Hydrogen Peroxide Generated During Cellular Insulin Stimulation
Is Integral to Activation of the Distal Insulin Signaling Cascade
in 3T3-L1 Adipocytes

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In a variety of cell types, insulin stimulation elicits the rapid production of H$_2$O$_2$ which causes the oxidative inhibition of protein-tyrosine phosphatases (PTPases) and enhances the tyrosine phosphorylation of proteins in the early insulin action cascade (Mahadev et al., J.Biol.Chem. 276:21938, 2001). In the present work, we explored the potential role of insulin-induced H$_2$O$_2$ generation on downstream insulin signaling using diphenyleneiodonium (DPI), an inhibitor of cellular NADPH oxidase that blocks insulin-stimulated cellular H$_2$O$_2$ production. DPI completely inhibited the activation of phosphatidylinositol (PI) 3’-kinase activity by insulin and reduced the insulin-induced activation of the serine kinase Akt by up to 49%; these activities were restored when H$_2$O$_2$ was added back to cells that had been pretreated with DPI. Interestingly, the H$_2$O$_2$-induced activation of Akt was entirely mediated by upstream stimulation of PI 3’-kinase activity, since treatment of 3T3-L1 adipocytes with the PI 3’-kinase inhibitors wortmannin or LY294002 completely blocked the subsequent activation of Akt by exogenous H$_2$O$_2$. Preventing oxidant generation with DPI also blocked insulin-stimulated glucose uptake and GLUT4 translocation to the plasma membrane, providing further evidence for an oxidant signal in the regulation of the distal insulin signaling cascade. Finally, in contrast to the cellular mechanism of H$_2$O$_2$ generation by other growth factors, such as platelet-derived growth factor, we also found that insulin-stimulated cellular production of H$_2$O$_2$ may occur through a unique pathway, independent of cellular PI 3’-kinase activity. Overall, these data provide insight into the physiological role of insulin-dependent H$_2$O$_2$ generation which is not only involved in the regulation of tyrosine phosphorylation events in the early insulin signaling cascade, but also has important effects on the regulation of downstream insulin signaling, involving the activation of PI 3’-kinase, Akt and ultimately cellular glucose transport in response to insulin.
INTRODUCTION

Major advances in our understanding of the regulation of the insulin action pathway have focused on the key role of tyrosine phosphorylation of the insulin receptor and its cellular substrate proteins (1). Insulin binding leads to autophosphorylation of specific residues of the transmembrane insulin receptor and activation of the intrinsic tyrosine kinase activity of its intracellular domains (2). The insulin signal is then transmitted further into the cell through the tyrosine phosphorylation of specific sites on cellular substrate proteins (e.g., IRS and Shc), which act as docking sites for the binding and activation of a variety of src-homology 2 (SH2) domain-containing signaling proteins (3). Much of insulin’s downstream signaling to metabolic events involves the activation of phosphatidylinositol (PI) 3'-kinase activity by the docking of its p85 subunit to tyrosine phosphorylated IRS-1 and IRS-2 (4-6), which is linked to a number of distal responses in adipocytes including the activation of the protein kinase Akt and subsequent vesicle translocation and glucose transport activation (7).

This reversible protein-tyrosine phosphorylation of components in the insulin signaling pathway has been shown to be regulated in a variety of ways. A major regulatory influence is exerted by specific cellular protein-tyrosine phosphatases (PTPases) which are involved in balancing the steady-state tyrosine phosphorylation of the insulin receptor and its substrate proteins (8). In turn, the enzymes in the PTPase superfamily are themselves regulated by oxidation/reduction reactions in vivo, since they require a reduced form of the thiol side chain of the catalytic cysteine residue for phosphotyrosine hydrolysis (9-11). In a recent study, we also showed that a burst of intracellular H2O2 resulting from insulin stimulation results in the reversible oxidative inhibition of cellular PTPase activity (12). The rapid inhibition of PTPases that negatively regulate insulin signaling was associated with enhanced insulin-stimulated tyrosine phosphorylation of the insulin receptor and high Mr IRS proteins, and was found to play an important role in the propagation of the early insulin signal.

In addition to the oxidative inactivation of thiol-dependent PTPases, it has been intriguing to speculate that reactive oxygen species, especially the H2O2 burst generated shortly after insulin binding, may be involved in the regulation of more distal cellular insulin signaling. There are an increasing number of examples in the literature indicating that at least some of the effects of various hormones, growth factors and cytokines on specific signaling pathways can
involve superoxide and H$_2$O$_2$ (13-15). For example, in the case of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), evidence has been accumulating to suggest that ligand binding is integrally associated with the generation of a cellular oxidant signal and also that redox species can mimic ligand-mediated signaling by the growth factors themselves (13;16). One report suggested that H$_2$O$_2$ may be involved in “redox priming” of the insulin receptor in muscle cells, which may render receptor kinase activation more efficient (17). However, little is known about how reactive oxygen species may affect downstream components of the insulin action pathway and a fuller characterization of these potential effects in various types of insulin-sensitive cells is needed.

To explore how the oxidant signal arising from insulin stimulation might be involved in the transmission of distal post-receptor insulin signaling, we used diphenyleneiodonium chloride (DPI), an inhibitor of cellular NADPH-oxidases (18), which we found to fully block the insulin-stimulated generation of H$_2$O$_2$ in 3T3-L1 adipocytes. This inhibitory effect was used to explore the role of the insulin-induced oxidant burst on distal insulin signaling, including glucose transport. We found that insulin-stimulated H$_2$O$_2$ was essential for the activation of Akt via stimulation of PI 3’-kinase activity, which also subsequently enhanced glucose transport activation in 3T3-L1 adipocytes. Furthermore, the mechanism by which insulin elicits cellular H$_2$O$_2$ production was shown to be independent of cellular PI 3’-kinase activity, unlike PDGF, which has been reported to generate cellular H$_2$O$_2$ by a PI 3’-kinase-mediated pathway (16). Thus, insulin-dependent H$_2$O$_2$ generation appears to occur by a unique metabolic pathway and has a cellular role not only in enhancing the early activation of insulin receptor autophosphorylation and kinase activity, but is also integrally involved in the regulation of events in the distal insulin signaling cascade.
EXPERIMENTAL PROCEDURES

Materials - Dulbecco’s modified Eagle’s medium (DMEM), Penicillin-streptomycin and fetal calf-serum were obtained from Cellgro (Herndon, VA), recombinant human insulin was obtained from Sigma (St. Louis, MO). 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCF) was from Molecular Probes, Inc. (Eugene, OR), enhanced chemiluminescence (ECL) reagents were from NEN Life Science Products (Boston, MA). Monoclonal anti-phosphotyrosine (4G10) and polyclonal antibodies to the insulin receptor β-subunit, IRS-1 and the p85 subunit of PI 3’-kinase were from Upstate Biotechnology (Lake Placid, NY). Antibodies to phosphorylated Akt (Ser473) and Akt protein (not isoform specific) and the Akt kinase activity kit were purchased from New England Biolabs (Beverly, MA). Para-nitrophenylphosphate (pNPP), diphenyleneiodonium chloride (DPI), and wortmannin were from Sigma chemical (St. Louis, MO). LY204002 (2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) was from Calbiochem (La Jolla, CA) and horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit IgG antibodies were obtained from Amersham Pharmacia Biotech (Piscataway, NJ), 2-deoxy-[3H]-D-glucose and [γ-32P] ATP was purchased from ICN (Costa Mesa, CA), Silica-coated thin layer chromatography plates obtained from VWR (Bridgeport, NJ), Phosphatidylinositol (PI) from Avanti Polar Lipids Inc. (Alabaster, AL) all other chemicals and reagents unless otherwise noted, were obtained from Sigma.

