

# Prolonged Oxidative Stress Impairs Insulin-Induced GLUT4 Translocation in 3T3-L1 Adipocytes

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**Prolonged exposure of 3T3-L1 adipocytes to micromolar concentrations of H<sub>2</sub>O<sub>2</sub> results in an impaired response to the acute metabolic effects of insulin. In this study, we further characterized the mechanisms by which oxidative stress impairs insulin stimulation of glucose transport activity. Although insulin induced a 2.5-fold increase in plasma membrane GLUT4 content and a 50% reduction in its abundance in the low-density microsomal (LDM) fraction in control cells, oxidation completely prevented these responses. The net effect of insulin on 2-deoxyglucose uptake activity was reduced in oxidized cells and could be attributed to GLUT1 translocation. Insulin stimulation of insulin receptor substrate (IRS) 1 tyrosine phosphorylation and the association of IRS-1 with phosphatidylinositol (PI) 3-kinase were not impaired by oxidative stress. However, a 1.9-fold increase in the LDM content of the p85 subunit of PI 3-kinase after insulin stimulation was observed in control, but not in oxidized, cells. Moreover, although insulin induced an increase in IRS-1-associated PI 3-kinase activity in the LDM in control cells, this effect was prevented by oxidation. These findings suggest that prolonged low-grade oxidative stress impairs insulin-stimulated GLUT4 translocation, potentially by interfering with compartment-specific activation of PI 3-kinase. *Diabetes* 47:1562-1569, 1998**

One of the primary actions of insulin is to stimulate the transport of glucose into adipose and skeletal muscle tissues by inducing translocation of GLUT4 from an intracellular membrane pool to the plasma membrane (1). Resistance to this stimulatory effect of insulin is a major pathological feature of diabetes (2). Although the understanding of the mechanisms of insulin action has increased significantly in the past decade, the exact processes responsible for insulin resistance have not been fully elucidated.

Recent studies have provided evidence that the diabetic state is associated with an antioxidant/pro-oxidant imbalance

(3,4). In addition to impaired antioxidant defense mechanisms (5), increased production of reactive oxygen species (ROS) has been proposed, although the exact source(s) for this increased production has yet to be elucidated (3). Recent data have stressed the role of glucose auto-oxidation and increased nonenzymatic glycation processes as major sources of ROS production (6,7). Increased plasma levels of lipid and DNA oxidation products, including lipid hydroperoxides and malondialdehyde, have been demonstrated in diabetic patients, further suggesting an association between oxidative stress and diabetes (8-11). Increased oxidative stress has been recognized to play a role in the pathogenesis of accelerated atherosclerosis observed in diabetic patients (12,13). However, the possible involvement of increased oxidative stress in the pathogenesis and progression of insulin resistance, though suggestive, currently remains controversial (14,15). This hypothesis is mainly supported by studies in which oxidative stress and metabolic parameters have been correlated (8,16), and by several clinical trials that have reported beneficial metabolic effects of antioxidant supplementation (17,18). However, neither experimental approach could prove a clear cause-and-effect relationship between oxidative stress and insulin resistance.

In the past 15 years, several studies have demonstrated various effects of H<sub>2</sub>O<sub>2</sub> on the insulin-signaling machinery. It is generally accepted that H<sub>2</sub>O<sub>2</sub> exerts insulinomimetic effects, particularly by inducing tyrosine phosphorylation events (19-21). However, these studies mostly used short-term exposure (<60 min) to millimolar concentrations of H<sub>2</sub>O<sub>2</sub>, and in some studies, H<sub>2</sub>O<sub>2</sub> effects were noted mainly in the presence of the tyrosine phosphatase-inhibitor vanadate (21,22). To the best of our knowledge, these studies did not address the possibility that prolonged exposure to H<sub>2</sub>O<sub>2</sub> may result in an insulin-resistant state by impairing insulin-induced translocation of glucose transporters. This could potentially occur by mimicking the well-documented effects of prolonged insulin stimulation (23-25) or by affecting potential oxidation sensitive steps in GLUT4 trafficking and fusion processes.

To investigate the possibility that prolonged, low-grade oxidative stress may induce a condition of insulin resistance, 3T3-L1 adipocytes (26) and L6 myotubes (27,28), representing the two main peripheral targets for the metabolic effects of insulin, were exposed to an H<sub>2</sub>O<sub>2</sub> enzymatic-generating system. Addition of glucose oxidase to glucose-containing medium generated an H<sub>2</sub>O<sub>2</sub> concentration in the low micromolar range, which remained stable for 24 h (26,27). GLUT1 gene expression was upregulated by oxidative stress in both L6 myotubes and 3T3-L1 adipocytes. In 3T3-L1 adipocytes, glucose oxidase treatment caused a reduction of insulin-stimulated glucose uptake, glycogenesis, and lipogenesis. The

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2DG, 2-deoxyglucose; DMEM, Dulbecco's modified Eagle's medium; IRS, insulin receptor substrate; LDM, low-density microsomal; NEM, *N*-ethylmaleimide; PAO, phenylarsene oxide; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PM, plasma membrane; ROS, reactive oxygen species.

present study was undertaken to investigate the cellular mechanisms underlying the impairment in insulin-stimulated glucose transport caused by prolonged exposure of 3T3-L1 adipocytes to oxidative stress. We report that in addition to a reduction in GLUT4 expression, oxidative stress selectively impairs insulin-induced GLUT4 translocation, while sparing GLUT1 translocation. The major oxidation-sensitive step under these conditions may be located distal to phosphatidylinositol (PI) 3-kinase activation through its interaction with insulin receptor substrate (IRS) 1, and may involve defective activation of PI 3-kinase activity in the low-density microsomal (LDM) fraction.

