Impact of Oxidative Stress on Signal Transduction Control by Phosphotyrosine Phosphatases

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Phosphotyrosine phosphatases (PTPs) serve as important regulators of cellular signal transduction pathways. PTPs are sensitive targets of oxidative stress and may be inhibited by treatments that induce intracellular oxidation. The effects of PTP inactivation under oxidizing conditions are amplified by the redox-linked activation of key protein tyrosine kinases (PTKs), thus leading to the initiation of phosphotyrosine-signaling cascades that are no longer under normal receptor control. These ligand-independent signals result in the accumulation of protein phosphotyrosine, the generation of second messengers, the activation of downstream kinases, and the nuclear translocation of nuclear factor kappa B (NF-κB). In this review we consider the relative contribution of oxidative stress to the effects of PTP inhibition by vanadium-based compounds in lymphocytes. Although the inactivation of PTPs can lead to NF-κB mobilization in the presence of antioxidants, the other effects noted appear to require a threshold of intracellular oxidation. The combined effects of oxidative stress on signal transduction cascades reflect a synergy between the initiation of signals by PTKs and the loss of control by PTPs. This suggests a mechanism by which environmental agents that cause oxidative stress may alter the course of cellular responses through induction or enhancement of signaling cascades leading to functional changes or cell death. — *Environ Health Perspect* 106(Suppl 5):1179–1184 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1179-1184krejsa/abstract.html*

Key words: phosphotyrosine phosphatase, oxidative stress, lymphocyte signal transduction, protein tyrosine kinase, vanadate, pervanadate, pV(phen), glutathione, CD45

Introduction

The role of aberrant signal transduction in chemical toxicity is emerging as an important area of environmental health research. In particular, numerous studies have shown that oxidative stress, induced by chemical prooxidants or ionizing radiation, can result in ligand-independent stimulation of cellular phosphorylation pathways. Oxidative stress results from an excess of reactive oxygen species (ROS) within the cell, which overwhelms the capacity of cellular antioxidant systems, leading to damage of cellular macromolecules (*1*). Oxidative stress can result from generation of ROS by the biochemical interactions of the prooxidant, as seen in the case of metal redox cycling, or from depletion of cellular antioxidants, which allows endogenously produced ROS to increase to toxic levels, as seen after glutathione (GSH) depletion by some alkylating agents (*2,3*). In addition, mechanisms exist for the induction of oxidative stress at sites of inflammation as a part of host defense (*4*). A growing body of literature suggests that changes in intracellular redox status may be an essential part of normal signal transduction control; therefore, shifting the balance of cellular oxidative stress may co-opt redox-sensitive pathways and alter the fate of the cell. In light of this hypothesis, significant effort has been committed to identifying redox-sensitive signal transduction pathways and the cellular effects of their perturbation.

As mechanisms of subacute toxicity are established, the epigenetic effects of oxidative stress are being strongly implicated in the development of diseases including atherosclerosis, autoimmunity, neurodegenerative disorders, and cancer (*5–8*). The activation of signaling pathways and the induction of suites of genes by oxidative stress can alter the functional development of affected cells, leading to such diverse outcomes as cellular proliferation and programmed cell death (*9*). Increasingly, toxicologists are working to understand these epigenetic effects in the context of normal signal transduction control by key kinases, phosphatases, and transcription factors. Since many environmental toxicants exert their effects through an oxidative mechanism, the induction of intracellular oxidative stress can serve as a general model for the impact of several classes of chemicals on signal transduction.

The activation of tyrosine phosphorylation pathways by oxidative stress has been the subject of much investigation (for reviews see Schieven and Ledbetter (*10*), Schieven (*11*), and Suzuki et al. (*12*)]. Oxidative stress in many cell types can activate kinases, induce Ca^{2+} influx, and stimulate the nuclear mobilization of transcription factors (*12*). Our research has focused on the action of oxidative stress in lymphocytes. Several key kinases, including the *src* family kinase Lck, which is essential for mediating T-cell receptor signals, and the downstream kinases Syk and ZAP-70, are activated by treatments that induce intracellular oxidation. These signaling cascades result in the generation of second messengers, including intracellular Ca^{2+} and inositol 1,4,5-triphosphate, and lead to the mobilization of the important transcription factor NF-κB (*13–16*). Our recent work has focused on a role for impairment of phosphotyrosine phosphatase (PTP) function in many of these effects (*17,18*).

