Original Article

Ceramide- and Oxidant-Induced Insulin Resistance Involve Loss of Insulin-Dependent Rac-Activation and Actin Remodeling in Muscle Cells

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In muscle cells, insulin elicits recruitment of the glucose transporter GLUT4 to the plasma membrane. This process engages sequential signaling from insulin receptor substrate (IRS)-1 to phosphatidylinositol (PI) 3-kinase and the serine/threonine kinase Akt. GLUT4 translocation also requires an Akt-independent but PI 3-kinase– and Rac-dependent remodeling of filamentous actin. Although IRS-1 phosphorylation is often reduced in insulin-resistant states in vivo, several conditions eliciting insulin resistance in cell culture spare this early step. Here, we show that insulin-dependent Rac activation and its consequent actin remodeling were abolished upon exposure of L6 myotubes beginning at doses of C2-ceramide or oxidant-producing glucose oxidase as low as 12.5 μmol/l and 12.5 mU/ml, respectively. At 25 μmol/l and 25 mU/ml, glucose oxidase and C2-ceramide markedly reduced GLUT4 translocation and glucose uptake and lowered Akt phosphorylation on Ser473 and Thr308, yet they affected neither IRS-1 tyrosine phosphorylation nor its association with p85 and PI 3-kinase activity. Small interfering RNA–dependent Rac1 knockdown prevented actin remodeling and GLUT4 translocation but spared Akt phosphorylation, suggesting that Rac and actin remodeling do not contribute to overall Akt activation. We propose that ceramide and oxidative stress can each affect two independent arms of insulin signaling to GLUT4 at distinct steps, Rac–GTP loading and Akt phosphorylation. *Diabetes* 56:394–403, 2007

Insulin promotes dietary glucose disposal into skeletal muscle through recruitment of GLUT4-containing vesicles to the cell surface. Signaling to GLUT4 requires tyrosine phosphorylation of the insulin receptor substrate (IRS)-1 isoform, which recruits and activates phosphatidylinositol (PI) 3-kinase (1). The latter triggers activation of several serine/threonine kinases, notably Akt, which, through its substrate AS160, regulates GLUT4 vesicle mobilization to and/or fusion with the plasma membrane (2).

Along with proper signaling, GLUT4 translocation and stimulation of glucose uptake require dynamic changes in the actin cytoskeleton. Insulin induces actin filament remodeling in mature skeletal muscle (3) and muscle cells in culture (4) that manifest as mesh-like structures beneath the plasma membrane. Actin filament–disrupting drugs (e.g., cytochalasin D and latrunculin B) and actin-stabilizing drugs (e.g., jasplakinolide) inhibit GLUT4 translocation and its consequent glucose uptake in muscle (4,5) and adipose (6,7) cells, as do toxins that inhibit Rho family GTPases that control actin dynamics (8). Interestingly, under these conditions, IRS-1 phosphorylation and PI 3-kinase activity remain unaffected (9–11). In muscle cells, actin remodeling was also prevented by wortmannin or expression of a dominant-negative mutant of the p85 subunit of class I PI 3-kinase (5), but not by a dominant-negative Akt mutant (12). Hence, insulin signaling bifurcates downstream of PI 3-kinase, one arm leading to actin remodeling and another to Akt activation, both converging to promote GLUT4 translocation. Recently, we demonstrated that insulin induces GTP loading of the Rho family GTPase Rac and that dominant-negative Rac1 prevents both actin remodeling and GLUT4 translocation in myoblasts (13,14). Like Akt activation, Rac–GTP loading was largely prevented by inhibitors of PI 3-kinase, suggesting that Akt and Rac are downstream effectors of PI 3-kinase involved in insulin-induced GLUT4 translocation. Whereas Akt is not upstream of actin remodeling in this sequence of events, it is not known whether Rac and its consequent actin remodeling impinge on Akt activation.

Reduced insulin-dependent GLUT4 translocation and glucose uptake into muscle and fat cells are hallmarks of insulin resistance accompanying type 2 diabetes and obesity, but the molecular mechanisms are not fully understood. Insulin resistance can be induced in simplified culture systems by exposing cells to environments emulating the conditions prevailing in obesity or diabetes.

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GLUT4myc, c-*myc* epitope–tagged GLUT4; IRS, insulin receptor substrate; NAC, *N*-acetyl-L-cysteine; PAK, p21-activated kinase; PI, phosphatidylinositol; siRNA, small interfering RNA.

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These include: *1*) incubation with high fatty acids or its derivative ceramide (a lipid metabolite that accumulates in muscle of insulin-resistant rodents and humans [15]) and *2*) exposure to low-grade oxidative stress (because reactive oxygen species have a causal role in multiple forms of insulin resistance [16,17]). These experimental conditions have been validated as paradigms of insulin resistance in cell cultures (18–22). Interestingly, the insulin resistance of glucose uptake caused by ceramide in adipose and muscle cells, or by peroxide in adipose cells, is not accompanied by defects at the total cellular level of IRS proteins or their associated PI 3-kinase activity (23–25), and yet Akt phosphorylation is reduced in all cases.

Here, we investigated whether Rac-dependent actin remodeling, a phenomenon essential for insulin-induced GLUT4 translocation in muscle cells, is targeted in insulin resistance induced by ceramide or oxidant produced by extracellular glucose oxidase, and we explore whether Rac activation is required for Akt phosphorylation.

RESEARCH DESIGN AND METHODS

Monoclonal anti-Rac1, polyclonal anti–IRS-1, and anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology. Polyclonal anti-p85 (Z-8) and anti-*myc* (A-14) antibodies were from Santa Cruz Biotechnology. Phospho-specific anti–Akt Ser473 and anti-Thr308 were from Cell Signaling. Monoclonal antibody to β-actin, glucolactone, glucose oxidase, *N*-acetyl-L-cysteine (NAC), OPD (o-phenylenediamine dihydrochloride), and ATP were from Sigma Chemical. C2-ceramide and C2-dihydroceramide were from Calbiochem. Small interfering RNA (siRNA) to Rac1 (sense sequence: 5′-GUUCUUAAUUUGCUUUUCCTT-3′; antisense sequence: 5′-GGAAAAG CAAAUUAAGAAC-3′) (26) and unrelated oligonucleotide (sense sequence: 5′-UAAGGCUAUGAAGAGAUACUU-3′; antisense sequence: 5′-GUAUCUCU UCAUAGCCUUAUU) were from Dharmacon.

Cell culture, treatments, and siRNA transfection. L6 muscle cells expressing c-*myc* epitope–tagged GLUT4 (GLUT4myc) were maintained in myoblast monolayer culture or differentiated into multinucleated myotubes as previously described (13). For all experiments, cells were incubated in serum-free α-MEM containing 5 mmol/l glucose for 3–5 h. For glucose oxidase or glucolactone treatment, myotubes were incubated in the last 2 h of serum deprivation with glucose oxidase or glucolactone, in the presence or absence of 20 mmol/l NAC, and then switched to serum-free medium containing 100 nmol/l insulin (10–20 min) as indicated. For C2-ceramide or C2-dihydroceramide treatments, cells were incubated with 12.5, 25, 50, or 100 μmol/l of agent during the last 2 h of serum deprivation and during the acute insulin challenge. Cell viability was not compromised under any of these conditions. Transfection of 200 nmol/l of unrelated siRNA or siRNA targeting Rac1 was performed twice on days 4 and 5 of culture, using oligofectamine as specified by the manufacturer, and myotubes were used for experimentation 24 h later (total transfection 72 h).

