The role of intracellular oxidative stress in the mechanism of action of phosphotyrosine phosphatase (PTP) inhibitors was studied using three vanadium-based compounds. Sodium orthovanadate (Na$_3$VO$_4$), sodium oxodiperoxo(1,10-phenanthroline)vanadate(V) (pV(phen)), and bis(maltolato)-oxovanadium(IV) (BMOV) differentially induced oxidative stress in lymphocytes. Treatment with pV(phen), which caused intracellular oxidation, induced strong protein tyrosine phosphorylation compared with Na$_3$VO$_4$ and BMOV. Syk family kinases and the mitogen-activated protein kinase erk2 were rapidly activated by pV(phen) but not by BMOV or Na$_3$VO$_4$. In contrast, both BMOV and pV(phen) strongly activated NF-xB. The antioxidant pyrrolidine dithiocarbamate (PDT) greatly diminished the intracellular oxidation and protein phosphotyrosine accumulation induced by pV(phen). Pretreatment of cells with PDT reduced and delayed the activation of Syk kinases and erk2. However, NF-xB activation by pV(phen) was markedly enhanced in lymphocytes pretreated with PDT, and another antioxidant, N-acetylcysteine, did not prevent the activation of NF-xB by BMOV. These results indicate a role for oxidative stress in the biological effects of some PTP inhibitors, whereas NF-xB activation by PTP inhibitors is mediated by mechanisms independent of intracellular redox status.

Lymphocyte signal transduction requires the activation of protein tyrosine kinases (PTKs), with subsequent assembly of signaling complexes, generation of second messengers, activation of transcription factors, and gene expression (1, 2). The balance of protein tyrosine phosphorylation within the cell is controlled by the relative activities of the PTKs and PTPs in the signaling network (3). Besides dephosphorylating a variety of PTK substrates, PTPs have been shown to directly modulate the activities of PTKs (4, 5). Thus PTPs serve a crucial function in lymphocytes by controlling both the initiation and termination of receptor-based signals.

The inhibition of PTPs reveals PTK substrates on which phosphotyrosine accumulates in the absence of receptor engagement (6). Some of these substrates are key phosphoproteins in lymphocyte signal transduction pathways, suggesting that PTKs involved in transmission of receptor signals are activated by the absence of PTP regulation (6–9). However, many of the PTP inhibitors used thus far to explore lymphocyte signal transduction pathways are redox-active compounds. For example, phenylarsine oxide, a thiol-reactive compound, and H$_2$O$_2$, which generates hydroxyl radicals, both act as potent PTP inhibitors (6, 10). The role of intracellular oxidation in the mechanism of action of PTP inhibitors is unknown, a question this study addresses.

Vanadium-based PTP inhibitors, which have been extensively studied as insulin mimetic agents, stimulate glucose uptake and fatty acid synthesis in adipocytes and mimic receptor-based signals in lymphocytes (11–15). The widely used PTP inhibitor pervanadate is a peroxovanadium compound generated by reaction of H$_2$O$_2$ with orthovanadate. Pervanadate is much more potent than H$_2$O$_2$ or Na$_3$VO$_4$, causing strong and rapid accumulation of intracellular protein phosphotyrosine and activating Src family kinases in T-lymphocytes (7, 8). Cytokine signaling via the transcription factor Stat1a is also mimicked by pervanadate but not orthovanadate or vanadyl sulfate (16). Since pervanadate is highly unstable and must be used within minutes of generation (17, 18), the molecular mechanism of its action has not been extensively studied but rather has been attributed solely to effects on PTP activity.

The development of stabilized peroxovanadium compounds (19) such as pV(phen) (Fig. 1A) provides the opportunity to more fully characterize the action of peroxovanadium within the cell. The action of vanadium can also be modified by organic ligands that facilitate cellular targeting and biochemical specificity (20, 21); for example, we found that BMOV (Fig. 1B) induced apoptosis in B cell lineages but enhanced the activation of T cell lineages (15). In this study, we compare the action of pV(phen), BMOV, and Na$_3$VO$_4$ on lymphocyte signal transduction pathways and show that redox-dependent effects such as early kinase activation occur in concert with the redox-independent activation of NF-xB.

