BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 316–321 (1997)
ARTICLE NO. RC977455

Phenylarsine Oxide Inhibits Insulin Activation of Phosphatidylinositol 3′-Kinase

Yuan-Ping Han[1] and R. A. Kohanski[2]
The Department of Biochemistry, Mount Sinai School of Medicine, New York, New York 10029

Received August 29, 1997

Two early events downstream of insulin receptor autophosphorylation that are necessary for activation of glucose transport in adipocytes appear to be: (1) The tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) which (2) recruits and activates phosphatidylinositol 3′-kinase (PI3′-K). Phenylarsine oxide (PAO) has long been known to inhibit glucose transport, without inhibiting insulin receptor auto- or substrate phosphorylation. However, the PAO-sensitive site downstream of these early regulatory eventshas not been identified. Here we provide evidence that exposure of 3T3-L1 adipocytes to PAO inhibits PI3′-K activation, but it does not decrease either IRS-1 tyrosine-phosphorylation or the recruitment of PI3′-K to IRS-1 after insulin stimulation. PAO is also shown to inhibit PI3′-K activity *in vitro.* Therefore, since PI3′-K activation is essential for insulin stimulation of glucose transport, our results demonstrate that PI3′-K is a PAO-sensitive target of the insulin signaling pathway regulating glucose transport. © 1997 Academic Press

Insulin stimulated glucose transport results from the translocation of glucose transporters from an intracellular compartment to the plasma membrane (1,2), a pathway known to begin with insulin-induced receptor autophosphorylation (3,4). To elucidate subsequent steps in this pathway many investigators have addressed the question of whether or not other *known* signaling molecules participate. The majority of evidence now available has ruled out individual components of the Ras-to-mitogen activated protein kinase pathway as important to insulin stimulation of glucose transport(5-7), although there is some evidence to the contrary (8). Attention has therefore been focused on one or another of the several proteins that interact with insulin receptor substrate-1 (IRS-1; (9)), and/or IRS-2 (formerly 4PS; (10)) which apparently function as the principle - but not exclusive- conduit for most insulin dependent signal transduction (11).

Phosphatidylinositol 3′-kinase (PI3′-K) associates with tyrosine-phosphorylated IRS-1 after insulin stimulation and its activity is dramatically increased (12-15).Two seminal experiments indicated that PI3′-K activation is essential for the signal transduction pathway leading from insulin-stimulated receptor autophosphorylation to the acceleration of glucose transport: *First,* wortmannin inhibited both processes with the same IC_{50} (16,17). *Second,* micro-injection of a mutant regulatory subunit of PI-3′-K blocked insulin stimulated glucose transport in 3T3-L1 adipocytes (18). Thus PI3′-K is an important and apparently necessary intermediate in the signaling pathway of insulin-regulated glucose transport. However, a pathway collinear to the PI3′-K pathway may also be required, since PI3′-K activation is not sufficient to fully activate transport (19,20).

This collinear pathway could involve a phosphotyrosine phosphatase (PTPase), based upon the study by Frost and Lane (21) that first demonstrated that phenylarsine oxide (PAO) inhibited specifically insulin stimulated glucose transport, and later work showing that PAO was a PTPase inhibitor (22-25) However, PAO is known to inhibit enzymes other than PTPases (26), and therefore we addressed the issue of whether or not PAO affects the known steps in insulin stimulated glucose transport. Since it was already demonstrated that PAO affected neither insulin receptor auto- nor substrate phosphorylation *in vivo* and *in vitro,* respectively, (3), we focused on the potential effects of PAO on the recruitment and activation of PI3-K.

EXPERIMENTAL PROCEDURES

Materials. Cell culture reagents were from Gibco/BRL; Streptomycin sulfate, dexamethasone, methyl isobytylxanthine, penicillin G, Bovine serum albumin, Tween-20, sodium orthovandate, and Non-

[1] Present address: Department of Pediatrics, USC School of Medicine, Children's Hospital, Los Angeles, CA.
[2] Corresponding author.

