Redox Paradox:

Insulin Action Is Facilitated by Insulin-Stimulated Reactive Oxygen Species With Multiple

Potential Signaling Targets

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Abstract

Propelled by the identification of a small family of NADPH oxidase (Nox) enzyme homologs that produce superoxide in response to cellular stimulation with various growth factors, renewed interest has been generated in characterizing the signaling effects of reactive oxygen species (ROS) in relation to insulin action. Two key observations made >30 years ago—that oxidants can facilitate or mimic insulin action and that H_2O_2 is generated in response to insulin stimulation of its target cells— have led to the hypothesis that ROS may serve as second messengers in the insulin action cascade. Specific molecular targets of insulin-induced ROS include enzymes whose signaling activity is modified via oxidative biochemical reactions, leading to enhanced insulin signal transduction. These positive

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DPI
diphenyleneiodonium

DTT
dithiothreitol

EGF
epidermal growth factor

IRS
insulin receptor substrate

LAR
leukocyte antigen-related protein tyrosine phosphatase

MAP
mitogen-activated protein

Nox
NADPH oxidase

PDGF
platelet-derived growth factor

PI
phosphatidylinositol

PTP
protein-tyrosine phosphatase

ROS
reactive oxygen species.

responses to cellular ROS may seem "paradoxical" because chronic exposure to relatively high levels of ROS have also been associated with functional β-cell impairment and the chronic complications of diabetes. The best-characterized molecular targets of ROS are the protein-tyrosine phosphatases (PTPs) because these important signaling enzymes require a reduced form of a critical cysteine residue for catalytic activity. PTPs normally serve as negative regulators of insulin action via the dephosphorylation of the insulin receptor and its tyrosine-phosphorylated cellular substrates. However, ROS can rapidly oxidize the catalytic cysteine of target PTPs, effectively blocking their enzyme activity and reversing their inhibitory effect on insulin signaling. Among the cloned Nox homologs, we have recently provided evidence that Nox4 may mediate the insulin-stimulated generation of cellular ROS and is coupled to insulin action via the oxidative inhibition of PTP1B, a PTP known to be a major regulator of the insulin signaling cascade. Further characterization of the molecular components of this novel signaling cascade, including the mechanism of ROS generated by insulin and the identification of various oxidation-sensitive signaling targets in insulin-sensitive cells, may provide a novel means of facilitating insulin action in states of insulin resistance.

Cellular reactive oxygen species (ROS; superoxide and H_2O_2), especially when chronically raised to high levels and associated with hyperglycemia, have been widely recognized to have an important pathophysiological role in the vascular complications of diabetes as well as in the development of the disease itself (1-3). In contrast, transient bursts of small amounts of ROS are triggered in response to stimulation with a variety of growth factors, cytokines, and hormones, including insulin, making it seem "paradoxical" that localized effects of ROS can enhance the cellular responses to these ligands. The involvement of an oxidation step in the action of insulin has been suggested for decades, but only recently have potential molecular mechanisms been identified that enable researchers to test the hypotheses that insulin-induced ROS serve a second messenger function and play an important role in facilitating the insulin action cascade. Among the signaling enzymes potentially susceptible to inhibition by biochemical oxidation are those that contain reduced cysteine thiol side chains essential for their catalytic activity. These effects have been best demonstrated for the protein-tyrosine phosphatases (PTPs), but other important enzymatic signal regulators may be susceptible to oxidative inhibition, as discussed below. Furthermore, the recent cloning of a novel family of NADPH oxidase (Nox) enzymes has provided evidence that the homolog Nox4 may be a source of the rapid generation of ROS in insulin-stimulated cells. A full understanding of this signaling network may provide a novel means of facilitating insulin action in states of insulin resistance and, potentially, of differentially regulating some of the pleiotropic cellular actions of insulin.

Reversible tyrosine phosphorylation in insulin signaling. Insulin is a critical regulator of pleiotropic metabolic and mitogenic cellular responses (4). The earliest defect in type 2 diabetes is insulin resistance in peripheral tissues, which is also a common pathophysiological feature of related conditions including obesity and the "metabolic" syndrome, which are associated with increased cardiovascular risk (5). Although advances are continually being made in our understanding of insulin action, we still do not have a complete picture of how reversible tyrosine phosphorylation in the insulin signaling pathway is regulated (4,6,7).

Insulin action is initiated by binding to a specific plasma membrane receptor whose tyrosine kinase activity is essential for insulin's growth-promoting and metabolic effects (4,6). Substrate proteins of the insulin receptor kinase, especially adapters of the insulin receptor substrate (IRS) family and Shc, are efficiently tyrosine phosphorylated (6,7). These sites act as docking scaffolds for the binding and activation of a variety of signaling and adaptor proteins, including the p85 subunit of phosphatidylinositol (PI) 3′-kinase, that are linked to the activation of downstream insulin responses, including glucose transport, p70 S6 kinase, nuclear DNA synthesis, and gene expression.

Role of PTPs in the regulation of insulin signaling. The cellular mechanisms that regulate the steady-state balance of reversible protein-tyrosine phosphorylation in the insulin signaling cascade involve the reciprocal effects of the insulin receptor kinase and the action of PTPs (6,8). In addition to serving as steady-state regulators, PTPs appear to be required for receptor deactivation because purified insulin receptors retain their autophosphorylation state after insulin is removed from the ligand binding site in vitro (9). In vivo, dissociation of insulin from the receptor is followed by its rapid dephosphorylation and by deactivation of its kinase activity (10,11). Specific PTPs, in particular PTP1B, have become important targets for therapeutic intervention in disease states associated with clinical insulin resistance (12-15).