H_2O_2, surface GLUT4myc, and 2-deoxyglucose uptake determinations. Glucose oxidase catalyzes the conversion of glucose to glucolactone, which is further converted to glucuronic acid and H_2O_2. H_2O_2 generation was quantified as reported (19). 2-Deoxyglucose uptake and surface *myc*-tagged GLUT4 were measured as previously reported (5,27).

Filamentous actin and Rac activation. Cells were fixed and actin filaments labeled as previously described (14). Images were acquired by confocal fluorescence microscopy and examined at 63× or 100× with a Zeiss LSM 510 laser scanning confocal microscope. Acquisition parameters were adjusted to exclude saturation and kept constant among the various conditions. The relative proportion of F- to G-actin was also determined biochemically by actin stabilization and detergent partitioning using an in vivo assay kit from Cytoskeleton. Activated Rac was detected using a glutathione-*S*-transferase–fusion protein of the CRIB (Cdc 42/Rac interactive binding) domain of p21 kinase conjugated to glutathione beads that specifically binds activated forms of Rho GTPases, as described earlier (13).

Protein immunoblotting, IRS-1 phosphorylation, and associated p85 and PI 3-kinase activity. Cells were washed twice with ice-cold PBS containing 1 mmol/l Na_3VO_4, and total cell lysates were subjected to 10% SDS-PAGE, electrotransferred and immunoblotted as specified in the figure legends, and quantified using National Institutes of Health Image graphics software version 1.61. Alternatively, whole-cell extracts were prepared and IRS-1 immunoprecipitated, followed by immunoblotting with anti-phosphotyrosine (pY) or anti-p85 antibodies or PI 3-kinase activity assay, as previously described (28). Total IRS-1 content was determined by stripping and reprobing with anti–IRS-1 antibody.

Statistical analysis. Statistical analyses were performed with Prism 4.0 software (San Diego). Experiments with more than two groups of cells were analyzed by ANOVA with Tukey's post hoc analysis.

RESULTS

Glucose oxidase or ceramide inhibit insulin-dependent GLUT4myc translocation. Although insulin can generate a rapid burst of peroxide that inhibits tyrosine phosphatases (29), sustained exposure of adipose cells to micromolar concentrations of oxidants via glucose plus glucose oxidase causes insulin resistance of glucose uptake and GLUT4 translocation in direct response to changes in the intracellular redox state (25,30). Such resistance is reversible by preincubation with antioxidants (20,31). Similarly, exposing adipose cells to 100 μmol/l of the lipid metabolite analog C2-ceramide or long-chain ceramides leads to insulin resistance of glucose uptake and/or GLUT4 translocation (21–23,32). Glucose oxidase and C2-ceramide also elicit insulin resistance of glucose uptake in muscle cells (19,20,22,24). We validated the insulin resistance potential of glucose oxidase and C2-ceramide on GLUT4 translocation to the surface of muscle cells as a preamble to studying the underlying mechanisms of insulin resistance. L6 muscle cells stably expressing *myc*-tagged GLUT4 were used because these cells have been repeatedly shown to display insulin-regulated GLUT4 traffic (12,27). L6 myotubes were serum deprived and pretreated with increasing concentrations of glucose oxidase for 2 h, followed by insulin stimulation for 20 min and determination of peroxide production (Fig. 1*A*) and surface GLUT4 levels (Fig. 1*B*). To selectively examine the insulin-dependent portion of the response, we present specifically the insulin effect (i.e., the difference, or delta, between insulin and basal values) at each concentration of glucose oxidase or C2-ceramide as the percent of the maximal insulin-dependent response (i.e., the delta in the absence of glucose oxidase or C2-ceramide). Basal values are shown in supplemental Table 1, which can be found in the online appendix (available at http://dx.doi.org/10.2337/db06-0823). Under the conditions used, H_2O_2 production increased linearly with glucose oxidase, whereas 25 mU/ml glucose oxidase generated ~60 μmol/l H_2O_2, representing the balance between H_2O_2 generation in the medium and its inactivation by cellular peroxidases and catalase. Notably, these H_2O_2 concentrations were more than an order of magnitude lower than those exogenously added to induce insulin-mimicking effects (29).

Figure 1*B* illustrates the change in surface GLUT4myc caused by insulin over a range of glucose oxidase dosages, expressed as the percent of the maximal insulin response elicited by each dose of glucose oxidase. Glucose oxidase levels of 6.25 mU/ml evidently reduced GLUT4 translocation, and nearly complete and statistically significant resistance occurred at 25 mU/ml glucose oxidase. In contrast, basal-state surface GLUT4 levels were not reduced by glucose oxidase (supplemental Table 1). Glucose oxidase also significantly inhibited insulin-stimulated 2-deoxyglucose uptake in a concentration-dependent manner with nearly full inhibition at 25 mU/ml glucose oxidase (Fig. 1*C*), but no significant effect on basal-state glucose uptake (supplemental Table 1). To ensure that the effect of glucose oxidase on insulin action was caused by the oxidant produced, muscle cells were incubated with 20 mmol/l NAC, a sulfhydryl reagent capable of protecting