Cell culture – 3T3-L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s medium containing 25 mM glucose (DMEM) plus 10% fetal calf serum in an 5% CO2 atmosphere and were differentiated to adipocytes as previously described (19). Briefly, confluent cells were placed in differentiation medium (DMEM containing 10% fetal bovine serum, 100 nM insulin, 0.25 µM dexamethasone and 500 µM isobutylmethylxanthine) for 2 days. The medium was then changed to DMEM containing 10% fetal bovine serum and 100 nM insulin. After an additional 6 days, cells were starved overnight in DMEM containing 0.5% (w/v) bovine serum albumin (BSA) and used for the experiments.

Assay of intracellular H2O2 in 3T3-L1 adipocytes – Intracellular generation of H2O2 was visualized as described (12;20). At various times indicated (0, 1, 5, 10 minutes) after stimulation with 100 nM insulin with and without prior treatment with 10 µM DPI for 30 minutes, dishes of differentiated 3T3-L1 cells were washed with MEM medium (lacking phenol red) and then
incubated in the dark for 10 minutes with 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCF). The fluorescence of CM-DCF was measured by Bio-Rad confocal microscope at an excitation wavelength of 488 nm and emission at 515-540 nm. To avoid photooxidation of the indicator dye, the fluorescence image was collected by a single rapid scan with identical parameters for all samples. Where indicated, the fluorescence intensity was quantitated from sampled images using Scion Image software (Scion Corporation, Fredrick, MD).

**PTPase enzyme activity using pNPP as substrate in 3T3-L1 adipocytes** – 3T3-L1 adipocytes after overnight serum starvation, treated with or without 10 µM DPI for 30 minutes, 1 mM H$_2$O$_2$ for 10 minutes or together with DPI and H$_2$O$_2$. Following 100 nM insulin stimulation for 1 or 5 minutes, cells were snap-frozen with liquid nitrogen, introduced into the anaerobic work station (Forma Scientific, Model # 901024) in a frozen state and lysed in the anaerobic condition using ice-cold deoxygenated homogenization buffer (150 mM NaCl, 5 mM EDTA, 5 mM EGTA, in 50 mM Hepes, pH 7.5, 1% (v/v) Triton X-100 containing a protease inhibitor cocktail (Sigma) followed by brief sonication. The whole cell lysate was cleared by centrifugation at 15,000 x g for 20 minutes and supernatant was saved for the PTPase enzyme assay. Protein was estimated using the method of Bradford (21). Aliquots of 20 µg protein were incubated in a final volume of 100 µl at 37°C for 30 minutes in reaction buffer containing 10 mM para-nitrophenylphosphate (pNPP; Sigma) and 2mM EDTA in 20 mM 2-(N-Morpholino)ethanesulfonic acid (MES) at pH 6.0. The reaction was stopped by the addition of 1 ml of 0.2 M NaOH and the absorption was determined at 410 nm (22). The initial rate of pNPP hydrolysis was estimated from the linear portion of the earliest time points of the enzymatic reaction.

**PTPase enzyme activity using $^{32}$P-RCM-lysozyme as substrate** - Recombinant human insulin receptors from transfected CHO cells (23) were partially purified on wheat germ lectin-agarose (Vector Laboratories, Burlingame, CA) as described (24). RCM-lysozyme was radioactively labeled on tyrosine by phosphorylation with the insulin receptor preparation and [$\gamma$-$^{32}$P]-ATP (25). The reaction was initiated with the addition of 0.5 mg of RCM-lysozyme and incubated at 25°C for 16 hrs. The reaction was terminated with the addition of trichloroacetic acid (TCA) to a final concentration of 20% (w/v) and centrifuged at 30,000 x g for 15 minutes at
4°C. The pellet was washed 3 times with 20% TCA and dialyzed overnight against 50 mM imidazole-HCl, pH 7.2. PTPase activity was assayed using the indicated amount of cell fraction protein in 50 mM HEPES, pH 7.0 and 2 mM EDTA, without DTT, as indicated. The reaction was initiated by the addition of 20 µl of [32P]-phosphotyrosyl RCM-lysozyme (~20,000 dpm) and terminated by the addition of 0.9 ml of acidic charcoal mixture, consisting of 0.9 M NaCl, 90 mM sodium pyrophosphate, 2 mM NaH₂PO₄ and 4% (w/v) Norit A activated charcoal (26). After centrifugation in a microfuge, the amount of radioactivity in 0.4 ml of supernatant was measured by Cerenkov counting in a liquid scintillation counter. The initial rate of RCM-lysozyme hydrolysis was estimated from the linear portion of the earliest time points of the enzymatic reaction where less than 20% of the RCM-lysozyme was hydrolyzed during the 5 minute reaction period.

**Immunoblotting** – After the indicated experimental treatments, cells were lysed in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 mM sodium fluoride, 1 mM EGTA, 1 mM EDTA, 2 mM sodium vanadate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Sigma). The lysates were briefly sonicated, centrifuged at 13,000 x g for 10 minutes, and 75 µg protein of the cleared supernatant was resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane using a semidry western blotting apparatus (AP Biotech). PVDF membranes were subjected to immunoblotting with either monoclonal antibody for phosphotyrosine (4G10) to detect insulin receptor β-subunit and IRS tyrosine phosphorylation, polyclonal antibody to detect phospho-Akt or additional antibodies to detect total protein levels of the insulin receptor β-subunit, IRS-1 and Akt, where indicated. Following incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were visualized by enhanced chemiluminescence, according to the instructions provided by the manufacturer. The immunoblotting signals were quantitated using an ImageStation 440 (Kodak).

**Insulin receptor autophosphorylation in vitro** - Recombinant human insulin receptors from transfected CHO cells were partially purified on wheat germ lectin-agarose as indicated above. Receptor autophosphorylation was performed with 20 µg of column eluate protein in 50 µl of reaction buffer (50 mM Hepes pH 7.6, 1 µM insulin, 5 mM MnCl₂, 0.1 mM ATP, 0.1% (v/v) Triton X-100) in the presence or absence of 10 µM DPI for 2 hrs at 4°C. The reaction was
stopped using Laemmli gel sample buffer (27). Samples were boiled at 100°C for 3 minutes and resolved by SDS-polyacrylamide gel electrophoresis. Protein was transferred to PVDF membrane and the membranes were subjected to immunoblotting with the monoclonal antibody for phosphotyrosine (4G10). Following incubation with horseradish peroxidase-conjugated secondary antibody, insulin receptor tyrosine phosphorylation was visualized by enhanced chemiluminescence.

