Activation of Mitogen-activated Protein (MAP) Kinase Pathway by Pervanadate, a Potent Inhibitor of Tyrosine Phosphatases*

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Rapid tyrosine phosphorylation of key cellular proteins is a crucial event in signal transduction. The regulatory role of protein-tyrosine phosphatases (PTPs) in this process was explored by studying the effects of a powerful PTP inhibitor, pervanadate, on the activation of the mitogen-activated protein (MAP) kinase cascade. Treatment of HeLa cells with pervanadate resulted in a marked inhibition of PTP activity, accompanied by a drastic increase in tyrosine phosphorylation of cellular proteins. The increased tyrosine phosphorylation coincided with the activation of the MAP kinase cascade as indicated by enzymatic activity assays of MEK (MAP kinase/ERK-kinase) and MAP kinase and gel mobility shift analyses of Raf-1 and MAP kinase. The activation was sustained but reversible. Upon removal of pervanadate, both tyrosine phosphorylation and MAP kinase activation declined to basal levels. Therefore, inhibition of PTP activity is sufficient per se to initiate a complete MAP kinase activation program.

Protein tyrosine phosphorylation plays a crucial role in signal transduction. The steady state of such phosphorylation is controlled by coordinate actions of protein-tyrosine kinases (PTKs) and phosphatases (PTPs) (1). Growth factors, hormones, cytokines, and antigens shift the balance by rapidly stimulating PTK activity, thus inducing tyrosine phosphorylation and initiating signal transduction. On the other hand, inhibition of the phosphatases has been shown to mimic certain aspects of signal transduction that are normally triggered by tyrosine kinase activation. Among various PTP inhibitors, vanadate and its derivative, pervanadate, have been found widespread use as inhibitors of PTPs (2). These compounds cause increases in tyrosine phosphorylation and various cell signaling responses. For example, vanadate induces transformation of NRK-1 cells (3). Vanadate and pervanadate are able to mimic insulin in stimulating glucose transport, lipogenesis, protein synthesis, and inhibiting lipolysis (4–8). In T cells and platelets, pervanadate induces phospholipid hydrolysis and increases intracellular Ca\(^{2+}\) (9–12), while in rat pheochromocytoma PC12 and human neuroblastoma SH-SY5Y cells, vanadate stimulates differentiation and neurite outgrowth (13). Recently, it was shown that pervanadate is able to activate the Stat proteins (14, 15).

Despite intense investigation in this area, effects of these PTP inhibitors on MAP kinase activation, a pleiotropic signaling pathway playing crucial role in cell proliferation, differentiation, and transformation, have not been demonstrated. MAP kinase, lying in a convergence center for cell signaling, is known to be activated by a variety of stimuli. A well-defined pathway leading to its activation is the receptor PTK and Ras-coupled signaling cascade (16–20). Autophosphorylation of the receptor PTKs on tyrosine as a consequence of ligand binding provides docking sites for Grb2, which then interacts with the nucleotide exchange factor mSOS and activates Ras. GTP-bound Ras recruits Raf-1 to the plasma membrane where it is activated and further activates dual specific protein kinase MEK by phosphorylating it on seryl residues. MEK, in turn, activates MAP kinase, which requires phosphorylation on both tyrosyl and threonyl residues. The whole MAP kinase activation program is not only controlled by protein tyrosine phosphorylation but also by phosphorylation on serine/threonine, activation of G-protein, protein-protein interactions, and perhaps protein-lipid interactions. Thus, studying the role of PTP inhibitors in this process is of great significance in understanding the function of PTPs. This study demonstrates that inactivation of PTPs by pervanadate not only increases tyrosine phosphorylation of proteins that are regulated by tyrosine phosphorylation but also lead to activation of the whole MAP kinase cascade.

