Oxidative Stress Disrupts Insulin-induced Cellular Redistribution of Insulin Receptor Substrate-1 and Phosphatidylinositol 3-Kinase in 3T3-L1 Adipocytes

A PUTATIVE CELLULAR MECHANISM FOR IMPAIRED PROTEIN KINASE B ACTIVATION AND GLUT4 TRANSLOCATION*

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In a recent study we have demonstrated that 3T3-L1 adipocytes exposed to low micromolar H₂O₂ concentrations display impaired insulin stimulated GLUT4 translocation from internal membrane pools to the plasma membrane (Rudich, A., Tirosi, A., Potashnik, R., Hemi, R., Kannety, H., and Bashan, N. (1998) Diabetes 47, 1562–1569). In this study we further characterize the cellular mechanisms responsible for this observation. Two-hour exposure to ~25 μM H₂O₂ (generated by adding glucose oxidase to the medium) resulted in disruption of the normal insulin stimulated insulin receptor substrate (IRS)-1 and phosphatidylinositol (PI) 3-kinase cellular redistribution between the cytosol and an internal membrane pool (low density microsomal fraction (LDM)). This was associated with reduced insulin-stimulated IRS-1 and p85-associated PI 3-kinase activities in the LDM (84 and 96% inhibition, respectively). The effect of this finding on the downstream insulin signal was demonstrated by a 90% reduction in insulin stimulated protein kinase B (PKB) serine phosphorylation and impaired activation of PKBa and PKBβ. Both control and oxidized cells exposed to heat shock displayed a wortmannin insensitive PKB serine phosphorylation and activity. These data suggest that activation of PKB and GLUT4 translocation are insulin signaling events dependent upon a normal insulin induced cellular compartmentalization of PI 3-kinase and IRS-1, which is oxidative stress-sensitive. These findings represent a novel cellular mechanism for the induction of insulin resistance in response to changes in the extracellular environment.

The information regarding the biological actions of reactive oxygen and nitrogen species has increased considerably in recent years, revealing diverse functions (1, 2). Exposure of tissues to free radicals in a variety of experimental systems leads to apoptosis and to cell damage (2). Paradoxically, reactive oxygen species have been demonstrated to participate in normal cellular responses including in signal transduction pathways and in gene regulation (1, 3, 4). H₂O₂, for example, has been shown to be produced intracellularly following stimulation with platelet-derived growth factor and to mediate the normal response to this growth factor (5). Similarly, insulin was reported to activate an adipocyte membrane bound NADPH oxidase (6, 7), further supporting a potential role for reactive oxygen species as second messengers. In agreement with this concept, direct exposure of cells to H₂O₂ has been demonstrated to result in insulinomimetic effects, as demonstrated both by mimicking the metabolic response to insulin as well as by activating components of its signal transduction machinery (8–11). However, prolonged exposure of 3T3-L1 adipocytes to micromolar concentrations of H₂O₂ resulted in impaired insulin-stimulated lipogenesis, activation of glycogen synthase, glucose transport, and GLUT4 translocation to the plasma membrane (PM) (12, 13).

Insulin-stimulated GLUT4 translocation has been suggested to depend upon the activation of phosphatidylinositol 3-kinase (PI 3-kinase), which in fat cells occurs in various cellular fractions (14, 15). Recently, the concept that the activation of PI 3-kinase in the low density microsomal fraction (LDM) or in GLUT4 containing vesicles is necessary for the specific ability of insulin to promote GLUT4 translocation has been suggested (16–19). In addition, overexpression of a constitutively active p110 (the catalytic subunit of PI 3-kinase), which resulted in increased total and LDM PI 3-kinase activity in primary adipocytes or in 3T3-L1 adipocytes, dramatically stimulated glucose uptake and GLUT4 translocation (18, 20). In a recent study we observed that impaired insulin-stimulated GLUT4 translocation following oxidation could not be attributed to defects in activation of PI 3-kinase as detected in total cell lysate (13). Thus, impaired compartment-specific activation of PI 3-kinase by insulin may represent a putative cellular mechanism for oxidation induced insulin resistance.