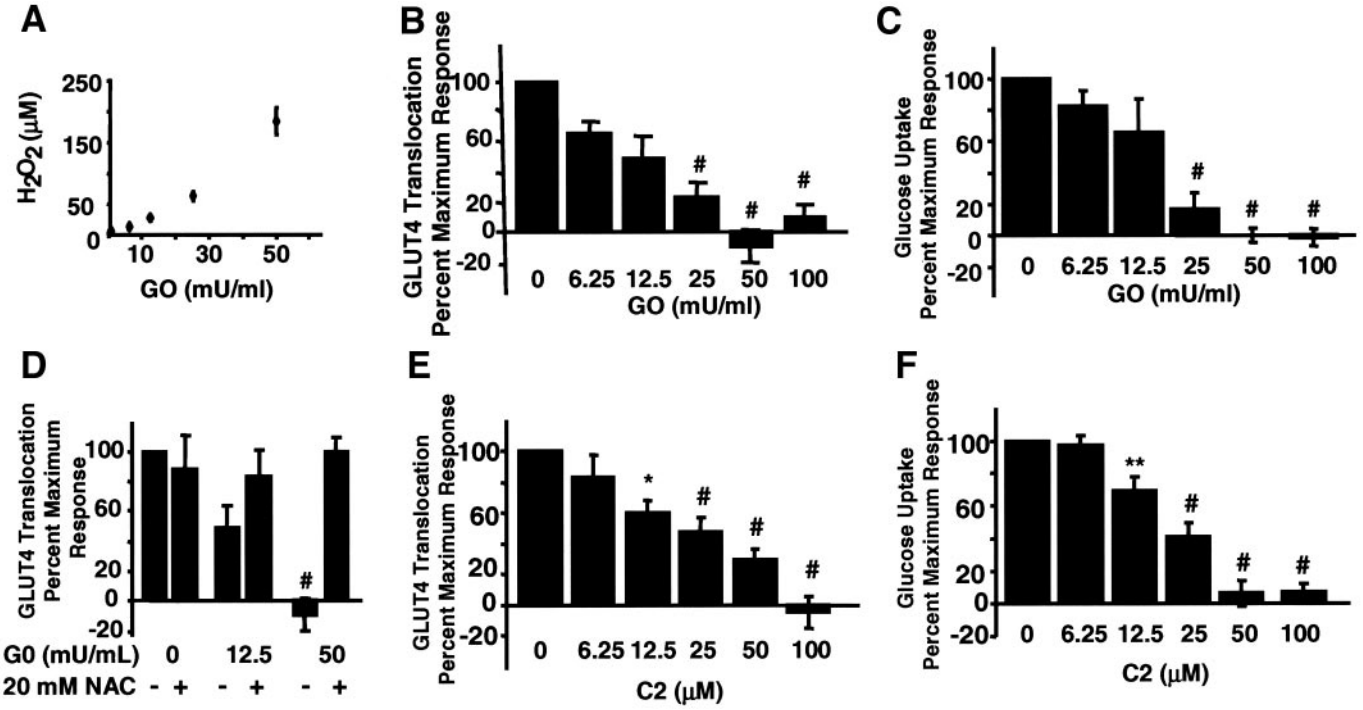

FIG. 1. Glucose oxidase (GO) and ceramide (C2) reduce GLUT4 translocation and glucose uptake in L6 myotubes. *A*: Glucose oxidase caused a dose-dependent increase in H_2O_2 in the medium. *B* and *C*: Glucose oxidase dose-dependent inhibition of insulin-induced gain in surface GLUT4myc (*B*) or in 2-deoxyglucose uptake (*C*). *D*: NAC prevents the glucose oxidase–induced decrease in insulin-induced GLUT4 translocation. *E* and *F*: C2-ceramide dose-dependent inhibition of insulin-induced gain in surface GLUT4myc (*E*) or in 2-deoxyglucose uptake (*F*). Results in *B–F* are expressed as the percent of maximal insulin response observed in the absence of glucose oxidase or C2-ceramide. #$P < 0.001$, *$P < 0.05$, *$P < 0.01$ ($n = 4–13$), relative to the insulin response in absence of glucose oxidase or C2-ceramide. Basal surface GLUT4myc levels were not reduced by glucose oxidase or C2-ceramide (see supplemental Tables 1 and 2).

cells against disulfide formation in response to oxidizing conditions. As shown in Fig. 1*D*, NAC protected insulin-dependent GLUT4 translocation against the decrease induced by a range of glucose oxidase concentrations. Furthermore, confirming that the effect of glucose oxidase was not caused by glucolactone generated by glucose plus glucose oxidase, GLUT4 translocation was not affected in muscle cells directly exposed to glucolactone at concentrations equimolar to the generated H_2O_2 (supplemental Fig. 1).

The second condition eliciting insulin resistance tested was exposure to cell-permeating ceramide. Treating L6 myotubes with increasing concentrations of C2-ceramide caused insulin resistance of GLUT4 translocation (Fig. 1*D*) and 2-deoxyglucose uptake (Fig. 1*F*). At 12.5 μmol/l C2-ceramide, there was ~40% inhibition of these responses, and 50 μmol/l C2-ceramide caused nearly complete insulin resistance without affecting basal levels (supplemental Table 2). Qualitatively similar effects on insulin action were produced by the longer-chain ceramides C6 and C8 (not shown). Interestingly, these effects of C2-ceramide were not attributable to oxidant production because NAC did not recover insulin-stimulated GLUT4 translocation when L6 myotubes were pretreated with 20 mmol/l NAC and 50 μmol/l C2-ceramide (data not shown).

Glucose oxidase and ceramide inhibit insulin-induced actin remodeling. As reported previously (5,11,14), insulin induces rapid and dynamic changes in cortical actin filaments that manifest as mesh-like structures along the dorsal surface of myotubes. Figure 2 is a collection of images representing cells treated with increasing concentrations of glucose oxidase (each representative of four to six experiments). Increasing concentrations of glucose oxidase progressively prevented the insulin-induced formation of these actin structures noticeable at 12.5 mU/ml (where less than half of the fields examined showed any of the small actin bundles illustrated in panel Fig. 2*F*). At 25 mU/ml glucose oxidase and beyond, actin filaments in insulin-stimulated cells resembled those in unstimulated (basal) cells. The inhibitory action of glucose oxidase on insulin-induced actin remodeling was prevented by 20 mmol/l NAC (supplemental Fig. 2), arguing that oxidant stress was the cause for the interference with insulin action on actin dynamics.

Similarly, increasing doses of C2-ceramide progressively prevented insulin-induced actin remodeling, with marked reductions detected in most fields of myotubes treated with 12.5 μmol/l C2-ceramide, where shorter actin clusters were observed only occasionally, as in the field illustrated in Fig. 3. Other than averting the insulin response, the morphologic appearance of stress fibers remained unaffected with no discernible thinning or disarray in the basal or insulin-stimulated states even at the highest doses of glucose oxidase or C2-ceramide (Figs. 2 and 3). The specific action of 12.5 mU/ml glucose oxidase or 12.5 μmol/l C2-ceramide on insulin-induced actin remodeling is further illustrated in supplemental Fig. 3. In supplemental Fig. 3*A–E*, (basal) actin filaments are not visibly different from those in supplemental Fig. 3*F–J* (basal plus 12.5 mU/ml glucose oxidase) or *K* and *L* (basal plus 12.5 μmol/l C2-ceramide) when focusing on the first three planes of optical sectioning (0.4 μm per section), respectively, near the ventral surface, at the middle of the myotubes, or at the dorsal plane. Conversely, the insulin response that typically manifests as dorsal actin remodeling (supplemental Fig. 3*DD* and *EE*) was selectively abolished by 12.5