Treatment of a B cell line (Ramos) or a T cell line (Jurkat) with pV(phen) caused strong increases in intracellular oxidation, while equimolar doses of Na$_3$VO$_4$ or BMOV produced only small changes in cellular redox status. pV(phen) caused strong and rapid induction of protein tyrosine phosphorylation in both cell lines. By comparison, BMOV or Na$_3$VO$_4$ did not appreciably affect global tyrosine phosphorylation in the doses and...
times assayed. Syk tyrosine kinase was activated in cells by pV(phen) treatment via an indirect mechanism, since the isolated kinase was not activated by treatment in vitro. The MAPK erk2 was activated by pV(phen) but not by BMOV or Na3VO4 in both cell lines. Both BMOV and pV(phen) strongly activated the transcription factor NF-xB. Pretreatment with antioxidants prevented development of intracellular oxidation and greatly diminished both the pV(phen)-induced accumulation of protein tyrosine phosphorylation and the activation of Syk, ZAP-70, and erk2 following pV(phen) treatment. Surprisingly, antioxidant treatment did not prevent the activation of MAPK erk2 by pV(phen) but not by BMOV or pV(phen). These results indicate that for many of the effects of pV(phen), the generation of oxidative stress is necessary. Here, we bring together results from vanadium compounds that differ in their ability to induce intracellular oxidation, along with effects of antioxidants on PTP inhibitor action, to provide novel evidence for the redox-independent activation of NF-xB triggered by PTP inhibition.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—The human B cell lymphoma line Ramos and the human T cell leukemia line Jurkat were cultured in RPMI 1640 media (Life Technologies, Inc.) supplemented with 10% fetal bovine serum.

Sodium oxodiperoxo(1,10-phenanthroline)vanadate(V) (pV(phen)) and bis(maltolato)oxovanadium(IV) (BMOV) were synthesized as described previously (15, 19), and the products were characterized by infrared and mass spectroscopy. Pyrrolidine dithiocarbamate (PDTC) and sodium orthovanadate were purchased from Aldrich.

**Infrared and Mass Spectroscopy.** Pyrrolidine dithiocarbamate (PDTC) serum.

**Cells and Reagents—**The human B cell lymphoma line Ramos and the human T cell leukemia line Jurkat were cultured in RPMI 1640 (no phenol red) supplemented with 10 mM HEPES buffer (Life Technologies, Inc.). Cells were incubated at 37 °C for 2 h and then washed with phosphate-buffered saline. The development of DCF fluorescence was measured on a Cytofluor 2300 fluorescence plate reader (Molecular Dynamics, Sunnydale, CA; excitation = 485 nm, emission = 530/20 nm). Alternatively, DCF fluorescence was measured in cells using flow cytometry (FACSCAN, Becton Dickenson, Mountain View, CA). For PTD-treated cells, a 60-min incubation with PTDTC preceded the addition of pV(phen). Following each plate assay, cell viability was determined by trypan blue exclusion; in flow cytometric studies non-viable cells were gated out of the sample population on the basis of light scattering characteristics.

**Analysis of Phosphate Activity—**Activity of a GST-phosphotyrosine phosphatase 1B fusion protein (PTPB1B) was assessed against the phosphatase substrate pNPP (Boehringer Mannheim). The PTP1B was supplied bound to reduced glutathione beads. For IC50 determinations, the PTPB1B was eluted from beads with three changes of elution buffer (5 mM reduced glutathione, 5 mM DTT, 1% bovine serum albumin, 150 mM NaCl, 25 mM imidazole, pH 7.2), and activity against pNPP was assayed in PTP buffer (60 mM MES, 5% glycerol, 3 mg/ml pNPP) in the absence or presence of varying concentrations of inhibitor. The rate of pNPP formation was measured over 15 min following the addition of substrate, and the Vmax (A405–725/min) was recorded. In a second set of experiments, the enzyme-agarose was incubated on ice for 30 min in the presence of 5 μM inhibitor and then repeatedly washed with buffer containing 10 mM EDTA and no inhibitor. The PTP1B was then suspended in PTP buffer without inhibitors and activity against pNPP was assayed as above.

**Immune Complex Phosphatase Assays—**For CD45 studies, cells were pretreated with PTDTC for 1 h, followed by incubation with pV(phen) for up to 3 h. Proteins were extracted with Nonident P-40 lysis buffer, and the mAb ITA6 was used to immunoprecipitate CD45. After extensive washing, the immune complex was resuspended in PTP buffer containing no inhibitors, and activity against pNPP was assayed as described above.

**Analysis of Tyrosine Phosphorylation—**Cells were lysed and blotted by standard methods. Polyvinylidene difluoride membranes were stained with affinity purified anti-phosphotyrosine antibodies, with antibody binding detected by [32P]protein A followed by autoradiography, as described previously (25). Alternatively, some blots were stained with the monoclonal antibody 4G10 (Upstate Biotechnology, Lake Placid, NY) followed by enhanced chemiluminescence (ECL, Amersham Corp.).