The events leading to the specific activation of PI 3-kinase in the LDM are not clear. Recently, insulin-induced redistribution of insulin receptor substrates (IRS) was suggested to play a role in the compartment-specific activation of PI 3-kinase in 3T3-L1 adipocytes (21). Insulin stimulation was found to induce a reduction in the amount of IRS1/2 in the LDM, while IRS tyrosine phosphorylation was elevated. The tyrosine-phosphorylated IRS in the LDM was suggested to serve as a docking molecule for PI 3-kinase, leading to a rapid translocation of the p85 subunit from the cytosol to the LDM, resulting in increased PI 3-kinase activity in this fraction (21, 22).

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‡ The abbreviations used are: PM, plasma membrane; PI, phosphatidylinositol; LDM, low density microsomal fraction; IRS, insulin receptor substrate; PKB, protein kinase B; PBS, phosphate-buffered saline.
Although the insulin signaling steps toward GLUT4 translocation distal to PI 3-kinase activation are currently not fully understood, a number of downstream targets for PI 3-kinase have been identified (23, 24). Among them, a serine/threonine kinase of 60 kDa (24, 25) termed protein kinase B (PKB) also known as RAC protein kinase or Akt (26–28). PI 3-kinase is both necessary and sufficient for insulin-dependent phosphorylation and activation of PKB, although the exact mode of activation has not been fully elucidated (reviewed in Ref. 29). It was suggested that binding of phosphatidylinositol 3,4,5-trisphosphate or phosphatidylinositol 3,4,6-trisphosphate to the pleckstrin homology domain of PKB and its translocation to the membranes are necessary for PKB phosphorylation on Thr308 and Ser473 by its upstream kinases PDK1 and PDK2, respectively. In the search for its relevant biological activity, PKB was found to mediate some of insulin’s effects, such as the inhibition of glycosynthase kinase-3 (30, 31), the stimulation of glucose and amino acids uptake (23), and protein synthesis (32). Several lines of evidence strongly support a crucial role for PKB in mediating GLUT4 translocation. In agreement with this notion, insulin activation of PKB precedes the hormonal effect on glucose transport (33). Moreover, overexpression of a constitutively active form of PKB resulted in enhanced glucose transport and GLUT4 translocation (32–36), while inhibition of PKB activity by transfecting rat adipocytes with a dominant PKB-inactive mutant, significantly inhibited insulin-stimulated GLUT4 translocation (34). The respective role of the different isoforms of PKB in mediating insulin-stimulated GLUT4 translocation in various cell types is as yet unclear. Although the involvement of IRS, PI 3-kinase, and PKB cellular redistribution and activation in the normal response to insulin is increasingly recognized, their relevance for the understanding of the cellular mechanisms leading to insulin resistance is largely unclear. In this study we report that impaired insulin-stimulated GLUT4 translocation induced by oxidative stress is associated with disruption of insulin-induced IRS-1 and PI 3-kinase intracellular trafficking and with inhibition of PKBα and PKBβ activation. This represents a novel cellular mechanism for the understanding of insulin resistance in response to a change in the extracellular environment.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture medium, serum, and antibiotic solutions were obtained from Biological Industries (Beit-Haemek, Israel). Recombinant human insulin was from Novo Nordisk (Bagsvaerd, Denmark). Anti-GLUT4 antibodies were from Chemicon International Inc. (Temecula, CA). Crosstide, anti-sheep IgG, anti-IRS-1, anti-p85, anti-phosphotyrosine (4G10), anti-PKBα (pleckstrin homology domain) antibodies were from Upstate Biotechnology (Lake Placid, NY). Anti-phosphospecific PKB (Ser473) antibodies were from New England Biolabs Inc. (Beverly, MA). Anti-PKBβ and -PKBγ- were a kind gift from Dr. D. Alessi (University of Dundee, Dundee, United Kingdom) and were used as described previously (37). Anti-PKB (C-terminal) antibodies were kindly provided by Dr. R. Segel (Weizmann Institute, Rehovot, Israel). Peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and [γ-32P]ATP were from Amersham Life Sciences (Buckingham, United Kingdom). Protein G-Sepharose and protein A-Sepharose were from Pharmacia Biotech (Uppsala, Sweden). 2-Deoxy-3H-glucose was purchased from Nuclear Research Center-Negev (Dimona, Israel). All other chemicals were obtained from Sigma.