**EXPERIMENTAL PROCEDURES**

Materials—HeLa cells were obtained from the American Type Culture Collection. Epidermal growth factor (EGF) was from Upstate Biotechnology, Inc. Phorbol 12-myristate 13-acetate (PMA), sodium vanadate, sodium molybdate, and sodium tungstate were purchased from Sigma, and hydrogen peroxide was purchased from Fisher. Peroxide derivatives of vanadate, molybdate, and tungstate were prepared freshly by mixing equal volumes of 0.1 M H\(_2\)O\(_2\) and 0.1 M Na\(_2\)VO\(_4\) and incubated for 10–20 min before use. Antibodies used were: monoclonal anti-MEK2 and anti-Grb2 from Transduction Laboratory, polyclonal sheep-anti-EGF-receptor and monoclonal anti-phosphotyrosine from Upstate Biotechnology Inc., and polyclonal anti-Raf-1 from Santa Cruz Biotechnology Inc. Polyclonal anti-MAP kinase serum 7884 provided by Dr. Jean Campbell (University of Washington, Seattle) was raised in rabbits against a 22-amino acid peptide (21). Buffer A consists of: 50 mM \(\beta\)-glycerophosphate, pH 7.3, 2 mM EDTA, 1 mM EGTA, 5 mM \(\beta\)-mercaptoethanol, 1% Triton X-100, 0.2 mM Na\(_2\)VO\(_4\), 0.1 \(\mu\)M microcystin, 1.0 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 20 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml pepstatin A, and 1 \(\mu\)g/ml aprotinin.

Cell Culture, Stimulation, and Extraction—HeLa cells were maintained in Earl’s minimal essential medium supplemented with 10% fetal calf serum, 1% nonessential amino acid mixture, and 50 \(\mu\)g/ml each of streptomycin and penicillin. Cells (~90% confluence) were ei-
other serum-fed or 0% serum-starved for 24 h before treatment with EGF, PMA, and PTP inhibitors. The stimulation reactions were stopped by washing with ice-cold phosphate-buffered saline. Cells were lysed in Buffer A except otherwise indicated, and the extracts were cleared by centrifugation.

Immunoprecipitation and Western Blotting—Cells were incubated with antibodies prebound to protein A-Sepharose overnight. The beads were washed three times with Buffer A supplemented with 0.3 M NaCl. To immunoprecipitate MEK2 and MAP kinase, cell extracts were boiled in 0.5% SDS for 10 min and then diluted to 0.05% SDS with Buffer A before addition of antibodies. For Western blot analyses, samples were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore). The membranes were probed with various primary antibodies and detected by use of the ECL system with horseradish peroxidase-conjugated secondary antibodies (Amersham Corp.) according to the manufacturer’s protocol.

Activity Assays of PTPs, MEK, and MAP Kinase—To analyze PTP activity, cell extracts were made in Buffer A without sodium vanadate. Assays were performed with p-nitrophenyl phosphate (p-NPP) at pH 5.0, peptide ENdYINASL at pH 6.0, reduced-carboxamidomethylated-maleylated lysozyme (RCML), and myelin basic protein (MBP) at pH 7.0 as described previously (22). The cell extracts made in Buffer A were directly used to analyze the activities of MEK and MAP kinase. MEK activity was determined by ability of the enzyme to stimulate the MBP kinase activity of recombinant ERK2 in a coupled assay system, and MAP kinase was assayed with MBP as substrate in the presence of PKI peptide and calmidazolium (21, 23).

RESULTS

Pervanadate Inhibits PTP Activity and Stimulates Protein Tyrosine Phosphorylation and Activation of MEK and MAP Kinase—HeLa cells were treated with vanadate, H2O2, pervanadate, EGF, and PMA. The former three are well known PTP inhibitors, while the latter two are notorious MAP kinase activators. PTP activity in the extracts of treated cells was analyzed by employing four commonly used PTP substrates as described in the legend to Fig. 1A. Pervanadate proved to be a much more powerful inhibitor of PTPs than vanadate and H2O2. Treatment of cells with 0.1 mM pervanadate almost totally abolished PTP activity toward ENdYINASL, RCML, and MBP and reduced the activity by over 80% when analyzed with p-NPP, a relatively nonspecific phosphatase substrate. The remaining activity toward p-NPP may be due to phosphatases other than PTPs that are not sensitive to pervanadate. Vanadate and H2O2 alone, each at 0.1 mM, had essentially no effect (data not shown). At a concentration of 1 mM, they showed moderate inhibition of 5–30%. These data suggest that the prominent inhibitory effects of pervanadate cannot be attributed to the additive effects of individual vanadate and H2O2 but to the pervanadate complex itself. In contrast to the inhibitory effects of these compounds, EGF and PMA had no significant effects on PTP activity. If anything, they caused a slight increase.