PTPs comprise an extensive group of homologous proteins involved in a variety of signal transduction pathways (16). Receptor and nonreceptor forms of the classical tyrosine-specific PTPs have in common a ~230 amino acid phosphatase domain that contains the tightly conserved signature catalytic motif VHCSxGxGR[T/S]G (17). The nonclassical, dual-specificity (phosphotyrosine and phosphoserine/phosphothreonine) phosphatases, e.g., mitogen-activated protein (MAP) kinase phosphatase and others (18) and PTEN (phosphatase and tensin homolog deleted on chromosome 10), which dephosphorylates the 3′-phosphate of inositol phospholipids generated by PI 3′-kinase and exhibits dual phosphatase activity in vitro (19,20), are structurally related, sharing a less tightly conserved catalytic motif that retains the essential $C(x)_5 R$ core structure (21). This sequence contains a reduced cysteine residue required for catalysis that is involved in the formation of a cysteinyl-phosphate intermediate (22,23). Modification of this catalytic cysteine by oxidation or disulfide conjugation has recently been recognized as a critically important mode of PTP regulation in vivo, including in the insulin action cascade, as discussed in detail below (24-27).

Among several candidate enzymes expressed in insulin-sensitive cells, compelling experimental evidence has best implicated the intracellular single-domain enzyme PTP1B as having a physiological role in the negative regulation of the insulin action cascade (15,28, 29). PTP1B is active in vitro against the autophosphorylated insulin receptor (30,31), and it also has a relatively high specific activity toward IRS-1 compared with other candidate PTPs (32,33). Several studies have characterized the unique molecular interactions underlying the close interaction between the insulin receptor and PTP1B, which are facilitated by the presence of a second phosphotyrosine binding site in the PTP1B catalytic region that interacts with the multiple phosphotyrosine residues of the receptor kinase (34-36).

Numerous studies have demonstrated that PTP1B modulates tyrosine phosphorylation and activation of the insulin receptor itself and that it plays a key role in the regulation of early and late steps in postreceptor insulin signaling (rev. in 12,15). The most compelling data supporting a key role for PTP1B in insulin signaling come from studies in independently derived lines of PTP1B knockout mice, which exhibit no obvious disease phenotype but have enhanced glucose tolerance and heightened insulin sensitivity (37,38). Studies of insulin signaling in the knockout mice revealed enhanced insulin-stimulated phosphorylation of the insulin receptor and IRS-1 in skeletal muscle and liver but, interestingly, not in adipose tissue. The knockout mice exhibit decreased adiposity and a striking resistance to weight gain when fed a high-fat diet, with an increased basal metabolic rate (37,38), which has been attributed to enhanced leptin signaling (39,40). Although enhanced epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptor activation can be demonstrated in PTP1B$^{-/-}$ fibroblasts isolated from the knockout mice, downstream signaling is not significantly affected, suggesting that compensatory regulatory mechanisms for other growth factor pathways exist (41). PTP1B can also regulate growth hormone signaling by diminishing JAK2 phosphorylation, which may influence the regulation of adiposity under various metabolic conditions (42).

ROS as second messengers for cellular tyrosine kinase signaling. Superoxide and H_2O_2, collectively called "reactive oxygen species" (ROS), are now well recognized to play an integral role in several growth factor and cytokine signal transduction pathways (43-51). Superoxide $[O_2 \cdot^-]$, hydroxyl $[\cdot OH]$ ions, and H_2O_2 generated by cellular redox reactions have a complex physiology (46,52) and are ultimately converted to thioredoxin, $H_2O + O_2$ cellular catalase, glutathione by peroxidase, and/or peroxiredoxins (53-56). Relatively low levels of H_2O_2, generated in response to growth factor stimulation, occur in a concerted fashion with specific signaling targets in the cell, suggesting that it serves as a second messenger (44,51, 57). H_2O_2 activates tyrosine phosphorylation cascades in cultured cells in a manner that mimics ligand-mediated signaling by PDGF and EGF (44,45,58). However, an actual second messenger function of cellular H_2O_2 generated transiently during stimulation of cells with growth factors has been convincingly demonstrated in pivotal experiments by Sundaresan et al. (44) and Bae et al. (57), showing that autophosphorylation of PDGF and EGF receptors, respectively, and their distal signaling effects are dependent on intracellular H_2O_2 production. Numerous studies have also recently emerged supporting the hypothesis that cellular oxidant signaling is mediated by discrete localized redox circuitry, which is distinct from the notion of a generalized "oxidative stress" effect (51). For example, overexpression of the Nox homolog Nox1 increases cellular ROS and specifically activates an oxidant-sensitive reporter gene via activation of c-Jun NH_2-terminal kinase (JNK) and extracellular signal-related kinase 1/2 without affecting the overall redox state of glutathione and thioredoxin, the major thiol antioxidant substances in the cell (59).

