1 vs. insulin siIRS-1/JAK2. In *D*, Ser473 phosphorylation of Akt was measured in myotubes transfected with siNR or siJAK2. Cells were deprived of serum for 5 h and then stimulated with 100 nmol/l wortmannin for 20 min, followed by 5 min of insulin stimulation. A representative blot is illustrated, representing four to five independent experiments.

JAK2 contribution to Akt downregulation in insulin resistance does not occur at the level of IRS-1. The above experiments with ceramide examined the effect of JAK2 in a model of insulin resistance that spares the early insulin-signaling steps. It was therefore of interest to examine how insulin resistance that reduces early signaling at the level of IRS is affected by reduction in JAK2. We have recently shown that knockdown of IRS-1 in muscle cells by siIRS-1 leads to impaired insulin-mediated Akt phosphorylation and glucose uptake (22) (confirmed in Fig. 8*A* and *B*). Hence, loss of IRS-1 directly causes metabolic insulin resistance in muscle cells. When cells were doubly transfected with siRNAs against IRS-1 and JAK2, the drop in insulin-mediated Akt phosphorylation and glucose uptake was partially rescued (Fig. 8*A* and *B*). These results suggest that in this model of insulin resistance, even when IRS-1 is markedly downregulated, JAK2 negatively regulates signaling further downstream at the level of Akt. As was the case with ceramide treatment, the reduced MAPK phosphorylation brought out by siIRS-1 was not relieved by JAK2 depletion, shown here for JNK phosphorylation (Fig. 8*C*). These results highlight that JAK2 inhibition impinges specifically on the metabolic rather than the mitogenic axis of insulin signaling in two insulin-resistant states.

JAK2 effects on Akt are independent of PI 3-kinase. To examine whether the JAK2 modulation of Akt takes place at the level of PI 3-kinase, cells were pretreated with the PI 3-kinase inhibitor wortmannin (100 nmol/l for 20 min). As expected, PI 3-kinase inhibition markedly impaired insulin-induced Akt phosphorylation (Fig. 8*D*). Notably, JAK2 depletion partially prevented such a drop in Akt phosphorylation. Therefore, the contribution of JAK2 to the downregulation of Akt phosphorylation occurs downstream of PI 3-kinase, potentially at the level of PDK1/PDK2 or of phosphatases targeting Akt.

DISCUSSION

JAK2 is the key mediator of cytokine signaling. This cytoplasmic kinase is activated by insulin in cultured cells or tissues of rats and is markedly upregulated in some insulin-resistant states (15–17). Moreover, in cells overexpressing insulin receptor, JAK2 directly phosphorylates IRS-1 and IRS-2. In vitro, tyrosine phosphorylation elicited by the insulin receptor or by JAK2 occurs on different sites of IRS-1 (15). It has not been feasible to analyze the biologically relevant functions of JAK2 in insulin receptor signaling in adult tissues due to the early lethality of JAK2 conventional knockout mice (39–41). However, JAK2^{-}/$^{+}$ mice do not display any noticeable phenotypic abnormalities (39), and there are no reports addressing insulin signaling or sensitivity in these animals. Therefore, JAK2 involvement in insulin signaling and function remains to be investigated.

JAK2 knockdown does not affect insulin-mediated IRS or Shc phosphorylation. In this study, we made use of siRNA-mediated gene silencing to specifically knock down JAK2 expression in L6 myotubes to investigate the contribution of JAK2 to insulin signaling. We found that reducing endogenous JAK2 protein by 75% did not affect the insulin-induced phosphorylation of the insulin receptor–interacting proteins IRS-1, IRS-2, or Shc. Even though the insulin receptor and JAK2 phosphorylate IRS-1 on distinct tyrosine residues (15), it appears that IRS1/2 phosphorylation by JAK2 is not necessary for insulin-induced responses. Numerous studies have demonstrated that hyper-phosphorylation of IRS-1 on serine and threonine residues inhibits insulin-stimulated IRS-1 tyrosine phosphorylation and subsequent downstream signaling cascades (42–44). Insulin signaling itself can stimulate Ser/Thr phosphorylation of IRS-1, providing a mechanism to finely tune signal transduction via tyrosine phosphorylation (rev. in 27). In JAK2-depleted L6 myotubes, we found that insulin-stimulated IRS-1 serine phosphorylation was significantly reduced and correlated with a decrease in SOCS3 protein expression, a well-described negative modulator of IRS-1 tyrosine phosphorylation (9,31). Intriguingly, the overall level of IRS-1 tyrosine phosphorylation was not improved by suppression of these negative modulators via JAK2. This lack of correlation between