**PI 3'-kinase activity in 3T3-L1 adipocytes** – PI 3'-kinase activity was determined as previously described (28). Briefly, 1 mg of 3T3-L1 adipocyte cell lysate was incubated overnight with 5 µg of anti-p85 antibody at 4°C. The samples were then adsorbed to protein A-trisacryl beads and washed sequentially with 1% (v/v) Nonidet P-40 in PBS followed by 100 mM Tris, pH 7.5, containing 5 mM LiCl and 100 µM sodium vanadate, and finally in 10 mM Tris, pH 7.5, containing 100 mM NaCl, 1 mM EDTA and 100 µM sodium vanadate. Each wash buffer also contained 100 mM sodium fluoride, 10 mM pyrophosphate, and 1 mM phenylmethylsulfonyl fluoride. The beads were then resuspended in 60 µl of kinase assay buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 20 mM MgCl₂), and the kinase reaction was initiated by the addition of 20 µg of phosphatidylinositol and 50 µM ATP containing 30 µCi of [γ-³²P] ATP. The samples were incubated for 10 minutes at 37°C, and the reactions were terminated by the addition of 20 µl of 8 N HCl. The samples were then extracted with 160 µl of chloroform-methanol (1:1). The resultant 50 µl lipid fractions were resolved by thin-layer chromatography in chloroform-methanol-water-ammonium hydroxide (60:47:11.3:2). The phosphorylated products were then visualized by autoradiography.

**Glucose uptake and GLUT4 protein translocation in 3T3-L1 adipocytes** – Differentiated 3T3-L1 adipocytes were starved for 2 hours in serum-free DMEM containing 25 mM glucose plus 0.2% (w/v) BSA at 37°C. The cells were washed with KRPH buffer (5 mM Na₂HPO₄, 20 mM HEPES, pH 7.4, 1 mM MgSO₄, 1 mM CaCl₂, 136 mM NaCl, 4.7 mM KCl, 0.2% (w/v) BSA) and treated with 100 nM insulin after prior treatment with 10 µM DPI for 30 minutes, 1 mM H₂O₂ for 10 minutes, or both, in KRPH buffer as indicated. Following 11 minutes of incubation with insulin, glucose uptake was assessed by the addition of 100 µM 2-deoxy-D-glucose containing 0.5 µCi of [³H] 2-deoxy-D-glucose as described previously (29). The reaction was stopped 4 minutes later by washing the cells three times with ice-cold PBS. The cells were
then solubilized in 0.05% sodium dodecyl sulfate at 37°C for 30 minutes, and aliquots were subjected to scintillation counting. Nonspecific uptake (<10% of the total) was determined in the presence of cytochalasin B (50 µM), and was subtracted from the total uptake.

Translocation of GLUT4 to the cell surface was visualized using the plasma membrane sheets assay using polyclonal anti-GLUT4 antibodies as described previously (29;30).

**Statistical Analyses:** Quantitative data are presented as the mean ± SEM for 3-5 experiments. Statistical analysis was based on Student’s t test for comparison of two groups and one-way analysis of variance for multiple group comparisons. A p value less than 0.05 was used to determine statistical significance.
RESULTS

The insulin-induced burst of intracellular oxidant is abolished by DPI – To visualize the intracellular generation of reactive oxygen species in response to insulin, 3T3-L1 adipocytes were incubated with 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCF), a sensitive oxidant indicator dye that readily diffuses into cells and is trapped after cleavage by cellular esterases. After oxidation by cellular reactive oxygen species, and H₂O₂ in particular, CM-DCF becomes highly fluorescent as detected by confocal microscopy (12;20). Following stimulation of cells with 100 nM insulin, a rapid increase in CM-DCF fluorescence was detected within 1 minute that begins to diminish at 5 minutes (Figure 1). In the presence of 10 µM DPI, an inhibitor of NADPH oxidases (31), the insulin-induced CM-DCF fluorescence is completely abolished, consistent with previous observations that the insulin effect is mediated by an NADPH oxidase system in adipocytes (32;33). In our previous work, this oxidant signal was also completely blocked by catalase, identifying H₂O₂ as one of the relevant reactive oxygen species that is generated in response to insulin (12).

Insulin-induced inhibition of PTPase activity is reversed by DPI – We recently demonstrated that one of the major consequences of the insulin-induced generation of H₂O₂ was to inhibit cellular PTPase activity by a mechanism that involved oxidation of the catalytic PTPase thiol residue. To further assess this effect, we tested how DPI, as an inhibitor of the oxidant generated by cellular insulin stimulation, affected the inhibition of cellular PTPase activity by insulin. Using our recently reported technique involving strict anaerobic conditions for cell lysate preparation as well as the PTPase assays to prevent the oxidation and enzyme inhibition that occurs on exposure to air, we measured changes in the cellular PTPase activity that accompanies insulin stimulation (34). These experiments were performed without reducing agents added during the assay to evaluate the endogenous level of enzyme activity dependent on the oxidation state of the PTPase catalytic thiol residues.

Following treatment of 3T3-L1 adipocytes with 100 nM insulin, using the pNPP assay there was a significant reduction in PTPase enzyme activity of 32 and 62% of the basal level observed in the control cell lysates after 1 or 5 minutes, respectively (Figure 2A). When the insulin-induced H₂O₂ was blocked by pre-treatment of the cells with 10 µM DPI for 30 minutes prior to stimulation with insulin, the inhibition of PTPase activity in the 3T3-L1 adipocytes was
dramatically inhibited and reduced by only 11 and 24% of the basal level at 1 and 5 minutes, respectively (Figure 2A). Similar results were obtained with the PTPase assay involving hydrolysis of $^{32}$P-RCM lysozyme (Figure 2B), where the inhibition of cellular PTPase activity induced by insulin treatment for 5 minutes was diminished from 43% of control to 28% of control after cellular pretreatment with 10 μM DPI (n=4; p=0.04). This important finding further confirmed that cellular H$_2$O$_2$ production mediated the oxidative inhibition of cellular PTPase activity associated with insulin stimulation. DPI alone had no significant effect on the basal level of cellular PTPase activity.

To further evaluate the effect of oxidant exposure on the cellular PTPase activity, the cells were then treated with 1 mM H$_2$O$_2$ for 10 minutes followed by stimulation with or without insulin for 1 or 5 minutes prior to cell lysis. As expected, these results showed a sharp reduction in PTPase activity to 28% of the basal level in the control cells (Figure 2A and 2B). The inhibition of cellular PTPase activity by H$_2$O$_2$ was independent of the addition of DPI, since cell incubation with DPI prior to H$_2$O$_2$ exposure and stimulation with insulin for 1 or 5 minutes did not alter the observed reduction of PTPase activity in basal and insulin-stimulated samples. These results supported the finding that the reduction of PTPase activity by insulin treatment in the 3T3-L1 adipocytes is mediated by H$_2$O$_2$, and that by preventing the insulin-induced generation of oxidant, cellular exposure to DPI leads to enhanced PTPase activity.