0006-291X/97 $25.00

idet P-40 were from Sigma; Tris, glycine, acrylamide, bis-acrylamide, ammonium persulfate, Triton X-100, and dithiothreitol were from Boehringer/Mannheim; Phenylarsine oxide was from Aldrich; Dimethyl sulfoxide was from Fisher (Certified or HPLC grades) Bovine insulin (Zn salt at 28 U/mg) was from Eli Lily Co.; anti-phosphotyrosine (clone 4G10) was from Upstate Biotechnology Inc.; anti-PI3′K (rabbit polyclonal antibodies against the 85kDa regulatory subunit) was from Santa Cruz (catalog no. SC-423); horseradish-peroxidase conjugated secondary antibodies were from Caltag: Rabbit polyclonal antiserum against IRS-1 was provided by Dr. J. Pierce, National Cancer Institute. All other reagents and chemicals were from Fisher or Sigma.

Cell culture and treatment of 3T3-L1 adipocytes with growth factors and drugs. The cell culture conditions and differentiation protocol of Frost and Lane (21) was used. 3T3-L1 adipocyte monolayers in 10-cm dishes were incubated in 8 ml serum-free Dulbecco's Modified Eagle's Medium (DMEM) for 2-3 hours at 37°C in an atmosphere of 10% CO_2. These serum-starved cells were transferred to a 37°C warm-room, and the DMEM was replaced with 8ml pre-warmed Krebs Ringer's Phosphate supplemented with radioimmuno-assay grade bovine serum albumin at 0.1mg/ml. PAO, prepared fresh in DMSO, and 5 minutes before the addition of 1 μM insulin, PAO was added to monolayers to give the final concentrations indicated in the Figures or text. Monolayers for the basal-state condition were treated as above, but neither PAO nor insulin was added. After these incubations, monolayers were washed twice in ice-cold saline-*T*ris-*v*anadate buffer (STV) containing 1.0 mM Na_3VO_4, 50 mM NaCl and 50 mM Tris-base at pH 7.6. Cells were lysed in 4ml STV containing 1% Nonidet P-40 if used for immunoprecipitation. If samples were taken only for SDS-PAGE, then cells were washed and lysed in 4ml STV at pH 10.5 without detergents; the cytosolic and membrane fractions were separated by ultracentrifugation.

Immunoprecipitation and Western blot. Proteins were immunoprecipitated or co-immunoprecipitated using 2μg rabbit polyclonal antibodies or 2 μl antisera, and 60 μl of a 50% suspension of protein A-Sepharose (Pharmacia) or protein G-agarose (Boehringer/Mannheim). All steps were performed at 4°C or on ice. The Protein A-adsorbed immune complexes were washed 6 times with 1-ml each time of STV/7.6 containing 1% Triton X-100. Bound proteins were eluted by heating in a boiling water bath for 3min in 80μl Laemmli sample buffer containing 100 mM DTT. Proteins were resolved by SDS-PAGE (8%T and 3%C) and detected by Western blot with selected antibodies.

For antiPTyr Western blots the blocking solution was 10% BSA in 20 mM Tris base at pH 7.4, 50 mM NaCl, (TBS) containing 0.2% Tween-20 (TBST), and the primary and secondary antibodies were bound in 2.5% BSA in TBST. For other monoclonal and/or polyclonal antibodies, the blocking solution was 10% (w/v) nonfat dry milk, and the antibodies were bound in 2.5% dry milk in TBST. Detection was done using the enhanced chemiluminescence reagents from Kierkegaard and Perry Laboratories.

PI-3′ kinase assay. The method of Fukui *et al.* (27) was used to measure PI3′-K activity in anti-IRS-1 and/or anti-p85 immunoprecipitates. Adipocyte monolayers in 10-cm culture dishes were treated with growth factors and/or drugs as described above. Cells were lysed in 4ml of ice-cold buffer: 150 mM NaCl, 20 mM Tris-HCl, 1mM PMSF, 1mM Na_3VO_4, 1% Nonidet P-40, and 5 mM EDTA, pH7.5, and the lysates clarified by centrifugation at 12,000 rpm in an SS-34 rotor in a Sorvall RC-5B high speed centrifuge. Immunoprecipitations were done as above, but the immunoprecipitates were washed with the following protocol: 4 times with 1ml of the Nonidet P-40 buffer described above; once with 1ml PBS; once with 1ml 0.5M LiCl in 20 mM Tris-HCl pH7.5; once with 1ml water; once with 1ml 20mM Tris-HCl, 1mMEDTA, 100mM NaCl, pH7.5, and once with 1ml TGN buffer (20mM Tris-HCl, 0.5mM EGTA, 100mM NaCl, pH7.5). PI-3′-K activities in these washed immunoprecipitates were measured in 15min reactions, the phospholipid products extracted in organic solvents, and resolved by thin layer chromatography exactly as described by Fukui *et al.* (27). ^{32}P-labeled phosphatidylinositol was quantified on the dried plates using a Phosphorimager.