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decreased serine/increased tyrosine phosphorylation of IRS-1 is in accordance with a recent study showing that, in primary adipocytes, inhibition of ERK1/2 substantially decreased insulin-stimulated phosphorylation of IRS-1 Ser312 and Ser316 but did not enhance insulin-stimulated tyrosine phosphorylation of IRS-1 (45). It is possible that JAK2 exerts some positive contribution on IRS-1 tyrosyl phosphorylation that obscures detection of the improved effect of insulin receptor–dependent phosphorylation.

JAK2 participates in the insulin-stimulated mitogenic pathway. Insulin stimulates the MAPK ERK, p38, and JNK proteins mainly involved in proliferation and differentiation (24,43). Activated MAPKs translocate to the nucleus and initiate a transcriptional program that leads to cell growth. Reducing endogenous JAK2 abated insulin-stimulated cell proliferation by 50%. IRS-1, IRS-2, and Shc, proteins proximal to the insulin receptor and required for the mitogenic responses, were not affected by siJAK2. In contrast, siJAK2 provoked a marked reduction in insulin-elicited ERK, p38, and JNK phosphorylation and on their corresponding upstream kinases MKK1/2, MKK3/6, and MKK4/7. JAK2 input to the mitogenic pathway may occur further upstream at the level of the GTPases, as it was shown here that AG490 significantly lessened Rac activation. Alternatively, JAK2 may act as a modulator of insulin action by phosphorylating and inactivating phosphatases required for signal termination. One such phosphatase is protein phosphatase-2A (PP2A). In L6 muscle cells, insulin rapidly activates JAK2, and this is accompanied by an increase in tyrosine phosphorylation and inhibition of the phosphatase activity of PP2A in JAK2 immunoprecipitates (46). PP2A also mediates dephosphorylation and inactivation of ERK, p38, and JNK and their respectively upstream MKKs in platelets, neutrophils, and fibroblasts (47–49). In this way, JAK2 depletion could lead to a rise in PP2A activity and a consequent drop in phosphorylation of the MKKs, followed by their downstream targets, the MAPKs. Regardless of the mechanism, our results highlight the possibility that JAK2 pathway may play a role in the control of insulin-induced cell proliferation.

JAK2 is not required for the insulin-mediated metabolic pathway in the absence of insulin resistance. Contrary to the results on the mitogenic pathway, insulin-stimulated Akt phosphorylation was normal in JAK2-depleted cells, even when individual Akt isoforms were examined. Moreover, GLUT4 translocation to the cell surface and insulin-stimulated glucose uptake were also unaffected by siJAK2. These results suggest that the kinase activity of the insulin receptor is sufficient to elicit acute insulin metabolic functions and that the level of acute insulin activation of JAK2 is insufficient to modulate metabolic signaling via Akt.

