Peroxovanadate Induces Tyrosine Phosphorylation of Multiple Signaling Proteins in Mouse Liver and Kidney

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The intraperitoneal injection of a vanadate/H$_2$O$_2$ mixture (peroxovanadate) into mice resulted within minutes in the appearance of numerous tyrosine-phosphorylated proteins in the liver and kidney. These effects are presumably due to the inhibition of phosphotyrosine phosphatase activity. Three of the tyrosine-phosphorylated proteins have been identified as the receptors for epidermal growth factor, insulin, and hepatocyte growth factor. The injection of peroxovanadate also enhanced the tyrosine phosphorylation of many of the proteins known to function downstream of these receptors, including SHC, signal transducer and activator of transcription (Stat) 1α,β, Stat 3, Stat 5, phospholipase C-γ, insulin receptor substrate 1, GTPase-activating protein, β-catenin, γ-catenin, p120cas, SHP-1, and SHP-2. The administration of peroxovanadate also induced nuclear translocation of a number of tyrosine-phosphorylated Stat proteins. In addition, the global effects on tyrosine phosphorylation permitted the detection of a number of novel intracellular protein interactions, including an association of Tyk2 with β-catenin. The in situ administration of peroxovanadate may prove useful in the search for novel tyrosine-phosphorylated proteins and the identification of new interactions between previously identified tyrosine-phosphorylated substrates.

Many of the cellular responses induced by hormones, growth factors, and cytokines are mediated by the activation of intracellular kinases. Some of these kinases are capable of autophosphorylation and of phosphorylating specific proteins on tyrosine residues. The timely appearance and disappearance of these tyrosine-phosphorylated proteins is critical for proper cell function. The tight regulation of this process is accomplished by the ubiquitous presence of tyrosine phosphatases (21). To circumvent these problems we combined our in situ system, which provides large quantities of material, with the potent phosphatase inhibitor peroxovanadate. In this report we demonstrate that the intraperitoneal injection of vanadate/H$_2$O$_2$ mixtures (peroxovanadate) into mice, in the absence of any added ligand, induces the rapid and massive tyrosine phosphorylation of multiple cellular proteins in both the liver and kidney. Not only were previously identified EGF-responsive tyrosine-phosphorylated proteins detected after peroxovanadate treatment, but we also were able to detect the enhanced tyrosine phosphorylation of receptors and downstream effectors of other hormones, growth factors, and cytokines. In addition, the extensive tyrosine phosphorylation facilitated the detection of novel intracellular interactions.

**EXPERIMENTAL PROCEDURES**

Materials—ND4 Swiss Webster mice were obtained from Harlan-Sprague-Dawley. Immobilon-P membranes were from Millipore. Prestained molecular weight standards were from Life Technologies, Inc. The following polyclonal antibodies were used: Tyk2, Stat 5, and Met (Santa Cruz); GTPase-activating protein (UBI); For (a gift from T. W. Wang, University of Medicine and Dentistry of New Jersey); insulin receptor and IRS-1 (gifts from C. R. Kahn, Joslin Diabetes Center); EGF and EGF receptor (this laboratory); PLC-γ (a gift from G. Carpenter, Vanderbilt University); SHP-1 and SHP-2 (a gift from Z. Zhao, Vanderbilt University); Stat 1 (a gift from J. Larner, U.S. Food and Drug

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The abbreviations used are: IRS-1, insulin receptor substrates; EGF, epidermal growth factor; PAGE, polyacrylamide gel electrophoresis; BC20H, horseradish peroxidase-conjugated recombinant antibody fragment specific for phosphotyrosine; PBS, Dulbecco’s calcium-free magnesium-free phosphate-buffered saline; Stat, signal transducer and activator of transcription; PLC-γ, phospholipase C-γ.

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Administration, Bethesda, MD); horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were obtained from Cappel. The following monoclonal antibodies were used: RC20H, Stat 1, Stat 5, β-catenin, γ-catenin, and tensin (Transduction Laboratories); anti-mouse IgG was obtained from Transduction Laboratories. Sodium vanadate (orthovanadate) was from Fisher, enhanced chemiluminescence reagent (ECL) was from Amer sham Corp., and protein A-Sepharose and all other reagents were from Sigma.

