Insulin-stimulated Hydrogen Peroxide Reversibly Inhibits Protein-tyrosine Phosphatase 1B in Vivo and Enhances the Early Insulin Action Cascade*

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The insulin signaling pathway is activated by tyrosine phosphorylation of the insulin receptor and key post-receptor substrate proteins and balanced by the action of specific protein-tyrosine phosphatases (PTPases). PTPase activity, in turn, is highly regulated in vivo by oxidation/reduction reactions involving the cysteine thiol moiety required for catalysis. Here we show that insulin stimulation generates a burst of intracellular H$_2$O$_2$ in insulin-sensitive hepatoma and adipose cells that is associated with reversible oxidative inhibition of up to 62% of overall cellular PTPase activity, as measured by a novel method using strictly anaerobic conditions. The specific activity of immunoprecipitated PTP1B, a PTPase homolog implicated in the regulation of the insulin signaling pathway, was also strongly inhibited by up to 88% following insulin stimulation. Catalase pretreatment abolished the insulin-stimulated production of H$_2$O$_2$ as well as the inhibition of cellular PTPases, including PTP1B, and was associated with reduced insulin-stimulated tyrosine phosphorylation of its receptor and high $M_r$ insulin receptor substrate (IRS) proteins. These data provide compelling new evidence for a redox signal that enhances the early insulin-stimulated cascade of tyrosine phosphorylation by oxidative inactivation of PTP1B and possibly other tyrosine phosphatases.

Protein-tyrosine phosphatases (PTPases) play a key role in the regulation of reversible tyrosine phosphorylation in the insulin action pathway. Insulin signaling is initiated by the phosphorylation of specific tyrosyl residues of the cell surface insulin receptor, which activates its exogenous kinase activity and promotes the phosphorylation of IRS proteins on specific tyrosine residues (1). These activation steps are balanced, in turn, by specific cellular PTPases that dephosphorylate and inactivate the receptor kinase and reverse the adapter function of the receptor substrate proteins (2). The cellular role of PTPases is apparent from the observation that highly purified insulin receptors and IRS proteins retain their tyrosine phosphorylation and activation state in vitro (3, 4), while in intact or permeabilized cells, receptor activation and substrate tyrosine phosphorylation are rapidly reversed (5–7).

Since PTPases are high turnover number enzymes, physiological suppression of PTPase catalytic activity has been postulated to be a key feature of their regulation within the cellular environment to allow tyrosine phosphorylation to proceed in a balanced manner (8). PTPases have in common a conserved ~230-amino acid domain that contains the cysteine residue that catalyzes the hydrolysis of protein phosphotyrosine residues by the formation of a cysteiny1-phosphate intermediate (9, 10). Several laboratories have recently provided evidence that reactive oxygen species, including H$_2$O$_2$, can oxidize and inactivate PTPases in vivo (11, 12). Since only the reduced form of the catalytic site is enzymatically active, stepwise and progressively irreversible oxidative inhibition is emerging as an important means by which PTPase activity can be suppressed in specific signal transduction pathways (13, 14).

In the present work, we show that insulin stimulation of hepatoma and adipose-like cells causes the rapid formation of intracellular H$_2$O$_2$, which is associated with significantly decreased overall PTPase activity as well as a reduction in the specific activity of PTP1B, a PTPase that has been strongly implicated in the regulation of the insulin signaling pathway. Inhibition of the insulin-stimulated production of H$_2$O$_2$ by catalase treatment blocks the PTPase inactivation and reduces insulin-stimulated receptor autophosphorylation and tyrosine phosphorylation of IRS proteins. These findings reveal a novel regulatory mechanism integral to the early steps in insulin signaling that contribute to the steady-state balance and propagation of the insulin action cascade.

MATERIALS AND METHODS

Cell Culture—Murine 3T3-L1 preadipocytes were differentiated with insulin, dexamethasone, and isobutylmethylxanthine as described previously (15). Cells were serum-starved overnight in medium containing 0.5% (w/v) bovine serum albumin prior to insulin stimulation.