## RESEARCH DESIGN AND METHODS

**Chemicals.** Tissue culture medium, serum, and antibiotic solutions were obtained from Biological Industries (Beit-Haemek, Israel). Recombinant human insulin was obtained from Novo Nordisk (Bagsvaerd, Denmark). Anti-GLUT1 antibodies were a gift from Dr. Pekala (East Carolina University, Greenville, NC). Anti-GLUT4 antibodies were obtained from Chemicon (Temecula, CA); anti-IRS-1 and anti-p85, from Upstate Biotechnology (Lake Placid, NY); anti-phosphotyrosine antibodies (PY20), from Transduction Laboratories (Lexington, KY); peroxidase conjugated anti-rabbit IgG, anti-mouse IgG, and  $\gamma$ -[<sup>32</sup>P]ATP, from Amersham Life Sciences (Buckingham, U.K.); 2-deoxy-[H]-glucose, from Nuclear Research Center-Negev (Dimona, Israel); and all other chemicals, from Sigma (St. Louis, Mo).

**Cell culture.** 3T3-L1 pre-adipocytes (American Type Culture Collection) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/l glucose, as previously described (26). Then, 48 h after confluence cells were induced to differentiate to adipocytes by changing the medium to DMEM supplemented with 10% fetal calf serum, 5  $\mu$ g/ml recombinant human insulin, 0.5 mmol/l 3-isobutylmethylxanthine, and 0.25  $\mu$ mol/l dexamethasone sodium phosphate for 48–72 h. Cells were used 9–10 days after differentiation was induced when they exhibited >90% adipocyte phenotype. H<sub>2</sub>O<sub>2</sub> was generated by adding 25 mU/ml glucose oxidase (type 2 from *Aspergillus niger*; 20,000 U/g solid in non-oxygen-saturated conditions; Sigma) to serum-free DMEM supplemented with 0.5% radioimmunoassay-grade bovine serum albumin (BSA). The addition of 25 mU/ml glucose oxidase resulted in a medium H<sub>2</sub>O<sub>2</sub> concentration that achieved a steady state of 7.5  $\pm$  0.8  $\mu$ mol after 0.5 h, as previously reported (26). After an 18-h incubation, medium glucose concentrations determined with hexokinase and glucose-6-phosphate dehydrogenase (29) were 18.2  $\pm$  2.1 and 15.5  $\pm$  2.4 mmol/l for control and glucose oxidase-treated cells, respectively.

**Hexose transport determinations.** 2-Deoxyglucose (2DG)-uptake measurements were performed as previously described (26). Assays were performed for 10 min using 50  $\mu$ mol/l 2-deoxy-[3H]-glucose (1  $\mu$ Ci/ml). Nonspecific uptake (<10% of the total) was determined in the presence of cytochalasin B (50  $\mu$ mol/l), and was subtracted from the total uptake.

**Cellular membrane preparations.** Following treatment with or without glucose oxidase, cells were rinsed three times with phosphate-buffered saline (PBS) and incubated for 20 min with or without 100 nmol/l insulin. Membrane preparations (4  $\times$  10 cm plates per condition) were performed following the procedure described by Clancy and Czech (30). The activity of the plasma membrane (PM) marker 5'-nucleotidase (EC 3.1.3.5) was determined, as previously described (30). The specific activity (nanomole per minute per milligram of protein) of PM 5'-nucleotidase for control and glucose oxidase-treated cells was 28.1  $\pm$  3.3 and 29.3  $\pm$  0.8, respectively, which reflected purification of >10-fold versus homogenate. Protein recovery of the different membrane fractions was not altered by glucose oxidase. Taken together, these results indicate that glucose oxidase treatment did not affect subcellular fractionation.

**Cell lysates and Western blots.** Cells (1  $\times$  10 cm plate per condition) were rinsed three times with PBS and incubated in the absence or presence of insulin for 7 min. For preparation of lysates for phosphotyrosine immunoblots, cells were scraped into 0.6 ml ice-cold lysis buffer (1% Triton X-100, 10 mmol/l sodium pyrophosphate, 50 mmol/l NaF, 10% glycerol, 80 mmol/l  $\beta$ -glycerophosphate, 2 mmol/l EDTA, 2 mmol/l EGTA, 2 mmol/l sodium vanadate, 50 mmol/l HEPES [pH 7.4], 1 mmol/l phenylmethylsulfonyl fluoride [PMSF], 1  $\mu$ mol/l aprotinin, 1  $\mu$ mol/l leupeptin, 1  $\mu$ mol/l pepstatin A), gently shaken for 15 min at 4°C, and centrifuged (12,000g, 15 min, 4°C). The lysate was collected, protein content determined (BCA method; Pierce, Rockford, IL) and added to Laemmli buffer. Aliquots of 150  $\mu$ g protein were resolved on 7.5% SDS-PAGE and subjected to Western blot, followed by quantitation by video densitometry analysis, as described (26,31).

**Immunoprecipitation and PI 3-kinase assay.** Cells were prepared as described in the above section, only that a different lysis buffer was used (50 mmol/l Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/l NaCl, 1

mmol/l EGTA, 1 mmol/l sodium vanadate, 1 mmol/l NaF, 1 mmol/l PMSF, 1  $\mu$ mol/l aprotinin, 1  $\mu$ mol/l leupeptin, 1  $\mu$ mol/l pepstatin A). For immunoprecipitation, 1 mg protein of cell lysate or 0.1 mg protein of LDM was used, and assayed as described (31). PI 3-kinase assay was performed following the protocol described by Hadari et al. (19), using PI and  $\gamma$ -[<sup>32</sup>P]ATP as substrates followed by thin-layer chromatography.