Image processing and quantitation. Figures were prepared by scanning on an Agfa color scanner and acquired through Adobe Photoshop. Quantification was done using NIH Image.

RESULTS

Rapid activation of PI3′-K by insulin. It has been shown previously, by us (3) and others (4) that the autophosphorylation of the insulin receptor reached its activated level within one minute after cells were challenged with insulin. The same is true for the activation of PI3′-K (Fig. 1). Two methods of immunoprecipitation were used as the basis for these assays: Either direct immunoprecipitation of PI3′-K through its 85kDa regulatory subunit, or by co-immunoprecipitation through IRS-1 of the complex with PI3′-K was done. *In vitro* assays of PI3′-K activity in these immunoprecipitates showed that PI3′-K became activated to nearly its maximum level within one minute after stimulation by insulin. As internal controls for these assays, we did Western blots with antiPTyr to determine the level of phosphotyrosyl-IRS-1 present (Fig. 1B and C). There was an approximately constant level of phosphotyrosyl-IRS-1 in each series of immunoprecipitates; the slightly higher level of phosphotyrosine in IRS-1 at 1 min has been described by others (11) and has been reproducible in our hands as well. The level of phosphotyrosyl-IRS-1 in these respective immunoprecitates was about 10:1, matching the absolute levels of PI3′-K activity assayed from each immunoprecitiate (Fig. 1A). The lag previously reported for the activation of glucose transport itself (3) was not observed for the activation of PI3′-K, suggesting that this event is several steps upstream of the final steps in transporter recruitment to the plasma membrane. Also, PI3′-K can be assayed after 10min of insulin stimulation, since it is in the activated steady-state at this time.

Inhibition of PI3′-K activation by phenylarsine oxide. PAO was shown first to be an inhibitor of insulin stimulated glucose transport (21), and later shown to inhibit directly protein tyrosine phosphatases (22-25) but not receptor tyrosine kinase autophophosphorylation nor substrate phosphorylation (28). However, PAO has been shown to inhibit NADPH-oxidase (26). To determine whether or not PAO would be diagnostic for a collinear pathway - parallel to but not dependent on PI3′-K - we tested for the ability of PAO to inhbit PI3′-K after its activation reached a steady-state level. To do this, we tested for the inhibition of insulin-dependent PI3-K activation following PAO treatment of intact cells. The *in vitro* assay of PI3′-K activity was measured in immunoprecipitates prepared from lysates of these cells, using either anti-IRS-1 or anti-p85 antibodies. We observed very strong inhibition of PI3′-