A novel regulatory paradigm: PTPs are thiol-dependent enzymes regulated by cellular ROS. In parallel with developments in the cellular physiology of H_2O_2 generation, studies have also characterized the biochemical inhibition of PTPs by progressive oxidation of their catalytic cysteine thiol moiety by cellular ROS to more inert forms in vivo (Fig. 1) (24,25, 27,60,61). The activity of PTP1B is dependent on the oxidation state of its cys-215 residue, which is required for catalytic activity via the formation of a phospho-enzyme intermediate (23,24,62,63). The catalytic cysteine residue in the PTP active site is particularly sensitive to oxidation because of hydrogen bonding of neighboring side chains, which lowers the thiol pK_a to ~5.5, >3 units below that of a typical sulfhydryl (-SH) group, rendering it in an ionized state at physiological pH. Compared with other typical protein sulfhydryl side chains, the catalytic PTP thiol can be readily oxidized by locally generated H_2O_2, even in the presence of high cytosolic concentrations (millimolar) of the cysteine-containing tripeptide glutathione (51).

The catalytic cysteine thiol is initially oxidized to the sulfenic (-SOH) form, which can be reversed by cellular enzymatic mechanisms or with reducing agents in vitro (Fig. 1) (62,64). Sequential steps of progressive oxidation, to the sulfinic (-SO$_2$H) and sulfonic (-SO H) forms can lead to irreversible PTP inactivation (65-67). [3]However, the partially oxidized sulfenic acid intermediate of PTP1B can also be rapidly converted to other forms that may stabilize the molecule and protect it from further irreversible oxidation. One potentially stabilizing modification is conjugation with glutathione, which may be enzymatically reactivated in the cell by glutaredoxin (63). The catalytic cysteine of PTP1B has recently been shown to be reversibly converted to a previously unknown intramolecular sulfenyl-amide species, in which it becomes linked to the main chain nitrogen of an adjacent residue, rendering the enzyme inactive and inducing large conformational changes that inhibit substrate binding (68,69). This novel protein modification not only protects the enzyme catalytic site from irreversible oxidation to sulfonic acid but also permits redox regulation of the enzyme by promoting its reversible reduction by thiols. The conformation of the catalytic cleft assumes a more open structure in the sulfenyl-amide derivative of PTP1B and renders it particularly amenable to reduction by glutathione (68,69). This suggests that this unique protein derivative may, in fact,

be an obligatory intermediate in the generation of the glutathionylated form of the oxidized enzyme.

Generation of H_2O_2 by cellular insulin stimulation. The potential involvement of oxidant species in insulin signaling was initially explored >30 years ago, with the observation by Czech and colleagues (70,71) and May (72) that certain metal cations interacting with albumin could transfer electrons to a cellular target and enhance glucose utilization by adipocytes. Livingston et al. (73) also showed that polyamines and related insulin mimickers acted via the generation of H_2O_2, and H_2O_2 itself stimulates lipid synthesis in adipocytes (74). Stimulation of glucose uptake in adipose cells by insulin was also found to be accompanied by sulfhydryl oxidation (75,76), and sulfhydryl blocking agents enhance insulin receptor function (77). Studies have also shown that oxidized vanadate derivatives can irreversibly inactivate PTPs and potently enhance insulin action (78-80).

In the late 1970s, insulin itself was shown to elicit the generation of H_2O_2 in adipocytes (81). An early characterization of the enzymology of this process revealed that insulin activated a plasma membrane enzyme system with the properties of an Nox, resulting in the downstream production of H_2O_2 (82,83). Further biochemical studies of this activity accounted for insulin-stimulated ROS production in rat adipocyte plasma membranes (84,85) also present in 3T3-L1 adipocytes (86). Nox catalyzes the reduction of oxygen to a superoxide radical:

$2 O_2 + NADPH \rightarrow 2O_2{}^- + NADP^+ + H$. Although a superoxide anion itself can react with thiols, it is rapidly converted spontaneously or by superoxide dismutase in the cell to generate H_2O_2 (51).

Closing the loop: insulin stimulation generates H_2O_2, which negatively regulates PTP1B. Related findings from several research approaches noted above have now been synthesized into a novel regulatory mechanism for insulin action (87). This process involves a plasma membrane oxidase activity stimulated by insulin that generates cellular ROS which, in turn, facilitate the insulin signaling cascade via the oxidative inhibition of cellular PTP activity. As discussed below, these inhibitory oxidative modifications may also affect additional cellular targets that normally serve as negative regulators of insulin action. Consistent with the previous work cited above, we reported that differentiated 3T3-L1 adipocytes that were loaded with a redox indicator dye based on dichlorodihydrofluorescein generated cellular H_2O_2 within 1 min of stimulation by 0.1-10 nmol/l insulin (25,26). The oxidant signal peaked at 5 min and began to dissipate by 10 min. Blocking the generation of cellular H_2O_2 with catalase or diphenyleneiodonium (DPI), an inhibitor of cellular Nox activity, reduced the insulin-stimulated autophosphorylation of the insulin receptor and the IRS proteins by up to 48%, suggesting that the oxidant signal inhibited cellular PTPs that serve as negative regulators of the insulin signaling cascade.