Treatment of Animals and Preparation of Tissue and Nuclear Extracts—A 5 mM solution of sodium vanadate in PBS was prepared by heating to boiling (22). Fifteen minutes prior to use, 30% H2O2 was added to the vanadate solution at room temperature to a final concentration of 50 mM. Solutions of this peroxovanadate or PBS were injected intraperitoneally into adult mice at a dose of 10 μl/g of body weight. Mice were sacrificed in a CO2 chamber at the indicated times and the tissues were removed and immediately frozen in liquid nitrogen. A 10% (wet weight/volume) tissue lysate was prepared by Dounce homogenization in Buffer A (50 mM Tris, pH 7.4, 0.15 mM NaCl, 1% Triton X-100, 0.25% deoxycholate, 1 mM EDTA, 1 mM sodium vanadate, and 50 μM sodium molybdate). When EGF was used as a ligand, solutions of EGF (1 mg/ml) were injected intraperitoneally at a dose of 10 μl/g of body weight.

Mouse liver nuclei were isolated, purified by centrifugation through 2.2 M sucrose, and extracted with 0.2 M sodium chloride as described previously (18).

Western Blotting and Immunoprecipitation—Portions (10 μl) of tissue extracts were separated by SDS-PAGE (7.5%), transferred to Immobilon-P membranes, and probed with RC20H (1:2500). Antibody binding was detected by ECL. Aliquots of the nuclear extracts (25 μl) were similarly resolved by SDS-PAGE (7.5%), transferred to Immobilon-P membranes, and probed with RC20H. Blots were stripped and reprobed sequentially with monoclonal antibodies to Stat 1, Stat 3, and Stat 5. In each instance antibody binding was detected by incubation with horseradish peroxidase-conjugated goat anti-mouse antibody and ECL.

For immunoprecipitation, lysates of liver from control and peroxovanadate-treated animals were centrifuged at 100,000 x g for 30 min and precleared by incubating 1 ml of extract with 50 μl of packed protein A-Sepharose for 30 min at 4 °C. When monoclonal antibodies were used for precipitation, aliquots of cleared lysate (300 μl) were incubated with 3 μl of the specific antibody (0.75 μg) for 2–4 h or overnight at 4 °C followed by the addition of 3 μg of goat anti-mouse IgG for 1 h and 50 μl of protein A-Sepharose (50% slurry) for 1 h. The resulting precipitates were washed three times in Buffer A, and the bound proteins were eluted by boiling in 100 μl of 2 × Laemmli buffer for 5 min. When polyclonal antibodies were used for immunoprecipitation, 300 μl of cleared lysate were incubated with 3 μl of specific antibody (3 μg) for 2 h followed by the addition of 50 μl of protein A-Sepharose (50% slurry) for 1 h. Aliquots were resolved by SDS-PAGE (7.5%), transferred to Immobilon-P membranes, and immunoblotted with RC20H or the specified antibody.

RESULTS

Intraperitoneal Injection of Peroxovanadate Induces Extensive Tyrosine Phosphorylation of Proteins in Liver and Kidney—Adult mice were treated by intraperitoneal injection of PBS alone or of PBS containing 5 mM sodium vanadate, 50 mM H2O2, or a mixture of 5 mM sodium vanadate and 50 mM H2O2 were injected into mice at a dose of 10 μl/g of body weight. Mice were sacrificed after 20 min of treatment. The organs were excised and processed as described under “Materials and Methods.” Aliquots (10 μl) of the clarified homogenate were resolved by SDS-PAGE, transferred to Immobilon-P membranes, and immunoblotted with antibodies to phosphotyrosine.

Intraperitoneal injection of mixtures of potassium chromate, sodium tungstate, or potassium permanganate with H2O2 at similar dosages resulted in no significant alteration of tyrosine-phosphorylated protein content in liver (data not shown).

Identification of Liver Proteins Whose Tyrosine Phosphorylation Is Induced by Peroxovanadate—To understand the biochemical interactions that led to the massive enhancement of tyrosine phosphorylation induced by peroxovanadate in the absence of any added activating ligand, we sought to identify the proteins affected. These exploratory experiments were carried out by preparing detergent lysates of livers from control and peroxovanadate-treated adult mice and immunoprecipitating them with an antibody specific for the protein of interest. The precipitated proteins were separated by SDS-PAGE and analyzed by α-phosphotyrosine immunoblot.

We have identified the presence of the tyrosine-phosphorylated EGF receptor (pp170), the β-subunit of the insulin receptor (pp85), and the receptor for hepatocyte growth factor (Met, pp140) in livers of peroxovanadate-treated animals (Fig. 2). The 52-kDa tyrosine-phosphorylated protein that is present in antibody precipitates of the EGF receptor was identified as SHC by reprobing the blot with antibodies specific for SHC. Immunoprecipitation using anti-SHC antibodies resulted in a pattern of tyrosine phosphorylation qualitatively identical to that shown for anti-EGF receptor in Fig. 2 (data not shown).

Thus, the in situ response to peroxovanadate resembles the in situ response to EGF (17).