Visualization of Intracellular H$_2$O$_2$—Intracellular H$_2$O$_2$ production was detected by fluorescence of 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCF-DA; catalog number C-6827, Molecular Probes) on confocal microscopy (Bio-Rad) at an excitation wavelength of 488 nm and emission at 515–540 nm. To avoid photobleaching, the fluorescence image was collected by a single rapid scan with identical parameters for all samples (16).

Measurement of Cellular PTPase Activities—Endogenous PTPase activities as isolated from the cellular environment were measured using a novel anaerobic technique to avoid air oxidation as we described recently (41). Briefly, an enclosed anaerobic work station (Forma Scientific number 901024) provided an oxygen-free environment (gas mixture of N$_2$:H$_2$:CO$_2$ = 85:10:5) for cell homogenization, sealing tubes for
centrifugations, immunoprecipitations, and PTPase enzyme assays. After the indicated treatments, cells were snap-frozen in liquid N₂, introduced into the anaerobic chamber in a frozen state, and disrupted by scraping into ice-cold, deoxygenated homogenization buffer (150 mM NaCl, 5 mM EDTA, 5 mM EGTA, in 50 mM Hepes, pH 7.5, containing a protease inhibitor mixture (Sigma) followed by brief sonication. The whole cell lysate was prepared by adding 1% (v/v) Triton X-100, mixing on ice for 45 min, and clearing of the lysate by centrifugation at 15,000 × g for 20 min. Prior to solubilization, the supernatant resulting from centrifugation of the homogenate at 100,000 × g for 45 min at 4 °C was taken as the cytosol. The solubilized particulate fraction was prepared by adding Triton X-100 to the pellet from the ultracentrifugation step and incubating and clearing as above by centrifugation. Protein was measured using the method of Bradford (17).

PTPase Assay—PTPase activity was determined in cell fractions containing 30 µg of protein in a final volume of 100 µl at 30 °C for 30 min in reaction buffer containing 10 mM para-nitrophenyl phosphate (pNPP, Sigma) and 2 mM EDTA in 20 mM MES at pH 6.0. Where indicated, assay samples were incubated with 1 mM DTT on ice for 10 min prior to enzyme assay. The reaction was stopped by the addition of 50 µl of 1 M NaOH, and the absorption was determined at 410 nm (18). PTPase activity is reported as the optical density from hydrolysis of pNPP.

Specific Activity of PTP1B—Under strictly anaerobic conditions, PTP1B was immunoprecipitated from cell lysates with a monoclonal antibody directed at a C-terminal epitope that preserves its enzymatic activity (Oncogene Sciences; Ab-2) followed by adsorption to Trisacryl protein G (Pierce). PTPase activity was measured by the hydrolysis of pNPP in the anaerobic chamber in washed immunoprecipitates as described above. Control samples using non-immune mouse IgG showed minimal background PTPase activity (~5% of the activity with Ab-2).

Immunoblot Analysis of Protein Tyrosine Phosphorylation—Samples of 3T3-L1 cell lysates containing 75 µg of protein were subjected to Western blot analysis using a monoclonal anti-phosphotyrosine antibody (4G10, Upstate Biotechnology) or polyclonal antibodies to IRS-1 or insulin receptor β-subunit proteins (Transduction Laboratories) as described previously (19). Labeled proteins were visualized with horseradish peroxidase-conjugated anti-mouse IgG for 4G10 or with conjugated anti-rabbit IgG for the polyclonal antibodies, using conditions supplied by the manufacturer (Pierce). The blots were quantitated using an ImageStation 440 (Eastman Kodak Co.).

RESULTS

An oxidant signal in response to insulin was demonstrated in 3T3-L1 adipocytes loaded with CM-H₂DCF-DA, a redox indicator dye that is trapped intracellularly after cleavage by cellular esterases (Fig. 1). When oxidized in situ, DCF generates a signal that can be visualized by fluorescence confocal microscopy (16). Following stimulation of 3T3-L1 adipocytes with 100 nM insulin, a strong oxidant signal was detected by DCF fluorescence at the indicated concentration of insulin for 5 min, rinsing with serum-free medium prior to measurement of DCF fluorescence by confocal microscopy. Where indicated, the cells were preincubated with 0.008% (w/v) catalase (Sigma, C-100) for 10 min prior to insulin stimulation as described previously (16). The dose-response study (B) was performed in 3T3-L1 adipocytes by incubating the serum-starved cells with the indicated concentration of insulin for 5 min, rinsing with serum-free medium, and then incubating the cells in the dark with DCF-DA for 10 min prior to confocal microscopy.