**Analysis of Syk Kinase Activity—**Syk was immunoprecipitated from Ramos cell lysates with rabbit anti-human Syk (N-terminal region), and the immune complex was incubated for 10 min at room temperature in the presence of [32P]ATP as described previously (25). Proteins were separated by gel electrophoresis, transferred to polyvinylidene difluoride membrane, then incubated for 1 h in 1 M KOH at 55 °C to hydrolyze phosphoserine and phosphothreonine. The blots were dried, and autoradiography was performed. Following this exposure, the blots were rewetted and stained with rabbit anti-porcine Syk, followed by ECL to quantify Syk protein levels.

**Detection of NF-xB Activation—**Jurkat cells were washed once in phosphate-buffered saline followed by preparation of nuclear extracts using a modification of the procedure of Dignam et al. (26). Briefly, 1 × 106 cells were lysed in buffer A (10 mM HEPES, 1.5 mM MgCl2, 10 mM NaCl, 0.25% Nonidet P-40, pH 7.5) for 5 min at 4 °C, followed by centrifugation for 10 min. The supernatant (cytosol) was removed and the nuclei extracted with buffer C (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM MgCl2, 0.25% Nonidet P-40, pH 7.5). The nuclei were vortexed vigorously several times over 20 min, followed by centrifugation at 14,000 rpm for 5 min. The supernatant (nuclear extract) was removed and diluted 1:2 with buffer D (20 mM HEPES, 50 mM KCl, 0.2 mM EDTA, 20% glycerol, pH 7.5) and frozen at −80 °C until use. Gel mobility shift assays were carried out on 5–15 μg of nuclear extract. Samples were mixed with 1.5 μg of poly(dI-dC) and 2–3 × 104 cpm of double-stranded oligonucleotide probe (5'-AGT-TAGGGGACTTCCCCAGG-3') for 30 min at room temperature prior to loading on the gel. Samples were resolved on a 6% non-denaturing Tris borate EDTA polyacrylamide gel (Novex, San Diego, CA); the gels were dried and labeled protein was detected by PhosphorImager analysis (Molecular Dynamics, Sunnydale, CA).

**RESULTS**

**pV(phen) Strongly Induces Lymphocyte Tyrosine Phosphorylation and Intracellular Oxidation Relative to Na3VO4 and Na3VO4**
BMOV—Treatment with micromolar concentrations of pV(phen) led to dose-dependent increases in protein phosphotyrosine in Jurkat (Fig. 2A) and Ramos cells (Fig. 2B). Treatment with BMOV resulted in slightly increased phosphotyrosine levels in Ramos cells; however, the induction was substantially less than previously observed with longer treatment times (15).

Treatment with Na₃VO₄ caused very little change in either cell line.

pV(phen) differs from Na₃VO₄ and BMOV by the presence of two peroxy groups, which alter its chemical reactivity and may contribute to the inhibition of PTP activity. We considered that generation of oxidative stress might be important, as the peroxy groups might participate in intermolecular redox cycling. The reduced fluorescein compound DCF-DA has been widely used to assess intracellular oxidation (24); we employed this probe to compare the effects of the three vanadium PTP inhibitors on cellular oxidation state. Jurkat cells were treated with DCF-DA and exposed to Na₃VO₄, BMOV, or pV(phen) for up to 2 h, and DCF oxidation was measured by flow cytometry. The pV(phen) treatment greatly induced intracellular DCF fluorescence compared with controls and to cells treated with equimolar concentrations of Na₃VO₄ or BMOV (Fig. 3A). Cells pretreated with the antioxidant pyrrolidine dithiocarbamate (PDTC, 100 μM) had lower basal oxidation and did not develop high DCF fluorescence following 2-h treatments with pV(phen), orthovanadate, or BMOV (Fig. 3B). In both Jurkat and Ramos cells, the increased DCF fluorescence in pV(phen) treated cells was time-dependent (see Fig. 4B) and dose-dependent (data not shown). Pretreatment of the cells with PDTC in a range of concentrations from 25 to 300 μM effectively eliminated the intracellular oxidation (data not shown).