We also showed that the enhancement of insulin signaling by the oxidant signal was associated with PTP inhibition, using a novel approach that includes sample handling and analysis under anaerobic conditions to preserve the endogenous activity of PTPs isolated from cultured cells and avoiding the cysteine oxidation that occurs on exposure to air (88). In HepG2 hepatoma cells, stimulation with 100 nmol/l insulin for 5 min resulted in up to a 52% reduction in overall PTP activity in the cell homogenate, the cytosol, and the solubilized particulate fraction (25). Biochemical reduction of the enzyme samples with dithiothreitol (DTT) before PTP assay fully restored the reduced PTP activity of the insulin-treated samples, indicating that they had been reversibly oxidized and inactivated by insulin exposure. Similarly, in 3T3-L1 adipocytes, insulin caused a 62% drop in PTP activity in the cell lysate, which was restored to within control levels by treatment of samples with DTT before enzyme assay. Catalase also blocked the reduction of PTP activity in the 3T3-L1 adipocyte cell lysate induced by insulin to a level that

was not significantly different from the control samples, indicating that H_2O_2 mediated the oxidative inhibition of cellular PTP activity (25). Insulin-stimulated generation of $H_2 O_2$ also affected the specific activity of endogenous 2^2PTP1B isolated from intact cells. 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 with DTT before PTP assay (25). In the continued presence of insulin, this effect was sustained for at least 10 min. Catalase pretreatment of the cells abolished the insulin-induced inhibition of PTP1B. Oxidative inactivation of PTP1B is thus associated with enhanced insulin signal transduction via the insulin-stimulated $H_2 O_2$ signal.

Blocking the insulin-stimulated cellular production of H_2O_2 with DPI also completely inhibited the activation of PI 3′-kinase activity by insulin and reduced the insulin-induced activation of the serine kinase Akt by up to 49% (26). The H_2O_2-induced activation of Akt was mediated by upstream stimulation of PI 3′-kinase activity because treatment of 3T3-L1 adipocytes with the PI 3′-kinase inhibitors wortmannin or LY294002 completely blocked the subsequent activation of Akt by exogenous H_2O_2. Interestingly, the effects of H_2O_2 on downstream insulin signaling we observed in the adipocyte model were similar to prior observations in other cell types, including fibroblasts, embryonic kidney cells, vascular smooth muscle cells, and astrocytes, where activation of Akt by H_2O_2 is also abrogated by inhibition of PI 3′-kinase (89-91). These important findings suggest that cellular PI 3′-kinase is a critical upstream mediator of Akt activation by oxidative molecules in a variety of signaling pathways, including insulin action.

Identification of the Nox homolog Nox4 as a potential mediator of insulin-stimulated ROS. In phagocytic cells, the prototypic Nox complex that generates copious $O_2 \cdot {}^-$ for bacterial killing has a multisubunit structure complex and is highly regulated (92). Among its six subunits are two plasma membrane-associated proteins, the catalytic gp91*phox* protein (now designated as Nox2, which contains the FAD [flavin-adenine dinucleotide] and NADPH binding domains) and p22*phox*, which together form the flavocytochrome b_{588}, and four regulatory cytosolic factors, p47*phox*, p67*phox*, p40*phox*, and rac, which form the active holoenzyme at the plasma membrane. Using the Nox2 sequence, a small family of five homologous Nox catalytic subunits has recently been cloned (rev. in 93), each encoding a predicted transmembrane protein of ~65 kDa with functional homology domains similar to Nox2. The Nox enzymes exhibit unique patterns of cellular expression by Northern analysis: Nox1 is predominantly expressed in colon and vascular smooth muscle cells, Nox2 in phagocytes, Nox3 in fetal tissues and the inner ear, and Nox5 in spleen, sperm, mammary glands, and cerebrum.

Nox4 is prominently expressed in kidney (94-96) but was also shown by RT-PCR analysis to be widely expressed among other cell types, including liver, skeletal muscle, and osteoclasts (95,97). Using RT-PCR as well as Northern and Western blot analyses, we found that Nox4 is present in mature adipocytes and differentiated 3T3-L1 cells (98). Evidence for an integral role of Nox4 in the insulin-induced oxidant signal was obtained by adenovirus-mediated expression of Nox4 deletion constructs lacking either the NADPH binding domain or the combined FAD/NADPH domains, which acted in a dominant-negative fashion and attenuated insulin-stimulated generation of H_2O_2. Functionally, expression of the deletion constructs led to an inhibition of insulin receptor and IRS-1 tyrosine phosphorylation, activation of downstream serine kinases, and glucose uptake.

In parallel studies, transfection of specific small interfering RNA oligonucleotides reduced Nox4 protein abundance by up to 50% in the 3T3-L1 adipocytes and reduced insulin receptor autophosphorylation up to 64% compared with control cells transfected with scrambled small interfering RNA constructs (98). Reduced Nox4 mass was also associated with decreased

insulin-stimulated Akt phosphorylation by up to 48%. Together with the wild-type and mutant Nox4 transduction studies noted above, these results suggest that Nox4 overexpression potentiates, and reduced Nox4 mass diminishes, insulin signal transduction in the 3T3-L1 cells. In related work using other cell systems, reduction of Nox4 expression with antisense DNA oligonucleotides markedly decreased the cellular generation of ROS in osteoclasts and mesangial cells (99,100). Lowering Nox4 mass also abolished the activation of Akt by angiotensin II, implying that the oxidant signal is integral to downstream signaling in the mesangial cells (100).

