Hepatic tyrosine-phosphorylated proteins identified and localized following in vivo inhibition of protein tyrosine phosphatases: effects of H$_2$O$_2$ and vanadate administration into rat livers

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**Summary**

Injection of a combination of H$_2$O$_2$ and vanadate (H/V) into the portal vein of rat livers resulted in inhibition of protein tyrosine phosphatase activity and led to a dramatic enhanced in vivo protein tyrosine phosphorylation. Some of the phosphorylated proteins were identified as the $\beta$-subunit of the insulin receptor, the insulin receptor substrate 1 ($\sim$183, PLC-γ (pp145), and a 100 kDa PLC-γ-associated protein. Immunofluorescence and immune electron microscopy of frozen liver sections with anti-P-Tyr antibodies revealed that most of the tyrosine-phosphorylated proteins are localized in close proximity to the plasma membrane in intercellular adherence junctions and tight junction regions. This close in vivo association between membranal protein tyrosine kinases, their target proteins, and cytoskeletal elements could enable formation of ‘signaling complexes’ which may play a role in transmembrane signal transduction. By affinity chromatography over immobilized anti-P-Tyr antibodies, a large number of these tyrosine-phosphorylated proteins were partially purified.

**Introduction**

A significant problem in identifying physiological protein substrates for protein tyrosine kinases (PTKs) is the very low abundance of tyrosine-phosphorylated proteins in the cell, probably due to potent protein tyrosine phosphatases (PTPs) activity (Cooper and Hunter, 1981). Detection of such substrates could thus be facilitated if PTP activity were inhibited under in vivo conditions. From studies done in our laboratory (Heffetz and Zick, 1989; Zick and Sagi-Eisenberg, 1990; Heffetz et al., 1992) and in others (Shriner and Brautigan, 1984; Chan et al., 1986; Kadota et al., 1987a,b), H$_2$O$_2$ and vanadate were found to be appropriate agents for this purpose.

Vanadate and H$_2$O$_2$ are known insulinomimetic agents. Vanadate stimulates glucose uptake and oxidation (Dubyak and Kleinzeller, 1980; Schecter and Karlish, 1980) and activates glycogen synthase (Tamura et al., 1984). Similarly, H$_2$O$_2$ enhances glucose transport and oxidation (Czech et al., 1974; May and De Haen, 1979), stimulates glycogen synthase (Lawrence and Larner, 1978), activates pyruvate dehydrogenase (Czech et al., 1974), and inhibits hormone-stimulated lipolysis (Little and De Haen, 1980). Both H$_2$O$_2$ and vanadate increase the phosphorylation of the insulin receptor, and when used in combination, their insulinomimetic activity is synergistic (Shriner and Brautigan, 1984; Chan et al., 1986; Kadota et al., 1987a,b; Heffetz et al., 1990). We have previously demonstrated that when added to cultured Fao hepatoma cells a combination of H$_2$O$_2$ and vanadate (H/V) effectively inhibits intracellular PTPs activity (Heffetz et al., 1990) and as a result, potentiates the insulin-dependent tyrosine phosphorylation of the insulin receptor substrate 1 (IRS-1) (White et al., 1985; Rothenberg et al., 1991; Sun et al., 1991).
and of other as yet unidentified tyrosine-phosphorylated proteins (Heffetz and Zick, 1989; Heffetz et al., 1990). Furthermore, H/V injection into intact rat livers leads to increased association between phosphatidylinositol 3'-kinase (PI3K) (Endemann et al., 1990; Ruderman et al., 1990; Backer et al., 1992) and IRS-1 and induces an in vivo activation of the latter (Hadari et al., 1992).

Since inhibition of intracellular PTPs is expected to enhance indirectly the activity of PTKs that are stimulated through autophosphorylation, tyrosine kinases such as the insulin and IGF-1 receptor kinases are expected to maintain a high basal activity in H/V-treated cells even in the absence of a ligand. Thus, H/V could be used as a tool to amplify in a rapid and reversible manner the extent of protein tyrosine phosphorylation for such kinases. Indeed, at least some of the proteins which undergo enhanced tyrosine phosphorylation in H/V-treated CHO cells are likely to represent direct or indirect substrates for the insulin receptor kinase (IRK) as their phosphorylation occurs only in the presence of a functional active receptor (Heffetz et al., 1992).

In this work we have extended our studies on the in vivo mode of action of H/V. The marked tyrosine phosphorylation that resulted from its perfusion into rat livers has enabled us to localize, characterize, and partially purify new potential in vivo substrates for the IRK and related tyrosine kinases.