Intracellular Oxidation by pV(phen) Is Independent of Kinase Activation—As the mechanism for development of intracellular oxidation following pV(phen) treatment was not known, we considered the possibility that the redox changes could be triggered by a kinase-dependent event. Examples of this type of mechanism have been found in other cell-signaling systems (27, 28). We pretreated Jurkat and Ramos cells with the tyrosine kinase inhibitor herbimycin A, which blocks antigen-receptor induced tyrosine phosphorylation (2). Herbimycin A treatment completely prevented the induction of protein tyrosine phosphorylation by pV(phen) (Fig. 4A). The DCF-DA assay was performed on cells pretreated with herbimycin A and control cells treated with Na₃VO₄, BMOV, or pV(phen). The dose- and time-dependent pattern of intracellular oxidation was similar for herbimycin A-pretreated cells and control cells treated with each of the different PTP inhibitors, although the herbimycin A-treated cells developed slightly enhanced intracellular oxidation compared with cells with no pretreatment (Fig. 4B). This finding confirms that the accumulation of protein phosphotyrosine following pV(phen) treatment requires kinase activation; however, intracellular oxidation occurs by a mechanism that is independent of PTK activity.

pV(phen)-induced Phosphotyrosine Accumulation Is Dependent Upon Intracellular Oxidation—Pretreatment of Ramos or Jurkat cells with the antioxidant PDTC inhibited the induction of protein phosphotyrosine by pV(phen). A time course was performed on Ramos cells; treatment for 2–8 h with pV(phen) resulted in the time-dependent accumulation of intracellular phosphotyrosine. Pretreatment with PDTC both delayed and decreased the response (Fig. 5A). The widely used antioxidants NAC and DTT could not be used because they rapidly degraded the pV(phen) in vitro. Analysis of the absorbance spectra of pV(phen) indicated no degradation of the pV(phen) by PDTC at concentrations 10-fold higher than those used in this study.
control PTP1B activity was slightly decreased if the enzyme through thiol oxidation. Preliminary experiments showed that could target this catalytic cysteine and inactivate the enzyme must be maintained in reduced form for catalysis to occur (10, PTPs contain an active site thiol which Oxidative Mechanism—

BMOV; L m (data not shown). Pretreatment with 300 pV(phen) or receptor engagement this effect was small. compared with the phosphotyrosine increases stimulated by herbimycin A prior to treatment with pV(phen) at the indicated doses. open symbols), or cells pretreated overnight with herbimycin A (closed symbols) prior to 2 h treatment with PTP inhibitors (25 μM). □, control; Δ, Na3VO4; ○, BMOV; ●, pV(phen). 

(data not shown). None of the PDTC doses employed in this study interfered with protein tyrosine phosphorylation following antigen receptor stimulation in B cells (Fig. 5B) or T cells (data not shown). Pretreatment with 300 μM PDTC prior to receptor stimulation did not interfere with Syk, Zap-70 activation, or erk2 activation.2 The PDTC treatment induced the tyrosine phosphorylation of a few protein substrates; however, compared with the phosphotyrosine increases stimulated by pV(phen) or receptor engagement this effect was small. 

1,10-Phenanthroline Increases Intracellular Oxidation but Not Protein Tyrosine Phosphorylation—The ligand 1,10-phenanthroline (PA) has been shown to chelate a variety of redox-active metals including copper (29); we therefore considered the possibility that PA was dissociating from the pV(phen) complex and inducing the generation of reactive oxygen within the cell. PA treatment did enhance intracellular oxidation, albeit to lower levels than pV(phen) (data not shown). However, protein tyrosine phosphorylation within the cell was not increased by PA even in combination with equimolar Na2VO4 (data not shown). Our results suggest that 1) the presence of oxidative stress alone is not enough to account for the massive protein tyrosine phosphorylation seen in pV(phen)-treated cells; and 2) the action of pV(phen) is particular to its structure and cannot be duplicated by the combination of PA plus Na2VO4.