In view of this similarity we directly compared the pattern of tyrosine-phosphorylated proteins in liver extracts from PBS-, EGF-, and peroxovanadate-treated mice. As demonstrated previously (16), 20 min following the administration of EGF an increase in the phosphotyrosine content of a number of liver proteins was detectable (Fig. 3). It is readily apparent that liver extracts from peroxovanadate-treated animals contain more...
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Fig. 2. Peroxovanadate-induced tyrosine phosphorylation of receptor/kinases. Detergent lysates of livers from control and peroxovanadate-treated animals were prepared as described in Fig. 1. Aliquots (300 µl) of the liver lysates were immunoprecipitated with the specified polyclonal antibody for 4 h at 4 °C followed by addition of protein A-Sepharose for 30 min. The resulting precipitates were analyzed by α-phosphotyrosine immunoblot as described under “Materials and Methods.”

Fig. 3. Comparison of peroxovanadate- and EGF-induced tyrosine phosphorylation. Solutions of peroxovanadate, EGF (1 mg/ml), or PBS were injected into mice at a dose of 10 µg/kg of body weight. Mice were sacrificed after 20 min of treatment. The livers were excised, processed, and analyzed by α-phosphotyrosine immunoblot as described in Fig. 2.

Fig. 4. Identification of peroxovanadate-induced tyrosine phosphorylation of several signal-related proteins. Detergent lysates of livers from control and peroxovanadate-treated animals were prepared, immunoprecipitated with the specified polyclonal antibody, and analyzed by α-phosphotyrosine immunoblot as described in Fig. 2. The arrowheads indicate the positions of the antigens.

Tyrosine-phosphorylated proteins than extracts from animals induced by EGF (Fig. 3).

In view of the enhanced tyrosine phosphorylation (and possible activation) of receptors for EGF, hepatocyte growth factor, and insulin in livers of peroxovanadate-treated animals, it was of interest to determine whether known downstream substrates of these receptor/kinases (reviewed in Refs. 23–25) were also tyrosine-phosphorylated. Livers of peroxovanadate-treated animals were found to contain markedly higher levels of tyrosine-phosphorylated Stat 5 (pp92), Stat 1α (pp91), PLC-γ (pp150), IRS-1 (pp185), and GTPase-activating protein (pp120) (with its associated proteins of 190 and 64 kDa) than controls (Fig. 4). The identities of the other tyrosine-phosphorylated proteins detected in these immunoprecipitates are not known.

Proteins that mediate cell adhesion, such as the cadherin-associated proteins β-catenin (pp92), γ-catenin (pp82), and pp120cas and cytoskeletal proteins such as talin (pp120), also have been shown to be tyrosine-phosphorylated following the activation of EGF or hepatocyte growth factor receptors in cell cultures (26–30). Enhanced tyrosine phosphorylation of β-catenin, γ-catenin, and several isoforms of p120cas are demonstrable in the liver of mice following intraperitoneal injection of EGF (data not shown). All of these putative signal transducing proteins are tyrosine-phosphorylated in the livers of peroxovanadate-treated animals (Fig. 5). The bands noted in immunoprecipitates of pp120cas are presumably isoforms (25). The identities of the higher molecular weight bands associated with γ-catenin in Fig. 5 are not known.

Among the possible cellular targets responsible for initiating the enhanced tyrosine phosphorylation observed following treatment with peroxovanadate are the intracellular tyrosine phosphatases. Tyrosine phosphatases that contain Src homology 2 domains (SHP-1 and SHP-2) have been implicated as regulators of signal transduction and tyrosine phosphorylation of SHP-1, and SHP-2 has been demonstrated in growth factor- and cytokine-stimulated cells (reviewed in Refs. 31 and 32). Livers of peroxovanadate-treated animals contain both SHP-1 (pp68) and SHP-2 (pp72) in a tyrosine-phosphorylated form (Fig. 6). Both phosphatases appear to be associated with major tyrosine-phosphorylated proteins in the 120–140-kDa range. The association of SHP-1 and SHP-2 with receptors and other tyrosine-phosphorylated proteins in various cell culture systems has been reported (32). The tyrosine-phosphorylated bands labeled a and b (Fig. 6) were identified as SHP-1 and SHP-2, respectively, by Western blotting with antibodies specific for these proteins and were present in both control and peroxovanadate-treated animals.