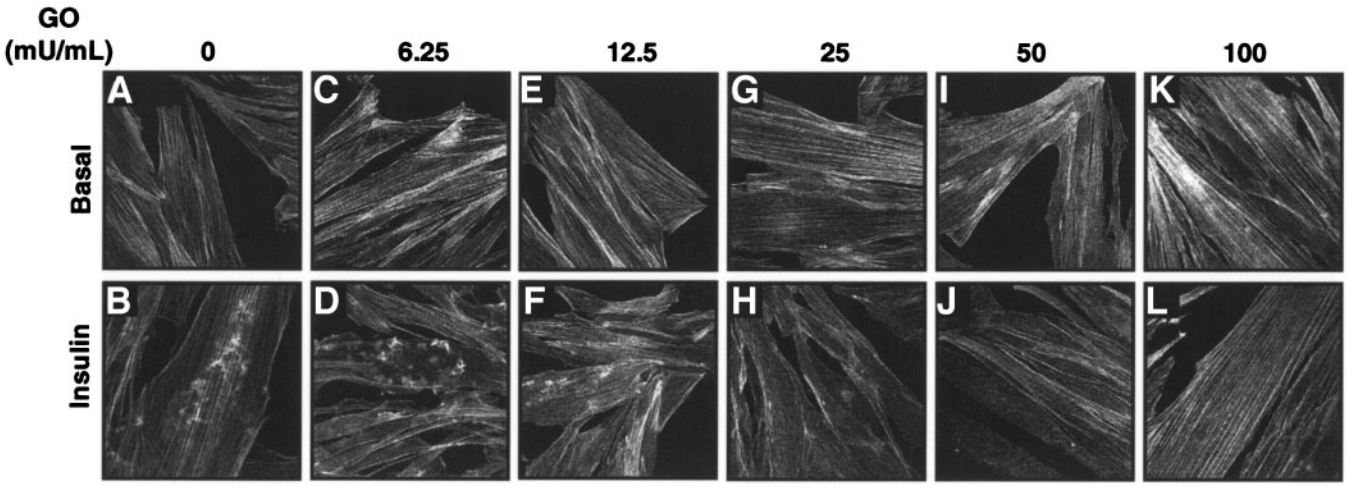

FIG. 2. Glucose oxidase treatment prevents insulin-induced actin remodeling. Myotubes pretreated without or with 6.25–100 mU/ml glucose oxidase (GO) were stimulated for 10 min with 100 nmol/l insulin where indicated. Cells were then fixed and actin filaments stained with rhodamine-phalloidin, and then images were collected by confocal fluorescence microscopy. *A*: Organized actin stress fibers in unstimulated (Basal) cells. *B*: Insulin-induced actin remodeling. Glucose oxidase doses progressively inhibited insulin-induced actin remodeling (*F*, *H*, *J*, and *L*) without affecting basal-state stress fibers (*E*, *G*, *I*, and *K*). Less than half of the fields examined showed any of the small actin bundles illustrated in *F*. See also supplemental Figs. 2 and 3.

mU/ml glucose oxidase (*II* and *JJ*) or 12.5 μmol/l C2-ceramide (*NN* and *OO*). In insulin-stimulated cells, there was no effect of 12.5 mU/ml glucose oxidase or 12.5 μmol/l C2-ceramide on the stress fibers prominent at the ventral region of the cell (supplemental Fig. 3*FF–HH* and *KK–MM*, respectively). Furthermore, the relative proportions of filamentous and free globular actin assessed biochemically were 55 and 45%, respectively, in unstimulated untreated myotubes and were not appreciably changed by insulin stimulation (filamentous 56% and globular 44%) or 12.5 μmol/l C2-ceramide pretreatment (basal: 43 and 57%; insulin: 55 and 45%, respectively). Collectively, these results suggest that insulin-induced actin remodeling involves a small fraction of G-actin or of preexisting filaments that reconfigure near the dorsal surface of the myotubes. In any case, because there was no discernible increase in G-actin, the results confirm that glucose oxidase and C2-ceramide do not cause generalized actin depolymerization.

Glucose oxidase and ceramide inhibit insulin-induced Rac activation. We recently reported that the small G-protein Rac is rapidly activated (GTP loaded) in response to insulin in both muscle and adipose cells and that a dominant-negative Rac mutant unable to bind GTP prevented insulin-induced actin remodeling (13). Hence, we next determined whether the glucose oxidase and C2-ceramide prevention of actin remodeling was linked to inhibition of insulin-induced Rac activation. Rac activation was substantial in response to insulin (5.2 fold ± 2.2 above basal). Pretreatment with 12.5 mU/ml glucose oxidase (Fig. 4*A*) or 12.5 μmol/l C2-ceramide (Fig. 4*B*) nearly abolished insulin-induced Rac activation without a statistically significant reduction in basal Rac-GTP levels (supplemental Tables 1 and 2). Concomitantly to the prevention of Rac activation, glucose oxidase and C2-ceramide treatments eliminated Rac signaling to its target p21-activated kinase (PAK) (data not shown).

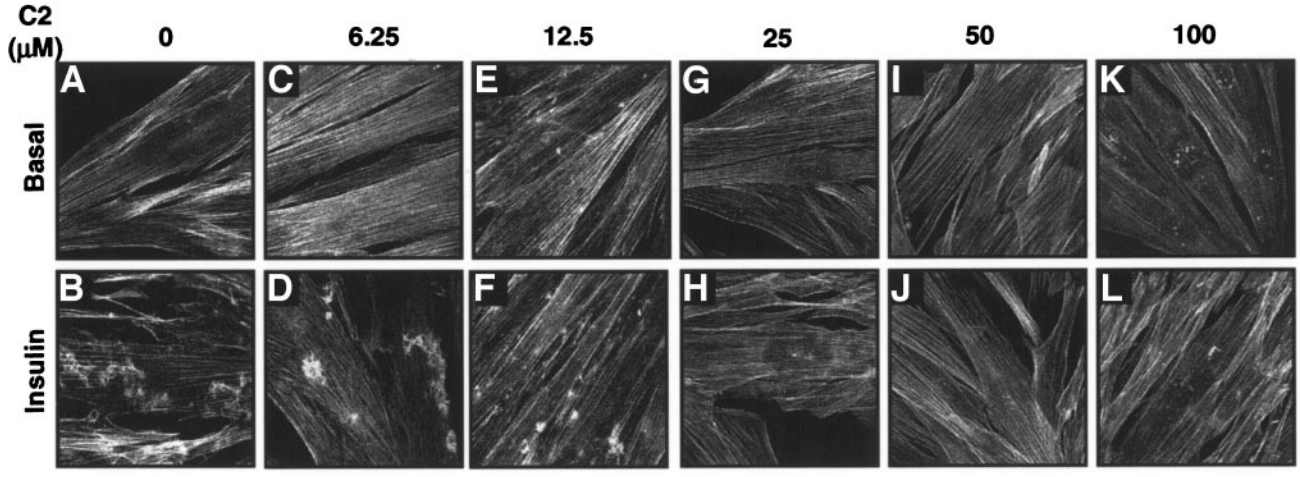

FIG. 3. C2-ceramide (C2) treatment prevents insulin-induced actin remodeling. Myotubes pretreated without or with 6.25–100 μmol/l C2-ceramide were stimulated for 10 min with 100 nmol/l insulin where indicated, and otherwise they were processed as in Fig. 2. *A*: Organized actin stress fibers in unstimulated (Basal) cells. *B*: Insulin-induced actin remodeling. C2-ceramide doses progressively inhibited insulin-induced actin remodeling (*F*, *H*, *J*, and *L*) without affecting basal-state stress fibers (*E*, *G*, *I*, and *K*). See also supplemental Fig. 3.