**Statistical analysis.** Data are expressed as means  $\pm$  SE. Each treatment was compared with controls, and statistical significance between two groups was evaluated using Student's *t* test. One-way analysis of variance (ANOVA) was used for the data presented in Fig. 4, with post-hoc multiple comparison by a Bonferroni test. The criterion for significance was set at *P* < 0.05.

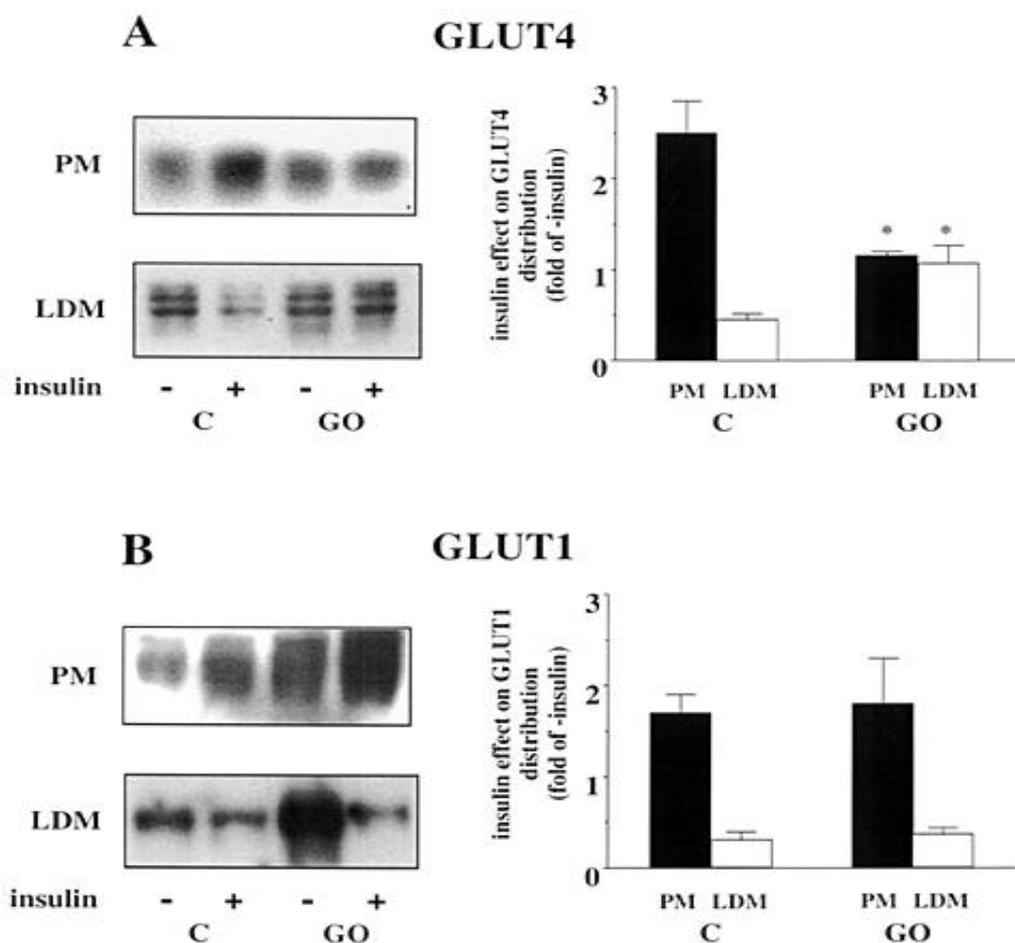
## RESULTS

### Oxidative stress impairs insulin-stimulated translocation of GLUT4 but not GLUT1 to the plasma membrane.

In previous studies, we demonstrated that exposure of 3T3-L1 adipocytes to prolonged, low-grade oxidative stress results in impaired response to the metabolic effects of insulin. Following an 18-h exposure to micromolar concentrations of H<sub>2</sub>O<sub>2</sub>, produced by adding glucose oxidase to the culture medium, a 3.5-fold increase in basal glucose transport activity was observed, associated with the reduced ability of insulin to increase glucose transport above basal levels. In addition, protein and mRNA levels of GLUT1 were increased ~3.5-fold, whereas those of GLUT4 were reduced by 45% after glucose oxidase treatment (26).

To investigate the effect of prolonged oxidative stress on glucose transporter translocation, control and glucose oxidase-treated cells were rinsed, stimulated with insulin for 20 min, and subjected to subcellular fractionation. GLUT4 and GLUT1 proteins in the PM and LDM fractions were identified using specific antibodies. Quantitation of four independent experiments is depicted in Fig. 1 (*right*). Although in control cells insulin induced a 255  $\pm$  35% increase in PM GLUT4 and a 55  $\pm$  1% reduction in LDM GLUT4 (Fig. 1A), in glucose oxidase-treated cells it induced neither a significant increase in PM GLUT4 nor a reduction in LDM GLUT4. These findings indicated that oxidative stress inhibited insulin-stimulated GLUT4 translocation. In contrast, insulin-induced GLUT1 translocation from the LDM to the PM was not impaired by glucose oxidase treatment (Fig. 1B). The increased total membrane GLUT1 content after prolonged oxidative stress (26) was reflected in both LDM and PM fractions, suggesting that GLUT1 transporters synthesized in response to oxidative stress displayed normal cellular distribution. After insulin stimulation, both control and glucose oxidase-treated cells exhibited a 170% increase in PM content and a 70% reduction in GLUT1 protein in LDM, consistent with translocation of GLUT1 protein to the PM. Taken together, these data suggest that oxidative stress may selectively impair insulin-stimulated GLUT4 translocation but not affect the translocation of GLUT1 to the PM.