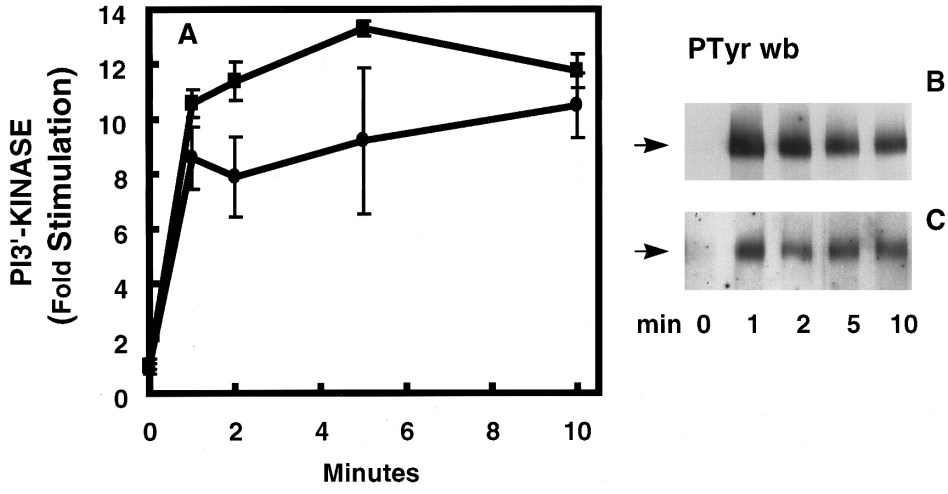

FIG. 1. Time course of PI3′-K activation. Insulin-stimulated PI3′-K activity was measured in αIRS-1 and in α-p85 immunoprecipitates. 3T3-L1 adipocytes were serum-starved for 2.5h and then challenged with 100nM insulin. After the indicated times, the monolayers were lysed in STV/7.6 containing 1.2% Triton X-100 (w/v). Immunoprecipitations were done with either αIRS-1 (■) or α-p85 (●) rabbit polyclonal antibodies. *Left panel.* PI3′-K activities were measured in these immunoprecipitates as described under Experimental Procedures. Results are the averages of duplicate determinations from duplicate immunoprecipitations, and are normalized to show fold-stimulation above each basal value. The relative levels of PI3′-K activity from unstimulated cells were ~ 10:1 in the αIRS-1 *versus* the α-p85 immunoprecipitates. *Right panels.* The levels of phosphotyrosyl-IRS-1 were determined in separate immunoprecipitates from these same cell lysates, by Western blot with anti-PTyr antibodies. The *upper panel* shows phosphotyrosyl-IRS-1 immunoprecipitated with anti-IRS-1 antibodies, and the *lower panel* shows phosphotyrosyl-IRS-1 co-immunoprecipitated with antibodies against the 85kDa regulatory subunit of PI3′-K. The mobility of IRS-1 was confirmed by stripping and reblotting these membranes with anti-IRS-1 antibodies (not shown).

K by PAO (Fig. 2). 30 μM PAO was sufficient to produce full inhibition of insulin stimulated glucose transport (21), and at this concentration the residual PI3′-K was reduced to basal levels. This is in contrast to the effects of wortmannin on PI3′-K activation (16), which are not preserved following cell lysis because of the ready reversibility of inhibition (29).

Recruitment of PI3′-K to IRS-1 in the presence of PAO. The loss of PI3′-K activation in these *in vitro* assays might be caused by a failure of PI3′-K to immunoprecipitate in a complex with phosphotyrosyl-IRS-1. We showed first that PAO treament of the adipocytes did not change the level of insulin stimulated phosphotyrosyl-IRS-1 immunoprecipitated directly with anti-IRS-1 antibodies (Fig. 3A). We next showed that PAO treament of the cells did not reduce the amount of phosphotyrosyl-IRS-1 co-immunoprecipitating with anti-p85 antibodies (Fig. 3B). Consistant with the first report that PAO did not lower substrate phosphorylation by the insulin receptor in vitro (28), we now observe that in whole cells PAO did not decrease - or increase - the level of phosphotyrosyl-IRS-1. Furthermore, PAO did not diminish the recruitment of PI3′-K to phosphotyrosyl-IRS-1.

Activation of PI3′-K by growth factors such as PDGF involves recruitment of the p85-p110 heterodimer to the receptor and may also be accompanied by tyrosine-phosphorylation of the p85 regulatory subunit (30,31). While insulin activates PI3′-K in adipose cells (32,33) there is more evidence to support recruitment to IRS-