As a consequence of the inactivation of PTPs, tyrosine phosphorylation of cellular proteins following pervanadate treatment displayed a drastic increase as shown in Fig. 1B. In fact, the overall level of tyrosine phosphorylation induced by pervanadate was −200-fold greater than that observed upon EGF stimulation of the cells (Fig. 1C). Vanadate and H2O2, which had only moderate effects on PTP activity, resulted in a much lower degree of tyrosine phosphorylation, supporting the view that protein tyrosine phosphorylation induced by pervanadate resulted from the inhibition of PTP activity.

Accompanying the drastic increase in protein tyrosine phosphorylation induced by pervanadate was the activation of MEK and MAP kinase as shown in Fig. 1D. The magnitude of activation was higher than that obtained with EGF or PMA stimulation. Vanadate and H2O2, which caused only moderate tyrosine phosphorylation, failed to activate the kinases, implying that a threshold of tyrosine phosphorylation has to be reached to activate the MAP kinase cascade.

Activation of MAP kinase was also apparent in gel mobility shift assays (Fig. 1E). Mobility shift of MAP kinase (ERK1 and ERK2) on SDS gel reflects the phosphorylation of the enzyme, which is required for its activation. The total shift of MAP kinase upon pervanadate stimulation suggests that the enzyme was fully activated. Mobility shift of Raf-1, which was thought to parallel its activation (24), was observed in pervanadate- and PMA-treated cells, but not in cells stimulated with EGF for 5 min, although activation of MEK and MAP kinase was apparent. Lack of a mobility shift presumably indicates an absent or insufficient phosphorylation of the protein.

Time Course of Activation of the MAP Kinase Cascade by Pervanadate—Fig. 2A illustrates the time courses of MEK and MAP kinase activation upon treatment of HeLa cells with pervanadate in comparison with those obtained with EGF. In
analyses with anti-MAP kinase and anti-Raf-1. EGF-treated serum-starved HeLa cells were subject to Western blotting kinase and Raf-1. Cell extracts from pervanadate-treated serum-fed or serum-starved and serum-fed HeLa cells detected by anti-phosphotyrosine Western blotting analyses.

Tyrosine phosphorylation of cellular proteins in pervanadate-induced tyrosine phosphorylation of cellular proteins in normal growing cells possess a higher level of tyrosine phosphorylation than the serum-fed cells as shown in Fig. 3 only shows the activation of MEK. Similar results were observed for the activity of MAP kinase.

In contrast to the biphasic pattern observed with EGF, the activity profiles of both kinases after pervanadate treatment were sustained and correlated with the sustained tyrosine phosphorylation of cellular proteins. The activities reached plateau level within 12 min of treatment and stayed thereafter. It should be noted that full activation of MEK and MAP kinase was achieved ahead of the maximum induction of tyrosine phosphorylation, which was seen until 24 min of incubation, indicating that activation of MEK and MAP kinase was a rather early event in the pervanadate-induced cell response. Furthermore, while serum starvation of cells was necessary to see maximum stimulation of MAP kinase by pervanadate (data not shown), it attenuated the stimulatory effects of pervanadate. In fact, the serum-starved cells displayed a slower tyrosine phosphorylation than the serum-fed cells as shown in Fig. 2B. This indicates that normal growing cells possess a higher level of tyrosine kinase activity than starved quiescent cells, thus give rise to a higher level of tyrosine phosphorylation. In both pervanadate- and EGF-treated cells, the activation profile of MAP kinase followed essentially exactly that of MEK, indicating a rapid transduction of the signal from MEK to MAP kinase.