**Experimental procedures**

**Insulin and H/V treatment**

Male Sprague-Dawley rats, weighing between 200 and 300 g, were injected intraperitoneally with Nembutal (60 mg/g of body weight) and were operated on 10–15 min later, as soon as anesthesia was complete. The abdominal cavity was opened, the portal vein exposed, and 200 µl of insulin (2 × 10^{-7} M) or 5 ml of phosphate-buffered saline (PBS) with or without H/V were perfused through a 21-gauge needle connected to a mechanical syringe pump. After infusion, the liver was excised rapidly and frozen in liquid nitrogen.

Liver extracts were prepared by homogenization in buffer A (5 ml/g), containing 50 mM Hepes, 150 mM sucrose, 80 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 2 mM Na_{3}VO_{4}, 10 mM sodium pyrophosphate, 50 mM NaF, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 5 µg/ml leupeptin, 10 µg/ml trypsin inhibitor, pH 7.6. To obtain cytosolic extracts, the homogenate was centrifuged for 1 h at 4°C at 100 000 × g and aliquots of the supernatants were normalized per protein, mixed with concentrated (×5) Laemmli sample buffer (Laemmli, 1970), boiled for 5 min, and resolved on 7.5% SDS-PAGE under reducing conditions (Laemmli, 1970). Liver extracts were prepared as above using buffer A supplemented with 1% Triton X-100.

**Preparation of subcellular fractions**

Subcellular fractionation of rat livers was performed essentially as described by Graham (1984). Rat liver homogenates were centrifuged at 12 000 × g for 15 min at 4°C, and the supernatant (S1) was further centrifuged at 26 000 × g at 4°C for 30 min. This pellet (P2) contains the high density microsomes (HDM). The resultant supernatant (S2) was then centrifuged at 100 000 × g at 4°C for 1 h; the pellet (P3) contains low density microsomes (LDM), while the supernatant (S3) contains cytosolic proteins (Graham, 1984). The first pellet (P1) was resuspended in buffer A, centrifuged at 1500 × g at 4°C for 10 min after which the pellet was discarded. The supernatant (S4) was then centrifuged at 26 000 × g at 4°C for 30 min. This pellet (P4), which contains the plasma membrane (PM) (Graham, 1984), was saved while the supernatant was discarded.

**Western immunoblotting**

Blotting of proteins to nitrocellulose papers was performed as previously described (Heffetz and Zick, 1989), using the technique of Burnet et al. (1981). Affinity-purified anti-phosphotyrosine (anti-P-Tyr) antibodies were generated as previously described (Heffetz and Zick, 1989). Rabbit polyclonal anti-insulin receptor antibodies were generated against a synthetic peptide corresponding to positions 1305–1324 of the human insulin receptor. Anti-PLC-γ antibodies were a gift of Dr. Sue Ghoo Rhee, Bethesda, MD. Detection of bound antibodies was carried out with the ECL kit, purchased from Amersham (Amersham, Buckinghamshire, UK), according to the manufacturer's instructions, or by using 125I-labeled second (i.e. goat anti-rabbit) antibodies.

**Immunoprecipitation**

Antibodies were added to 30 µl of protein A-Sepharose (Uppsala, Sweden) in 0.1 M Tris, pH 8.5, and incubated for 1 h at 4°C. The complex was precipitated at 12 000 × g (5 min) and washed three times with 0.1 M Tris, pH 8.5, and once with buffer A containing 1% Triton X-100. 500 µl liver extracts (0.8 mg) (prepared in buffer A, as described above) from control, insulin or H/V-treated rats, were incubated for 2 h with the antibody-protein A Sepharose complex. The immunocomplexes were pelleted by centrifugation at 12 000 × g (5 min) and washed twice with buffer A containing 1% Triton X-100, and twice with IITN buffer (150 mM NaCl, 50 mM Hepes, 0.1% Triton X-100, pH 7.6). The final pellets were then suspended in Laemmli sample buffer (Laemmli, 1970), resolved by 7.5% SDS-PAGE and transferred to nitrocellulose filters for Western immunoblotting.
**Immunofluorescence labeling**

Frozen liver sections of 5–10 mm were cut at -20°C in a Frigocyt 2800 cryostat (Jung, Reichert, FRG). The sections were recovered on a clean glass slides, air-dried, fixed with acetone at -20°C and immunolabelled as described (Volberg et al., 1991). Antibody-stained sections were recovered on a clean glass slides, air-dried, fixed with acetone at -20°C, and immunolabelled as described (Volberg et al., 1991). Antibody-stained sections were briefly dehydrated in absolute ethanol, mounted in Entellan and examined in a Zeiss Axiophot photo microscope equipped for epifluorescent observations, using X40/1.0 or X100/1.3 oil-immersion plan neofluar objectives.