To determine whether the insulin-stimulated generation of intracellular H₂O₂ affected the endogenous activity of cellular PTPases, we applied a novel approach recently established in our laboratory that involves sample handling and analysis under anaerobic conditions (41). This method preserves the activity of PTPases as isolated from the cultured cells and avoids oxidation and artifactual enzyme inhibition that otherwise occurs on exposure to air. Treatment of HepG2 cells with 100 nM insulin for 5 min resulted in a 32–52% reduction in overall PTPase activity in the cell homogenate, the cytosol, and the solubilized particulate fraction (p < 0.001) (Fig. 2A). Biochemical reduction of the enzyme samples with DTT prior to PTPase assay had no significant effect on the control samples prior to insulin treatment, but fully restored the reduced PTPase activity of the insulin-treated samples, indicating that they had been reversibly oxidized and inactivated by insulin exposure. A similar but more striking effect was observed using 3T3-L1 adipocytes, where insulin treatment caused a 62% drop in PTPase activity in the cell lysate (p < 0.001), which was restored to within control levels by treatment of the assay samples with DTT (Fig. 2B).

To determine the role of insulin-induced H₂O₂ in the oxidative inhibition of cellular PTPase activity, cells were preincubated with catalase prior to insulin stimulation and PTPase assay. Catalase had no significant effect on the basal level of PTPase activity. However, the presence of catalase blocked the reduction of PTPase activity in the 3T3-L1 adipocyte cell lysate induced by insulin to a level that was not significantly different from the control samples (Fig. 2). This important finding indicated that H₂O₂ mediated the oxidative inhibition of cellular...
HepG2 hepatoma cells and 3T3-L1 adipocytes. NPP. The mean indicated, and after snap-freezing with liquid N2, introduced into the lysate by centrifugation at 15,000 × g for 20 min. Prior to solubilization, the supernatant resulting from centrifugation of the homogenate at 100,000 × g for 45 min at 4 °C was taken as the cytosol. The solubilized particulate fraction was prepared by adding Triton X-100 to the pellet from the ultracentrifugation step and incubating and clearing by centrifugation. Protein was measured using the method of Bradford (17). PTPase activity was determined under anaerobic conditions in samples from the indicated fractions containing 30 μg of protein in a final volume of 100 μl at 30 °C for 30 min in reaction buffer containing 10 mM pNPP and 2 mM EDTA in 20 mM MES at pH 6.0. Where indicated, assay samples were incubated with 1 mM DTT on ice for 10 min prior to enzyme assay. The reaction was stopped by the addition of 50 μl of 1 M NaOH, and the absorption was determined at 410 nm (18). PTPase activity is reported as the OD from hydrolysis of pNPP. The mean ± S.E. of four independent experiments is shown.

PTPase activity associated with insulin stimulation.

To explore whether the insulin-stimulated generation of H2O2 affected the specific activity of PTP1B itself as isolated from intact cells, we immunoprecipitated PTP1B from snap-frozen HepG2 cell lysates under anaerobic conditions and assayed its activity within the anaerobic chamber. Following insulin treatment for 2 or 5 min, the activity of immunoprecipitated PTP1B was reduced to 46 and 29% of control, respectively (Fig. 3A). In the continued presence of insulin, this effect was sustained for at least 10 min (not shown).