JAK2 contributes to Akt downregulation in insulin-resistant states. A central component of type 2 diabetes and the metabolic syndrome is insulin resistance, whereby the hormone is unable to elicit anabolic responses in muscle and adipose tissues. Numerous studies have demonstrated that aberrantly increased cellular production of ceramide contributes to the pathogenesis of insulin resistance and to impaired glucose utilization (37,38,50). To further investigate the involvement of JAK2 in insulin signaling, we questioned whether JAK2 contributes to insulin resistance of Akt signaling, GLUT4 translocation, and glucose uptake. Our results revealed that pretreatment of myotubes with ceramide inhibits insulin-stimulated Akt phosphorylation and glucose uptake. These ceramide effects were partially reverted in JAK2-depeleted cells, suggesting that JAK2 contributes in part to the downregulation of insulin-stimulated Akt phosphorylation and glucose uptake in this model of insulin resistance. Several groups have shown that ceramide does not exert any effect on IRS and/or PI 3-kinase but rather that it directly blocks activation of Akt (37,38,50). How ceramide inhibits the insulin-mediated activation of Akt is a current matter of debate, because PP2A (50) and protein kinase Cξ (51) have been independently implicated as culprit negative regulators of Akt. JAK2 activation by insulin inactivates PP2A (46), thus one might predict that JAK2 knockdown would maintain PP2A activity in the presence of insulin (as demonstrated using the inhibitor AG-490 [46]) and consequently further curb Akt phosphorylation on Ser473. However, this was contrary to our observation (Fig. 5*A* and *B*), and hence it is unlikely that PP2A is the JAK2 mediator of ceramide effects on Akt. A second possible mediator of JAK2 action on Akt could be SHP-1, because SHP-1 binds JAK2 and because PC12 cells expressing a dominant-inhibitory SHP-1 mutant show improved Akt phosphorylation (52). Furthermore, because the drop in insulin-mediated Akt phosphorylation and glucose uptake provoked by reduction of IRS-1 or wortmannin are also partially rescued by JAK2 knockdown, the present study supports the hypothesis that JAK2 impinges on Akt phosphorylation via an adjacent pathway independent of insulin-regulated upstream signals. The fact that siJAK2 did not revert the decline in the MAPK phosphorylation provoked by ceramide or siIRS-1 corroborates the hypothesis that JAK2-dependent inhibition of Akt occurs at the level of this serine kinase and does not involve signaling proteins that can also regulate the mitogenic arm. We cannot rule out that JAK2 modulates feedback signaling upstream of Akt, such as JNK-dependent phosphorylation of Ser307 on IRS-1. However, in cells with IRS-1 knocked down, the remaining IRS-1 is unlikely to be relatively less phosphorylated on Ser307, because JNK activation is not further reduced upon concomitant depletion of JAK2 (Fig. 8*C*). Similarly, JAK2 knockdown is unlikely to affect ceramide signaling to Akt via JNK input into IRS-1, because ceramide itself markedly reduces JNK activation (Fig. 6*D*). Defining the exact mechanism by which JAK2 knockdown alleviates negative regulation of Akt phosphorylation and activity is of significant interest for future studies and may suggest mechanisms to alleviate insulin resistance.

Taken together, our results suggest that in muscle cells, JAK2 differentially participates in the mitogenic and metabolic signals by insulin. Insulin-dependent JAK2 activation contributes to MAPK signaling and cell proliferation, whereas Akt and glucose uptake have no contribution from this intracellular kinase under normal conditions (Fig. 9*A*). We hypothesize that, in states of insulin resistance, heightened JAK2 activation may cause an unbalance in insulin metabolic signaling such that this kinase now inhibits signal transduction at the level of Akt impacting on glucose uptake (Fig. 9*B*). From a clinical perspective, the results presented implicate JAK2 as a potential and promising therapeutic target because its ablation may decrease the undesirable insulin-mitogenic effects related to several chronic complications in insulin-resistant states and may conversely ameliorate insulin-dependent glucose uptake.

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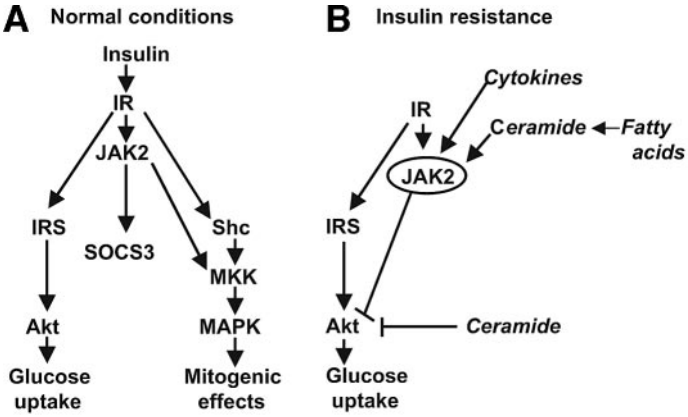

FIG. 9. Suggested implications of signal transduction downstream of JAK2 in normal (*A*) and in insulin-resistant (*B*) states. *A*: In normal conditions, JAK2 contributes to the activation of MAPKs and cell proliferation by insulin but spares Akt and glucose uptake. *B*: In insulin-resistant states, heightened JAK2 activation may contribute to Akt and glucose uptake downregulation and cause an imbalance in insulin metabolic signaling.

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