**Immunoelectron microscopy**

H/V-treated livers were perfused with 'fixative solution' (5 mM CaCl₂, 1% glutaraldehyde, 3% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.5) and dissected into small blocks (~1 mm). The blocks were further fixed for 3 h at 22°C, washed 4 times in 0.1 M cacodylate buffer, immersed in 2.3 M sucrose solution for 60 min, and frozen in liquid nitrogen. Ultra-thin frozen sections (700–1000 Å, Å = 0.1 nm) were cut at -120°C using a Reichert-Jung FS-4D ultramicrotome. The sections were recovered from the knife on microscope grids in a 2.3 M sucrose droplet as described in (Tokuyasu and Singer 1976). The samples were immunogold labeled with affinity-purified antibodies to P-Tyr and gold-conjugated goat anti-rabbit antibodies. Sections were examined with CM-12 electron microscope (Philips, Eindhoven, Holland) at 100 kV.

**Assay of protein tyrosine phosphatase (PTP) activity**

Livers were extracted in phosphatase buffer containing 250 mM Sucrose, 5 mM EDTA, 0.5 mM EGTA, 50 mM Heps, 2 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, pH 7.4. The assay was based on measurements of the release of 32P from 32P-labeled poly(Glu,Tyr) 4:1. All samples were normalized for equal amounts of protein. PTP activity was assayed by mixing 40 μl (~40 μg) of cytosolic livers extracts from control or H/V-treated rats with 40 μl of a reaction mixture containing 25 mg/ml 32P-poly(Glu,Tyr) 4:1, 5 mM ATP, and 50 mM Heps, pH 7.4. Reactions were carried out for 8 min at 30°C and terminated by applying 60 μl aliquots onto Whatman 3MM filter papers. The papers were extensively washed in 10% trichloroacetic acid (TCA), rinsed in ethanol, dried, and counted by liquid scintillation. The extent of reduction of TCA-precipitable 32P-poly(Glu,Tyr) 4:1 was taken as a measure of PTP activity.

**Purification of tyrosine-phosphorylated proteins**

40 mg of affinity-purified rabbit anti-P-Tyr were adsorbed to 10 ml (dry volume) of Affi-gel 10 by slowly mixing the complex at 4°C overnight in 0.1 M Heps, pH 7.6. The complex was washed with 10 bed volumes of PBS and packed in a 120 ml column. In order to preserve the tyrosine-phosphorylated state of the proteins, the cytosolic (S3) fractions from H/V-treated rat livers were boiled in the presence of 1% Triton X-100 and 1% SDS for 30 min. Before application to the column, the solution was diluted 1:10 in a buffer containing 50 mM Heps and 1% Triton X-100, pH 7.6. The column was washed with 10 bed volumes of buffer containing 50 mM Heps, pH 7.6, 150 mM NaCl, 1% Triton X-100 and 0.1% SDS, and finally with ten bed volumes of HTN buffer containing 1 M NaCl, and finally with ten bed volumes of HTN. 100 ml of diluted denatured protein extract (1.6 mg/ml) was applied onto the column, and incubated 2 h at 4°C with vigorous mixing. The effluent was collected and the column was washed with ten bed volumes of buffer containing 50 mM Tris, 0.1% Triton X-100, 0.1% SDS, and 150 mM NaCl, pH 7.6, followed by ten bed volumes of buffer containing 50 mM Tris, 1% Triton X-100, 0.1% SDS, pH 7.6. The last wash was with ten bed volumes of 50 mM Tris, pH 7.6. The elution of the phosphoproteins was carried out in one bed volume of buffer containing 50 mM Tris, 150 mM NaCl, 0.025% SDS and 40 mM p-nitrophenylphosphate (PNPP) pH 7.6. One ml fractions were collected, and the presence of phosphotyrosine-containing proteins was determined by immunoblotting with anti-P-Tyr antibodies as described above.