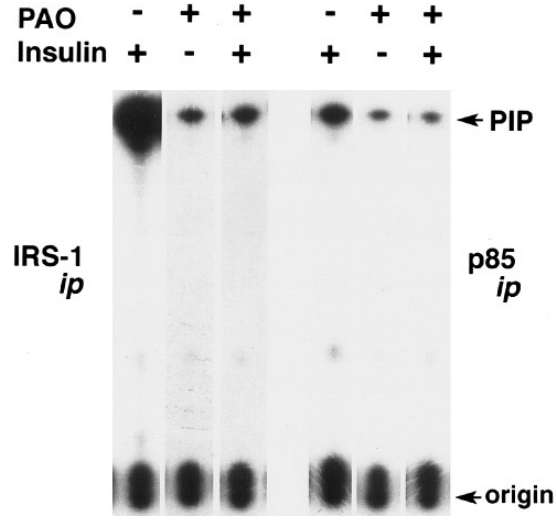

FIG. 2. Effects of phenylarsine oxide on IRS-1-associated PI3′-K activity. PI3′-K activity was assayed in αIRS-1 immunoprecipitates prepared from Triton X-100 lysates of 3T3-L1 adipocytes. The *In vivo* effects of PAO were measured using *in vitro* assays of PI3′-K activity in αIRS-1 (*left panel*) or αp85 (*right panel*) immunoprecipitates prepared from monolayers exposed to 100nM insulin for 15 min, to 30 μM PAO for 5min, or to 30 μM PAO for 5 min followed by 100nM insulin for 15min. Neither carrier DMSO, PAO, nor insulin were present when the monolayers were then washed or lysed, nor during any subsequent step in the immunoprecipitation or assay. Representative assays are shown in these lanes from autoradiograms of the chromatographic separation of reactants and products of the PI3′-K assay, as described under experimental procedures. Further details are given in the text.

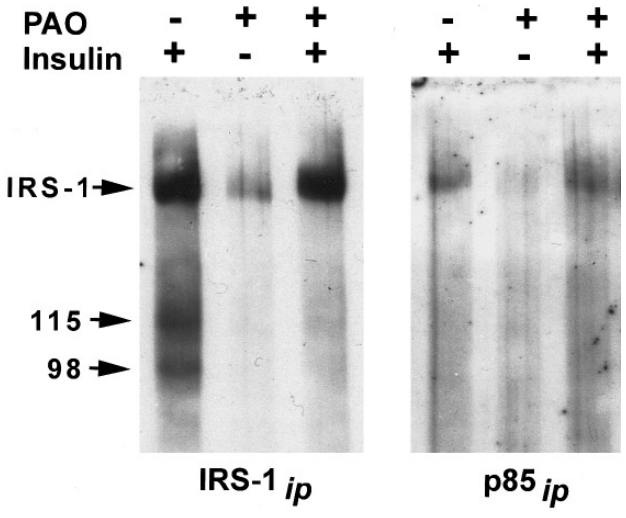

FIG. 3. Effects of phenylarsine oxide on the co-immunoprecipitation of IRS-1 and PI3′-K. 3T3-L1 adipocyte monolayers were serum-starved for 2h and then treated with 100nM insulin, 30μM PAO, or both, as indicated above the panels and as described under Figure 2. The cells were lysed in STV/7.6 containing Triton X-100, and then matched samples were taken for immunoprecipitation. The presence of phosphotyrosyl-IRS-1 in αIRS-1 (*left panel*) and in αp85 (*right panel*) immunoprecipitates was determined by Western blot with αPTyr antibodies, as described under Figure 1. The electrophoretic mobility of phosphotyrosyl IRS-1 is marked by the arrow.

1(34-37) than there is evdience that PI3′-K binds directly to the insulin receptor (38,39). It is also not generally agreed that p85 is (40) or is not (41) phosphorylated in response to insulin. While we have not addressed these issues directly, the only phosphotyrosyl protein detected by Western blot after anti-p85 immunoprecipitation was phosphotyrosyl-IRS-1 (Fig. 2C), and we found no evidence for 85-95kDa phosphotyoryl-proteins in these anti-p85 immunoprecipitates. Therefore, our findings do not support either the insulin-induced tyrosine-phosphorylation of p85, or the association of p85 with the autophosphorylated insulin receptor's 95kDa β-subunit.

PAO inhibits PI3′-K in vitro. Assays were done to test if PAO inhbits PI3′-K in vitro. PI3′-K was co-immunoprecipitated with anti-IRS-1 antibodies from cells stimulated with insulin for 15min. Using a dilution series of PAO added to these assays, it was determined that PAO inhibited PI3′-K activity itself, with an $IC_{50} \approx 20\mu$M and complete inhibition at about 50μM. These results correlate fairly well with the same parameters for the inhibition of insulin-actiated glucose transport that we measured here ($IC_{50} \approx 10\mu$M), and the inhibition of PI3′-K ($IC_{50} \approx 10\mu$M) reported by Okaka *et al.* In these same 3T3-L1 aipocytes (16).