We have used vanadium-based compounds to investigate the differential effects of PTP inhibition and oxidative stress in the activation of lymphocyte signaling pathways.

Vanadium is a transition metal of environmental health concern, as chronic respiratory exposures are common in the steel, mining, petrochemical, and utilities industries. Epidemiologic evidence indicates that workers exposed to vanadium in fumes and particulates develop bronchopulmonary diseases, increased pulmonary infections, and lung cancer (19). Rats exposed by intratracheal infusion to vanadium salts or residual oil fly ash, an industrial particulate containing high levels of vanadium, exhibited pulmonary inflammation, increased chemokine production, and altered cytokine responses compared with controls (20,21). Subacute tests with rats exposed to ammonium metavanadate (NH$_4$VO$_3$) in aerosol form suggest that these effects are due to alterations in pulmonary macrophage function (19). The contribution of tyrosine phosphatase inhibition and oxidative stress to these effects has not yet been established, although current understanding of inflammatory processes suggests that both factors may be important. Vanadium compounds have also been used therapeutically as components of metal alloys in joint-replacement prosthetics, and are currently being investigated for the treatment of insulin-resistant diabetes mellitus (22,23). The action of vanadium as a PTP inhibitor is thought to be responsible for its ability to mimic insulin in vivo (23).

PTP Control in Lymphocyte Signal Transduction

In lymphocytes, the PTPs act as both positive and negative regulators of signal transduction (24). For example, the membrane-bound PTP CD45 dephosphorylates the negative regulatory tyrosine on Lck, allowing the kinase to adopt an active conformation and initiate T-cell antigen receptor (TCR) signals (25). CD45 also exerts negative control by dephosphorylation of the TCR ζ chain and the associated kinase ZAP-70 (24,26). The src homology (SH2) domain containing PTPs SHP-1 and SHP-2 also plays an important role in signal termination from a variety of receptors on cells of both lymphoid and myeloid lineages. SHP-1 in particular was found to be defective in the motheaten mouse, resulting in the severe inflamation and lymphoproliferative disorder seen in that phenotype (27). Other PTPs and dual-specificity phosphatases exert control on downstream pathways, acting on the regulatory tyrosines of Raf1, SHC, erk1, erk2, JNK, and various transcription factors (28–33). While the discovery of important PTPs has lagged behind that of tyrosine kinases, it is clear that PTPs perform crucial functions in the orchestration of immune responses in vitro and in vivo [reviewed by Neel (34)].

Biological Effects of PTP Impairment

Studies with the motheaten mouse have demonstrated that SHP-1 impairment causes a failure in signal termination from the antigen receptors on lymphoid cells, leading to hypergammaglobulinemia and severe lymphoproliferative disorder (27,34). CD45-negative cell lines and mice have been generated; these show a lack of responsiveness to antigen-receptor signals, resulting in deficits in thymocyte development, and T-cell-mediated immune responses, perturbed B-cell development, and impaired B-cell proliferation (34). Clinical studies with CD4$^+$ T cells from HIV$^+$ patients showed reduced CD45 activity and failure to proliferate compared to noninfected controls; these deficits could be alleviated by culturing the cells with antioxidants (35). This suggests that oxidative stress plays a role in the loss of T-cell function seen in HIV$^+$ patients. Treatment of T-cells or macrophages with peroxovanadium PTP inhibitors also induced activation of the HIV-1 long terminal repeat and production of virions in infected cells (36,37).

PTPs and Oxidative Stress

The active sites of the PTPs and dual-specificity phosphatases contain an acidic cysteine that must remain in reduced form for catalysis to occur. Briefly, the cysteine exerts a nucleophilic attack on the phosphorous of a phosphotyrosine substrate, resulting in the formation of a thiol-phosphate intermediate. The reaction is completed by hydrolysis and the cysteine is regenerated with water as the electron donor (38). PTPs are sensitive targets of oxidative stress, and many of the best PTP inhibitors are sulfhydryl reactive agents (39,40). While the PTP active site appears to be deeply buried in the protein, its microenvironment is sensitive to intracellular oxidation. Approximately 80% of PTP activity in hepatocytes is sensitive to hydrogen peroxide (H$_2$O$_2$) treatment (40). In addition to determining substrate specificity, the arrangement of residues surrounding the PTP active site may also confer more or less sensitivity to oxidative stress.