**Decreased GLUT4 expression after oxidation is independent of the increase in GLUT1 content.** To further determine the respective roles of GLUT1 and GLUT4 in regulating glucose transport activity after oxidative stress, the increase in GLUT1 protein content observed after oxidation was prevented using the protein synthesis inhibitor cycloheximide. As seen in Fig. 2A, in control cells treated for 18 h with 5  $\mu$ g/ml cycloheximide (Fig. 2A, *upper blot, lane 3*), total membrane GLUT1 protein content was significantly reduced compared with untreated cells (Fig. 2A, *upper blot, lane 1*). Total membrane GLUT4 protein content was not significantly affected by cycloheximide in control cells (Fig.

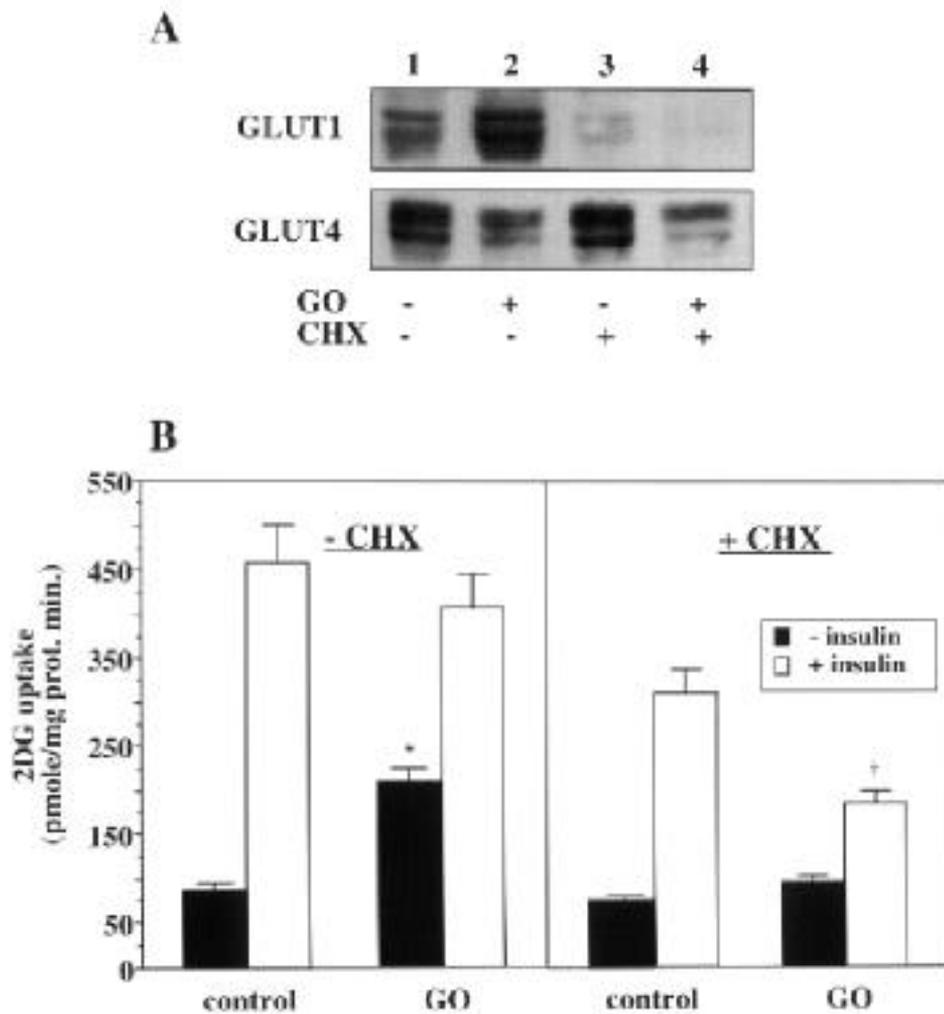


**FIG. 1.** Oxidative stress impairs GLUT4 but not GLUT1 insulin-stimulated translocation to the PM. Differentiated 3T3-L1 adipocytes were rinsed three times with PBS and incubated for 18 h without (control cells; C) or with 25 mU/ml glucose oxidase (GO) added to DMEM supplemented with 0.5% radioimmunoassay-grade BSA. Cells were then rinsed again with PBS, and further incubated for an additional 20 min with serum-free medium with or without 100 nmol/l insulin. Subcellular fractionation was performed, followed by Western blot analysis using anti-GLUT4 antibodies (A) or anti-GLUT1 antibodies (B), as described in METHODS. Blots represent four independent experiments. Video densitometry analyses of four independent experiments are presented in right panels. Results (means  $\pm$  SE) were normalized by arbitrarily setting the densitometry of each membrane fraction in the absence of insulin to 1.0. \* $P < 0.01$  vs. fold increase by insulin in the respective membrane fraction of control cells.