The concentrations of PAO that produced complete inhibition of each process were 30μM PAO for glucose transport, and 50μM PAO for PI3′-K activation. These values are only 3- to 5-fold greater than the IC_{50} for each process, as reported above. More typically, normal saturation would be observed with maximal inhibiton at concentrations of inhibitor at least 10-fold greater than those that yielded the IC_{50}. However, the "shortened titration range" reported here has been seen frequently with PAO in these cells and in other systems and pathways: Frost and Lane reported an IC_{50} against insulin stimulated glucose transport of 7μM, but maximum inhibition ocurred at $\geq$ 25μM PAO (21). Wiley and Cunningham showed an $IC_{50} \approx 3\mu$M PAO but maximum inhibition at $\geq$ 10μM PAO for EGF receptor internalization(42). The inhibition of NADPH oxidase was 50% complete at 0.2μM PAO and 100% complete at 1μM PAO when added to intact neutrophils(26). Thus it appears typical of several systems that the titration of inhibition by PAO is characterized by a 3- to 5-fold difference between IC_{50} and the lowest concentration giving maximum inhibition.

DISCUSSION

The molecular mechanism for activation of PI3′-K by growth factors requires recruitment of the p85-p110 heterodimer to a tyrosine-phosphorylated protein. In most cases this is the receptor tyrosine kinase itself, but for insulin PI3′-K is recruited primarily to IRS-1 as part of its activation(12-14,43). The two steps upstream of PI3′-K activation by insulin, receptor autophosphorylation and IRS-1 phosphorylation, are thus potential targets for inhbitors of glucose transprot that act by blocking insulin activation of PI3′-K. Using intact cells treated with PAO, insulin receptor autophosphorylation was shown previously to be unaffected by PAO (28), and here we demonstrated that IRS-1 phosphorylation was unperturbed by PAO treament as well (Fig. 4A). We also showed that the association between phosphotyrosyl-IRS-1 and PI3′-K was not inhibited by PAO (Fig. 3). This narrowed the target for inhibition to PI3′-K itself, which was inhibited *in vitro* by PAO.

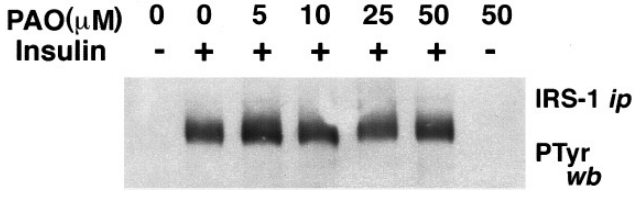

FIG. 4. Effects of phenylarsine oxide on insulin-induced tyrosine phosphorylation of IRS-1. 3T3-L1 adipocyte monolayers were serum-starved for 2h, and then treated with the indicated concentration of PAO (or carrier DMSO alone) for 5min, and then challenged (or not) with 100nM insulin for an additional 15min. Monolayers were lysed in STV/10.5 and clarified by centrifugation. Samples were taken for immunoprecipitation with αIRS-1 antibodies; the pH was adjusted to 7.5-8.0 before addition of the antibodies. Insulin stimulation of the tyrosine phosphorylation of IRS-1 was determined by Western blot with αPTyr after immunoprecipitation with αIRS-1.

These findings are consistant with PAO inhibiting those exocytic and endocytic phases of vesicle trafficking that are regulated by PI3′-K; *i.e.*, glucose transporter recruitment to the plasma membrane, and receptor endocytosis, respectively.