ROS generation by Nox4 in the adipocytes was also associated with oxidative inhibition of cellular PTP1B activity (98). Overexpression of recombinant PTP1B inhibited insulin-stimulated tyrosine phosphorylation of the insulin receptor, which was significantly reversed by co-overexpression of active Nox4. The effect of overexpression of Nox4 on receptor autophosphorylation was closely associated with inhibition of PTP1B catalytic activity, as measured in enzyme immunoprecipitates. Interestingly, PTP1B inhibition was relatively specific because it was evident with no measurable reduction in the overall PTP activity in cell lysate fractions (M.K., X.W., B.J.G., unpublished observations). These findings suggest that Nox4 provides a novel link between the insulin receptor and ROS generation that enhance insulin signal transduction, at least in part via the oxidative inhibition of cellular PTPases, including PTP1B.

Nox2 itself does not appear to be a major mediator of insulin-induced ROS production. Nox2 knockout mice exhibit a severe deficiency of H_2O_2 production in phagocytes and aortic fibroblasts, but not in vascular smooth muscle cells (101). We recently found that lymphoblasts from Nox2-deficient human subjects (Coriell Institute) had reduced overall H_2O_2 generation, but they retained a 44% increase in ROS production after insulin treatment, suggesting that Nox2 is not involved in insulin-stimulated ROS generation (X.W., B.J.G., unpublished observations).

Regulation of Nox4 signaling in the insulin action cascade. Although structurally related, the Nox homologs vary markedly in their signaling properties and modes of regulation. Nox1 has mitogenic activity and promotes growth (102), whereas Nox4 has been implicated in cellular senescence (94). In vascular smooth muscle cells, Nox1 and Nox4 show opposite regulation by angiotensinII, and Nox1 colocalizes with caveolin, whereas Nox4 colocalizes with vinculin in focal adhesions (103). Nox1, Nox2, and Nox4 are physically associated with p22*phox*, an essential component of the catalytic flavocytochrome complex, suggesting a potential commonality in their subunit structure (104). Several groups have very recently reported the cloning of novel Nox1-interacting proteins in nonphagocytic cells (especially colonic epithelia) that dramatically enhance superoxide production by Nox1, termed NOXO1 (Nox organizer 1, or p41) and NOXA1 (Nox activator 1, or p51), which are closely homologous to the well-characterized Nox2 regulatory proteins p47*phox* and p67*phox* (105-108). Superoxide production by Nox3 is also enhanced by interactions with regulatory subunits (109,110). Nox5 is regulated by intracellular calcium levels (111). Less is known about the mechanisms underlying the regulation of Nox4 activity. NIH 3T3 fibroblasts transfected with Nox4 exhibit constitutively increased superoxide generation (94), suggesting that Nox4 may not to depend on activation by regulatory subunits as is evident with several other homologs in the Nox family.

H_2O_2 generated by PDGF and EGF stimulation in various cell models via a Nox1-mediated pathway appears to require PI 3′-kinase activation (112,113). Similarly, in skin fibroblasts, Ceolotto et al. (114) found that insulin-stimulated ROS production was also dependent on PI 3′-kinase activity. In contrast, we reported that insulin-stimulated ROS generation in 3T3-L1 adipocytes, presumably via a Nox4-mediated pathway, was not sensitive to zPI 3′-kinase

inhibition (26). These data suggest that Nox catalytic and regulatory components may differ in various cell types, including those responsive to insulin. For example, fibroblast cells are likely to express several Nox homologs as well as some of the regulatory components of the classical Nox2 (gp91*phox*) system, including p47*phox*, which apparently undergoes an apocynin-sensitive translocation from the cytosol to the cell membrane in response to insulin stimulation (114). A better understanding of the expression of regulatory components for the Nox system in the classical insulin target tissues and their responsiveness to insulin is clearly required.

Potential role of G-proteins in insulin-stimulated H_2O_2 and PTP regulation

Rac. This protein is a key component of the Nox complex in a variety of cell types. Rac2 is expressed predominantly in phagocytic cells, and Rac1 is expressed in other cell types (115). The Rac effector domain has been mapped, and a chimeric Rac1-p67*phox* protein increases Nox2 activity (116,117). Expression of a dominant-negative Rac (T17N) inhibits the rise in ROS seen after stimulation by growth factors or cytokines, and a constitutively active Rac (G12V) stimulates ROS formation in NIH 3T3 cells and in renal mesangial cells (100,118). Because superoxide generation in the renal cell system is likely to involve Nox4, these data also implicate Rac as a potential regulator of this Nox homolog (100).

Is Rac an effector of Nox4 in insulin-sensitive cells? Rac is expressed in adipocytes and skeletal muscle cells and is activated by insulin, albeit in a manner at least partly dependent on PI 3′-kinase activity (119). Although Rac does not couple distal insulin signaling to glucose transport (120), its strong association with Nox activity in other systems warrants its testing as a candidate regulatory protein for Nox4 in insulin target cells (Fig. 2).

Protein complex formation has a major regulatory influence on Nox function, increasing the catalytic activity of some of the Nox homologs, playing a key role in Nox subcellular localization and possible access to substrates. In this regard, it is of interest that the guanine nucleotide exchange factor Trio, which can activate the phagocyte Nox in the absence of GDP-to-GTP exchange on Rac (121), is a widely expressed multidomain protein that also forms a complex with leukocyte antigen-related PTP (LAR) (122). LAR is a receptor-type transmembrane PTP localized to focal adhesions that has been implicated in the regulation of insulin receptor dephosphorylation and insulin resistance (29,123,124). Trio has two functional guanine nucleotide exchange factor domains, one with specificity for Rac and the other for Rho, as well as a protein serine kinase domain. Although highly speculative, it is possible that Trio, with or without the involvement of Rac, interacts with one or more Nox homologs at the plasma membrane and directs the oxidative inhibition of the catalytic PTP domain of LAR. The localization of Nox4 to focal adhesions, demonstrated at least in vascular smooth muscle cells (103), raises the possibility that these regulatory protein interactions may involve this Nox homolog. Studies of the subcellular localization of Nox4 in insulin-sensitive cell types is thus a high priority for further work in this area.