pV(phen) Irreversibly Inhibits PTP1B and CD45 Through an Oxidative Mechanism—PTPs contain an active site thiol which must be maintained in reduced form for catalysis to occur (10, 18, 30). We postulated that the peroxy groups on pV(phen) could target this catalytic cysteine and inactivate the enzyme through thiol oxidation. Preliminary experiments showed that control PTP1B activity was slightly decreased if the enzyme was assayed in the absence of antioxidants; however, pV(phen) was a much more potent PTP inhibitor under these conditions. Therefore we tested the ability of pV(phen) to irreversibly inhibit the PTP1B enzyme-agarose complex under nonreducing conditions. PTP1B was completely inhibited by incubation with 5 μM of pV(phen), BMOV, and Na3VO4. However, PTP1B activity was recovered if the BMOV- or Na3VO4-treated enzyme-agarose was washed repeatedly in buffer containing 10 mM EDTA, whereas pV(phen) inhibited PTP1B irreversibly (Fig. 6A). This inhibition appears to be due to thiol oxidation because washing the pV(phen)-treated enzyme with 5 mM DTT or 1% 2-mercaptoethanol in the wash buffer could partially restore enzyme activity.2 To test the relevance of this finding in lymphocytes, the PTP CD45 was immunoprecipitated from control Jurkat and Ramos cells and cells treated with 25 μM pV(phen) for 3 h prior to lysis. After extensive washing, immune complex phosphatase assays were performed. Jurkat cells had significantly higher CD45 activity than did Ramos; however, CD45 from both cell types was completely inactivated if the cells were treated with pV(phen) (Fig. 6B). To test the ability of PDTC to protect CD45 activity in situ, Jurkat cells were pretreated with 100 μM PDTC prior to treatment with pV(phen). Cells were lysed, and CD45 was immunoprecipitated and assayed as described above. Pretreatment of the lymphocytes with PDTC prevented the complete inactivation of CD45 in pV(phen)-treated cells, although substantial inhibition was observed. PDTC did not alter the CD45 activity from control cells (Fig. 6C). These results suggest that the antioxidant treatment may inhibit the phosphotyrosine accumulation within the cell by

2 C. Krejsa, unpublished results.
protecting PTPs against irreversible inhibition.

The antioxidant PDTC decreased both the induction of oxidative stress and the accumulation of phosphotyrosine in cells treated with pV(phen). To test if PDTC were interfering with the ability of pV(phen) to inhibit phosphatases, PTP1B activity against the substrate pNPP was assessed in the presence of PDTC and the three vanadium PTP inhibitors. PTP1B was inhibited by nanomolar concentrations of pV(phen), BMOV, and Na3VO4 in the presence of 300 μM PDTC (Fig. 6D). This result is consistent with assays using PTP1B against a phosphotyrosine peptide substrate (data not shown). Taken together, our results indicate that PDTC does not block the direct inhibition of PTPs by the vanadium compounds but rather prevents irreversible inhibition by protecting against the oxidation of sensitive thiols in the PTPs.

pV(phen) Activation of Protein Kinases Is Oxidation-dependent—Oxidative stress is known to alter lymphocyte signal transduction pathways by activating protein kinases (for review see Ref. 31), including activation of the PTKs Syk and ZAP-70 (23, 25) as well as MAP kinases (32). The combination of H2O2 and vanadate, but neither compound alone, was shown to activate the Src family kinases p59vιιο and p56Δικ (9). We hypothesized that since pV(phen) increases intracellular oxidation, it might be activating PTKs in a similar manner to H2O2; this kinase activation combined with phosphatase inhibition could be responsible for the massive accumulation of phosphotyrosine seen in pV(phen)-treated cells. To address this question, we first performed immune complex kinase assays on Syk PTK immunoprecipitated from Ramos cell lysates. Cells were treated with pV(phen) overnight prior to cell lysis, or, alternatively, the pV(phen) was added to the immunoprecipitated Syk kinase just prior to the kinase assay. Syk from pV(phen)-treated cells was highly activated, much more than Syk from cells activated by receptor engagement (Fig. 7A). The protein levels of immunoprecipitated Syk did not change due to treatment (Fig. 7B). Interestingly, addition of pV(phen) directly to the kinase assay did not activate Syk, indicating the activation of Syk by pV(phen) is an indirect effect.

We also examined the role of intracellular oxidation in the activation of Syk and ZAP-70 by pV(phen). As Syk and ZAP-70 are activated by tyrosine phosphorylation following antigen receptor engagement, the phosphotyrosine status of these proteins was used as a marker for their activation as has been previously described (25, 33). Treatment with pV(phen) resulted in the time-dependent activation of Syk (Fig. 8A, top panels) and ZAP-70. Pretreatment with PDTC inhibited the activation of these kinases (Fig. 8A), although some activation of Syk occurred at later time points, consistent with the late induction of tyrosine phosphorylation in whole cell lysates (Fig. 8A). The blots were stripped and stained with anti-Syk; recovery of Syk protein was similar for all treatments (Fig. 8A, bottom panels).
bottom panel). These results, taken together with the finding that pV(phen) does not directly activate Syk, indicate that there is a redox-sensitive step in the signaling pathway which occurs upstream of Syk family kinases.