**Results**

**Effects of H/V on protein tyrosine phosphorylation in perfused rat livers**

Consistent with our previous studies (Nadiv et al., 1992), injection of insulin into the portal vein of rats induced tyrosine phosphorylation of a 95 kDa polypeptide (Fig. 1A), whose identity as the β-subunit of the insulin receptor was confirmed by immunoprecipitation using anti-insulin receptor antibodies and immunoblotting with anti-P²-Tyr antibodies (Fig. 1C). H/V, on the other hand induced tyrosine phosphorylation of a number of proteins (Fig. 1A), ranging from 40–200 kDa. Some (e.g. pp95), but not all, of these proteins could be precipitated by anti-P-Tyr antibodies (Fig. 1B), suggesting that they could generate tight complexes with anti-P-Tyr antibodies when present in their native, non-denatured conformation. Maximal phosphorylation was obtained after 30 min of perfusion with 1–1.5 mM H₂O₂ together with 0.33–0.5 mM vanadate (Fig. 2). At higher concentrations the phosphorylation of all proteins decreased in parallel.

**Subcellular distribution of the tyrosine-phosphorylated proteins**

**Biochemical studies.** The subcellular distribution of the tyrosine-phosphorylated proteins (TPPs) was next eval-
uated. Following H/V perfusion, liver extracts were fractionated, resolved on SDS-PAGE and immunoblotted with anti-P-Tyr antibodies. As seen in Fig. 3, TPPs were detected in each subcellular fraction. The pattern of the TPPs obtained from the plasma membranes (PM) and the HDM fractions were identical, and similar to that obtained from the cell extracts. The TPPs in these fractions ranged in molecular mass from 55 to 200 kDa. The LDM fraction exhibited a different pattern of TPPs. Save for a common 185 kDa, three TPPs (pp15, pp22 and pp45) were detected exclusively in this fraction, while most other TPPs, present in the PM and LDM were missing. The cytosolic fraction contained the lowest percentage of TPPs. The most abundant protein in this fraction had a molecular mass of 120 kDa. It should be noted however, that when proteins from the subcellular fractions were resolved on SDS-PAGE according to their original fraction's volume 52% of the TPPs were found in the cytosolic fraction, 25% in the HDM fraction, 13% in the PM fraction and 12% in the LDM fraction (not shown).

Immunofluorescent labeling of TPPs. To better localize the distribution of the TPPs following H/V perfusion, frozen sections of liver were prepared and analyzed by immunofluorescently labeled anti-P-Tyr antibodies. P-Tyr labeling was undetectable in sections derived from PBS-treated livers (not shown). In contrast, sections from H/V-treated livers revealed that the highest density of TPPs is localized within, or in close proximity to the plasma membrane (Fig. 4a). Taken together with the results of the subcellular fractionation, it seems that even the cytosolic TPPs are found in close proximity to membranal proteins.

Immunoelectron microscopy analysis of TPPs. Electron microscopic examination of thin sections from H/V-treated livers indicated that the TPPs were mainly localized along the plasma membrane in the sub luminal region, close to the bile canaliculi (Fig. 4c). Consistent with previous immunofluorescence studies in cultured cells (Volberg et al., 1991), intercellular adherens junctions and tight junctions were specifically enriched with TPPs, while no particular staining of gap junctions (Fig. 4c) or desmosomes (not shown) could be detected. TPPs were sparsely distributed in other organelles such as cytoplasmic vesicles. Binding of the anti P-Tyr antibodies, detected either by immunofluo-
rescence or electron microscopy, was specific, and could be completely abolished by excess P-Tyr (compare Fig. 4a vs. 4b, and 4c vs. 4d). These findings suggest that intercellular junctions are major sites of action of PTKs, which leads to the accumulation of tyrosine-phosphorylated proteins in these regions.

**Phosphorylation of PLC-γ following H/V treatment.**

Since PLC-γ is a known substrate for several PTKs [reviewed in (Cantley et al., 1991)] attempts were made to determine whether it undergoes enhanced in vivo tyrosine phosphorylation in H/V-treated livers. Cytosolic fractions from H/V-perfused rat livers were immunoprecipitated using anti-PLC-γ antibodies and immunoblotted using anti-P-Tyr or anti-PLC-γ antibodies. PLC-γ underwent a time-dependent in vivo phosphorylation on tyrosine residues in response to H/V injection (Fig. 5A). Similar amounts of PLC-γ were detected in the cytosolic fraction of control and H/V-treated rats (Fig. 5B), indicating that tyrosine phosphorylation of PLC-γ did not largely affect its in vivo redistribution between the cytosol and other subcellular organelles. Interestingly, PLC-γ co-precipitated with another tyrosine-phosphorylated protein with an apparent molecular mass of 100 kDa (Fig. 5A). It is not likely that pp100 is a degradation product of PLC-γ since it fails to react with anti-PLC-γ antibodies on immunoblots (Fig. 5B). pp100 could represent a 100 kDa phosphoprotein that has been previously demonstrated to form tight complexes with PLC-γ (Meisenhelder et al., 1989).