For comparison, direct treatment of HepG2 cells with 0.5 mM H2O2 for 5 min prior to cell lysis caused a 66% reduction of PTP1B enzyme activity (Fig. 3A). Further insight into the biochemical alterations induced in PTP1B following insulin stimulation of HepG2 cells was obtained by treating the immunoprecipitated enzyme with DTT prior to the PTPase assay (Fig. 3B). As in the previous experiment, insulin stimulation reduced the PTP1B enzyme activity to 28% of control. Treatment of the isolated enzyme with DTT restored the activity to 83% of control, indicating that the insulin-induced oxidative inactivation of PTP1B was largely reversible by biochemical reduction. In contrast, treatment of the cells directly with H2O2 reduced the catalytic activity of immunoprecipitated PTP1B to a similar degree (26% of control), but this effect was less reversible in vitro with DTT incubation, to only 61% of the control level (Fig. 3B). Thus, H2O2 itself caused a greater degree of PTP1B catalytic thiol oxidation compared with cell treatment with insulin. Treatment of immunoprecipitated PTP1B from the control HepG2 cells with DTT increased the enzyme activity slightly but significantly by 16% (p < 0.03), suggesting that in vitro, only a small fraction of the enzyme is present in an oxidized state that is activable by biochemical reduction in vitro.

In 3T3-L1 adipocytes, insulin treatment also potently reduced the activity of immunoprecipitated PTP1B to 12% of control, which was reversible to 72% of control by preincubation of the immunoprecipitated enzyme with DTT prior to PTPase assay (Fig. 3C). To further clarify the role of H2O2 in the reduced activity of PTP1B from the insulin-treated cells, 3T3-L1 adipocytes were preincubated with catalase, which completely blocks the insulin-stimulated generation of H2O2 in these cells, as shown above in the experiments utilizing DCF-DA fluorescence. Insulin stimulation of the 3T3-L1 adipocytes reduced PTP1B activity in the control cells by 64%, which was completely prevented by preincubating the cells with catalase (Fig. 3D). Catalase similarly prevented the insulin-stimulated reduction in PTP1B activity in HepG2 cells (not shown), indicating that in both of these cell types, the insulin-induced oxidative inhibition of PTP1B activity, mediated by H2O2, was proportionally greater than the decrease in overall PTPase activity elicited by insulin in the cell lysates.

A receptor-mediated effect of insulin on PTP1B activity was also suggested by treatment of HepG2 cells with staurosporine (20), which blocks insulin receptor autophosphorylation in the HepG2 cells (not shown), and reduced the insulin inhibition of PTP1B enzyme activity by 40% (Fig. 4A). To determine the reversibility of the insulin effect, insulin bound to the HepG2 cells was dissociated with a mild acid wash after various times of insulin stimulation, and after an additional 10 min, the cellular PTP1B activity was determined by immunoprecipitation under anaerobic conditions (Fig. 4B). At each time point, continued exposure to insulin led to a persistent reduction in PTP1B activity; however, removal of the bound insulin fully reversed the inhibition of PTP1B activity following incubations with insulin of up to 30 min.

Finally, we evaluated the effect of blocking the insulin-induced production of H2O2 with catalase on insulin-stimulated
tyrosine phosphorylation of the insulin receptor and IRS proteins in 3T3-L1 adipocytes (Fig. 5). Catalase treatment had no effect on the basal level of insulin receptor or IRS phosphorylation in the serum-starved cells. However, catalase reduced the autophosphorylation of the insulin receptor by 48 and 44% and similarly reduced the insulin-stimulated tyrosine phosphorylation of IRS proteins by 34 and 43%, respectively, at 1 or 5 min of insulin treatment. The protein abundance of IRS-1 and the insulin receptor β-subunit was unchanged by incubation of the cells with catalase (Fig. 5A). Taken together, these data suggest that by preventing the insulin-induced inhibition of PTP1B, catalase treatment results in a sustained level of PTP1B activity, which appears to be inhibitory to insulin-stimulated autophosphorylation of its receptor and restricts the propagation of the early insulin signal to IRS proteins.