Tyrosine phosphorylation of the insulin receptor and IRS proteins is reduced during insulin signaling in the presence of DPI – The effect of the insulin-induced oxidant on the tyrosine phosphorylation of the insulin receptor and IRS-1/2 was then examined by measuring the insulin-stimulated autophosphorylation of its receptor and its high Mr substrate proteins in the 3T3-L1 adipocytes in the presence or absence of DPI using western blot analysis with anti-phosphotyrosine (Figure 3). Treatment of the cells with DPI had no effect on the low basal tyrosine phosphorylation of the insulin receptor or high Mr IRS proteins. However, in the presence of DPI, the tyrosine phosphorylation of IRS proteins was reduced to 61 and 40% of control at 1 and 5 minutes of insulin stimulation, respectively. Similarly, the tyrosine phosphorylation of the insulin receptor-β subunit in the cells pretreated with DPI was reduced to 70 and 47% of control at 1 and 5 minutes of insulin stimulation, respectively.
DPI was also tested for possible direct effects on insulin receptor autophosphorylation and PTPase activity in cell lysates in vitro. Recombinant human insulin receptors purified from Chinese Hamster Ovary cells overexpressing insulin receptors were exposed to 10 µM DPI and insulin-stimulated tyrosine autophosphorylation of the receptor was evaluated in vitro by western blot analysis with anti-phosphotyrosine antibodies (23). In addition, we assayed PTPase activity in the presence or absence of 10 µM DPI added to 3T3-L1 cell lysates in vitro. DPI had no direct effect in the cell lysates on the rate or extent of insulin receptor autophosphorylation or on PTPase activity (data not shown). These findings provided further support for our conclusion that the mechanism of DPI action on protein tyrosine phosphorylation and cellular PTPase activities involves its inhibition of NADPH oxidase in the intact cells which abrogates the insulin-induced production of oxidant species.

Inhibition of insulin-induced generation of H$_2$O$_2$ by DPI down-regulates PI 3'-kinase activity – In order to gain insight into how the oxidant signal from insulin stimulation might play a role in the downstream activation of kinases in the insulin action pathway, we tested whether inhibition of insulin-induced generation of H$_2$O$_2$ by DPI affected PI 3’-kinase activation induced by insulin (Figure 4). In control cells, without DPI exposure, 5 minutes of stimulation with 100 nM insulin increased the PI 3'-kinase activity in the 3T3-L1 adipocytes by 1.8 fold over basal (p=0.036; n=3). Cellular treatment with 10 µM DPI for 30 minutes did not affect basal PI 3’-kinase activity; however, DPI exposure completely blocked the increase in PI 3'-kinase activity observed with insulin stimulation at 5 minutes (p=0.006; n=3). These results suggested that the oxidant signal resulting from cellular insulin stimulation played an important role in the transmission of the insulin signal to PI 3’-kinase.

The role of H$_2$O$_2$ in PI 3’-kinase activation by insulin was further tested by adding back H$_2$O$_2$ to the cells that had been pretreated with DPI (Figure 4). Treatment of cells with 1 mM H$_2$O$_2$ had a strong activating effect on the basal level of PI 3’-kinase activity to 3.6-fold over the level in control cells. There was also a trend for insulin treatment of the cells exposed to H$_2$O$_2$ to further stimulate PI 3’-kinase activity, but this was not statistically significant. Pretreatment of the cells with DPI also was inhibitory to the H$_2$O$_2$-induced increase in PI 3’-kinase activity prior to insulin and also blocked the apparent insulin-stimulated increase in PI 3’-kinase activity in the H$_2$O$_2$-treated cells.
In additional control experiments, we demonstrated that DPI or H$_2$O$_2$ had no direct effects on cellular PI 3'-kinase activity. Activated PI 3'-kinase was immunoprecipitated with anti-p85 antibody from lysates of cells treated with insulin or H$_2$O$_2$. Following exposure to 10 µM DPI \textit{in vitro} for 30 minutes, there was no change in production of PI3P, assayed as described in the Experimental Procedures (data not shown). In addition, treatment of immunoprecipitated PI 3'-kinase from resting cells with 1 mM H$_2$O$_2$ did not affect enzyme activity. Overall, these results indicate that H$_2$O$_2$ plays an important role in the activation of the PI 3'-kinase pathway by insulin in 3T3-L1 adipocytes, and can act when added exogenously, as well as when generated with the target cells by insulin stimulation. Furthermore, the effects of DPI and H$_2$O$_2$ on PI 3'-kinase activity are indirect, mediated through the cellular signaling pathways present in the intact cells.

\textit{Abolition of H$_2$O$_2$ generation by DPI inhibits insulin-stimulated Akt activation in 3T3-L1 adipocytes} – Downstream of PI 3'-kinase, we next evaluated whether blockade of the insulin-stimulated oxidant signal with DPI affected the activation of Akt by insulin (Figure 5). 3T3-L1 cells were incubated with or without 10 µM DPI for 30 minutes prior to treatment with 100 nM insulin for 1 or 5 minutes. DPI treatment reduced the level of Akt phosphorylation by 38 and 49% at 1 and 5 minutes of insulin stimulation, respectively.

To confirm the involvement of H$_2$O$_2$ in the cellular activation of Akt, we then treated the cells with 1 mM H$_2$O$_2$ for 10 minutes and the phosphorylation of Akt protein was measured as above using immunoblotting with phospho-Akt antibodies. In the presence of H$_2$O$_2$, there was a dramatic increase in the basal level of Akt phosphorylation, which was not significantly increased further by insulin stimulation. Exposure of adipocytes to DPI prior to treatment with H$_2$O$_2$ also did not alter the level of Akt phosphorylation, indicating that DPI acted at a step prior to Akt activation by H$_2$O$_2$ and had no direct effect of its own on the intracellular phosphorylation of Akt (Figure 5).

\textit{H$_2$O$_2$-induced phosphorylation of Akt is mediated by activation of PI 3'-kinase} – Since activation of Akt in the insulin signaling pathway is known to be linked to the upstream activation of PI 3'-kinase, we then tested whether inhibition of Akt activation by DPI treatment was mediated by the effect of DPI to reduce the activation of PI 3'-kinase under these conditions. As shown above in Figure 5, H$_2$O$_2$ is a potent activator of Akt in an insulin-independent manner.
Interestingly, treatment of 3T3-L1 adipocytes with the PI 3’-kinase inhibitors wortmannin (100 nM) or LY294002 (50 µM) for 10 minutes completely blocked the subsequent activation of Akt phosphorylation in response to cell stimulation with exogenous H₂O₂ (Figure 6). These important results indicated that an oxidant signal in the cell is tightly coupled to the activation of PI 3’-kinase, which in turn, triggers Akt phosphorylation and activation.