Originally we had hoped to shed light on a potential collinear pathway for glucose transport activation by insulin. The existence of a collinear pathway activated by insulin but not by other growth factors that, in concert with the PI3′-K pathway, leads to translocation of GLUT4 transporters to the plasma membrane, has not been ruled out by this study. However, our findings may help resolve a paradox discussed specifically by Li *et al*. (44): The PTPase inhibitor vandate mimics insulin-stimulated glucose transport (45) but the PTPase inhbitor PAO blocks insulin-stimulated glucose transport(21,46). A similar paradox has been observed in the control of respiratory bursts in macrophages: Both vanadate and PAO increase protein tyrosine phosphorylation after triggering through FcγRI (47), and both agents thus appear to act as PTPase inhibitors. However, in granulocytes vanadate was found to transiently increase NADPH oxidase activity (48), apparently indirectly. In contrast, PAO was shown to inhibit NADPH oxidase activity in neutrophils andt his inhibitory effect of PAO occurs by direct action on NADPH oxidase(26). These counter-active effects of two "PTPase inhbitors" are remeniscent of their paradoxical effects on insulin action in fat cells, and in the case of NADPH-oxidase may be explained by indirect and direct effects on a single enzyme. Similarly, vanadate may increase the recruitment and activation of PI3′-K to docking proteins such as the PDGF receptor or to IRS-1, predominantly through its ability to inhibit the tyrosine-dephosphorylation of those proteins; *i.e.*, an indirect effect on PI3-K leading to activation. PAO, on the other hand, acts directly on PI3′-K to inhibit its activity. The seeming paradox between the actions of these two PTPase inhibitors may therefore be explained. Vanadate acts as an insulin-mimetic by inhibiting PTPases that would normally down-regulate insulin's stimulatory effects, whereas PAO inhibits directly a specific enzyme that would normally be activated by insulin. Thus, in addition to being an inhibitor of PTPase activity, PAO also inhibits NADPH oxidase (26) and PI3′-K. The rpesent study is therefore only the second report of an enzyme, other than a PTPase, that is inhibited directly by PAO. Our findings therefore demonstrate that while PAO does enhance levels of tyrosine phosphorylation, these effects are apparently secondary to its ability to inhibit PI3′-K in the insulin signaling pathway that regulates glucose transport.

REFERENCES

1. Cushman, S. W., and Wardzala, L. J. (1980) *J. Biol. Chem.* **255,** 4758–4762.
2. Suzuki, K., and Kono, T. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 2542–2545.
3. Kohanski, R. A., Frost, S. C., and Lane, M. D. (1986) *J. Biol. Chem.* **261,** 12272–12281.
4. Klein, H. H., Freidenberg, G. R., Kladde, M., and Olefsky, J. M. (1986) *J. Biol. Chem.* **261,** 4691–4697.
5. Robinson, L. J., Razzack, Z. F., Lawrence, J. C., Jr., and James, D. E. (1993) *J. Biol. Chem.* **268,** 26422–26327.
6. Hausdorf, S. F., Frangioni, J. V., and Birnbaum, M. J. (1994) *J. Biol. Chem.* **269,** 21391–21394.
7. Lazar, D. F., Wiese, R. J., Brady, M. J., Mastick, C. C., Waters, S. B., Yamauchi, K., Pessin, J. E., Cuatrecasas, P., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270,** 20801–20807.
8. Kozma, L., Baltensperger, K., Klarlund, J., Porras, A., Santos, E., and Czech, M. P. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4460–4464.
9. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) *Nature* **352,** 73–77.
10. Sun, X. J., Wang, L. M., Zhang, Y., Yenush, L., Myers, M. G., Jr., Glasheen, E., Lane, W. S., Pierce, J. H., and White, M. F. (1995) *Nature* **377,** 173–177.
11. Myers, M. G., Jr., Sun, X. J., and White, M. F. (1994) *Trends. Biochem. Sci.* **19,** 289–293.
12. Kelly, K. L., and Ruderman, N. B. (1993) *J. Biol. Chem.* **268,** 4391–4398.
13. Folli, F., Saad, M. J., Backer, J. M., and Kahn, C. R. (1992) *J. Biol. Chem.* **267,** 22171–22177.
14. Myers, M. G. J., Backer, J. M., Sun, X. J., Shoelson, S., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10350–10354.
15. Backer, J. M., Myers, M. G. J., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J., et al. (1992) *EMBO J.* **11,** 3469–3479.
16. Okada, T., Sakuma, L., Fukui, Y., Hazeki, O., and Ui, M. (1994) *J. Biol. Chem.* **269,** 3563–3567.
17. Clarke, J. F., Young, P. W., Yonezawa, K., Kasuga, M., and Holman, G. D. (1994) *Biochem. J.* **300,** 631–635.
18. Kotani, K., Carozzi, A. J., Sakaue, H., Hara, K., Robinson, L. J., Clark, S. F., Yonezawa, K., James, D. E., and Kasuga, M. (1995) *Biochem. Biophys. Res. Commun.* **209,** 343–348.
19. Wiese, R. J., Mastick, C. C., Lazar, D. F., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270,** 3442–3446.
20. Isakoff, S. J., Taha, C., Rose, E., Marcusohn, J., Klip, A., and Skolnik, E. Y. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10247–10251.
21. Frost, S. C., and Lane, M. D. (1985) *J. Biol. Chem.* **260,** 2646–2652.
22. Liao, K., Hoffman, R. D., and Lane, M. D. (1991) *J. Biol. Chem.* **266,** 6544–6553.
23. Garcia-Morales, P., Minami, Y., Luong, E., Klausner, R. D., and Samelson, L. E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9255–9259.
24. Shekels, L. L., Smith, A. J., Van Etten, R. L., and Bernlohr, D. A. (1992) *Protein Sci.* **1,** 710–721.
25. Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y., and Kasuga, M. (1994) *Mol. Cell Biol.* **14,** 6674–6682.
26. Le Cabec, V., and Maridonneau Parini, I. (1995) *J. Biol. Chem.* **270,** 2067–2073.
27. Fukui, Y., Kornbluth, S., Jong, S.-M., Wang, L., and Hanafusa, H. (1989) *Oncogene Res.* **4,** 283–292.
28. Frost, S. C., Kohanski, R. A., and Lane, M. D. (1987) *J. Biol. Chem.* **262,** 9872–9876.