Although many agents that induce oxidative stress may inhibit PTPs, it is of interest to compare treatments with different oxidative potentials to assess the relative contribution of intracellular oxidation to the action of the PTP inhibitors. For example, neither the PTP inhibitor sodium orthovanadate (Na$_3$VO$_4$) nor H$_2$O$_2$ alone can activate the protein tyrosine kinase (PTK) Lck, but treatment of lymphocytes with both agents together results in the strong Lck activation (13). Similarly, treatment of lymphocytes with Na$_3$VO$_4$ or another PTP inhibitor, molybdate, has a very minor effect on the Janus Kinase (Jak)/STAT (signal transducers and activators of transcription) pathways, which mediate cytokine signaling in lymphocytes; however the peroxoderivatives of these metals are extremely effective activators of the Jak/STAT pathways (33). There is evidence that vanadium and other transition metals may be oxidized intracellularly through redox cycling with NADPH (2,33,41). The generation of ROS in these redox cycles may be an important mechanism by which metals induce toxicity and may increase the susceptibility of PTPs by lowering the cellular pool of GSH and other reducing agents (2). Because oxidizing conditions can both inhibit PTPs and activate PTKs, the strong induction of signaling cascades by PTP inhibitors that also induce oxidative stress is likely to result from synergistic activation of the signaling molecules and the loss of regulation by PTPs.

Studies with Vanadium PTP Inhibitors

Pharmacologic PTP inhibitors have been widely used as probes to understand the role of phosphotyrosine signaling in lymphocytes, and numerous studies have shown that disruption of PTP control over signal transduction networks by pharmacologic agents alters lymphocyte function. Our recent studies focus on the role of oxidative stress in the action of PTP inhibitors, using vanadium-based complexes as model compounds. To differentiate the effects of direct PTP inhibition from other possible effects of oxidative stress, we have chosen three vanadium compounds that exhibit similar inhibition of major PTPs such as CD45 and PTP 1B in direct assays but differ markedly in their ability to induce oxidative stress within the cell.

Vanadium compounds have been widely used as PTP inhibitors, and their efficacy has been demonstrated in animal models and human clinical trials of insulin-resistant diabetes mellitis (23).

Different ligation of vanadium compounds enhances the action of the vanadium, stabilizes the vanadium in a particular oxidation state, and targets the compound to different tissues (42,43). Many studies have shown that pervanadate is an extraordinarily effective activator of cellular tyrosine phosphorylation in a wide variety of cells (11). Recent studies elucidated the mechanism of pervanadate action. Unlike vanadate, which acts as a reversible transition-state analogue of phosphate, pervanadate can also oxidize the PTP catalytic site cysteine to form cysteic acid, which cannot be reduced to regenerate an active enzyme (44). The rate of this reaction may vary between different PTPs, since in our hands PTP-1B activity following pV(phen) treatment could be partially restored by the reducing agents dithiothreitol or β-mercaptoethanol, whereas CD45 activity was irreversibly lost (18). This suggests that the effects of a particular metal–ligand complex on cellular PTP activity depend on both its affinity for specific PTP targets and the sensitivity of these targets to thiol oxidation.

A comparison of three structurally different vanadium-based PTP inhibitors on PTP-1B activity *in vitro* is shown in Figure 1. Na$_3$VO$_4$,bis(maltolato)-oxovanadium(VI) (BMOV), and sodium oxodiperoxo(1,10-phenanthroline)vanadate(V) (pV(phen) were used for this study. These compounds differ in their ability to induce intracellular oxidative stress, as measured by fluorescence of dichlorofluorescein (DCF), an intracellularly localized indicator of oxidative stress, in lymphocytes treated with equimolar amounts of the three inhibitors. Na$_3$VO$_4$ induced no intracellular oxidation, BMOV induced minor oxidation, and pV(phen) strongly increased the level of DCF-detectable intracellular oxidation compared to that in untreated controls. Consistent with the mechanism of action proposed for pervanadate, the pV(phen), a stabilized peroxovanadium complex, irreversibly inhibited the PTP-1B, whereas PTP activity was restored if the Na$_3$VO$_4$- or BMOV-treated enzyme was washed to remove the inhibitor prior to assay. Recovery of PTP activity in the Na$_3$VO$_4$- or BMOV-treated enzyme upon washing demonstrated that the mechanism of inhibition was reversible for these compounds in contrast to inhibition by treatment with pV(phen)(18).