**Inhibition of H₂O₂ production suppresses insulin-stimulated glucose uptake and GLUT4 plasma membrane translocation.** Since the oxidant signal from insulin action was involved in the activation of PI 3’-kinase and subsequently of Akt, we also evaluated how inhibition of H₂O₂ generation with DPI affected a key biological activity in the differentiated 3T3-L1 adipocyte model, glucose transport. In control cells, without DPI treatment, insulin stimulated a mean of 3.7-fold increase in glucose uptake (Figure 7). In the presence of DPI, basal glucose transport was not significantly affected (88% of control); however, insulin stimulation of glucose transport was completely nullified by prior incubation of the 3T3-L1 adipocytes with DPI, with only a 23% increase above basal levels. For comparison, cells were treated with H₂O₂, which caused a stimulation of glucose uptake to the same extent as insulin alone, to 3.5-fold over basal levels. Cell treatment with DPI prior to H₂O₂ partially inhibited both the basal stimulation of glucose uptake by H₂O₂ as well as the uptake stimulated by the combination of insulin and H₂O₂ (by 54% and 50%, respectively). Although differing somewhat in magnitude, the general pattern of response to DPI and H₂O₂ in glucose transport is similar to the profile observed in the PI 3’-kinase assay (Figure 4).

The glucose uptake data was also validated by the plasma membrane sheets assay to assess GLUT4 translocation (Figure 8). The visualization of GLUT4 appearance at the cell surface was highly stimulated by 15 minutes of treatment with 100 nM insulin. Similar to the results obtained for cellular glucose uptake, pre-treatment of the cells with DPI completely blocked the insulin-stimulated translocation of GLUT4. H₂O₂ treatment increased basal GLUT4 translocation, with a further increase by stimulation with insulin. Cellular pre-treatment with DPI effectively inhibited both the basal and insulin-stimulated increase in GLUT4 translocation elicited by H₂O₂. The translocation of GLUT4 thus closely follows the measured glucose uptake data presented in Figure 7, the only exception being the increase in basal glucose uptake elicited
by H₂O₂ in the absence of insulin which does not appear to be accompanied by translocation of GLUT4 to a similar degree.

**Role of cellular PI 3’-kinase activity in insulin-stimulated H₂O₂ production**- Previous studies by Bae et al. (16) demonstrated that PI 3’-kinase activity was integral to the production of cellular H₂O₂ by PDGF stimulation. To evaluate the potential role of PI 3’-kinase in cellular H₂O₂ production induced by insulin, we measured fluorescence by confocal microscopy in 3T3-L1 adipocytes that were loaded with CM-DCF and stimulated with insulin for 5 minutes in the presence and absence of preincubation with either 100 nM wortmannin or 50 μM LY294002 (Figure 9). Even though under these conditions these two PI 3’-kinase inhibitors were effective in blocking the activation of Akt (Figure 6), they did not prevent the cellular generation of H₂O₂ in response to insulin, indicating that insulin-stimulated H₂O₂ production is independent of PI 3’-kinase activation.
DISCUSSION

H$_2$O$_2$ and other oxidizing molecules have been recognized for many years to have a strong impact on insulin signal transduction, although the regulatory mechanisms and sites of interaction with specific components of the insulin action cascade have not been fully evaluated (35-37). In published studies, exposure of cells to exogenous H$_2$O$_2$ has alternatively been shown to either enhance insulin action or reduce cellular insulin responsiveness, depending to some extent on the concentration of oxidant, the duration of treatment, and the particular cellular models employed (38;39). For example, prolonged exposure of 3T3-L1 adipocytes for several hours to micromolar concentrations of H$_2$O$_2$ disrupts insulin-induced subcellular redistribution of IRS-1 and PI 3'-kinase between the cytosol and the low density microsomal fraction and impairs the insulin-stimulated translocation of GLUT4 (40;41). As discussed in the introduction, PTPase enzymes have been shown to be one important target of intracellular H$_2$O$_2$ and/or superoxide action in insulin-sensitive cells, since they can be transiently or permanently inhibited by stepwise oxidation of their catalytic thiol residue (10;11).

In addition to the effects of oxidants when added from outside of the cell, attention has recently been paid to the important role in cellular signaling played by endogenous H$_2$O$_2$ and other reactive oxygen species that are rapidly generated directly within target cells following treatment with various growth factors and hormones (13;16). Elaboration of H$_2$O$_2$ in response to insulin signal transduction has been known to occur in adipose tissue for many years, although the significance of reactive oxygen species for the insulin action cascade has not been identified (32;42;43). We recently showed that treatment of 3T3-L1 adipocytes and HepG2 hepatoma cells with insulin rapidly generates a burst of H$_2$O$_2$ , which has a major impact on the early transmission of the insulin receptor signal by modulation of the steady-state tyrosine phosphorylation of the insulin receptor and its cellular substrate proteins (12). In these studies, we showed that the effect of insulin-stimulated cellular H$_2$O$_2$ was mediated, at least in part, by inhibition of the catalytic activity of cellular PTPases. We demonstrated that this effect also specifically involved the single-domain intracellular PTPase, PTP1B, which has been strongly implicated in the negative regulation of the insulin action pathway (44).

In the present work, we capitalized on the observation that DPI, a flavoprotein NADPH oxidase inhibitor, blocked the production of intracellular H$_2$O$_2$ arising from stimulation with
insulin. This inhibitory agent was then used to determine which of a variety of steps in the post-receptor insulin signaling cascade might be affected by the loss of insulin-generated H$_2$O$_2$ in the 3T3-L1 adipocyte model. Initially, we confirmed the findings of our previous study to show that eradicating the oxidant signal from insulin diminished the insulin-induced inactivation of cellular PTPase activity, and that this effect was associated with enhanced tyrosine phosphorylation of the insulin receptor and its high molecular mass substrates, IRS-1 and 2 (Figures 2 and 3) (12). Thus, H$_2$O$_2$ coupled to the insulin-receptor interaction appears to be essential for the initiation of the early insulin signal, by reducing the rapid and potent dephosphorylating activity of cellular PTPases proximate to the receptor (45).

The novel finding reported in the present study is that the oxidant signal from insulin functions not only to enhance early insulin action, but also serves an essential role in the downstream insulin action cascade. In particular, the endogenous oxidant enhances the activation of PI 3’-kinase by insulin, since this process is inhibited by DPI. Furthermore, we have demonstrated that the oxidant effect on PI 3’-kinase activation is integral to the downstream activation of the Akt kinase, since the PI 3’-kinase inhibitors wortmannin and LY-294002 completely blocked the activation of Akt mediated by exogenous H$_2$O$_2$. Signaling subsequent to Akt activation was also blocked, notably involving the stimulation of glucose uptake and GLUT4 transporter translocation by insulin, indicating that there are multiple potential cellular signaling effects that arise from the insulin-stimulated generation of H$_2$O$_2$. Interestingly, the activation of Akt by H$_2$O$_2$ in fibroblasts, embryonic kidney cells and vascular smooth muscle cells was also abrogated by inhibition of PI 3’-kinase, suggesting that cellular PI 3’-kinase is a critical upstream mediator of Akt activation by oxidative stress in a variety of cell types and involving various signaling pathways, including insulin action (46;47). An additional consideration is that the serine protein phosphatase PP2A, which has been implicated in the negative regulation of Akt by dephosphorylation of Ser473, has a redox-sensitive cysteine residue that is potentially susceptible to inhibition by H$_2$O$_2$ (48). One of the possible effects of DPI in this regard could thus be to accentuate the activity of a phosphatase acting directly on Akt which would facilitate the inactivation of Akt. Thus, multiple influences, affecting upstream as well as more distal mechanisms may be influenced by the oxidant signal stemming from cellular insulin stimulation.