**DISCUSSION**

Cross-talk involving oxidative inactivation of PTPases and the signaling pathways they regulate has become evident in recent work showing that reactive oxygen species can be generated by stimulation of cells with growth factors and cytokines (12, 21). Specifically, \( \text{H}_2\text{O}_2 \) has been implicated in the activation of tyrosine phosphorylation cascades in a manner that mimics the effect of ligands such as epidermal growth factor and platelet-derived growth factor; also, \( \text{H}_2\text{O}_2 \) can serve as an mimics the effect of ligands such as epidermal growth factor and platelet-derived growth factor; also, \( \text{H}_2\text{O}_2 \) can serve as an

Recent data has also suggested that signal transduction pathways modulated by reactive oxygen species associated with the action of growth factors and cytokines may involve the stepwise oxidation and inactivation of PTPase enzymes (11, 12). The susceptibility of enzymes in the PTPase family to oxidative inactivation resides in the characteristic active site sequence motif, which requires the catalytic cysteine moiety to be in a reduced state (10). The catalytic thiol hydrogen lies in spatial proximity to amino acid side chains adjacent to the enzyme active site that strongly affect its ionization, effectively lowering its \( \text{pK}_a \) to more than 3 units below that found in a typical cysteine and facilitating its derivatization at physiological pH (9, 28). This cysteine residue is susceptible to oxidation to progressively more inert forms. First, this forms the sulfenic (-SOH) form, which is reversible and amenable to reduction by cellular mechanisms or by reducing agents in vitro; sequential steps of oxidation may then lead to sulfenic (-SO\(_2\)H) and sulfonic (-SO\(_3\)H) forms and can lead to irreversible PTPase inactivation. This general scheme may constitute a major regulatory mechanism for PTPases within the cellular environment.

PTP1B, in particular, appears to be a cellular target for oxidative inactivation possibly followed by disulfide conjugation with glutathione (glutathiolation), processes that are at least partially balanced by cellular reductases (13, 14, 29). PTP1B was one of the first specific PTPases to be implicated in the negative regulation of insulin receptor autophosphorylation and post-receptor insulin signaling (7, 18, 30–32). The most compelling evidence for a physiological role of PTP1B in insulin action has been the recent demonstration of enhanced insulin sensitivity and potentiation of insulin-stimulated protein-tyrosine phosphorylation in PTP1B knockout mice (33, 34). Changes in the intracellular enzymatic activity of PTP1B may also affect insulin action in adipose tissue from obese subjects (35). PTP1B has been recognized as a target for drug development to enhance insulin signaling through enhancing protein-tyrosine phosphorylation in the insulin action pathway (36), and novel, relatively specific inhibitors of PTP1B have been reported (37).

In the present work, we provide evidence for a close coupling between the insulin-induced generation of cellular \( \text{H}_2\text{O}_2 \) and the oxidative inactivation of the catalytic activity of PTP1B. In early studies of insulin action, it was recognized that \( \text{H}_2\text{O}_2 \) could mimic at least some of the actions of insulin (38), and more recent work has demonstrated that \( \text{H}_2\text{O}_2 \) is elaborated during physiological insulin signal transduction in adipose cells (39). Some years ago, Meyervitch and colleagues (40) made the observation that stimulation of Fao rat hepatoma cells with nanomolar concentrations of insulin over a time course of several minutes caused a significant reduction in cytosolic PTPase activity measured against an insulin receptor

![Oxidative Inhibition of PTP1B by Insulin](http://www.jbc.org/)
phosphopeptide. Among the novel findings in our present study is the demonstration that insulin stimulation generates a burst of H₂O₂ in multiple types of insulin-sensitive cells. In addition to the down-regulation of the specific activity of cellular PTP1B by oxidants elicited by insulin, it is possible that this burst of H₂O₂ also affects downstream insulin signaling, as shown in response to growth factors in other cell types (12, 21, 26). Further studies detailing the implications of this pathway for response to growth factors in other cell types (12, 21, 26).

In summary, we report here the novel observation that in both 3T3-L1 adipocytes and hepatoma cells, the elaboration of reactive oxygen species (H₂O₂) by insulin is associated with reversible oxidative inactivation of overall cellular PTPase activity and specifically of PTP1B. This effect, in turn, markedly influences insulin-stimulated receptor autophosphorylation and the tyrosine phosphorylation of IRS proteins. Given the growing body of evidence that PTP1B is integrally involved in the negative regulation of insulin signaling, this reciprocal regulation of the balance of protein-tyrosine phosphorylation may play a critical role in signal transduction in the insulin action pathway.

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