**H/V treatment inhibits PTP activity in vivo**

The enhanced and sustained tyrosine phosphorylation in H/V-perfused liver suggested that, as observed in cultured cells (Heffetz et al., 1990, 1992) the effects of these agents could be exerted, at least in part, through inhibition of PTPs activity. Indeed, injection of H/V inhibited cytosolic PTPs activity in a time-dependent manner (Fig. 6). Interestingly, the kinetics of inhibition showed a complex biphasic pattern. Following a rapid acute inhibitory phase (75% inhibition) that reached its maximum within 3 min, the extent of inhibition slowly decreased to 35% by 20 min where it increased and again reached maximal levels by 40 min. The reason for this complex pattern of inhibition is presently unknown.

**Partial purification of tyrosine-phosphorylated proteins using anti-P-Tyr antibodies coupled to Sepharose**

The dramatic increase in the content of hepatic tyrosine-phosphorylated proteins following treatment with H/V prompted us to initiate studies aimed at their purification and isolation by immunoaffinity chromatography over columns of anti-P-Tyr coupled to Sepharose. No major difference in the protein content could be detected when cytosolic extracts and the proteins present in the effluent fraction of the column were silver stained (Fig. 7A, lanes 1 vs. 2). This indicates that the majority of the cytosolic proteins were not retained by the affinity matrix. By contrast, when the pattern of the TPPs was compared (Fig. 7B, lanes 1 vs. 2), it became apparent that most of them (> 85%) remained bound to the affinity matrix, and were absent from the effluent fraction. These TPPs could be subsequently eluted with PNPP (Fig. 7B, lanes 6–10). When the PNPP eluates were silver-stained, bands corresponding to pp145, 95, 65, 55, and pp40 could be detected. Additional bands that stained weakly corresponded to pp185, 120, and pp70.

It should be noted that additional proteins that did not contain significant amounts of P-Tyr were also detected by silver staining, especially in the low molecular weight range. These proteins could either be contaminants that were bound and eluted non-specifically from the anti-P-Tyr antibody column, weakly tyrosine-phosphorylated proteins, or degradation fragments of the major P-Tyr proteins that were devoid of P-Tyr residues and remained non-covalently attached to the phosphorylated fragments. From 1 g (wet weight) of H/V-perfused rat liver, which produced 80 mg of cytosolic proteins, 12 μg of tyrosine-phosphorylated proteins were purified. This analysis indicates that a
single step immunoaffinity purification with anti-P-Tyr antibodies generated large amounts of in vivo TPPs that were purified about 2000-fold with a recovery of approximately 0.015% of the starting material.

Discussion

Protein tyrosine phosphorylation is a rare event in intact cells. Less than 0.01% of all phosphoproteins are
phosphorylated on tyrosine residues (Hunter, 1987). Potent PTPs have been implicated as playing an important role in maintaining the intracellular phosphotyrosine content at such low levels (Tonks et al., 1988; Lau et al., 1989). In the present study, we have demonstrated that a combination of H$_2$O$_2$ and vanadate (H/V) effectively inhibits PTPs activity in vivo in intact rat livers, similar to their effects in cultured cells (Heffetz et al., 1990; Bushkin et al., 1991). As a result of this inhibition, enhanced and sustained protein tyrosine phosphorylation takes place which enables the identification of a set of in vivo tyrosine-phosphorylated proteins.

The pattern of H/V-mediated inhibition of PTPs activity in the intact liver (Fig. 6) differs from that observed in Fao hepatoma cells (Heffetz et al., 1990). In the latter, maximal protein tyrosine phosphorylation correlates with a complete inhibition of PTPs activity, while in the perfused liver, a biphasic inhibitory pattern has been observed (Fig. 6). Interestingly, the par-
tial recovery of PTPs activity occurs concomitantly with
the increase in protein tyrosine phosphorylation (i.e.
between 5 and 30 min following the H/V perfusion
Hadari et al., 1992). The reason for this biphasic
kinetics is presently unknown. One might speculate,
however, that the recovery process might involve a
cascade of events in which acute inhibition of PTPs,
immediately following H/V perfusion, leads to activa-
tion of PTKs that reactivate certain PTPs in a feedback
control mechanism. This reactivation may involve con-
version of some PTPs to an H/V-insensitive form. This
tentative model is supported by recent findings where
insulin was shown to decrease the phosphotyrosine
content of phosphatase inhibitor 1, presumably by
stimulating a PTP activity (Williams, 1992).