**Cell Culture**—3T3-L1 pre-adipocytes (American Type Culture Collection) were grown to confluence in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, as described previously (12). After 12 h following confluence cells were differentiated to adipocytes by changing the medium to Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 5 µg/ml recombinant human insulin, 0.5 mM 3-isobutylmethylxanthine and 0.25 mM dexamethasone for 48–72 h. Cells were used 9–10 days following differentiation induction when exhibiting >90% adipocyte phenotype. H₂O₂ was generated by adding 100 milliunits/ml glucose oxidase (type II from Aspergillus niger, 20,000 units/g solid in non-oxygen-saturated conditions, Sigma) to serum-free Dulbecco’s modified Eagle’s medium supplemented with 0.5% radioimmunossay grade bovine serum albumin. The addition of 100 milliunits/ml glucose oxidase resulted in medium H₂O₂ concentration that achieved a steady state of 27.4 ± 0.3 µM after 15 min. Following a 2-h incubation, medium glucose concentrations determined with hexokinase and glucose-6-phosphatase (31) were 17.4 ± 1.3 mM for control and glucose oxidase-treated cells, respectively.

**Cellular Membrane Preparations**—Following treatment with or without glucose oxidase, cells were rinsed three times with phosphate-buffered saline (PBS) and incubated for 7 or 20 min with or without 100 mM insulin in freshly prepared medium supplemented with 0.5% bovine serum albumin (radioimmunossay grade). Membrane preparations were prepared from plasma membranes by homogenization in 2 M sucrose, 10 mM sodium phosphate, pH 7.5, 0.5 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotonin, 1 µM leupeptin, and 1 µM pepstatin A, with no addition of detergent, as described (13, 39).

**Plasma Membrane Label Preparation**—Plasma membrane sheets were prepared as described by Chen (40). Differentiated 3T3-L1 adipocytes were washed with PBS and incubated for 1 min with 0.5 mg/ml pol-y-b-lysine followed by three washes with hypotonic buffer (23 mM KCl, 10 mM HEPES, pH 7.5, 1.7 mM MgCl₂, 1 mM EDTA). The cells were then covered with sonication buffer (3 X hypotonic buffer containing 1 mM dithiothreitol and 0.1 mM phenethylsulfonyl fluoride) and sonicated with a probe membrane disrupter. Following sonication, the plasma membrane sheets were washed three times with sonication buffer and were used for immunoblotting as described below.

**Cell Lysates and Western Blots**—Cells (1 X 10⁶ cells per condition) were rinsed three times with PBS and incubated in the absence or presence of insulin for 7 min. Cells were then scraped into 0.6 ml of ice-cold lysis buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.05% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium vanadate, 1 mM NaF, 1 mM phenethylsulfonyl fluoride, 1 µM aprotinin, 1 µM leupeptin, 1 µM pepstatin A). Lysates were gently shaken for 15 min at 4 °C, centrifuged (12,000 X g, 15 min at 4 °C), supernatant collected, and protein content determined (BCA method, Pierce). Laemml buffer was added and samples boiled for 10 min. Protein samples were resolved on 7.5–10% SDS-polyacrylamide gel electrophoresis and subjected to Western blot, followed by quantitation using video densitometry analysis, as described (12).

**Glucose and amino acid uptake**—Uptake of [3H]glucose and [14C]amino acids was measured as described previously (12). Although the insulin signaling steps toward GLUT4 translocation had been identified (39), the activity of the PM marker 5'-nucleotidase (EC 3.1.3.5) was determined as described previously (39). The specific activity (nmol/min/mg protein) of PM 5'-nucleotidase for control and glucose oxidase-treated cells was 28.1 ± 3.3 and 27.9 ± 1.9, respectively, which reflected purification of more than 10-fold versus homogenate. Protein recovery of the different membrane fractions was determined by glucose uptake by various cellular compartments were resuspended in the original fractionation buffer (255 mM sucrose, 20 mM HEPES, pH 7.4, 1 mM EDTA, 0.2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotonin, 1 mM leupeptin, 1 mM pepstatin A), with no addition of detergent, as described (13, 39).

**Plasma Membrane Preparations**—Plasma membrane sheets were prepared as described by Chen (40). Differentiated 3T3-L1 adipocytes were washed with PBS and incubated for 1 min with 0.5 mg/ml poly-y-b-lysine followed by three washes with hypotonic buffer (23 mM KCl, 10 mM HEPES, pH 7.5, 1.7 mM MgCl₂, 1 mM EDTA). The cells were then covered with sonication buffer (3 X hypotonic buffer containing 1 mM dithiothreitol and 0.1 mM phenethylsulfonyl fluoride) and sonicated with a probe membrane disrupter. Following sonication, the plasma membrane sheets were washed three times with sonication buffer and were used for immunoblotting as described below.