In another set of experiments, CD45 was immunoprecipitated from T cells treated with pV(phen) or from cells that had been pretreated with the antioxidant pyrrolidine dithiocarbamate (PDTC) before pV(phen) treatment. CD45 activity was partially preserved in cells pretreated with PDTC, whereas cells treated with pV(phen) alone had no CD45 activity (18). This suggests that the antioxidant protected CD45 from irreversible inactivation by pV(phen) in cells. However, in direct assays, pV(phen) was an effective inhibitor of PTP-1B and CD45 in the presence of antioxidants (18). Further study of CD45 inactivation in pV(phen)-treated cells revealed that the PTP was totally inhibited at the earliest timepoint tested (Figure 2A), whereas the accumulation of protein phosphotyrosine lagged behind CD45 inhibition. It has been shown previously that peroxovanadium compounds do not directly activate PTKs in *in vitro* kinase assays (13,15,18). Therefore, we examined the relationship between the development of oxidative stress and the accumulation of
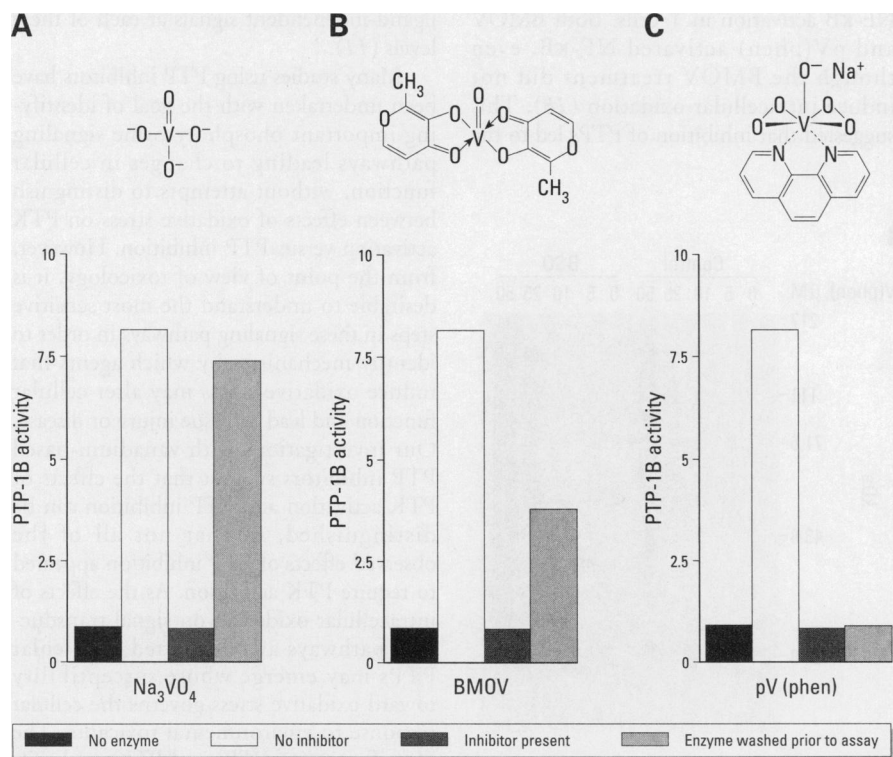
Figure 1. Structures and PTP-1B inhibition by three vanadium-based PTP inhibitors. PTP-1B-GST-agarose was incubated with 5 µM Na$_3$VO$_4$ (A), BMOV (B), or pV(phen) (C) and assayed for activity against *p*-nitrophenyl phosphate as previously described (18). The enzyme–agarose was either washed with buffer containing 5 mM EDTA prior to the assay or tested in the presence of 5 µM inhibitor.
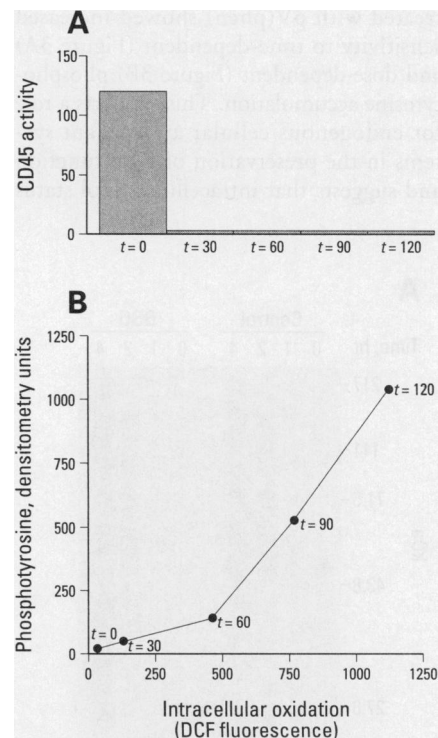
Figure 2. Intracellular phosphotyrosine accumulation is delayed with respect to CD45 inhibition. (A) CD45 activity from Jurkat T cells treated with 25 µM pV(phen) for 30 to 120 min [Krejsa et al. (18)]. (B) Correlation between development of intracellular oxidation and phosphotyrosine accumulation in Jurkat cells treated with 25 µM pV(phen) for 30 to 120 min.