In the present study, we also found that unlike H$_2$O$_2$ generated by cell stimulation with PDGF (16), insulin-stimulated production of H$_2$O$_2$ is not mediated by PI 3’-kinase, since the
CM-DCF fluorescence induced by insulin binding was not blocked by the PI 3'-kinase inhibitors wortmannin or LY294002 (Figure 8). Thus, the generation of H$_2$O$_2$ in response to insulin in adipocytes may be different from that found in other cell types or with other growth factors. This is consistent with work by Krieger-Brauer and colleagues showing that the elaboration of cellular H$_2$O$_2$ during the process of physiological insulin signal transduction is generated by a novel plasma membrane-bound Mn$^{2+}$-dependent NADPH oxidase that is coupled to G$\alpha_{i2}$ (32;33). Other recent work has provided further evidence in several in vivo models that G$\alpha_{i2}$ is closely linked to insulin action. For example, G$\alpha_{i2}$ deficiency in a transgenic mouse model produces insulin resistance and impaired glucose tolerance (49); conversely, conditional expression of a constitutively active mutant of G$\alpha_{i2}$ in insulin-sensitive tissues mimics insulin action in vivo with enhanced glucose tolerance and activation of adipocyte GLUT 4 recruitment, hexose transport and glycogen synthase(50). These data are consistent with the hypothesis that G$\alpha_{i2}$ plays a permissive role for insulin signaling, possibly involving a reactive oxygen species-coupled mechanism, although this has not yet been directly evaluated.

Our data also provides a contrast between the oxidant signal arising from insulin stimulation, inhibited in this work by DPI, and cellular treatment with exogenous H$_2$O$_2$, which has been used to potentiate or mimic insulin action in previously published studies noted above. We show that insulin-stimulated activation of PI 3'-kinase and glucose transport (Figures 4 and 7) are completely blocked by prior treatment with DPI, demonstrating an important regulatory role for the endogenous oxidant species in the transmission of the insulin signal. In contrast, in the same experiments, the stimulation of PI 3'-kinase activity or glucose transport by H$_2$O$_2$ in the presence or absence of insulin, is only partially inhibited by DPI. This may be due to several possible mechanisms, including the balance of individual reactive oxygen species, e.g., the proportion of cellular superoxide (reduced by DPI inhibition of NADPH oxidase) and H$_2$O$_2$ provided exogenously, which may differentially regulate various steps in the distal signaling cascade (11). In addition, cellular reactive oxygen species, especially when derived from receptor activation, appear to oxidize a restricted subset of susceptible proteins in the cell, perhaps by colocalization in cellular microdomains (14;51). Although technically challenging, sensitive methods to decipher the subcellular localization and the identity of the various reactive oxygen species in signal transduction are needed to help clarify these issues.
In summary, these data provide new insight into the physiological role of the oxidant signal that arises from cellular insulin stimulation, which affects the initial tyrosine phosphorylation of proteins in the early insulin signaling cascade by oxidative inhibition of PTPases. In addition, the insulin-induced generation of H$_2$O$_2$ is shown here to have important effects on the regulation of downstream insulin signaling, affecting the activation of Akt by a PI 3'-kinase mediated pathway, as well as subsequent steps leading to the activation of glucose uptake and GLUT4 transporter translocation in response to insulin. Defective cellular action of insulin in its target tissues is a fundamental pathophysiologic features of type 2 diabetes mellitus, a prevalent disorder with devastating health and economic consequences in developed societies (52). Thus, a full characterization of the regulation of insulin signal transduction is essential in order to decipher the underlying causes of this disease. Further studies on the formation and cellular role of reactive oxygen species at various steps in the insulin signaling cascade may also provide new targets for therapeutic intervention in diabetes and other insulin-resistant states.
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REFERENCES

1. White, M. F. (1998) *Recent Prog. Horm. Res.* **53**, 119-138

2. Patti, M. E. and Kahn, C. R. (1998) *J Basic Clin Physiol Pharmacol* **9**, 89-109

3. White, M. F. (1998) *Mol. Cell. Biochem.* **182**, 3-11

4. Sanchez-Margalet, V., Goldfine, I. D., Vlahos, C. J., and Sung, C. K. (1994) *Biochem. Biophys. Res. Commun.* **204**, 446-452

5. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., and Ui, M. (1994) *J.Biol.Chem.* **269**, 3568-3573

6. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) *Mol.Cell.Biol.* **14**, 4902-4911

7. Brady, M. J. and Saltiel, A. R. (1999) *J.Clin.Invest.* **104**, 675-676

8. Goldstein, B. J. (2000) In: LeRoith, D., Olefsky, J. M., and Taylor, S. I., editors. *Diabetes Mellitus: A Fundamental and Clinical Text*, Lippincott, Philadelphia, pp.206-217.

9. Zhang, Z. Y. (1998) *Crit.Revs.Biochem.Mol.Biol.* **33**, 1-52

10. Claiborne, A., Yeh, J. I., Mallett, T. C., Luba, J., Crane, E. J., Charrier, V., and Parsonage, D. (1999) *Biochemistry* **38**, 15407-15416

11. Barrett, W. C., DeGnore, J. P., Keng, Y. F., Zhang, Z. Y., Yim, M. B., and Chock, P. B. (1999) *J.Biol.Chem.* **274**, 34543-34546

12. Mahadev, K., Zilbering, A., Zhu, L., and Goldstein, B. J. (2001) *J.Biol.Chem.* **276**, 21938-21942

13. Herrlich, P. and Bohmer, F. D. (2000) *Biochem.Pharmacol.* **59**, 35-41

14. Finkel, T. (2000) *FEBS Lett.* **476**, 52-54

15. Griendling, K. K. and Ushio-Fukai, M. (2000) *Regulatory Peptides* **91**, 21-27

16. Bae, Y. S., Sung, J. Y., Kim, O. S., Kim, Y. J., Hur, K. C., Kazlauskas, A., and Rhee, S. G. (2000) *J.Biol.Chem.* **275**, 10527-10531

17. Schmid, E., Hotz-Wagenblatt, A., Hack, V., and Droge, W. (1999) *FASEB Journal.* **13**, 1491-1500

18. Li, Y. and Trush, M. A. (1998) *Biochem.Biophys.Res.Commun.* **253**, 295-299

19. Gagnon, A. and Sorisky, A. (1998) *Obes Res* **6**, 157-163
20. Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., and Rhee, S. G. (1997) *J Biol Chem* **272**, 217-221

21. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254

22. Goldstein, B. J., Bittner-Kowalczyk, A., White, M. F., and Harbeck, M. (2000) *J. Biol. Chem.* **275**, 4283-4289

23. Hashimoto, N., Feener, E. P., Zhang, W. R., and Goldstein, B. J. (1992) *J. Biol. Chem.* **267**, 13811-13814

24. Pike, L. J., Kuenzel, E. A., Casnellie, J. E., and Krebs, E. G. (1984) *J. Biol. Chem.* **259**, 9913-9921

25. Tonks, N. K., Diltz, C. D., and Fischer, E. H. (1991) *Methods Enzymol.* **201**, 442-451

26. Streuli, M., Krueger, N. X., Thai, T., Tang, M., and Saito, H. (1990) *EMBO J.* **9**, 2399-2407

27. Laemmli, U. K. (1970) *Nature* **227**, 680-685

28. Serunian, L. A., Auger, K. R., and Cantley, L. C. (1991) *Methods Enzymol.* **198**, 78-87

29. Fingar, D. C., Hausdorff, S. F., Blenis, J., and Birnbaum, M. J. (1993) *J. Biol. Chem.* **268**, 3005-3008

30. Hausdorff, S. F., Frangioni, J. V., and Birnbaum, M. J. (1994) *J. Biol. Chem.* **269**, 21391-21394

31. Stuehr, D. J., Fasehun, O. A., Kwon, N. S., Gross, S. S., Gonzalez, J. A., Levi, R., and Nathan, C. F. (1991) *FASEB J.* **5**, 98-103

32. Krieger-Brauer, H. I., Medda, P. K., and Kather, H. (1997) *J Biol Chem* **272**, 10135-10143

33. Krieger-Brauer, H. I. and Kather, H. (1992) *J Clin Invest* **89**, 1006-1013

34. Zhu, L., Zilbering, A., Wu, X., Mahadev, K., Joseph, J. I., Jabbour, S., Deeb, W., and Goldstein, B. J. (2001) *FASEB J* **15**, 1637-1639

35. Lavis, V. R. and Williams, R. H. (1970) *J Biol Chem* **245**, 23-31

36. Czech, M. P. and Fain, J. N. (1972) *J Biol Chem* **247**, 6218-6223

37. Czech, M. P., Lawrence, J. C. J., and Lynn, W. S. (1974) *J Biol Chem* **249**, 1001-1006

38. Blair, A. S., Hajduch, E., Litherland, G. J., and Hundal, H. S. (1999) *J Biol Chem* **274**, 36293-36299
39. Hansen, L. L., Ikeda, Y., Olsen, G. S., Busch, A. K., and Mosthaf, L. (1999) J Biol Chem 274, 25078-25084

40. Rudich, A., Tirosh, A., Potashnik, R., Khamaisi, M., and Bashan, N. (1999) Diabetologia 42, 949-957

41. Tirosh, A., Potashnik, R., Bashan, N., and Rudich, A. (1999) J Biol Chem 274, 10595-10602

42. May, J. M. and de Haen, C. (1979) J Biol Chem 254, 9017-9021

43. May, J. M. and de Haen, C. (1979) J Biol Chem 254, 2214-2220

44. Kennedy, B. P. and Ramachandran, C. (2000) Biochem. Pharmacol. 60, 877-883

45. Mooney, R. A. and Anderson, D. L. (1989) J Biol Chem 264, 6850-6857

46. Shaw, M., Cohen, P., and Alessi, D. R. (1998) Biochem J 336, 1-246

47. Ushio-Fukai, M., Alexander, R. W., Akers, M., Yin, Q. Q., Fujio, Y., Walsh, K., and Griendling, K. K. (1999) J Biol Chem 274, 22699-22704

48. Guy, G. R., Cairns, J., Ng, S. B., and Tan, Y. H. (1993) J Biol Chem 268, 2141-2148

49. Moxham, C. M. and Malbon, C. C. (1996) Nature 379, 840-844

50. Chen, J. F., Guo, J. H., Moxham, C. M., Wang, H. Y., and Malbon, C. C. (1997) Journal of Molecular Medicine 75, 283-289

51. Kim, J. R., Yoon, H. W., Kwon, K. S., Lee, S. R., and Rhee, S. G. (2000) Anal. Biochem. 283, 214-221

52. Pessin, J. E. and Saltiel, A. R. (2000) J Clin Invest 106, 165-169

53. Ahmad, F., Li, P. M., Meyerovitch, J., and Goldstein, B. J. (1995) J Biol Chem 270, 20503-20508
FIGURE LEGENDS

Figure 1. Effect of DPI on insulin-stimulated production of H$_2$O$_2$ in 3T3-L1 adipocytes. Confluent, differentiated 3T3-L1 adipocytes were starved overnight in serum-free medium prior to stimulation with 100 nM insulin for the indicated amount of time. Intracellular H$_2$O$_2$ production was detected by fluorescence of 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-DCF), which generates a fluorescent signal in situ visualized by confocal microscopy using fluorescein parameters for excitation and emission as we have described previously (12). Where indicated, cells were pre-incubated for 30 minutes with 10 µM DPI prior to insulin stimulation.

Figure 2. Effect of DPI on insulin-induced reduction in endogenous PTPase activity in 3T3-L1 adipocytes. 3T3-L1 adipocytes were starved for serum overnight and then pre-treated with 10 µM DPI for 30 minutes and/or 1 mM H$_2$O$_2$ for 10 minutes where indicated, followed by stimulation with 100 nM for 1 or 5 minutes. The cells were then snap-frozen with liquid nitrogen, introduced into the anaerobic work station in a frozen state and disrupted by scraping into ice-cold deoxygenated homogenization buffer followed by brief sonication under the anaerobic atmosphere. PTPase activity was measured within the anaerobic chamber in aliquots containing 20 µg of lysate protein using pNPP (Panel A) or $^{32}$P-RCM lysozyme (Panel B) as substrate as described in Experimental Procedures. *p=0.04, **p=0.008, ***p<0.001 vs. control with no DPI treatment.

Figure 3. Effect of DPI on insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit and IRS proteins in 3T3-L1 adipocytes. Upper panel, representative anti-phosphotyrosine (4G10) and anti-insulin receptor β-subunit (IR-β) and anti-IRS-1 immunoblots of 3T3-L1 cell lysates following stimulation of serum-starved cells with 100 nM insulin for the indicated period of time with and without pretreatment with 10 µM DPI for 30 minutes. Cells were lysed into buffer containing protease and PTPase inhibitors as described (53) and 75 µg samples of the cleared cell lysates were subjected to electrophoresis in SDS gels containing 7.5% (w/v) polyacrylamide. Following transfer to PVDF membranes and immunoblotting with the indicated antibodies, proteins were visualized with horseradish peroxidase-coupled secondary antibodies. The migration positions of the tyrosine-phosphorylated high M$_r$ IRS proteins (~185
kDa) and the insulin receptor β-subunit (95 kDa) are indicated as pY IRS-1/2 and pY IR-β, respectively. In the protein immunoblots, the migration positions of IRS-1 and insulin receptor β-subunit protein bands are indicated. Lower panels, mean data for the phosphotyrosine density of the insulin receptor β-subunit and IRS-1/2 from replicate immunoblots performed as shown in the upper panel after quantitation using an ImageStation 440 (Kodak).