2A, lower blot, lane 3 vs. lane 1). These results were consistent with the differences in protein half-life reported for the two transporters isoforms in 3T3-L1 adipocytes (19 and 50 h for GLUT1 and GLUT4, respectively) (32). Treatment of the cells for 18 h with glucose oxidase increased GLUT1 protein content as compared with non-oxidized cells (Fig. 2A, lane 2 vs. lane 1, respectively). However, in cycloheximide-treated cells, this effect of oxidation was completely prevented (Fig. 2A, upper blot, lane 4 vs. lane 3). In contrast, the reduction in GLUT4 protein content induced by oxidation was not prevented by cycloheximide (Fig. 2A, lower blot), suggesting that this process does not require de novo protein synthesis. **The insulin-stimulated glucose transport activity in oxidized cells is dependent on increased GLUT1 expression.** Cells exposed to glucose oxidase exhibited an increase in basal 2DG transport activity, whereas after acute insulin stimulation, transport activity was not significantly affected by oxidation (Fig. 2B, left panel). In cycloheximide-treated cells, GLUT1 protein content was markedly reduced (Fig. 2A, upper blot), but basal glucose transport activity was not inhibited. This was probably due to a cycloheximide-

induced increase in intrinsic activity of the glucose transporters, as previously suggested (33). Under these conditions, the increase in basal 2DG uptake induced by oxidation was inhibited, and insulin-stimulated glucose transport activity was reduced from  $315 \pm 26$  to  $188 \pm 13$  pmol 2DG  $\cdot$  mg $^{-1}$  protein  $\cdot$  min $^{-1}$  in nonoxidized and oxidized cells, respectively ( $P = 0.05$ ) (Fig. 2B, right panel). These data suggest that the normal glucose uptake activity in the presence of insulin observed in oxidized cells is dependent on the increase in GLUT1 content and its preserved ability to translocate to the PM (as shown in Fig. 1).

**Effect of prolonged oxidative stress on components of the insulin-signaling cascade.** Insulin-stimulated translocation of glucose transporters to the PM is dependent on an intact signaling cascade, including increased phosphorylation on tyrosine residues of the IRS family members and PI 3-kinase activation through its interaction with phosphorylated IRS proteins. To investigate which step(s) in this cascade may be perturbed by prolonged oxidative stress, resulting in the observed defect in GLUT4 translocation to the PM, protein tyrosine phosphorylation in total cell lysates as well as

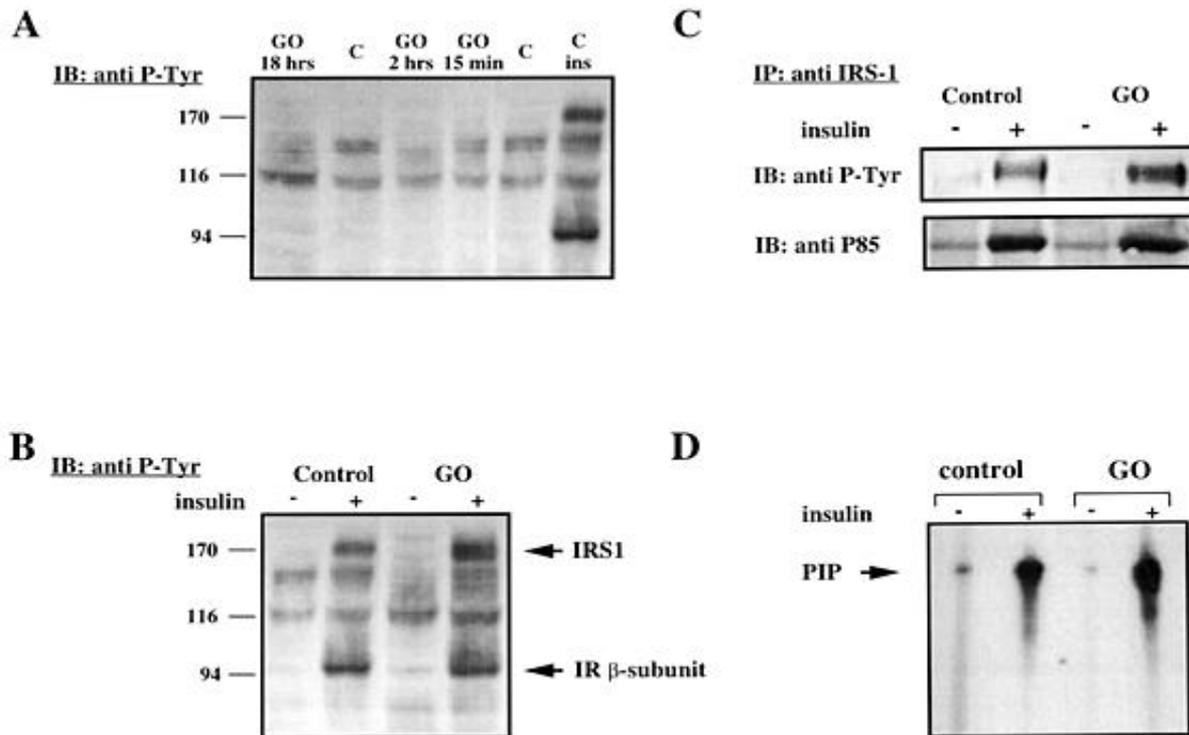


**FIG. 2.** Decreased GLUT4 expression after oxidation is independent of both the increase in GLUT1 expression and the associated increase in glucose flux into the cells. Cells were incubated without (control) or with 25 mU/ml glucose oxidase (GO) for 18 h in the presence or absence of 5  $\mu$ M cycloheximide (CHX), a protein synthesis inhibitor. Protein recovery and MTT tetrazolium tests revealed intact viability of the cells. **A:** Cells were rinsed three times and total membranes were isolated followed by Western blot analysis using anti-GLUT1 or anti-GLUT4 antibodies. Shown is a blot representing three independent experiments yielding similar results. **B:** Following treatment, cells were rinsed three times and exposed to fresh medium without (■) or with (□) 100 nmol/l insulin for an additional 20 min, after which 2DG uptake was measured. Results are means  $\pm$  SE of four independent experiments, each performed in duplicate. \* $P = 0.001$  vs. control in the absence of insulin; † $P = 0.05$  vs. CHX-treated control cells in the presence of insulin.