To assess the downstream effects of pV(phen) treatment, we investigated the activation of erk2, a p42 MAPK isoform, which phosphorylates the transcription factor Elk-1 (34). The Ras pathway has been shown to be sensitive to oxidative stress in rat PC12 cells (35), and in NIH3T3 cells the antioxidant NAC inhibits the activation of MAPK by H$_2$O$_2$, x-irradiation, and phorbol esters (32). Furthermore, the MAPK pathway is negatively regulated by the dual-specificity phosphatase MKP-1 (36, 37). MAP kinase activation state was detected by a shift in electrophoretic mobility of the phosphorylated protein. Treatment with pV(phen) resulted in a dose-dependent shift to the activated form of erk2 in both Jurkat T cells and Ramos B cells (Fig. 8B). Pretreatment with PDTC caused a very slight activation of MAPK but prevented any further activation by pV(phen). Neither Na$_3$VO$_4$ nor BMOV induced the activation of MAPK (data not shown).

**Accumulation of Phosphotyrosine Is Delayed Compared with CD45 Inhibition**—To assess the relative effects of phosphatase inhibition and kinase activation by pV(phen), we treated Jurkat cells with 25 $\mu$M PTP inhibitor and assayed CD45 activity and phosphotyrosine accumulation after 30, 60, 90, or 120 min treatment. CD45 was completely inactivated in cells treated with pV(phen) for 30 min or longer, whereas cells treated with Na$_3$VO$_4$ for 120 min had only partly inhibited CD45 activity (Fig. 9A). Recovery of CD45 from Jurkat cells was similar for all treatments (Fig. 9B). The accumulation of phosphotyrosine in pV(phen)-treated cells was delayed compared with inactivation of CD45 (Fig. 9C), suggesting some secondary mechanism was primarily responsible for the strong activation of cellular phosphorylation pathways. The induction of oxidative stress by pV(phen) is also time-dependent (see Fig. 4B) and is required both for kinase activation and for the accumulation of cellular phosphotyrosine. Furthermore, no phosphotyrosine accumulation was seen in Na$_3$VO$_4$-treated cells even though the CD45 from these cells was partially inhibited following 120 min of treatment. These results, taken together with the studies above, suggest that peroxovanadium compounds act through the generation of intracellular oxidative stress which induces cellular signaling pathways and that these effects are mediated by the activation of kinases.

**pV(phen) Induces Tyrosine Phosphorylation of I$\kappa$B**—The transcription factor NF-$\kappa$B, which controls many of the functional effects of lymphocyte activation, is bound to the inhibitory protein I$\kappa$B in the cytosol of unactivated cells. I$\kappa$B prevents translocation of NF-$\kappa$B to the nucleus and is degraded via the ubiquitin/proteosome pathway following a variety of treatments (38). Recently it has been reported that hypoxia induces the activation of NF-$\kappa$B by a mechanism which involves I$\kappa$B phosphorylation on tyrosine (39). Further studies indicated that treatment with pervanadate, but not orthovanadate or H$_2$O$_2$ alone, resulted in I$\kappa$B tyrosine phosphorylation and activation of NF-$\kappa$B without causing I$\kappa$B degradation (40). We found that in Jurkat cells treated with pV(phen), I$\kappa$B was tyrosine-phosphorylated and shifted to a lower mobility form, whereas in cells treated with BMOV, no tyrosine phosphorylation of I$\kappa$B was observed (Fig. 10). Treatment with 300 $\mu$M H$_2$O$_2$, which activated NF-$\kappa$B, did not cause tyrosine phosphorylation of I$\kappa$B (data not shown). Pretreatment of the cells with the antioxidant PDTC prevented the tyrosine phosphorylation of I$\kappa$B (Fig. 10A), whereas this pretreatment did not prevent the pV(phen)-induced activation of NF-$\kappa$B (see below).

Interestingly, we observed significant reduction of I$\kappa$B protein levels following pV(phen) or BMOV treatment (Fig. 10B). These results suggest that peroxovanadium compounds cause the induction of I$\kappa$B tyrosine phosphorylation via a mechanism...
which requires intracellular oxidation; however, IκBα tyrosine phosphorylation is not necessary for the activation of NF-κB by PTP inhibitors.