Figure 4. Effect of DPI on insulin-stimulated PI 3’-kinase activity in 3T3-L1 adipocytes. Following overnight serum starvation, 3T3-L1 adipocytes were pre-treated with 10 µM DPI for 30 minutes or 1 mM H2O2 for 10 minutes prior to stimulation for 5 minutes with 100 nM insulin. Cell lysates were prepared and incubated overnight with anti-p85 antibody at 4°C and PI 3’-kinase activity was measured as described in Experimental Procedures. The phosphorylated products were visualized by autoradiography. Upper panel, representative autoradiogram of a thin layer chromatogram demonstrating the PI 3’-kinase reaction products. Lower panel, mean data from replicate autoradiograms as shown in panel A quantitated using an ImageStation 440 (Kodak). *p=0.036 vs. control cells without insulin stimulation; **p=0.006 vs. control cells stimulated with insulin for 5 minutes but not treated with DPI.

Figure 5. Effect of DPI on insulin-stimulated Akt activity in 3T3-L1 adipocytes. Following overnight serum starvation, 3T3-L1 adipocytes were pre-treated with 10 µM DPI for 30 minutes or 1 mM H2O2 for 10 minutes where indicated prior to stimulation with 100 nM insulin for 1 or 5 minutes. Cell lysates were prepared and immunoblotting was performed with a polyclonal antibody to detect phospho-Akt (Ser473) or non-isoform-specific anti-Akt protein antibody. Following incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were visualized by enhanced chemiluminescence. Upper panel, representative immunoblots demonstrating the phosphorylation state of Akt Ser-473 and the Akt protein level. Lower panel, mean data from replicate immunoblots as shown in the upper panel quantitated using an ImageStation 440 (Kodak), demonstrating the effect DPI pre-incubation on insulin-stimulated Akt activity. *p=0.016, **p<0.001 vs. control cells not treated with DPI, but stimulated with insulin for 5 min or 1 min as shown.

Figure 6. Effect of PI 3’-kinase inhibitors on the activation of Akt by H2O2. Following overnight serum starvation, lysates were prepared from control 3T3-L1 adipocytes (lane a), or following treatment with insulin for 5 minutes (lane b), or from duplicate cultures of cells treated
with 1 mM H$_2$O$_2$ for 10 minutes (lanes c and d) that were also pre-incubated with the PI 3’-kinase inhibitors wortmannin (100 nM; lanes e and f) or LY294002 (50 µM; lanes g and h) for 10 minutes as shown. Following electrophoresis in SDS-polyacrylamide gels and transfer to PVDF membranes, immunoblotting was performed with a polyclonal antibody to detect phospho-Akt (Ser-473) or non-isoform-specific anti-Akt protein antibody as described in the legend to Figure 5.

**Figure 7. Effect of DPI on glucose uptake in 3T3-L1 adipocytes** – Differentiated 3T3-L1 adipocytes were starved for 2 hours in serum-free medium and then treated with 10 µM DPI for 30 minutes, 1 mM H$_2$O$_2$ for 10 minutes, or both, as indicated, prior to stimulation with 100 nM insulin. Glucose transport was assessed by the uptake of 2-deoxy-D-glucose as described in Experimental Procedures. **p<0.001 vs. control cells stimulated with insulin but not treated with DPI.

**Figure 8. Effect of DPI on translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes.** Differentiated 3T3-L1 adipocytes were starved for 2 hours in serum-free medium and then treated with 10 µM DPI for 30 minutes, 1 mM H$_2$O$_2$ for 10 minutes, or both, where indicated, prior to stimulation with 100 nM insulin for 15 minutes. The translocation of GLUT4 to the cell surface was then visualized using the plasma membrane sheets assay described in Experimental Procedures.

**Figure 9. Lack of effect of inhibitors of PI 3’-kinase on insulin-stimulated H$_2$O$_2$ production 3T3-L1 adipocytes.** Differentiated 3T3-L1 adipocytes were serum-starved overnight prior to treatment with the PI 3’-kinase inhibitors wortmannin (100 nM) or LY294002 (50 µM) for 10 minutes where indicated. The cells were then stimulated with 100 nM insulin for 5 minutes and intracellular H$_2$O$_2$ production was detected by fluorescence of CM-DCF as described in Experimental Procedures. The fluorescent confocal images were then quantitated using Scion Image software and the tabulated data is presented in the bar graph.
Figure 1
Figure 2

A. pNPP Assay

B. [\(^{32}\)P]-RCM-Lysozyme Assay

PTPase Activity (A\(_{\text{v,10}}\))

- Insulin: 0, 1, 5 (min)
- DPI: - - - + + + - - - + + +
- H\(_2\)O\(_2\): - - - - - + + + - - - + + +
Figure 3

| DPI  | - | - | - | + | + | + |
|------|---|---|---|---|---|---|
| Insulin | 0 | 1 | 5 | 0 | 1 | 5 (min) |

**Antibody**
- 4G10
- 4G10
- anti-IRS-1
- anti-IR-β

**IR-β subunit**
- DPI
- + DPI

**IRS-1/2**
- DPI
- + DPI

Graphs showing the effect of DPI on pY content of IR-β and IRS-1/2 subunits at 1 min and 5 min after insulin stimulation.
Figure 4

| H2O2  | - | - | - | - | + | + | + | + |
| DPI   | - | - | + | + | - | - | + | + |
| Insulin | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | (min) |

![PI3P Image]

![Phosphorimager density graph]

- 0 min Insulin
- 5 min Insulin

Control | DPI | H2O2 | DPI + H2O2

* | **
Figure 5

H$_2$O$_2$: - - - - - - + + + + + +

DPI: - - - + + + - - - + + + +

Insulin: 0 1 5 0 1 5 0 1 5 0 1 5 (min)

Phospho-Akt

Akt

Akt Phosphorylation (% of control)

| Time of Insulin Stimulation | - DPI | + DPI |
|----------------------------|-------|-------|
| 1 min                      | **    |       |
| 5 min                      |       | *     |

[Graph showing Akt phosphorylation at 1 min and 5 min with and without DPI treatment]
### Figure 6

| Treatment    | a | b | c | d | e | f | g | h |
|--------------|---|---|---|---|---|---|---|---|
| LY294002     | - | - | - | - | - | + | + | + |
| Wortmannin   | - | - | - | - | + | + | - | - |
| H2O2         | - | - | + | + | + | + | + | + |
| Insulin      | - | + | - | - | - | - | - | - |

**Phospho-Akt**

**Akt**
Figure 7

![Graph showing glucose transport (nmol/min/mg protein) for different treatments: Control, DPI, H2O2, and DPI + H2O2. The graph compares basal and insulin-treated conditions.](image)
Figure 8

- Insulin  + Insulin

Control

DPI

H2O2

DPI+H2O2
Figure 9

Fluorescence (% of basal)

Insulin:  -  +  +  +  +
Wortmannin: -  -  +  -  -
LY294002:  -  -  -  +  +
Hydrogen peroxide generated during cellular insulin stimulation is integral to activation of the distal insulin signaling cascade in 3T3-L1 adipocytes

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