protein phosphotyrosine (*18*). A correlation of phosphotyrosine accumulation with intracellular oxidation, as detected by DCF fluorescence, reveals a threshold effect, suggesting that the activation of kinases may require the induction of intracellular oxidation (Figure 2B). These results may help to distinguish effects typical of kinase activation by oxidative stress from those mediated solely by PTP inhibition; alternatively, they may reflect the differential sensitivity of key PTPs to intracellular oxidation, resulting in a sequential loss of control over cellular phosphorylation cascades as the oxidative stress increases.

The accumulation of phosphotyrosine and activation of downstream kinases by vanadium PTP inhibitors required a degree of intracellular oxidation and could be prevented by PDTC (*18*). To further investigate the synergy between intracellular oxidation and PTP inhibition, we studied the effect of the cellular reductant GSH on the efficacy of pV(phen) (Figure 3). GSH synthesis can be inhibited by treatment of cells with buthionine sulfoximine (BSO) (*45*). After overnight BSO treatment to deplete intracellular GSH, Ramos B cells treated with pV(phen) showed increased sensitivity to time-dependent (Figure 3A) and dose-dependent (Figure 3B) phosphotyrosine accumulation. This supports a role for endogenous cellular antioxidant systems in the preservation of PTP function and suggests that intracellular thiol status

could affect the activation of key kinases in Ramos cells.

Although Na_3VO_4, BMOV, and pV(phen) had equivalent potency in direct PTP assays, there were marked differences in their effects on intact lymphocytes. For example, Na_3VO_4 had little effect on either B or T lymphocytes, whereas BMOV and pV(phen) had distinct effects on these two cell types. BMOV induced apoptosis in B cells but enhanced the activation of T cells (*17*). These effects occurred without the induction of significant intracellular oxidation by BMOV. In contrast, pV(phen) induced strong oxidative stress in both T cells and B cells. The effects of pV(phen) on signal transduction resembled the reported effects of oxidative stress, including the activation of PTKs, accumulation of tyrosine phosphorylated proteins, and activation of erk2. Much of the response to pV(phen) was mitigated if the cells were pretreated with PDTC before pV(phen) treatment. Phosphotyrosine accumulation was mimimal, the activation of PTKs was greatly delayed and diminished, and the downstream kinase erk2 was not activated (*18*).

We have also examined the ability of vanadium-based PTP inhibitors to induce NF-κB activation in T cells. Both BMOV and pV(phen) activated NF-κB, even though the BMOV treatment did not induce intracellular oxidation (*18*). This suggested that inhibition of PTPs led to the

activation of NF-κB without a requirement for intracellular oxidation. To further address this finding, we pretreated T cells with antioxidants and found that both BMOV and pV(phen) still induced the activation of NF-κB (*18*). This confirms that NF-κB, a transcription factor well known for its sensitivity to oxidative stress, can also be activated by PTP inhibition independent of intracellular oxidation (*18*).

Discussion

Because the PTKs and PTPs act in concert to regulate phosphotyrosine-signaling pathways, it has been difficult to separate the effects of oxidative stress mediated by kinase activation from those caused by phosphatase inhibition. In addition, some of the end points commonly used to observe the induction of cellular signals by chemical agents are less sensitive to oxidative stress than others. It is therefore important to assay signaling pathways at multiple points, including the proximal activation of key kinases, the accumulation of phosphorylated substrates, and the activation of downstream signaling molecules and important transcription factors that integrate incoming messages into a cellular response. Oxidative stress can induce ligand-independent signals at each of these levels (*11*).