IRS-1 interaction with PI 3-kinase was evaluated (Fig. 3). Oxidative stress produced by glucose oxidase (for 15 min, 2 h, or 18 h) did not cause an increase in the total cellular tyrosine phosphorylation state, the phosphorylation of the insulin receptor  $\beta$  subunit, or IRS-1 (Fig. 3A). These findings suggest that the micromolar concentrations of  $H_2O_2$  used in this system did not produce detectable insulinomimetic effects. The nature or significance of the time-dependent reduction in tyrosine phosphate content of constitutively tyrosine-phosphorylated  $\sim$ 130-kDa protein(s) (Fig. 3A) is currently not clear. Insulin stimulation of control and glucose oxidase-treated cells resulted in increased tyrosine phosphorylation of both IRS-1 and the  $\beta$ -subunit of the insulin receptor (Fig. 3B and C). To evaluate the ability of insulin to induce activation of PI 3-kinase through its interaction with IRS-1, IRS-1 was immunoprecipitated and the amount of p85 regulatory subunit of PI 3-kinase as well as PI 3-kinase activity was assessed in the immunoprecipitate. As depicted in Fig. 3C

(lower blot), insulin increased the amount of p85 co-precipitable with IRS-1 to the same extent in oxidized as in control cells. In accordance with this, IRS-1-associated PI 3-kinase activity was increased by insulin in glucose oxidase-treated cells to a similar extent as in control cells (Fig. 3D). These data support intact insulin-stimulated tyrosine phosphorylation and PI 3-kinase activation in oxidized cells. Thus prolonged oxidative stress seems to impair insulin-stimulated GLUT4 translocation at stages that are distal to the activation of PI 3-kinase through its interaction with IRS-1.

**Activation of glucose transport by osmotic shock is not altered by oxidative stress.** Osmotic shock has recently been reported to increase glucose transport activity by inducing GLUT4 translocation through mechanisms independent of the insulin receptor-IRS-1-PI 3-kinase cascade (34). The existence of *N*-ethylmaleimide (NEM)-sensitive proteins within GLUT4-containing vesicles (35) suggests possible oxidation-sensitive steps in GLUT4 vesicular trafficking,



**FIG. 3.** Effect of prolonged oxidative stress on components of the insulin-signaling cascade. Cells were treated without (control; C) or with glucose oxidase (GO) for 15 min, 2 h, or 18 h, then rinsed and further incubated in fresh medium with or without insulin for 7 min. Cell lysates were prepared as described in METHODS, after which total cell lysates (150  $\mu$ g/lane) were subjected to Western blot analysis using anti-phosphotyrosine (anti P-Tyr) antibodies (A and B). C: Immunoprecipitation (1 mg cells lysate protein) using anti-IRS-1 antibodies was performed, after which immunoprecipitates were subjected to Western blot analysis using anti-phosphotyrosine or anti-p85 antibodies. D: PI 3-kinase activity in IRS-1 immunoprecipitates was measured as described in METHODS. Blots are representative of three to five independent experiments with similar results. IB, immunoblot; IP, immunoprecipitation; IR, insulin receptor; PIP, phosphatidylinositol phosphate.

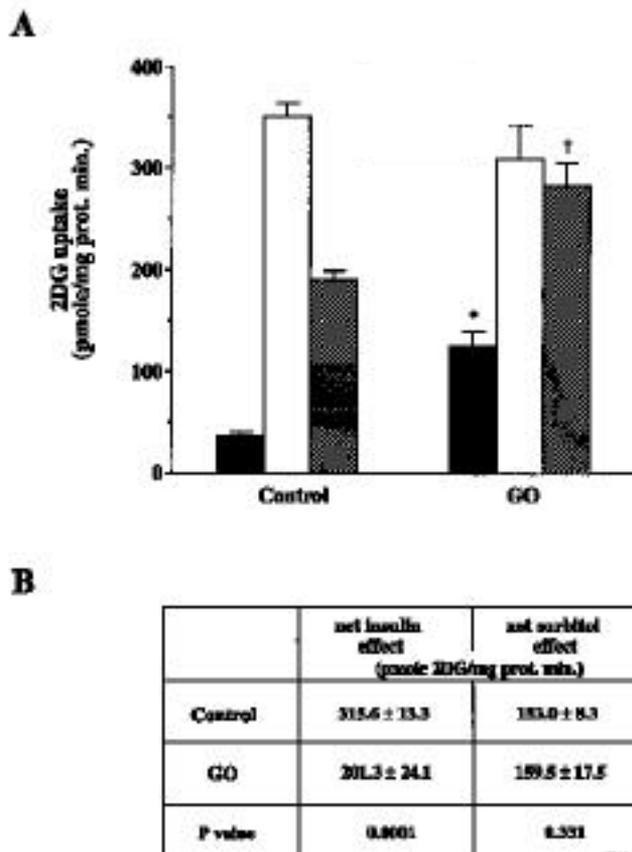
docking, or fusion with the PM. To assess the potential involvement of these processes in the effects of oxidative stress on GLUT4 translocation, the response of glucose uptake in control and glucose oxidase-treated cells to 600 mmol/l sorbitol for 30 min was evaluated. As demonstrated in Fig. 4A (left), in non-oxidized cells, osmotic shock increased 2DG uptake activity by ~50% of the value obtained with insulin. Following oxidation, the response to osmotic shock was additive to the increased basal 2DG uptake activity. Thus, although the net insulin effect was significantly reduced after oxidation, the net sorbitol effect was preserved in oxidized cells as compared with non-oxidized cells (Fig. 4B). This finding may suggest that GLUT4 vesicular trafficking and fusion events are probably not major targets for the oxidative stress produced by glucose oxidase. Hence the step(s) in insulin-mediated GLUT4 translocation that is sensitive to the oxidative stress condition used may be distal to the PI 3-kinase interaction with IRS-1, but proximal to GLUT4 vesicular trafficking-fusion processes.