Gαi2. Interesting data from diverse sources has also linked the small G-protein Gαi2 with insulin action and potentially with insulin-stimulated Nox activity (Fig. 2). There has been a steady flow of data over the years linking insulin action particularly with a pertussis toxin-sensitive G-protein (rev. in 125,126). Krieger-Brauer et al. (127) first reported that the insulin-stimulated plasma membrane Nox was coupled to Gαi2. In an insightful recent study using human adipocyte plasma membranes and recombinant protein components in an incubation buffer lacking added $MnCl_2$, this group demonstrated that insulin stimulation led to protein association between Gαi2 and the insulin receptor (128). Furthermore, insulin receptor autophosphorylation was stimulated by activated Gαi2 and blocked by pretreatment with pertussis toxin, consistent with an earlier study in Fao hepatoma cells (129). A recent report

also linked the attenuation of platelet activation by insulin with both tyrosine phosphorylation of Gαi2 and complex formation between IRS-1 and Gαi2, but not other Gα subunits (130).

In transgenic mice, Malbon and colleagues (131-133) showed that deficiency of Gαi2 in adipose tissue and liver caused hyperinsulinemia, impaired glucose tolerance, and insulin resistance, although conditional expression of a constitutively active Gαi2 mutant in insulin-sensitive tissues mimicked insulin action, with increased GLUT4 translocation in adipose tissue and skeletal muscle and enhanced overall glucose tolerance supporting a permissive role of Gαi2 in insulin signaling in vivo. In fact, transgenic expression of the Q205L constitutively activated mutant of Gαi2 ameliorates streptozotocin-induced insulin-deficient diabetes (134). Interestingly, the mice lacking Gαi2 also exhibited increased tissue PTP activity (131), implicating a potential loss of insulin-stimulated Nox activity in the Gαi2-deficient animals, with reduced oxidative inhibition of PTPs that regulate the insulin action pathway; however, this has not been directly tested in this model system. Overall, these studies are consistent with the hypothesis that the regulation of tyrosine phosphorylation in the insulin signal cascade is propagated by a wave of H_2O_2, possibly generated by a link between the insulin receptor and Gαi2, coupled to cellular Nox activity, which transiently inhibits PTP activities.

Novel targets of insulin-stimulated ROS that may potentially influence insulin action. In addition to PTPs that have been implicated in the regulation of insulin signaling (e.g., PTP1B and LAR, as discussed above) (29), a number of additional cellular enzymes are potential targets of oxidative inhibition by insulin-induced ROS (Table 1). A serine-threonine phosphatase, protein phosphatase 2A, implicated in the negative regulation of Akt by dephosphorylation of ser-473, has a redox-sensitive cysteine residue that is potentially susceptible to inhibition by H_2O_2 (135). The dual-specificity phosphatase MAP kinase phosphatase-1, which attenuates insulin-stimulated MAP kinase activity (136), is also dependent on a reduced thiol for activity. The lipid phosphatase PTEN can modulate downstream insulin signaling (137) and is also inactivated by oxidation of essential cysteine residues in its active site, which can be reactivated by thioredoxin in the cell (138,139). Further research is needed to determine the effects of the oxidative inhibition of these important signaling regulators on proximal and distal events in the insulin action cascade.

Perspective. Reversible tyrosine phosphorylation plays an essential role in the regulation of transmission of the insulin signal at receptor and postreceptor sites in the insulin action pathway. PTPs, in particular PTP1B, and other thiol-sensitive signaling proteins are integral to the negative regulation of insulin signaling. A growing body of data over the past 3 decades has led to the appreciation that cellular stimulation with insulin generates ROS that can inhibit these negative regulators by oxidative biochemical alterations that, in turn, can facilitate the insulin signaling cascade. With the recognition that a small family of Nox homologs catalyzes the generation of ROS at the plasma membrane, we have recently provided evidence in the 3T3-L1 adipocyte system that Nox4, which is expressed in insulin-sensitive cell types, is a novel molecular target that may mediate this process (Fig. 2).

Much remains to be learned about the mechanism of Nox-generated ROS in response to insulin and the ROS targets that influence intracellular insulin signaling responses. Research in this area must account for observations related to the cellular itinerary of the insulin receptor, including the localization of receptor activation and deactivation, and how receptor internalization may be involved in this process. Nox4 is localized to the plasma membrane in adipose cells (M.K., B.J.G., unpublished observations), which may facilitate its interaction with the insulin receptor during the process of insulin-stimulated ROS production. If Nox is also involved in the receptor internalization process, the ROS could access oxidation-sensitive targets at the plasma membrane and at intracellular sites.