Finally, we have examined liver extracts for the presence of tyrosine-phosphorylated cytoplasmic tyrosine kinases such as Jak1, Jak2, Tyk2, and Fer. Small amounts of all four were detected as tyrosine-phosphorylated proteins in peroxovanadate-treated animals (data not shown for Jak1 and Jak2). Perhaps the most interesting observations (Fig. 6) were that immunoprecipitates of Tyk2 (pp135, band c) also contained a tyrosine-phosphorylated protein identified as β-catenin (pp92, band d), and immunoprecipitates of Fer (pp95, band f) contained tyrosine-phosphorylated pp120cas (band e). All of these proteins were identified by reprobing the blots with appropriate antibodies. The association of β-catenin with Tyk2 and p120cas with Fer also were seen in extracts of livers from control animals in the absence of detectable tyrosine phosphorylation of these molecules (data not shown). The association of Fer with pp120cas in A431 cells stimulated by EGF or platelet-derived growth factor, as well as the association of Fer with unphosphorylated p120cas, were reported previously (33).
the tyrosine phosphorylation and nuclear translocation of Stat proteins in livers (18, 34), we compared the patterns of protein tyrosine phosphorylation in salt extracts of liver nuclei from control, peroxovanadate-treated, and EGF-treated animals. Both EGF and peroxovanadate induced the appearance of major tyrosine-phosphorylated bands of $M_r = 86,000–92,000$ (Fig. 7A). Additional bands were detected in nuclei from peroxovanadate-treated animals.

We have previously identified phosphorylated Stat1$\alpha$, Stat1$\beta$, Stat3, and Stat5 in liver nuclei as components of the 86–92-kDa tyrosine-phosphorylated bands detected after administration of EGF (20). Western blots of nuclear extracts from control and peroxovanadate-treated animals revealed the presence of Stat1$\alpha$, Stat1$\beta$, Stat3, and Stat5 proteins in nuclei from peroxovanadate-treated animals and not in nuclei from control animals (Fig. 7B). Thus, peroxovanadate mimics some of the nuclear effects induced by the administration of either EGF or growth hormone.

**DISCUSSION**

The activation of transmembrane and cytoplasmic tyrosine kinases by growth factors, hormones, and cytokines initiates many intracellular signaling events. Increased attention has recently been focused on the importance of protein tyrosine phosphatases in the regulation of cell function (reviewed in Refs. 31, 35, and 36).

Our experiments describing the *in situ* effects of peroxovanadate suggest that the control of protein tyrosine phosphatase activity may be of greater significance in cell signaling than heretofore realized. The intraperitoneal injection of a vanadate/\(H_2O_2\) mixture (peroxovanadate) into mice resulted within minutes in the appearance of many tyrosine-phosphorylated proteins in both the liver and kidney (Figs. 1 and 3). The differing patterns of phosphotyrosine-containing proteins in each organ may reflect different types and amounts of tyrosine kinases or substrates present in each tissue. The liver and kidney are the organs predominantly affected by peroxovanadate, suggesting that they are able to rapidly concentrate the biologically active component from the peritoneal cavity.

It was surprising that, in the absence of any added ligand, we were able to detect extensive tyrosine phosphorylation of not only receptors for EGF, insulin, and hepatocyte growth factor (Fig. 2) but also of many of the downstream substrates of these and other kinases. The cellular signaling components in liver whose tyrosine phosphorylation was enhanced by the peroxovanadate treatment include SHC, Stat5, Stat1$\alpha$, PLC-$\gamma$, IRS-1, GTPase-activating protein (with its associated proteins...
of 190 and 64 kDa), and the cadherin-associated proteins β-catenin, γ-catenin, and p120\textsuperscript{cas} (Figs. 4 and 5).

The Src homology 2-containing phosphotyrosine phosphatases (SHP-1 and SHP-2) are presumably among those tyrosine phosphatases inhibited by peroxovanadate. These two enzymes have been reported to be targets of a number of receptor and receptor-associated tyrosine kinases (37–41). The tyrosine phosphorylation of both SHP-1 and SHP-2 is enhanced in the receptor-associated tyrosine kinases (37–41). The tyrosine phosphatases and/or kinases. Although it is probable that the effects of peroxovanadate on protein tyrosine phosphorylation is due to its ability to inhibit intracellular protein tyrosine phosphatases, vanadate derivatives are known to bind and inhibit a variety of other enzymes (47). Furthermore, in view of the rapid equilibria among the many complex forms of vanadate in aqueous solutions (48), it is not possible to specifically define the structure of the relevant biologically active species. Despite these uncertainties, the potent biological effects of peroxovanadate may help to elucidate the biochemical mechanisms involved in the coordination of kinases and phosphatases in cell signaling and to uncover novel intracellular interactions. Our data also emphasize the importance of phosphatase activity on the steady-state levels of phosphotyrosine in cellular proteins and the extent to which the basal activity of cellular kinases is always "on" and is sufficient to activate many signaling pathways.

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