**BMOV and pV(phen) Activate NF-κB by a Mechanism Independent of Intracellular Oxidation**—Numerous reports have demonstrated that NF-κB is sensitive to intracellular redox changes, and the antioxidants PDTC and NAC have been widely used to inhibit NF-κB activation (41, 42). We have previously reported that the combination of H₂O₂ and vanadate can activate NF-κB (9). However, a recent study of PTP inhibitors showed that peroxovanadate and even the sulphydryl reactive compounds diamide and phenyl arsine oxide were inhibitors of TNF-induced NF-κB activation (43). Therefore it was of great interest to examine NF-κB responses to pV(phen) and BMov. We performed gel shift assays on nuclear extracts from Jurkat cells treated with PTP inhibitors, H₂O₂, and PDTC. Treatment with 25 μM pV(phen) activated NF-κB more strongly than did 300 μM H₂O₂ (Fig. 11A). In cells pretreated with 300 μM PDTC, as expected, the H₂O₂ activation of NF-κB was prevented. However NF-κB activation by pV(phen) was actually enhanced in cells pretreated with PDTC. We repeated the assay with the antioxidant NAC, which has been shown to block NF-κB activation following a variety of stimuli. BMov can be used in combination with NAC, whereas pV(phen) is not stable in the presence of NAC, preventing its use for this assay. NF-κB was strongly activated in Jurkat cells treated with 200 μM BMov, even though this PTP inhibitor does not induce strong intracellular oxidation. Although NAC completely prevented the activation of NF-κB in H₂O₂-treated cells, it only partially reduced the NF-κB activation following BMov treatment (Fig. 11B). These results demonstrate the activation of NF-κB by vanadium PTP inhibitors by a mechanism that is independent of intracellular oxidation.

**DISCUSSION**

We have characterized oxidation-sensitive and oxidation-independent effects of three structurally distinct vanadium PTP inhibitors, pV(phen), BMov, and Na₃VO₄. Each of these compounds was shown to inactivate PTP1B at nanomolar concentrations in vitro. In lymphocytes treated with the peroxovanadium compound pV(phen), large increases in intracellular oxidation were correlated with the strong induction of cellular tyrosine phosphorylation and activation of kinases. Treatment with BMov or Na₃VO₄, which do not contain peroxo groups, did not cause intracellular oxidation and resulted in very little tyrosine phosphorylation. However, BMov activated NF-κB without inducing strong phosphotyrosine accumulation or oxidative stress. Both the accumulation of phosphotyrosine and the activation of specific kinases were diminished when PDTC was employed to prevent intracellular oxidation. Whereas the H₂O₂-induced activation of NF-κB was inhibited by the antioxidants PDTC and NAC, these antioxidants failed to prevent NF-κB activation in cells treated with PTP inhibitors. This suggests a dual mechanism of action for redox-active PTP inhibitor compounds, in which the early activation of kinases requires the development of intracellular oxidation, while other important responses such as NF-κB activation occur through redox-insensitive pathways.

Oxidative stress is known to alter lymphocyte signal transduction pathways by activating PTKs (23, 25), p21<sup>ras</sup> and the MAPK pathway are also sensitive to changes in cellular redox status (32, 35). We found that the activation of kinases by pV(phen) was dependent upon its ability to induce oxidative stress. The effect required the intracellular milieu, however, as pV(phen) added directly to Syk kinase did not alter its activity in vitro. Similarly, the antioxidant PDTC did not prevent the inhibition of PTP1B by pV(phen) in vitro, but it partially protected CD45 from inactivation by pV(phen) in cells. This indicates the presence of a redox-active mediator that is triggered by pV(phen) but not BMov or Na₃VO₄. The redox-sensitive steps in lymphocyte signaling cascades are not known but could involve changes in the associations between key signaling proteins, alterations in protein conformation, or the generation of a second messenger such as a reactive oxygen species.

The transcription factor NF-κB, which is essential for T cell activation and B cell differentiation, is known to be activated by oxidative stress. NF-κB activation following a wide variety of stimuli has been prevented by the antioxidants NAC and PDTC (41, 44). Since a variety of signals leading to NF-κB
activation may be blocked by antioxidants, a reactive oxygen species has been the postulated second messenger in NF-κB activation pathways (45, 46). However, some studies indicate that more complex regulation of NF-κB exists, involving both oxidation-sensitive and redox-independent pathways. The role of oxidation in the activation of NF-κB has been shown to vary by cell type (47). For example, oxidative stress may play a role in NF-κB activation by the serine-threonine phosphatase inhibitor okadaic acid, but experiments using Jurkat cells and HeLa cells yielded contradictory results (48, 49).