Understanding the subcellular localization of insulin receptor dephosphorylation might provide clues as to how the oxidative regulation of PTP activity may enhance receptor signal transduction. PTP1B and LAR both interact with autophosphorylated insulin receptors (140-142). Insulin receptor activation triggers its internalization through an endosomal compartment accompanied by dynamic changes in the receptor phosphorylation and activation state (143). Biochemical evidence in liver has suggested that intracellular PTP activity in an endosomal fraction, rather than PTPs localized to the plasma membrane, are responsible for insulin receptor dephosphorylation (144); however, receptor internalization may not be obligatory because blocking internalization in 3T3-L1 adipocytes does not diminish the ability of PTP1B to dephosphorylate the cell surface receptor (145). Recent studies using novel markers for fluorescence or luminescence microscopy in HEK-293 cells have revealed that the insulin receptor is dephosphorylated within minutes after internalization—interacting most effectively with the form of PTP1B targeted to the endoplasmic reticulum, where it normally resides (146), rather than a soluble cytoplasmic form (147)—and that the receptor interaction with PTP1B occurs in a perinuclear endosomal compartment (148). Similarly, work with the EGF receptor has shown it to be under tonic phosphatase suppression at the plasma membrane, but after ligand-mediated activation and endocytosis it undergoes dephosphorylation at specific sites on the surface of the endoplasmic reticulum (149,150). Thus, the sites of interaction between PTPs and the insulin receptor may include the cell surface, where the receptor is kept inactive in a tonically dephosphorylated state, and PTP inhibition by ROS may help facilitate the initial receptor activation process. The major interaction appears to occur after internalization, when the autophosphorylated receptors undergo the bulk of dephosphorylation, at sites involving endosomes and the endoplasmic reticulum. One hypothesis is that Nox4, for example, may be internalized during the course of insulin receptor signaling and transiently inhibit PTPs in a process that facilitates the initial phases of signal transduction.

Insight into the disposition of growth factor-induced ROS has recently been gleaned from elegant studies by Reynolds et al. (151), who have modeled EGF receptor activation and signal propagation with PTP inhibition by ROS. ROS generated in response to EGF stimulation in MCF7 cells were spatially constrained to a layer below the plasma membrane, and they did not appear to occur at endosomal membranes. Using reaction constants gleaned from published experimental work, including PTP inhibition by H_2O_2 and related effects, a model was developed that was most consistent with a bistable activation state for PTP and receptor tyrosine kinase activity. The formation of a reaction "wavefront" is postulated that involves local cycles of EGF receptor activation, hydrogen peroxide production, and PTP inhibition, which propagates along the plasma membrane. The model proposes that signal initiation involving oxidative inhibition of PTPs adds a feedback control loop to a reaction network that responds in an amplified and switch-like manner, especially at low levels of ligand stimulus. Consistent with this model, they also showed experimentally that blocking ligand-dependent H_2O_2 generation with the Nox inhibitor DPI abolished the propagation of receptor phosphorylation as well as the amplification of receptor activation at low concentrations of EGF, converting the system to a "stable" steady state with a more linear phosphorylation response to ligand stimulation (151). Diminishing PTP inactivation with DPI also suppresses insulin receptor activation and several aspects of the downstream insulin signaling cascade (26,152). Thus, it would be of interest to use these types of novel imaging techniques to evaluate the role of similar regulatory networks in insulin-sensitive cells.

ROS production after ligand stimulation, including insulin, generates only a fraction of the ROS concentration observed in phagocytic cells and follows a brief time course, on the order of minutes (25). These features apparently account for the signaling role of insulin-induced ROS compared with the chronic exposure to ROS in patients with hyperglycemia that is associated with organ dysfunction and chronic complications of diabetes (1). The low levels of ROS in insulin signaling implies that there must be specific cellular protein targets that are

particularly susceptible to oxidative modification. Importantly, using novel protein labeling techniques, Rhee and colleagues (153,154) have shown that even in a cellular milieu containing millimolar concentrations of slowly reactive thiols like glutathione, only a limited set of proteins are rapidly oxidized by growth factor-stimulated ROS, including PTP1B and a few other proteins with reactive cysteines, including protein disulfide isomerases, thioredoxin reductase, and creatine kinase. We have confirmed similar findings after cellular insulin stimulation of insulin target cell types (X.W., B.J.G., unpublished observations). The biochemical evidence, therefore, also supports the notion of a discrete network of "redox circuitry" (51,59,155) with temporal and spatial influences that are likely to correspond to other regulatory aspects of the insulin action pathway (156).

Further work will help define the regulatory components and mechanism of Nox activation by insulin in various insulin-sensitive cell types and the effects of insulin-stimulated ROS on the insulin action cascade, with the identification of specific cellular targets susceptible to oxidative modification by insulin-stimulated ROS. Elucidation of these processes will determine how they are involved in the normal physiology of insulin signaling, if they contribute to insulin-resistant disease states, and whether elements of this system may emerge as novel targets for pharmaceutical intervention.