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**PKB Kinase Assay**—Cell lysates were prepared as described above using PKB lysis buffer (50 mM Tris-HCl pH 7.5, 0.1% (w/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM sodium b-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 0.1% (w/v) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 µM aprotonin, 1 µM leupeptin, 1 µM pepstatin A). Protein content was determined using the Bio-Rad Bradford procedure (41). Lysates were subjected to immunoprecipitation with the different PKB antibodies and were assayed for kinase activity using Crotide (GRPRTSSSAEG) as a substrate according to the manufacturer’s instructions as described previously (31).

**Hexose Transport Determinations**—2-Deoxyglucose uptake measurements were performed as described previously (12). Assays were performed for 10 min using 10 mM 2-Deoxy-3H-glucose. Non-specific uptake (less than 10% of the total) was determined in the presence of cytochalasin B (50 µM) and was subtracted from the total uptake.

**Statistical Analysis**—Data are expressed as mean ± S.E. Each treatment was compared with control, and statistical significance between two groups was evaluated using the Student’s t test. The criterion for significance was set at p < 0.01.
RESULTS

Oxidative Stress Impairs the Compartment-specific Activation of PI 3-Kinase by Insulin—In order to study the effect of oxidative stress on the ability of insulin to activate PI 3-kinase in various cellular fractions, fully differentiated 3T3-L1 adipocytes were exposed for 2 h to 27.4 ± 0.8 μM H_2O_2 continuously generated by addition of 100 milliunits/ml glucose oxidase to the culture medium. In agreement with recent reports (16, 21), insulin induced a 2.8-fold increase in p85 content in the LDM (Fig. 1A), which was associated with a 50% reduction in its abundance in the cytosolic fraction (Fig. 1C). PI 3-kinase activity measured in p85 immunoprecipitates revealed an approximately 7-fold increase by insulin in the LDM and a 50% reduction in the cytosolic fraction (Fig. 1, B and A). This may suggest that oxidative stress increased LDM PI 3-kinase activity primarily by inducing activation rather than translocation of this enzyme in the LDM. Yet, oxidative stress resulted in a significant reduction in the ability of insulin to increase p85 content in the LDM (Fig. 1A) or to reduce its cytosolic content (Fig. 1C). This was associated with inhibition of insulin-stimulated PI 3-kinase activation in the LDM (Fig. 1B), as well as in impaired insulin induced reduction in its cytosolic activity (Fig. 1D). In total cell lysates, both p85 content as well as the activation of p85-associated PI 3-kinase activity by insulin were not affected following oxidation (Fig. 1, E and F, respectively). Taken together these data suggest that both the insulin-stimulated translocation of PI 3-kinase to the LDM and its activation in this fraction are impaired following oxidative stress and are associated with retention of this enzyme in its cytosolic pool.

Oxidative Stress Alters the Pattern of Insulin-stimulated IRS-1 Redistribution—In 3T3-L1 adipocytes most of PI 3-kinase activation by insulin occurs via its interaction with tyrosine-phosphorylated IRS-1 (21). In order to assess whether altered IRS-1 cellular compartmentalization may account for the impaired PI 3-kinase activation in the LDM, IRS-1 content and interaction with PI 3-kinase were evaluated in the LDM and cytosolic fractions. In control cells, an approximately 50% reduction in IRS-1 LDM content following 7-min insulin stimulation was observed (Fig. 2A). In oxidized cells, IRS-1 content in the LDM was significantly reduced by approximately 35%, with no further reduction following insulin stimulation. In control cells, insulin induced an inhibition in IRS-1 gel mobility (Fig. 2A), which could be attributed to increased serine/threonine phosphorylation, since incubation of LDM with alkaline phosphatase diminished this change in gel mobility (data not shown). In addition, despite the reduction in IRS-1 LDM content (Fig. 2A), insulin induced a marked elevation in IRS tyrosine phosphorylation in this fraction (Fig. 2B). In LDM prepared from oxidized cells, insulin stimulation failed to induce the change in IRS gel mobility (Fig. 2A), suggesting inhibition of its insulin stimulated serine/threonine phosphorylation. Moreover, insulin-induced tyrosine phosphorylation of IRS-1 was significantly impaired following oxidation (Fig. 2B). In accordance with this finding, the 16-fold increase in IRS-1-associated PI 3-kinase activity observed in the LDM of control cells was markedly impaired by oxidative stress (Fig. 2C).