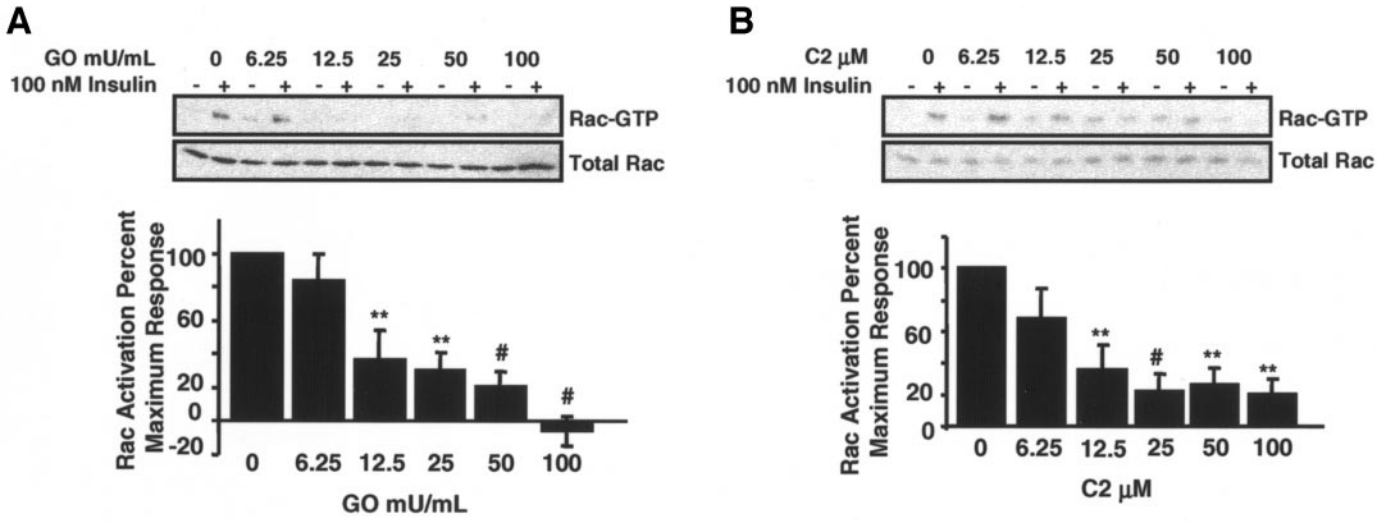

FIG. 4. Dose-dependent inhibition of insulin-induced Rac activation by glucose oxidase (GO) (*A*) or C2-ceramide (C2) (*B*). Myotubes were pretreated with glucose oxidase or C2-ceramide and exposed to insulin for 10 min, and then GTP-Rac bound to glutathione-*S*-transferase–CRIB (Cdc 42/Rac interactive binding) as well as total Rac in lysates were determined by immunoblotting. Representative gels with doses up to 50 mU/ml glucose oxidase or 50 μmol/l C2-ceramide are illustrated. Additional gels tested the effect of higher concentrations. These experiments were repeated 3–12 times, and the averaged results are expressed as a percent of the maximal insulin response in the absence of pretreatments. $^{\#}P < 0.001$, $^{**}P < 0.01$ relative to the stimulation in the absence of glucose oxidase or C2-ceramide. Basal levels were not altered by glucose oxidase or C2-ceramide (see supplemental Tables 1 and 2).

Effects of glucose oxidase and ceramide on IRS-1, PI 3-kinase, and Akt. Neither glucose oxidase (25,30) nor C2-ceramide (21,23) affect total cellular insulin-dependent IRS-1 tyrosine phosphorylation, nor its association with p85 and PI 3-kinase activity in 3T3-L1 adipocytes, and C2-ceramide also spares these signaling components in L6 myotubes (22). We further characterized the effect of glucose oxidase in L6 myotubes and found that insulin-induced IRS-1 tyrosine phosphorylation and its association with the p85 subunit of PI 3-kinase (Fig. 5*A*) and with PI 3-kinase activity (Fig. 5*B*) was unaltered by glucose oxidase, even at concentrations that caused marked insulin resistance of GLUT4 translocation, Rac activation, and actin remodeling. Only at the highest concentration of glucose oxidase (100 mU/ml) was there any appreciable diminution of PI 3-kinase activity, which, however, was not statistically significant. Confirming previous results in the literature (22), C2-ceramide had no effect on the ability of insulin to stimulate PI 3-kinase activity (results not shown).

In contrast, glucose oxidase treatment prevented insulin-stimulated Akt phosphorylation on Thr308 and Ser473

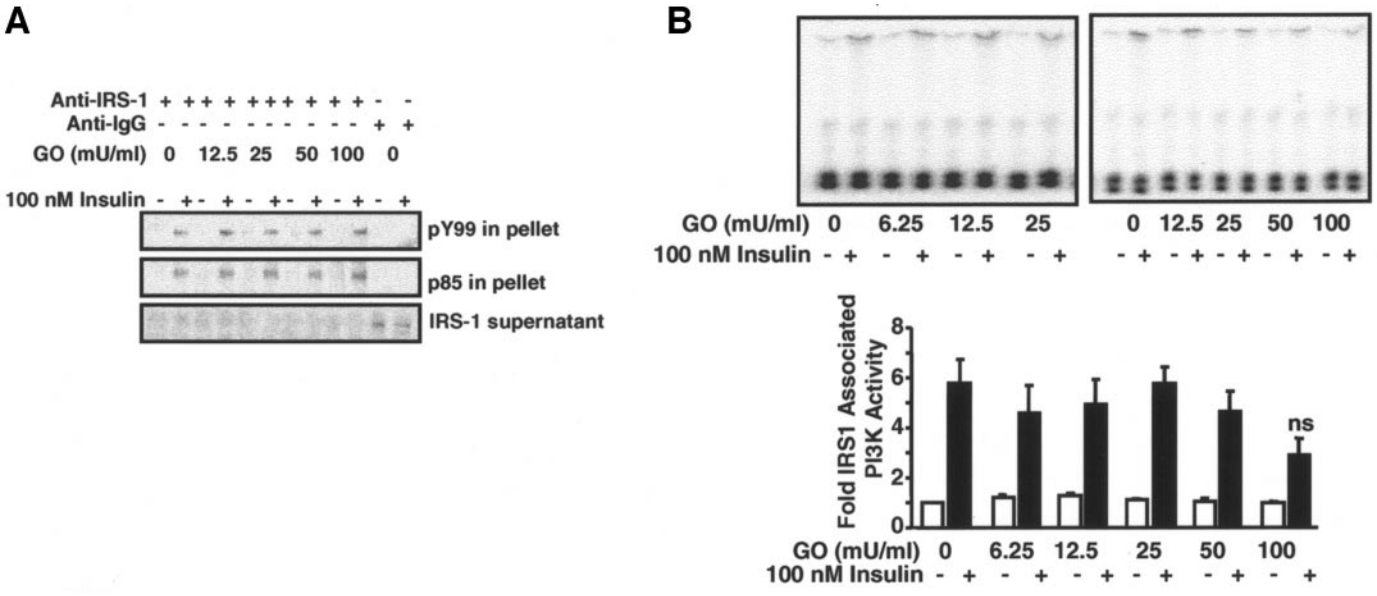

FIG. 5. Glucose oxidase (GO) does not alter whole-cell levels of insulin-induced IRS-1 tyrosine phosphorylation or its association with p85 or PI 3-kinase activity. *A*: Myotubes pretreated with glucose oxidase or C2-ceramide as in Fig. 1 were incubated without or with insulin for 10 min, followed by lysis, immunoprecipitation with polyclonal anti–IRS-1 or IgG, and immunoblotting for phosphotyrosine or p85. Supernatants were immunoblotted with anti–IRS-1 to ascertain immunoprecipitation efficiency. *B*: PI 3-kinase activity associated with anti–IRS-1 immunoprecipitates was determined by in vitro incorporation of radioactive ATP into PI. Shown are representative thin-layer chromatography plates and averaged results of seven experiments quantifying the fold insulin response normalized to basal activity in the absence of treatments. ns, not statistically significant.