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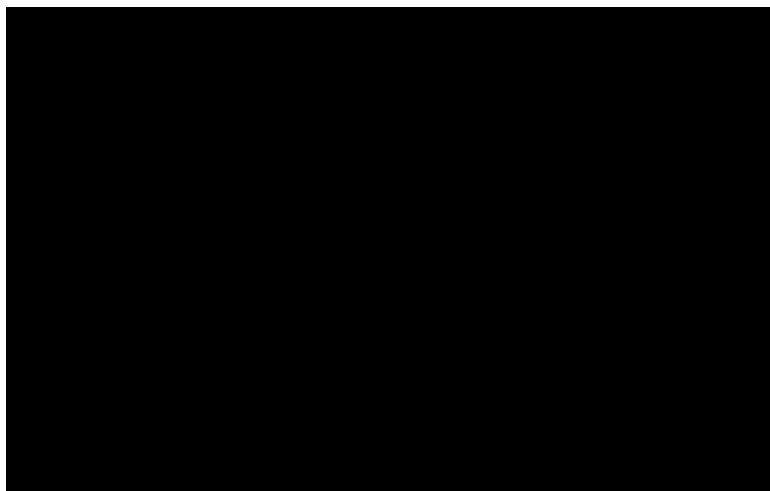
FIG. 1.
Regulation of PTP catalytic activity by oxidation, reduction, and conjugation. The catalytic cysteine residue of PTPs is especially reactive because the low pK_a of the sulfhydryl favors a relatively ionized state of the cysteinyl hydrogen (51). When subjected to ROS, including those elicited by cellular insulin stimulation, the cysteine side-chain undergoes stepwise oxidation to increasingly inert forms (27,60,157). The inactive sulfenic derivative may be reduced to regenerate the active thiol form of the protein. Alternatively, it may be directly conjugated with glutathione (GSH) in the cell, producing a catalytically inert PTP derivative that can be reactivated by biochemical reduction or through the action of glutathione reductases (63). Recently, the sulfenic derivative of PTP1B has been shown to undergo an intramolecular rearrangement, forming a novel sulfenyl-amide derivative that also sequesters the PTP in an inactive state (68,69). The sulfenyl-amide form may actually be an obligate intermediate in this reaction scheme because its altered protein conformation opens a groove adjacent to the catalytic center that may render it particularly susceptible to reduction with cytosolic glutathione compared with the sulfenic derivative. GSSH, oxidized glutathione.
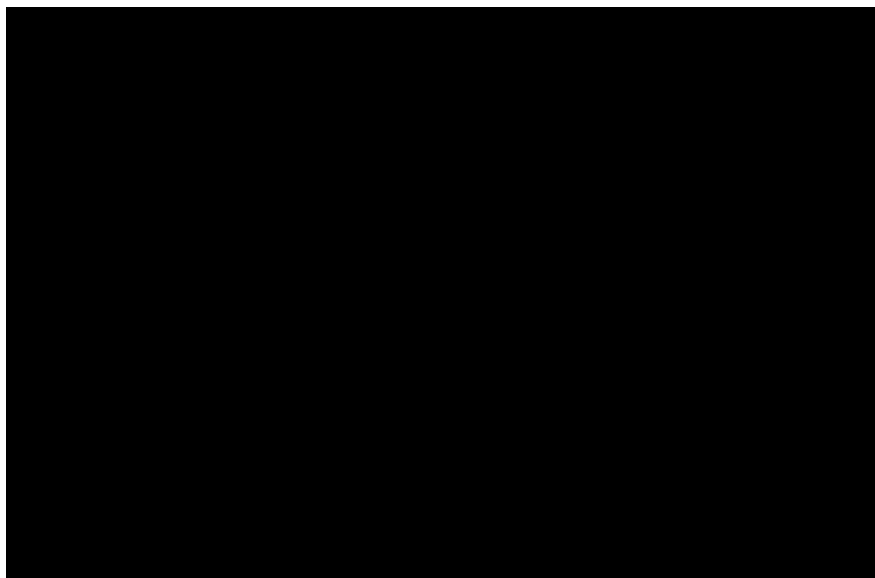
FIG. 2.
Postulated effectors of insulin-induced ROS via Nox4 and influences on downstream events in the insulin action cascade. Insulin stimulation of its target cells, especially adipocytes, elicits a burst of superoxide, with rapid generation of H_2O_2 catalyzed by cellular superoxide dismutase (SOD). As discussed in the text, the Nox homolog Nox4 may mediate a major part of the insulin-induced ROS generation in adipocytes (98). However, the mechanism(s) coupling Nox4 with the insulin receptor is not known. Potential effectors of the insulin activation of Nox activity may include the G-proteins rac and Gαi2, the flavocytochrome b$_{558}$ subunit p22*phox*, or interactions with novel Nox adaptor proteins (perhaps homologous to NOXO1 [Nox organizer 1] or NOXA1 [Nox activator 1]), among other possibilities. Insulin-stimulated ROS are believed to interact with a limited set of cellular proteins that contain catalytic thiol side-chains known to be particularly susceptible to biochemical oxidation (see Table 1). Inhibition of these signaling proteins, e.g., PTPs, leads to enhanced tyrosine autophosphorylation of the receptor and its substrate proteins as well as alterations in downstream signaling in the insulin action cascade. See text for further discussion and references.

TABLE 1

Potential cellular targets of insulin-induced ROS susceptible to biochemical regulation by oxidative inhibition

Acronym	Enzyme
PTP1B	Protein-tyrosine phosphatase 1B
PTPs	Other PTPs potentially involved in insulin action (e.g., LAR)
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PP2A	Protein serine/threonine phosphatase 2A
MKP-1	MAP kinase phosphatase-1; dual-specificity (serine/tyrosine) phosphatase
Dual-specificity PTPs	Other dual-specificity regulatory PTPs

See text for discussion.