In the cytosolic fraction, insulin stimulation of either control or oxidized cells did not result in changes in IRS-1 gel mobility, as in the LDM fraction (Fig. 2D). Yet, both the increase in cytosolic IRS-1 content induced by insulin (Fig. 2D), as well as the stimulation of IRS-1-associated PI 3-kinase activity in this fraction (Fig. 2E), were significantly impaired by oxidative stress. Taken together these results suggest that oxidation disrupted IRS-1 cellular redistribution and tyrosine phosphorylation in the LDM. This in turn resulted in the reduced ability of IRS-1 to serve as a LDM docking protein for PI 3-kinase.
Insulin-stimulated Activation of PKBα and PKBγ Is Impaired following Oxidative Stress—To assess whether the defect in PI 3-kinase redistribution is associated with impairment in the activation of PKB by insulin, PKB phosphorylation and activation were evaluated following oxidative stress. Immunoblot analysis of total cell lysates using anti-phospho-Ser473 PKB antibody demonstrated that oxidative stress induced a 3.47 ± 0.85-fold increase in PKB phosphorylation, which was wortmannin-sensitive (Fig. 3A). Exposure to insulin led to a rapid, time-dependent stimulation of PKB phosphorylation reaching maximal stimulation by 10 min in control cells, while in oxidized cells PKB phosphorylation was dramatically reduced (Fig. 3B). To correlate changes in PKB phosphorylation with its activity, total cell lysates were subjected to immunoprecipitation using anti PKB antibodies raised against its C-terminal tail. The efficiency of the immunoprecipitation protocol was not affected by oxidation, as assessed by immunoblotting the supernatant above the immunoprecipitate (data not shown). Fig. 3C demonstrates a 12.2 ± 2.3-fold increase in PKB activity following 7-min exposure to insulin. In accordance with the serine phosphorylation pattern, oxidative stress induced a 3.6 ± 1.7-fold increase in PKB activity, while the net insulin effect on PKB activity was markedly reduced from 24.10 ± 2.7 to 4.65 ± 0.9 milliunits/mg of protein/min (p < 0.001). Both basal activation of PKB induced by oxidative stress, as well as its stimulation by insulin, were completely inhibited by wortmannin, indicating the involvement of PI 3-kinase in both processes.

To further characterize the effect of oxidative stress on basal and insulin stimulated PKB activity, the various isoforms of PKB were studied using isoform specific antibodies. In 3T3-L1 adipocytes, all known PKB isoforms were found to be expressed, and their total cellular content was not significantly altered by oxidation (Fig. 4A). PKB activity was measured in immunoprecipitates of the different PKB isoforms from total cell lysates of control and oxidized cells before and after exposure to 100 nM insulin for 7 min. Fig. 4B demonstrates that in 3T3-L1 adipocytes the basal activity of PKBα, PKBβ, and PKBγ was 1.87 ± 0.25, 2.74 ± 0.42, and 6.02 ± 0.96 milliunits/mg of protein/min, respectively. Insulin increased the activity of PKBα and PKBγ by approximately 13- and 6-fold, respectively, while exerting only a minor effect on PKBβ activity. Exposure of the cells to oxidative stress resulted in a significant increase in basal PKBα activity. The ability of insulin to further activate PKBα was completely inhibited, while a significant reduction in insulin stimulated PKBγ activity was observed (from 32.3 ± 4.9 to 15.6 ± 2.6 milliunits/mg of protein/min, p < 0.01). These data demonstrate the role of PKBα in the induction of basal PKB activity observed in oxidized cells, while the reduction in insulin-stimulated PKB activity could be mainly related to PKBα and PKBγ isoforms.

To assess whether oxidative stress also impairs the ability of PKB to be activated through non-PI 3-kinase-mediated pathways, the phosphorylation and activation of PKB by heat shock were studied in oxidized cells. In control cells, exposure of 3T3-L1 adipocytes for 20 min to 44 °C resulted in a wortmannin-insensitive increase in both PKB serine phosphorylation (Fig. 5A) and in PKB activity (Fig. 5B). Cells treated for 2 h
with glucose oxidase, and subsequently exposed to heat shock
treatment, exhibited increased PKB phosphorylation and acti-
vation comparable with control cells. Taken together, these
results suggest that oxidative stress does not interfere with
activation of PKB through wortmannin-insensitive pathway(s).