**Oxidative stress impairs insulin-induced increase in LDM PI 3-kinase content and activity.** Recently it has been suggested that insulin-induced activation of PI 3-kinase is characterized by increased activity of this enzyme in PM, cytosolic, and LDM fractions (36,37). Furthermore, it has been suggested that PI 3-kinase translocation to and/or acti-

vation in the LDM fraction is a necessary step in insulin-mediated GLUT4 vesicular trafficking (37–39). Fig. 5A demonstrates the content of p85 in LDM and PM preparations from control and glucose oxidase-treated cells. In control cells, an increase in LDM p85 content after insulin stimulation was observed. Quantitation of three independent experiments revealed a  $188 \pm 16\%$  increase, which is consistent with recent observations (36,37). Following prolonged oxidative stress, the amount of p85 recruited to the LDM by insulin was  $52.0 \pm 3.5\%$  of the value obtained in insulin-treated control cells ( $P < 0.05$ ). To evaluate whether this finding was associated with a parallel reduction in PI 3-kinase activity in the LDM, IRS-1 was immunoprecipitated from LDM and a PI 3-kinase assay was performed (Fig. 5B). Although insulin induced a significant increase in phosphate incorporation into PI in control cell LDM, no similar effect could be observed in glucose oxidase-treated cells. These results may suggest that although total IRS-1-associated PI 3-kinase activation by insulin is normal after oxidation (Fig. 3C and D), it is impaired in the LDM fraction.

## DISCUSSION

In this study, we demonstrated that insulin-stimulated GLUT4 translocation to the plasma membrane is impaired after 18-h exposure of 3T3-L1 adipocytes to micromolar concentrations



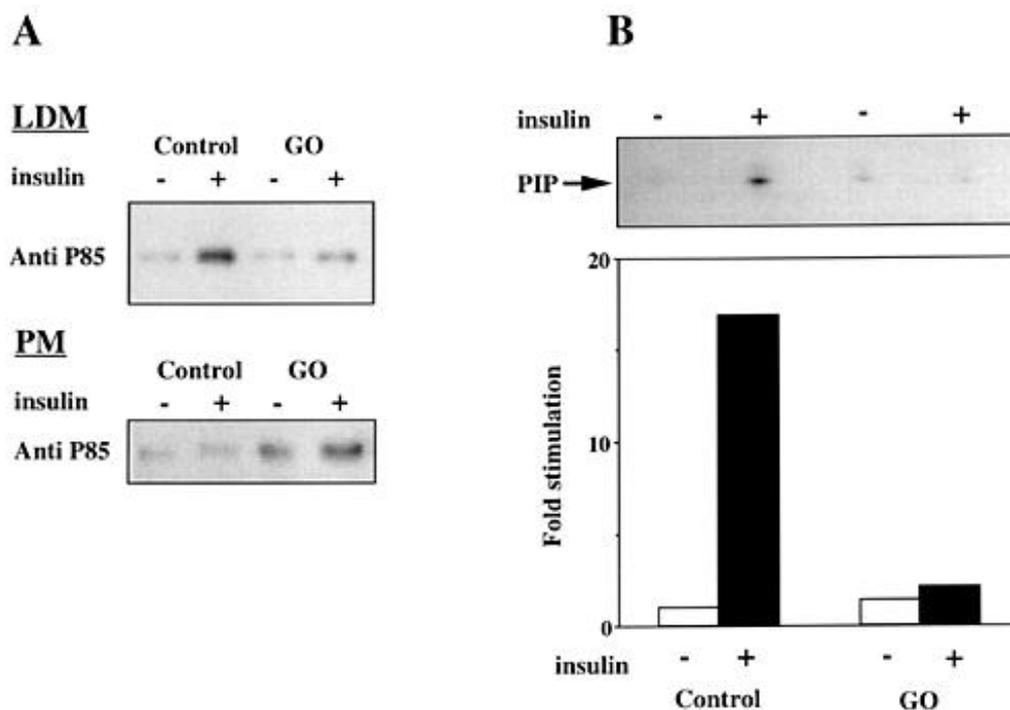
**FIG. 4.** Insulin and sorbitol stimulation of glucose transport activity in 3T3-L1 adipocytes exposed to prolonged oxidative stress. **A:** Differentiated 3T3-L1 adipocytes were exposed for 18 h to serum free medium without (Control) or with 25 mU/ml glucose oxidase (GO), then rinsed three times and exposed to fresh medium in the absence (■) or presence of 100 nmol/l insulin (□) or 600 mmol/l sorbitol (▨) for an additional 20 and 30 min, respectively. Subsequently, 2DG uptake was measured as described in METHODS. **B:** Net insulin or sorbitol effects were calculated as the difference between 2DG uptake after insulin or sorbitol stimulation and basal uptake activity, respectively. Values are means ± SE of five independent experiments, and expressed in picomoles per milligram of protein per minute. \* $P = 0.0001$  vs. controls in the absence of insulin; † $P = 0.0005$  vs. sorbitol-treated control cells. In addition, one-way ANOVA of the six values presented in **A** and the four values in **B** yielded  $P < 0.0001$ .