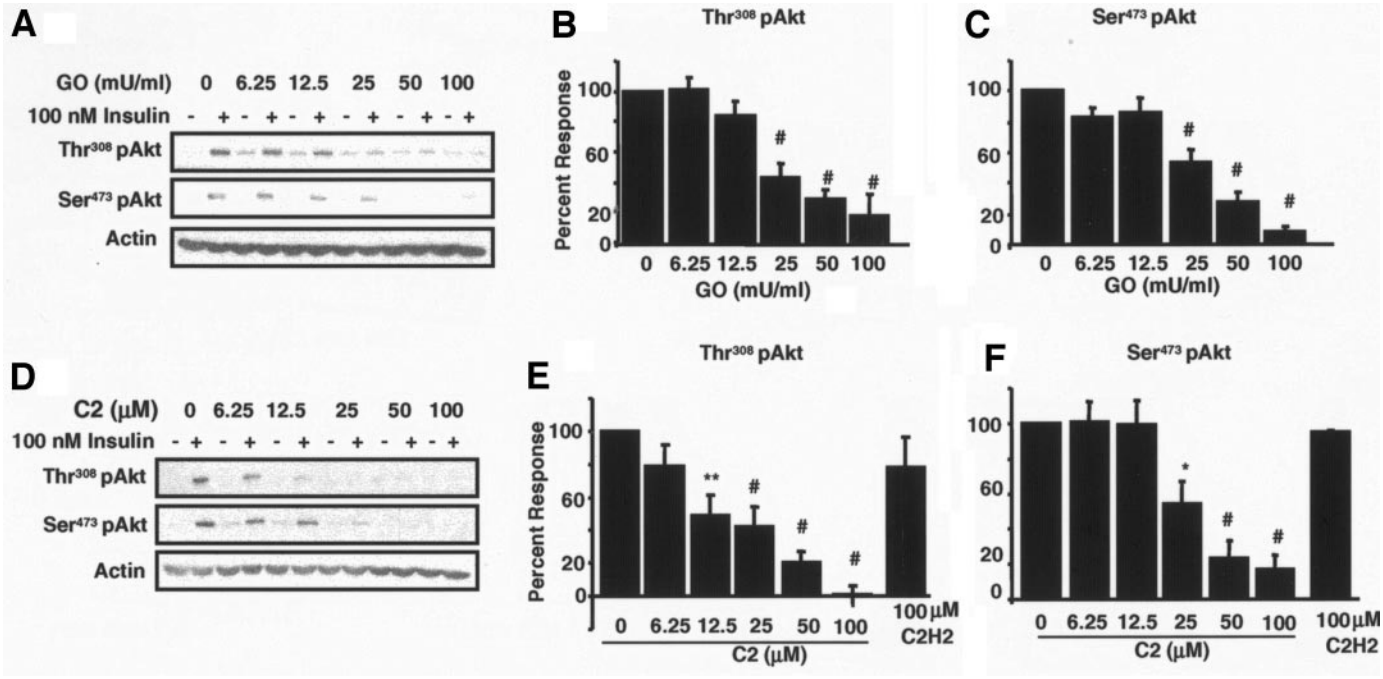

FIG. 6. Glucose oxidase (GO) and C2-ceramide (C2) inhibit insulin-induced Akt phosphorylation on Thr308 and Ser473. Myotubes retreated with glucose oxidase or C2-ceramide were incubated without or with insulin for 10 min, followed by lysis and immunoblotting for phosphorylated Akt (pAkt) on Thr308 and Ser473. *A* and *D*: Representative gels illustrate the response to 0–100 mU/ml glucose oxidase or 0–100 μmol/l C2-ceramide. Parallel experiments were carried out with 100 μmol/l C2-ceramide and C2-dihydroceramide. The results from 4–12 experiments analyzing Thr308 phosphorylated Akt (*B* and *E*) or Ser473 phosphorylated Akt (*C* and *F*) in cells pretreated with glucose oxidase (*B* and *C*) or C2-ceramide (*E* and *F*) were quantified and expressed as a percent of the maximal insulin response observed in the absence of treatments. $^{**}P < 0.01$,$^{*}P < 0.05$, $^{\#}P < 0.001$ ($n = 4$–12) relative to the stimulation in absence of glucose oxidase or C2-ceramide.

beginning at 25 mU/ml (Fig. 6*A*–*C*). Of note, there was minimal reduction in Akt phosphorylation in cells treated with 12.5 mU/ml glucose oxidase, yet under those conditions Rac activation and actin remodeling were notably attenuated.

In previous studies, treatment with various doses of C2-ceramide prevented Akt phosphorylation on Ser473 (22). Consistent with those studies, 25 μmol/l C2-ceramide caused a 50% reduction in insulin-stimulated Akt phosphorylation on Thr308 and Ser473, and both responses were completely abolished with 100 μmol/l C2-ceramide (Fig. 6*D*–*F*). However, Ser473 was not affected by 12.5 μmol/l C2-ceramide, a dose that markedly diminished Rac activation.

Rac1 silencing prevents actin remodeling and GLUT4 translocation but not Akt activation. The above results suggest that C2-ceramide and glucose oxidase affect two arms of insulin signaling that emanate from PI 3-kinase and are required for GLUT4 translocation: Rac-mediated actin remodeling and Akt activation. It was therefore important to explore whether the two signaling arms intersect. The participation of Rac in insulin-induced actin remodeling had been inferred from results obtained in L6 myoblasts transiently expressing a mutant Rac1 unable to bind GTP (14) and had not been confirmed in differentiated myotubes. Moreover, dominant-negative mutants may not directly reflect participation of a single Rho family protein because they can inhibit upstream guanine exchange factors, thereby potentially affecting diverse Rho proteins. Therefore, to further substantiate the contribution of Rac1 to insulin-dependent actin remodeling and GLUT4 translocation, particularly in differentiated myotubes, Rac1 expression was silenced using siRNA oligonucleotides. On transfection of 400 nmol/l siRNA targeting Rac1, Rac1 protein expression was reduced by 70% compared with cells transfected with unrelated siRNA (Fig. 7*A*). Concomitantly, there was a marked prevention of insulin-dependent actin remodeling determined by actin filament decoration with rhodamine phalloidin in myotubes (Fig. 7*B*) and myoblasts (data not shown). In contrast, stress fibers in unstimulated cells were not visibly affected (Fig. 7*B*). Along with the marked reduction in Rac1 expression, there was a 48% inhibition in insulin-stimulated GLUT4 translocation compared with cells expressing unrelated siRNA (Fig. 7*C*). The effectiveness of Rac1 knockdown in blocking downstream signaling was confirmed because this treatment readily inhibited insulin-mediated activation/phosphorylation of its downstream target, PAK (Fig. 7*D*). Strikingly, a 70% knockdown of Rac1 expression did not alter insulin-dependent Akt phosphorylation on Ser473 or Thr308 (Fig. 7*D*). These results suggest that Rac-dependent signaling (and likely its consequent actin remodeling) does not directly affect Akt activation, highlighting the independence of the Rac and Akt signals downstream of PI 3-kinase.