Many studies using PTP inhibitors have been undertaken with the goal of identifying important phosphotyrosine signaling pathways leading to changes in cellular function, without attempts to distinguish between effects of oxidative stress on PTK activation versus PTP inhibition. However, from the point of view of toxicology, it is desirable to understand the most sensitive steps in these signaling pathways in order to identify mechanisms by which agents that induce oxidative stress may alter cellular function and lead to tissue injury or disease. Our investigations with vanadium-based PTP inhibitors suggest that the effects of PTK activation and PTP inhibition can be distinguished, in that not all of the observed effects of PTP inhibition appeared to require PTK activation. As the effects of intracellular oxidation on signal transduction pathways are elucidated, particular PTPs may emerge whose susceptibility toward oxidative stress governs the cellular response to environmental toxicants. The identification of PTPs and PTKs with differential sensitivity to redox-regulated inhibition or activation may in turn shed light on the regulation of cellular function by endogenously produced oxidants.
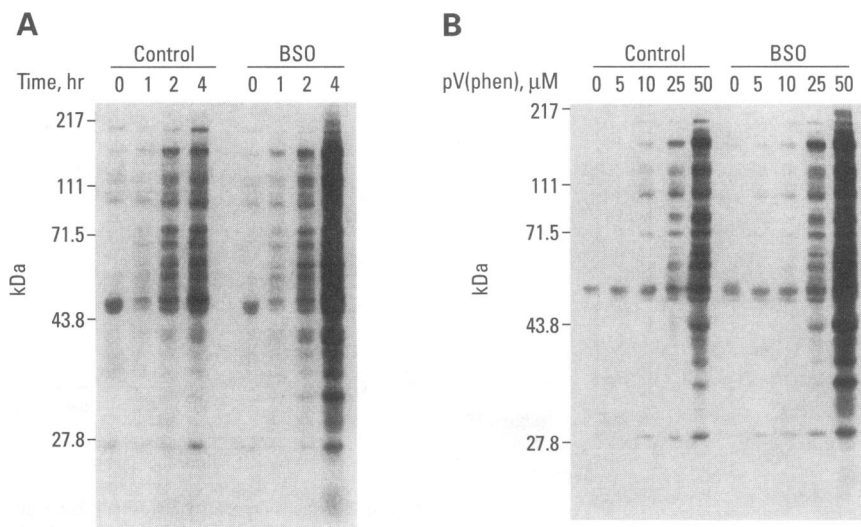
Figure 3. GSH depletion enhances intracellular phosphotyrosine accumulation after PTP inhibition. Ramos B cells were treated overnight with 100 μM BSO to deplete intracellular GSH before pV(phen) treatment. Cells were lysed and immunoblotted and blots were stained for phosphotyrosine. (A) Cells treated with 25 μM pV(phen) for 1 to 4 hr. (B) Cells treated for 2 hr with pV(phen) at indicated doses.

Conclusion

Signal transduction requires the concerted actions of a great many signaling molecules, and small changes in the activity of these molecules are amplified along signaling cascades. Therefore the impairment of function of only a few oxidation-sensitive PTPs may result in a shift in the balance of, phosphotyrosine along certain pathways at the expense of others. Communication between the cell surface and the nucleus might be altered subtly at each level along the signaling pathway, with message fidelity progressively lost as the signal passes on. In cases of stronger oxidative insult, the inhibition of PTPs synergizes with the oxidative induction of PTK activity, and signaling cascades normally under receptor control are initiated in a ligand-independent manner.

The long-term effects of oxidant initiated signaling cascades *in vivo* are unknown, but given the complexities of lymphocyte receptor interactions and the elaborate controls placed on induction of immune responses, it is expected that oxidant-induced signals will have detrimental effects. The studies to date suggest that these principles apply as well to other tissues in which phosphorylation pathways control cellular function. Oxidative stress has been implicated the etiology of a wide variety of diseases, many of which are believed to have environmental causes. Further research is needed to learn the mechanisms by which lymphocytes resist oxidative stress, and to identify effects of environmental oxidants, especially sulfhydryl-reactive transition metals, on the control of signal transduction cascades by PTPs. Studies of cellular interactions and *in vivo* effects can enhance understanding of how oxidative stress can increase susceptibility to immune disorders and reveal possible strategies for prevention or mitigation of such illnesses.

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