Activation of the MAP kinase cascade was also demonstrated by gel mobility shift analyses as shown in Fig. 2C. Mobility shifts of MAP kinase and Raf-1 are believed to be a consequence of protein phosphorylation on multiple sites. Upon pervanadate treatment, mobility shifts of MAP kinase (ERK1 and ERK2) matched the kinase activity profile of MEK and MAP kinase. In EGF-induced biphasic activation of the MAP kinase cascade, the pattern of the mobility shifts also followed the activation and inactivation of MEK and MAP kinase. In contrast, in both pervanadate- and EGF-stimulated cells, mobility shifts of Raf-1 clearly lagged behind the activation of MEK and MAP kinase and occurrence of the mobility shifts of MAP kinase. In pervanadate-treated cells, a total shift of MAP kinase was observed after 12 min of incubation corresponding to its full activation, but it took 24 min to see a clear shift of Raf-1. Similarly, upon EGF stimulation, maximum shifts of MAP kinase were found at 6 min corresponding to the peak activity of MAP kinase, but the shift of Raf-1 was not detected until after 24 min of incubation when MAP kinase shifted back the basal level. Additional data (not shown) indicated that EGF-induced mobility shift is transient, it disappeared after 40 min. Due to a lack of accurate ways to measure the activity of Raf-1, the correlation between the activity of the enzyme and its mobility shift is hard to ascertain. However, since Raf-1 is upstream of MEK and MAP kinase in the MAP kinase activation cascade, the mobility shift reflecting its phosphorylation on multiple sites unlikely represent a cause of the activation but rather a consequence of the activation. This result explained the absence of a mobility shift of Raf-1 in cells treated with EGF for 5 min as shown in Fig. 1E. A similar pattern of MAP kinase and Raf-1 mobility shifts was observed with PMA-treated cells (data not shown).

Dose Response of Pervanadate-induced Activation of MEK and MAP Kinase—To determine the potency of pervanadate in stimulation of MAP kinase activation, HeLa cells were treated with different doses of pervanadate. Activation of MEK following the treatment was shown in Fig. 3. Nearly full activation of MEK was obtained by as low as 12.5 μM pervanadate upon 20 min of incubation, indicating that pervanadate is a very potent activator. In comparison with pervanadate, peroxide derivatives of tungstate and molybdate, which are known to be potent inhibitors of PTPs in vitro, were much less effective. Only slight activation of MEK activity was observed when their concentrations reached 200 μM. The poor MEK activation was correlated with the weak induction of tyrosine phosphorylation induced by these two compounds (data not shown). For simplicity, Fig. 3 only shows the activation of MEK. Similar results were observed for the activity of MAP kinase.

Pervanadate-induced Tyrosine Phosphorylation of Proteins Involved in MAP Kinase Cascade—Tyrosine phosphorylation of
Pervanadate-induced Activation of MAP Kinase

Fig. 4. Tyrosine phosphorylation of proteins involved in the MAP kinase activation pathway. HeLa cells were treated with 0.1 mM pervanadate for 20 min. Cell extracts were immunoprecipitated with antibodies indicated. Western blotting was performed as described under “Experimental Procedures.” LC and HC stand for the light and heavy chains of IgG, respectively, and Anti-PY, for anti-phosphotyrosine.

Fig. 5. Reversion of pervanadate-induced tyrosine phosphorylation and the MAP kinase activation. Pervanadate (0.1 mM) was added to cells at time 0 (down arrow). After 20-min incubation, cells were washed three times with phosphate-buffered saline and further cultured in normal medium (up arrow). Anti-phosphotyrosine Western blotting and assays for MEK (■) and MAP kinase (▲) activity and were performed as described under “Experimental Procedures.”