The in vivo TPPs are virtually distributed among all
subcellular fractions (Fig. 3). The phosphoproteins pat-
tern is similar in both PM and HDM fractions, suggest-
ing that either the same proteins are present in both
der fractions, or else these fractions are cross contami-
ated. By contrast, the LDM and the cytosolic fra-
tions exhibit unique repertoire of TPPs. About 50% of
the total TPPs are localized to the cytosol, although
their abundance, when compared with other subcellu-
lar fractions, is the lowest in the cytosol. Most reveal-
ing are the immunoelectron microscopy and immu-
nunofluorescence studies (Fig. 4) which indicate that
the TPPs present in intact liver are not diffusely dis-
btributed in the cytosol but rather concentrate in close
proximity to the plasma membrane where they could
serve as target proteins for membranal growth factor
receptor that function as PTKs. In fact, a substantial
population of the TPPs in intact liver localize to de-
ined regions of intercellular adherens junctions and
tight junction areas (Fig. 4). These observations are
supported by immunofluorescence studies in trans-
formed cells and embryonic lines, where we (Volberg
et al., 1991) and others (Maher and Pasquale, 1988;
Takata and Singer, 1988; McBride et al., 1991) have
demonstrated that TPPs, such as PLC-γ (McBride et
al., 1991) concentrate in focal contacts and intercellu-
lar junction. Thus, a close association between mem-
branal PTKs, their target proteins, and cytoskeletal
elements could enable formation of signaling sites (Ullrich and Schlessinger, 1990; Geiger et al., 1993)
which may play a role in transmembranal signal trans-
duction. The association of the tyrosine-phosphory-
lated proteins with the cytoskeletal machinery might
also serve as means to mobilize the TPPs among differ-
ent subcellular organelles.

The nature of most of the TPPs in H/V-treated rats
is presently unknown. Using specific antibodies we
have so far identified just a few: pp95 as the β-subunit
of the insulin receptor (Fig. 1), pp185 as IRS-1 (Hadari
et al., 1992), and pp150 as PLC-γ (Fig. 5). Interestingly,
PLC-γ co-precipitates with a tyrosine-phosphorylated
protein of 100 kDa (pp100). Although the nature of
pp100 is presently unknown it could be the same
protein that co-immunoprecipitates with PLC-γ follow-
ing PDGF induction in cultured cells (Meisenhelder et
al., 1989).

PLC-γ is not phosphorylated following insulin stim-
ulation (Meisenhelder et al., 1989), nor is PLC-γ co-
immunoprecipitated with IRS-1 following insulin or
H/V treatment (Hadari et al., 1992). Thus, the in-
creased tyrosine phosphorylation of PLC-γ following
H/V perfusion suggests that H2O2 and vanadate,
which are potent insulinomimetic agents, may activate
tyrosine kinases other than the insulin receptor (Tobe
et al., 1990). Potential candidates are PTKs such as
the proto-oncogene c-MET (Giordano et al., 1989), that
is highly expressed in the liver (Naldini et al., 1991a), and
like the insulin receptor (Rosen, 1987; Zick, 1989), is
activated upon autophosphorylation (Naldini et al.,
1991b). Such autophosphorylation is markedly en-
hanced once intracellular PTPs are inhibited in H/V-
treated cells (Heffetz et al., 1990).

Inclusion of vanadate in the drinking water of dia-
abetic rats normalizes their blood sugar and cardiac
performance (Heyliger et al., 1985). While the in vivo
mode of action of vanadate is unknown, the present
study suggests that vanadate may exert its insuli-
nomimetic effects through an increase in the rate and
extent of tyrosine phosphorylation of proteins some of
which, like IRS-1, are potential targets for the insulin
receptor kinase. The exact role of these proteins in
mediating the activity of the insulin receptor and re-
lated PTKs still remains to be determined but their
initial purification (about 2000-fold) described here
(Fig. 7), forms the basis for their complete purification
and future characterization.

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