of  $H_2O_2$ . This effect may have been due to an oxidation-sensitive step(s) anywhere within the insulin-signaling cascade and GLUT4 translocation machinery. Various studies have suggested numerous potential oxidation-sensitive steps within this system by assessing the effects of direct exposure to  $H_2O_2$  or sulfhydryl reagents such as NEM and phenylarsene oxide (PAO). These studies yielded contradictory results. Although insulinomimetic effects of  $H_2O_2$  and PAO have been reported by some (21,40,41), others have observed inhibition of insulin actions by PAO (42–44). These discrepancies can be at least partly explained by the differences in the experimental models used and in concentrations of reagents. In the present study, we demonstrated that  $H_2O_2$  concentrations produced by 25 mU/ml glucose oxidase had no detectable insulinomimetic effect up to the activation of PI 3-kinase (Fig. 3). In addition, prolonged oxidation did not interfere with insulin-induced tyrosine phosphorylation of the insulin receptor and IRS-1, as well as the activation of IRS-1-associated PI 3-kinase

activity. Hence oxidation appears to affect a step(s) distal to the activation of PI 3-kinase by IRS-1, implying that the effect of oxidative stress is distinct from that reported for prolonged exposure of 3T3-L1 adipocytes to insulin (23). Recently some of the proteins shown to participate in cellular vesicular trafficking and fusion processes have been suggested to be NEM sensitive (the SNARE hypothesis) (35, 45–47). The presence of these proteins in GLUT4-containing vesicles may provide an explanation for the impaired GLUT4 translocation described. However, the fact that sorbitol-induced increase in 2DG uptake was not impaired after glucose oxidase (Fig. 4) may suggest that GLUT4 trafficking and fusion mechanisms are probably not major targets for the effect of oxidative stress in this experimental model. An alternative explanation is that sorbitol and insulin, which induce GLUT4 translocation through distinct signaling cascades (34), may cause the mobilization of GLUT4 from two different vesicular storage compartments (48).

In the 3T3-L1 adipocytes model, increased PI 3-kinase activity in the LDM was described after acute insulin but not after platelet-derived growth factor (36–38). It has been further suggested that LDM PI 3-kinase plays a critical role in insulin's ability to induce GLUT4 translocation (36,37), perhaps by its interaction with GLUT4-containing vesicles (38). The insulin-induced increase in the LDM content of the PI 3-kinase regulatory unit p85 as well as in PI 3-kinase activity in this fraction was impaired after glucose oxidase treatment (Fig. 5), suggesting that although PI 3-kinase was activated by insulin in oxidized cells, its compartment-specific activation was disrupted by prolonged oxidative stress. An interaction of p85 with cytoskeleton and cytoskeleton-associated proteins (e.g., focal adhesion kinase) has been reported to play a role in PI 3-kinase cellular redistribution (49–51). Whether any of these interactions are influenced in 3T3-L1 adipocytes by exposure to glucose oxidase and are related to the observed defect in GLUT4 translocation remains to be evaluated.

Although insulin-induced GLUT4 translocation was impaired by oxidative stress, our data suggest that GLUT1 translocation still occurred (Fig. 1) and resulted in insulin-stimulated glucose transport activity comparable with that of non-oxidized cells (Figs. 2 and 4). This is consistent with the notion that GLUT1 and GLUT4, or at least a certain percentage of them, may be located within the cell in separate compartments (47,52). Although increasing data suggest that GLUT4-containing vesicles are a specific vesicular subpopulation, GLUT1 may be more closely associated with the recycling endosomal compartment (53,54). This may explain the differences in trafficking kinetics and regulation (55), and hence the effect of oxidative stress on GLUT4 but not GLUT1 function. Reduced insulin-stimulated glucose transport activity occurs when GLUT1 upregulation is prevented (Fig. 2B), further emphasizing that GLUT1 compensates for defective GLUT4 translocation. In addition, it suggests that the defective GLUT4 function is not a consequence of the increased basal glucose flux to the cells (glucose toxic effect), but rather represents a direct and independent result of prolonged oxidative stress. In obese nondiabetic subjects and in nondiabetic first-degree relatives of NIDDM patients, impaired glucose transport activity in muscles and adipose tissue has been described (2). This is also consistent with the notion that abnormal GLUT4 expression and function may precede hyperglycemia, and thus are not solely a consequence of glucose toxicity.



**FIG. 5.** Oxidative stress impairs insulin-induced activation of PI 3-kinase in the LDM. Cells were treated and subjected to subcellular fractionation as described in the legend for Fig. 1. **A:** PM and LDM fractions were immunoblotted with anti-p85 antibodies, as described in METHODS. Blots represent three independent experiments yielding similar results. **B:** LDM protein (0.1 mg) was subjected to immunoprecipitation using anti-IRS-1 antibodies, followed by PI 3-kinase assay and thin-layer chromatography, as described in METHODS. Quantitation was performed by video scanning densitometry. Results represent three independent experiments.

Whether increased oxidative stress plays a pathogenic role in these subjects by altering GLUT4 expression and function remains to be confirmed.

In conclusion, prolonged low-grade oxidative stress results in impaired insulin-stimulated GLUT4 translocation and PI 3-kinase compartment-specific activation in 3T3-L1 adipocytes. Combined with the reduction in GLUT4 expression, this provides a potential cellular mechanism for the possible association between increased oxidative stress and reduced insulin responsiveness.

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## Author Queries (please see Q in margin and underlined text)

**Q1:** <<AU: Should this be  $\mu\text{mol/l}$ ?>

**Q2:** <<AU: Please spell out.>

Q3: <<AU: Please spell out and indicate to what "Pierce" refers.>

Q4: <<AU: **Addition correct?**>

Q5: <<AU: Are you referring to upper blot only?>

**Q6:** <<AU: Please spell out MTt.>

**Q7:** <<AU: Please define IB, IP, and PIP in the Figure 3 legend.>

Q8: <<AU: As meant for PDGF?>

Q9: <<AU: "Precede" instead of "proceed" correct?>

Figure 2: Please define MTt in the legend.