The present study indicates that inactivation of PTPs by pervanadate results in drastic tyrosine phosphorylation of cellular proteins, which is sufficient to turn on the MAP kinase pathway. Activation of MAP kinase per se could result from phosphorylation by activated MEK and/or from blockage of dephosphorylation by MAP kinase phosphatases such as the dual specificity protein phosphatases. However, activation of MEK by pervanadate requires activation of an upstream kinase, since activity of MEK is controlled by serine phosphorylation only. In fact, the inability of pervanadate to induce any tyrosine phosphorylation of MEK provides further evidence that tyrosine phosphorylation is not involved in the regulation of this enzyme. Raf-1 might be one of the upstream kinase involved in activation of MEK. Of course, involvement of other upstream elements such as MEKK cannot be ruled out (25). Pervanadate could cause tyrosine phosphorylation of Raf-1 by inhibiting the PTPs responsible for its dephosphorylation. However, although required for its activation, tyrosine phosphorylation alone is probably not sufficient to activate the enzyme (24). Therefore, activation of Raf-1 also requires an upstream element, which is presumably Ras. Phosphorylation of the EGF receptor and its association with Grb2 can serve just that purpose. Therefore, the pervanadate-induced activation of MAP kinase cascade is at least partly through the receptor PTK and the Ras-coupled pathway. Activation via the EGF receptor is only one typical example, other receptors could be activated in a similar way.

The pervanadate-induced MAP kinase activation is different from that induced by okadaic acid, a serine/threonine phosphatase inhibitor affecting pp1 and pp2a mainly (26). Okadaic acid presumably acts at the levels of MEK and MAP kinase, while pervanadate can turn on the whole MAP kinase pathway. However, pervanadate might also function through pp2a, since the latter is inactivated upon phosphorylation on tyrosine (27). Furthermore, it has been reported recently that pp2a plays a major role in the inactivation of MAP kinase in vivo (28). If pervanadate indeed induces tyrosine phosphorylation of pp2a and causes its inactivation, this could provide an explanation for the sustained activation of MAP kinase induced by pervanadate.

The activation of MAP kinase pathway by pervanadate is not limited to HeLa cells, an aggressively transformed cell line. Similar results were observed in other types of cells, which include transformed cell lines 293, HepG2, and HL-60, normal cell line NIH 3T3, and primary culture human colony-forming units-erythroid (data not shown). It seems that activation of MAP kinase pathway by pervanadate is a general phenomenon. As a nonspecific inhibitor, pervanadate could inactivate essentially all PTPs in cells. Therefore, it could have multiple...
effects on cell activity. However, no matter what other signaling pathways in addition to MAP kinase pathway can be turned on by the PTP inhibitor, the sustained activation of MEK and MAP kinase alone could have profound impact on cell behavior (29). This may explain the transformation of NRK-1 cells and differentiation of PC12 and SH-SY5Y cells by vanadate (3, 13).

This study further extends and amplifies the crucial role of PTPs as negative regulators of signal transduction. However, it does not imply that inhibition of PTPs can always be stimulatory. Since some PTPs play positive roles in cell signaling, inactivation of these PTPs could have the opposite effects. For example, vanadate both mimics and antagonizes the trans-
dynamicequilibrium. Tyrosinephosphorylationundergoescon-
activation results from interruption of the equilibrium by boosting a
PTK activity or diminishing a PTP activity. Thus, cell signaling can be triggered by inactivation of PTPs as well as by activation of PTKs. The ligand of a receptor PTK activates the kinase by enhancing its autophosphorylation. One might postulate that a factor or a ligand inactivating the PTP specific for this PTK could have the same effect. Regulatory mechanisms for PTPs are not known. It is not known whether ligands or effectors of these enzymes could induce inactivation of the enzymes, in contrast to the activation of receptor PTKs by their ligands. Understanding of the down-regulation of PTP activity has great biochemical and physiological significance, perhaps equal to that of the regulation of PTKs.

REFERENCES

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