DISCUSSION

Defects in insulin signaling in insulin-resistant states in vivo and in cell culture. In vivo, insulin resistance accompanying type 2 diabetes manifests as a drop in insulin-regulated glucose clearance. Insulin signaling defects arise in muscle of obese or type 2 diabetic individuals, notably involving drops in IRS-1 tyrosine phosphorylation and in PI 3-kinase and Akt activation (33). The defects appear to originate from multiple serine/threonine phosphorylations on IRS-1 brought about by diverse kinases (34). Intriguingly, in human subjects, de-

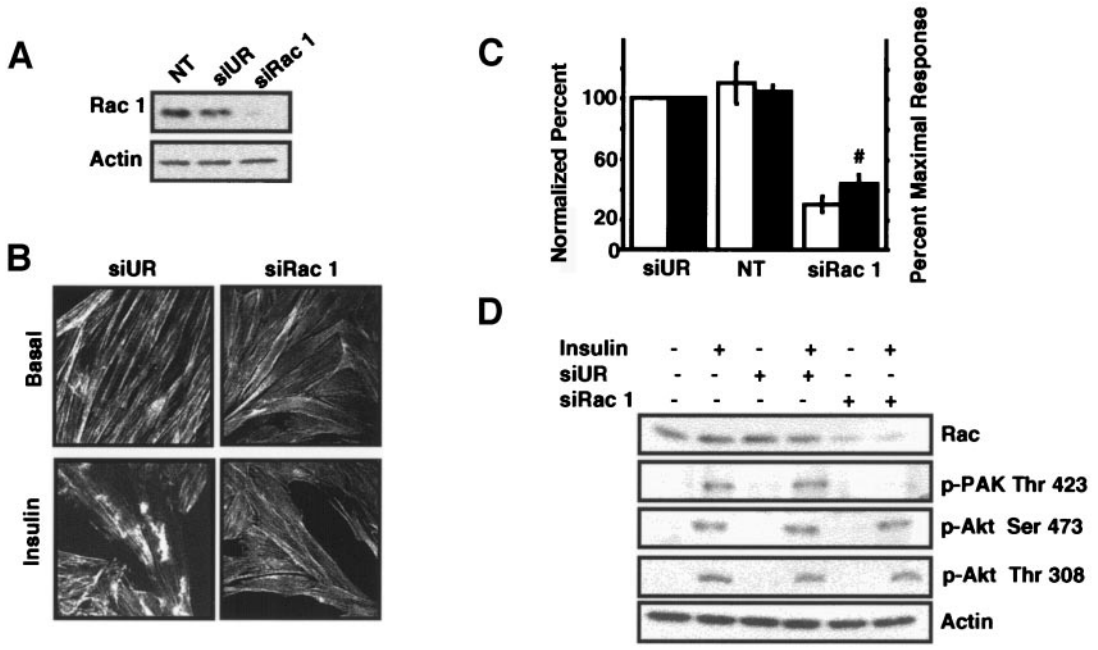

FIG. 7. Rac1 expression silencing via siRNA inhibits insulin-induced actin remodeling and GLUT4 translocation, but not Akt phosphorylation. Myotubes were exposed to 400 nmol/l of unrelated siRNA (siUR) or Rac1 siRNA (siRac1) for up to 72 h, incubated without or with insulin for 10 min, and used in the following assays. *A*: Rac expression assessed by immunoblotting of cell lysates. *B*: Filamentous actin visualization in fixed and permeabilized myotubes. *C*: Surface GLUT4myc in 15 experiments, expressed as a percent of the maximal insulin response in unrelated siRNA cells, and corresponding fractional expression of Rac. ■, surface GLUT4myc; □, fractional expression of Rac. #$P < 0.001$ relative to unrelated siRNA. *D*: Phosphorylation of Rac-target PAK on Thr423, phosphorylation of Akt on Ser423 and Thr308, and immunoblotting of actin levels to assess protein loading. NT, no treatment; p-Akt, phosphorylated Akt.

creased PI 3-kinase activation is not always accompanied by similar impairment in Akt (35), suggesting that additional signaling steps downstream of PI 3-kinase may contribute to the defective insulin signal propagation. Consistently, in simplified, isolated cell systems, several manipulations causing insulin resistance, such as exposure to high glucose (36), fatty acids (37), growth hormone (38) or nelfinavir (39), also spare IRS-1 tyrosine phosphorylation while affecting downstream signaling elements to elicit insulin resistance. Thus, the contribution of such a diversity of defects toward dampening the GLUT4 response requires further study.

In vivo, defects in fat and liver tissues contribute to muscle insulin resistance through secreted factors, complicating the analysis of dietary and environmental impact on skeletal muscle, which is facilitated by the use of simplified cellular systems. Accordingly, exposure to permeating forms of the fatty acid metabolite ceramide, specifically C2-ceramide (40), emulates the ceramide accumulation observed in muscle of insulin-resistant rodents and humans, and exposure to oxidative radicals emulates the oxidative stress accompanying type 2 diabetes. Indeed, elevated reactive oxidant species levels correlate with fasting plasma glucose (41), and insulin resistance may be reversed by diverse antioxidants (42,43). Increased oxidant production may arise from hyperglycemia-induced glucose auto-oxidation or glycation (44) and from the impaired mitochondrial function documented in type 2 diabetes (45).

Notably, both C2-ceramide administration and oxidant production through glucose oxidase spared insulin-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase association in adipose cells yet reduced insulin-dependent glucose uptake (23,30). Here, we extend these observations to muscle cells, where oxidant production by glucose oxidase again failed to reduce tyrosine phosphorylation of IRS-1 (Fig. 5). Although glucose oxidase caused serine phosphorylation and partial IRS-1 degradation in 3T3-L1 adipocytes, this was not prevented by the antioxidant α-lipoic acid, which, however, restored insulin-sensitive glucose uptake; conversely, rapamycin prevented IRS-1 degradation but did not restore glucose uptake (46). These results illustrate how oxidant dampens the GLUT4 insulin response independently of IRS-1 levels and tyrosine phosphorylation.

Collectively, these observations suggest that insulin resistance may arise from defects at several different levels of the insulin signaling chain, which requires analysis of the contribution by individual signals to GLUT4 translocation. Here, we analyzed the defects in insulin signaling in muscle cells exposed to C2-ceramide or oxidant-producing glucose oxidase, and we focused on the participation of insulin-dependent Rac activation and actin remodeling in conferring insulin resistance to GLUT4 translocation. Insulin causes Rac activation (GTP loading) downstream of PI 3-kinase, and overexpression of a Rac mutant unable to load GTP prevents insulin-induced actin remodeling and GLUT4 translocation in L6 myoblasts (13,14). Yet, actin remodeling is unaffected by dominant-negative Akt, suggesting that PI 3-kinase signaling bifurcates, leading, respectively, to Rac activation and Akt phosphorylation (12). The connection of Rac signaling with insulin resistance had thus far not been explored and was the focus of the current study.