Tyrosine kinase activity also appears to be necessary for the induction of NF-κB (9, 23, 50). However, the role of tyrosine phosphorylation in NF-κB activation is much less well defined than the classical serine phosphorylation pathway leading to ubiquitination and degradation of IκBα. Both the tyrosine phosphorylation of IκBα and activation of NF-κB following hypoxia have been reported to be prevented by PTK inhibitors (39). Pervanadate was reported to activate NF-κB through a mechanism that induced IκBα tyrosine phosphorylation and did not require the proteosomal degradation of IκBα (40). We found that pV(phen) likewise induced the tyrosine phosphorylation of IκBα; however, cytosolic levels of IκBα were substantially decreased by pV(phen) treatment (Fig. 10B). The degree of IκBα degradation in pV(phen)-treated cells was similar to that seen following BMOV treatment, which did not induce the tyrosine-phosphorylated form of IκBα. Antioxidant treatment reduced the apparent degradation of IκBα and inhibited its tyrosine phosphorylation without preventing the activation of NF-κB.

In the present study the activation of NF-κB by H2O2 was prevented by PDTC pretreatment, whereas NF-κB activation by pV(phen), which exerts many of its effects through an oxidative mechanism, was enhanced rather than prevented by the antioxidant. By comparison, BMOV induced much smaller amounts of intracellular oxidation, and pretreatment with the antioxidant NAC did not block NF-κB activation, although it was reduced. These results suggest that multiple pathways, including redox-sensitive and redox-insensitive mechanisms, can lead to the activation of NF-κB. It is also possible that pV(phen) and BMOV affect NF-κB responses differently following nuclear translocation, since DNA binding of NF-κB has been shown to be inhibited under oxidizing conditions (51, 52). The strong intracellular oxidation seen with pV(phen) may result in diminished DNA binding independent of nuclear translocation of NF-κB, leading to a less than maximal response. PDTC may potentially improve the NF-κB-DNA interaction by blocking oxidative alteration of the DNA-binding site. However, even in the absence of PDTC treatment with 25 μM pV(phen) induced greater NF-κB-DNA binding than treatment with 300 μM H2O2. The pV(phen)-induced binding was enhanced in the presence of PDTC, whereas H2O2-induced binding was totally abrogated. Taken together, these results suggest the existence of an efficient, redox-insensitive pathway for NF-κB activation, which can be stimulated by the inhibition of PTPs.

PDTC has been widely used as an antioxidant, and its ability to prevent oxidative stress may be determined by the combination of its functions as a metal chelator and a thiol-reducing agent (42). A recent report demonstrated that low dose treatments with PDTC could actually increase oxidative stress within thymocytes, due to increased cellular import of copper, a highly redox-active metal (53). We did not observe this effect within the PDTC dose and time regimes we tested; the PDTC acted only as an antioxidant in our hands. However, PDTC at high doses was toxic to cells despite its ability to prevent intracellular oxidation. The mechanism for this toxicity was not determined. In the short term, low dose experiments performed, the PDTC treatments did not result in cell death.

Vanadium PTP inhibitors have been extensively used as tools for elucidating the regulation of cellular signal transduction by phosphatases. These compounds have been shown to normalize glucose levels in animal models of diabetes mellitus and have achieved favorable responses in human clinical trials (54). Likewise, they could prove important in other applications for which the control of cellular tyrosine phosphorylation is desired. In the present study, we found that differences in the effects of three structurally distinct vanadium PTP inhibitors could be partially explained by their ability to induce intracellular oxidative stress. Our finding that antioxidant treatment can protect cellular CD45 from total inactivation by pV(phen) suggests a direct role for oxidative stress in the greatly increased potency of peroxo derivatives of vanadate. A recent publication (18) comparing pervanadate to Na3VO4 showed that in PTP1B the active site cysteine was oxidized to cysteic acid by pervanadate, whereas Na3VO4 did not permanently alter the active site thiol. We have shown that while the development of oxidative stress causes both the activation of kinases and the irreversible inactivation PTPs, the contribution of kinase activation is more important than PTP inhibition for the accumulation of phosphotyrosine within the cell. However, the finding that oxidative stress and phosphotyrosine accumulation are not required for NF-κB activation by vanadium PTP inhibitors suggests that PTP inhibition activates signaling cascades which are more difficult to observe, albeit quite important to understand. Our results demonstrate a role for oxidative stress in many of the effects of PTP inhibition and suggest the presence of a PTP-regulated, redox-independent pathway for NF-κB activation.

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