Fig. 3. PKB phosphorylation and activation by insulin in control and oxidized cells. A, cells were treated without or with 250 nM wortmannin for 15 min before and during the 2-h incubation without (C) or with glucose oxidase (GO). Subsequently, cells were lysed in PKB lysis buffer, and 50 μg of protein were separated on SDS-polyacrylamide gel electrophoresis and immunoblotted with phosphoserine 473-specific PKB antibody, as described under “Experimental Procedures.” B, control and oxidized cells were incubated without or with insulin for the indicated times, and the phosphorylation pattern was studied by Western blot analysis using anti-phospho-Ser473 antibody. C, 200 μg of cell lysate were subjected to immunoprecipitation with anti-PKB (C-terminal) antibody and assayed for PKB activity using Crosstide as a substrate. Wortmannin treatment was as described above and also during the 7-min insulin stimulation. *, p < 0.01 as compared with unstimulated control cells; **, p < 0.01 versus insulin-stimulated control cells.

Fig. 4. The effect of oxidation on the PKB isoforms expression and regulation by insulin. A, control or glucose oxidase (GO)-treated 3T3-L1 adipocytes were lysed using PKB lysis buffer, and 50 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-PKBα, anti-PKBβ, and anti-PKBγ antibodies. Each blot is a representative of at least three independent experiments. B, 200 μg of protein from control and GO-treated cells, stimulated without or with 100 nM insulin for 7 min, were lysed and subjected to immunoprecipitation using the specific PKB isoform antibodies. PKB activity was assayed on the immunoprecipitates using Crosstide as a substrate, as further detailed under “Experimental Procedures.” Values are expressed as milliunits of activity/mg of protein and are the mean ± S.E. of three independent experiments. *, p < 0.01 as compared with unstimulated control cells; **, p < 0.01 versus insulin-stimulated control cells.
translocation. Fully differentiated 3T3-L1 adipocytes exhibited an approximately 12-fold increase in glucose transport activity following 20-min stimulation with 100 nM insulin (Fig. 6A). When cells were exposed to glucose oxidase, a marked reduction in insulin-stimulated glucose transport activity was observed. This was associated with a significant 2.160.15-fold increase in basal glucose transport, resulting in a reduction in the net insulin effect on glucose transport activity above basal from 510617 to 77613 pmol/mg of protein/min. As observed with the activation of PKB (Fig. 3C), both the basal activation as well as the insulin stimulation of glucose transport were completely inhibited by wortmannin. Since insulin acutely activates glucose transport mainly by promoting the translocation of GLUT4 from internal membrane pools to the plasma membrane, GLUT4 translocation was assessed. PMs were prepared using the plasma membrane lawn technique, as described (40), and subjected to Western blot analysis (Fig. 6B, left side). Using this method, insulin induced a 4.8761.43-fold increase in PM GLUT4 content in control cells, while only a 1.660.3-fold increase was observed in cells treated with glucose oxidase prior to insulin stimulation. To further establish whether oxidative stress inhibits translocation of GLUT4 from LDM to the PM, GLUT4 content was evaluated in the subcellular fractions using the sucrose cushion methods (39). As can be evaluated, insulin induces 2.5560.35-fold increase in PM GLUT4 content in control cells (Fig. 6B, right side). This was associated with a 5561.2% reduction in its abundance in the LDM, indicating insulin-stimulated GLUT4 translocation. In glucose oxidase-treated cells, no significant increase in PM GLUT4 content was observed with insulin, while a nonsignificant 15% reduction in LDM GLUT4 was detected. Taken together these results indicate that the changes in normal insulin-induced PI 3-kinase redistribution and PKB activation by oxidative stress correlate with impairment in insulin-stimulated GLUT4 translocation and activation of glucose transport activity.