Ceramide and glucose oxidase inhibit Rac activation and actin remodeling more readily than Akt phosphorylation. An interesting observation of this study is that insulin-induced Rac activation and actin remodeling appear to be more sensitive to disruption by glucose oxidase than is Akt phosphorylation: Rac activation was

reduced by >60% at 12.5 mU/ml glucose oxidase (Fig. 4), whereas Akt phosphorylation at either Thr308 or Ser 473 had only begun to be affected by twice those concentrations (Fig. 6). A slight difference was also observed with C2-ceramide, which at 12.5 μmol/l effectively reduced Rac activation, yet Akt Ser473 phosphorylation remained intact. However, Thr308 phosphorylation was as sensitive to C2-ceramide as was Rac activation.

Insulin-induced GLUT4 translocation was also somewhat more susceptible to inhibition by glucose oxidase and C2-ceramide than was Akt phosphorylation. Defects in GLUT4 translocation were apparent at 12.5 mU/ml glucose oxidase or 12.5 μmol/l C2-ceramide, and complete inhibition was achieved with 50 mU/ml glucose oxidase or 50 μmol/l C2-ceramide, concentrations that spared between 20 and 40% of Akt phosphorylation. Using three distinct dominant-negative Akt mutants at different dosages, we had previously observed that GLUT4 translocation is normally maintained with only 40% of the maximal Akt activation, and only more severe inhibition of Akt impairs GLUT4 translocation (12). Hence, it is unlikely that the reduction in GLUT4 translocation caused by oxidant-producing glucose oxidase or by low doses of C2-ceramide is caused by partially diminished Akt activation. Instead, we found that the C2-ceramide and glucose oxidase dose-dependent sensitivity of GLUT4 translocation correlates better with the sensitivity of Rac–GTP loading and actin remodeling.

What could be the mechanism whereby oxidant-producing glucose oxidase or C2-ceramide prevent Rac activation? PI 3-kinase association with IRS-1 sampled in whole-cell lysates was not affected by either treatment, and neither was the activity of the lipid kinase toward exogenous PI (Fig. 5) (23,25). It is possible that PI 3-kinase is mislocalized as a result of either oxidant-producing glucose oxidase or C2-ceramide treatments, thereby failing to generate PI-(3,4,5)-trisphosphate in the appropriate locations required for Rac activation. Indeed, glucose oxidase treatment of 3T3-L1 adipocytes prevents PI 3-kinase migration from the cytosol to microsomes (30). As well, only a small fraction of IRS-1 may be defective, as supported by Bloch-Damti et al. (47), who identified two IRS-1 pools that are differentially phosphorylated on serine residues and have distinct levels of PI 3-kinase association. Furthermore, it is possible that glucose oxidase or C2-ceramide may interfere with the guanine exchange factors responsible for insulin-dependent Rac activation. Finally, it is plausible that glucose oxidase or C2-ceramide directly affect chemical characteristics or localization of Rac. Surprisingly, Rac translocation to membranes (presumably leading to Rac activation) occurred on sustained exposure of Rat2 fibroblasts to tumor necrosis factor-α (an agent that elevates ceramide levels) (48), and chronic high peroxide exposure activated Rac in epithelial cells (49). The latter result is consistent with the insulin-mimetic effect of peroxide levels higher than those generated in our study. Those observations suggest that under conditions of insulin resistance, there may be tonic activation of Rac. However, such activation was not observed during the shorter exposures to oxidant or ceramide used herein (Fig. 6 and supplemental Tables 1 and 2), which instead selectively prevented the insulin-induced activation of Rac.

Future studies should explore the mechanism whereby C2-ceramide and oxidants prevent acute insulin-dependent GTP loading of Rac and the consequences on actin polymerization, severing, and uncapping. In this context, reactive oxygen species produced on epidermal growth factor stimulation increased G-actin deglutathionylation in A431 cells (50), a modification that increases the rate of actin polymerization, F-actin content, and barbed-end exposure (50,51). As well, it is possible that C2-ceramide may not work in the same manner as endogenous ceramides in inducing insulin resistance, and exogenous C2-ceramide may perturb the membrane structure. Future studies should examine the effect of strategies that increase endogenous ceramides.

What could be the mechanism for the inhibition of Akt caused by glucose oxidase or C2-ceramide? Akt translocation to the plasma membrane is important for its activation, and C2-ceramide inhibited this process in 3T3-L1 adipocytes and L6 muscle cells in a protein kinase C-ζ–dependent manner (24). There is no equivalent analysis for the action of glucose oxidase. Future studies should analyze Akt localization to better understand the partial inhibition of Akt that occurs with glucose oxidase and C2-ceramide.

Rac is required for GLUT4 translocation but not Akt activation. The results of this study highlight the exquisite susceptibility of Rac activation to C2-ceramide and glucose oxidase, and its correlation with loss of actin remodeling and GLUT4 translocation and—to a lesser extent—of Akt phosphorylation. We therefore explored more directly whether elimination of Rac prevents GLUT4 translocation and Akt phosphorylation. Using siRNA targeting Rac1, we achieved substantial diminution in Rac expression and signaling, as evinced by the loss of PAK phosphorylation. Importantly, Akt phosphorylation in response to insulin remained intact, and yet GLUT4 translocation (and, as expected, actin remodeling) was abolished. These results highlight the requirement for Rac activation in insulin action leading to GLUT4, and they reveal the independence of Akt activation from Rac signaling leading to actin remodeling, despite the reduction in Akt activation observed on generic actin filament disruption by latrunculin B (10). It is conceivable that actin remodeling is required for events that are independent of Akt input, or that actin remodeling is required for events downstream of Akt. However, the latter possibility is less likely given the effective GLUT4 translocation caused by myristoylation signal–attached Akt in cells with disrupted actin filaments (52). The nature of the putative early event governed by Rac-dependent actin remodeling requires scrutiny, but it may include proper vesicle exposure to signals and molecular motors or vesicle docking at the membrane, whereas Akt may participate in subsequent vesicle fusion with the plasma membrane.

In conclusion, Rac activation is a key step in insulin signaling leading to GLUT4 translocation, which occurs independently of Akt signaling. It is plausible that Rac/actin and Akt may each contribute to different steps in GLUT4 translocation, e.g., mobilization toward the membrane and docking/fusion. Moreover, Rac activation is prevented by ceramide and oxidants, agents that cause insulin resistance in vitro and contribute to insulin resistance in vivo. Defects at the level of Rac activation (likely translating into defects in actin remodeling) and of Akt may separately and synergistically contribute to insulin resistance of GLUT4 translocation elicited by C2-ceramide and glucose oxidase. These mechanisms may potentially participate in diverse conditions causing muscle insulin resistance in humans.

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