DISCUSSION

This study was aimed at investigating the cellular mechanisms by which oxidative stress disrupts insulin action in 3T3-L1 adipocytes. The data presented demonstrate that while certain insulinomimetic effects of micromolar H2O2 concentrations could be observed, oxidative stress impaired the compart-
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The activation of the serine/threonine kinase PKB was dramatically inhibited by oxidative stress (Figs. 3 and 4). Insulin induced activation of the ERK1/2 MAP kinase was also impaired (unpublished results). Interestingly, insulin induced serine/threonine phosphorylation of IRS-1 in the LDM was markedly reduced by oxidation (Fig. 2A), and was associated with inhibition of its translocation from the LDM to the cytosol. These results are consistent with the notion that serine/threonine phosphorylation of LDM IRS-1 may be needed for its normal cellular redistribution. Nevertheless, insulin stimulated tyrosine phosphorylation of IRS-1 was also impaired in the LDM following oxidation (Fig. 2B), and was associated with reduced PI 3-kinase activity in this fraction (Fig. 1 and 2). Thus, the oxidation-induced reduction in tyrosine phosphorylated IRS-1 in the LDM may represent the cause rather than the effect of alterations in serine/threonine phosphorylation of LDM IRS-1. Moreover, reduced tyrosine-phosphorylated IRS-1 in the LDM appears to represent the limiting factor for the translocation and activation of PI 3-kinase in this fraction.

It is well established that PKB activation by insulin is dependent on PI 3-kinase (29). Yet whether insulin stimulation of PI 3-kinase in the LDM is required for the activation of the various known isoforms of PKB is largely unknown. Thus, the effect of oxidative stress on the basal and insulin-stimulated activity of PKBo, PKBβ, and PKBγ was evaluated. In 3T3-L1 adipocytes, similar to observations in L6 myotubes (23), most of the insulin-stimulated PKB activation could be attributed to PKBo and PKBγ (Fig. 4). Surprisingly, insulin barely affected PKBβ activity, which was demonstrated as the major PKB isoform regulated by insulin in primary rat adipocytes (57). Oxidative stress profoundly impaired both insulin-stimulated PKBo and PKBγ activities (Fig. 4), resulting in an overall reduction of total cellular PKB activity. The striking similarity between the effect of oxidation on PKB (Figs. 3C and 4) and on glucose uptake (Fig. 6A) suggests a potential cause and effect relationship between the impairment in both insulin stimulated PKB activation and GLUT4 translocation.

The ability to activate PKB through wortmannin-insensitive pathway(s) following oxidative stress was evaluated to exclude a direct inhibitory effect of oxidation on either PKB or its immediate upstream kinases PDK1 and PDK2 (29, 58). As demonstrated in COS-7 and NIH 3T3 cells (59), heat shock treatment of 3T3-L1 adipocytes induced phosphorylation and activation of PKB through wortmannin-insensitive mechanisms (Fig. 5, A and B, respectively). Following oxidative stress, this activation of PKB remained intact, supporting the notion that the impaired insulin-stimulated PKB activation induced by oxidation may be a consequence of the reduced ability of insulin to activate PI 3-kinase in the LDM. This, in turn, may offer the possibility that activation of PKB is also an insulin signaling event dependent on normal cellular compartmentalization of PI 3-kinase. In support of this concept is the observation that membrane-localized p110 was found to be sufficient to activate PKB in COS-7 cells (60).

This paper presents evidence that while micromolar concentrations of H2O2 inhibits acute metabolic response to insulin, they also induce a certain insulinomimetic effect. Exposure to H2O2 for 2 h resulted in wortmannin-sensitive increase in basal glucose transport and in PKB activities, as well as in basal PI 3-kinase activity in the LDM (Figs. 6A, 3C, and 1B, respectively). These seemingly opposing effects suggest several cellular targets for H2O2 along the multistep insulin signaling network. While one or more targets may be activated by H2O2, other can eventually become rate-limiting for additional insulin stimulation.

The potential relevance of findings presented herein may be in the understanding of the cellular mechanisms leading to...
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peripheral insulin resistance in various conditions. Increased oxidative stress has been suggested by various mechanisms to occur in diabetic or prediabetic individuals (61–63) and to contribute to the pathogenesis of diabetic late complications (64), as well as to peripheral insulin resistance (65, 66). Reduced GLUT4 expression and impaired translocation in response to insulin stimulation were reported in adipocytes of insulin-resistant individuals (67). The impaired insulin response was suggested to represent both receptor and postreceptor mechanisms. This study offers a potential mechanism by which oxidative stress can contribute to the development of impaired insulin-stimulated GLUT4 translocation in adipocytes of diabetic subjects.

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