29. Thelen, M., Wymann, M. P., and Langen, H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4960–4964.
30. Kavanaugh, W. M., Turck, C. W., Klippel, A., and Williams, L. T. (1994) *Biochemistry* **33,** 11046–11050.
31. Escobedo, J. A., Kaplan, D. R., Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1991) *Mol. Cell Biol.* **11,** 1125–1132.
32. Giorgetti, S., Ballotti, R., Kowalski Chauvel, A., Cormont, M., and Van Obberghen, E. (1992) *Eur. J. Biochem.* **207,** 599–606.
33. Kelly, K. L., Ruderman, N. B., and Chen, K. S. (1992) *J. Biol. Chem.* **267,** 3423–3428.
34. Uddin, S., Yenush, L., Sun, X. J., Sweet, M. E., White, M. F., and Platanias, L. C. (1995) *J. Biol. Chem.* **270,** 15938–15941.
35. Myers, M. G., Jr., Sun, X. J., Cheatham, B., Jachna, B. R., Glasheen, E. M., Backer, J. M., and White, M. F. (1993) *Endocrinology* **132,** 1421–1430.
36. Myers, M. G., Jr., Grammer, T. C., Wang, L.-M., Sun, X. J., Pierce, J. H., Blenis, J., and White, M. F. (1994) *J. Biol. Chem.* **269,** 28783–28789.
37. Myers, M. G., Jr., Backer, J. M., Sun, X. J., Shoelson, S., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B., and White, M. F. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 10350–10354.
38. Levy-Toledano, R., Taouis, M., Blaettler, D. H., Gorden, P., and Taylor, S. I. (1994) *J. Biol. Chem.* **269,** 31178–31182.
39. Hayashi, H., Nishioka, Y., Kamohara, S., Kanai, F., Ishii, K., Fukui, Y., Shibasaki, F., Takenawa, T., Kido, H., Katsunuma, N., and Ebina, Y. (1992) *J. Biol. Chem.* **268,** 7017–7117.
40. Hayashi, H., Nishioka, Y., Kamohara, S., Kanai, F., Ishii, K., Fukui, Y., Shibasaki, F., Takenawa, T., Kido, H., Katsunuma, N., et al. (1993) *J. Biol. Chem.* **268,** 7107–7117.
41. Sung, C. K., and Goldfine, I. D. (1992) *Biochem. Biophys. Res. Commun.* **189,** 1024–1030.
42. Wiley, H. S., and Cunningham, D. D. (1982) *J. Biol. Chem.* **257,** 4222–4229.
43. Yonezawa, K., Ueda, H., Hara, K., Nishida, K., Ando, A., Chavanieu, A., Matsuba, H., Shii, K., Yokono, K., Fukui, Y., et al. (1992) *J. Biol. Chem.* **267,** 25958–25965.
44. Li, J., Elberg, G., and Shechter, Y. (1996) *Biochim. Biophys. Acta* **1312,** 223–230.
45. Paquet, M. R., Romanek, R. J., and Sargeant, R. J. (1992) *Mol. Cell Biochem.* **109,** 149–155.
46. Yang, J., Clark, A. E., Harrison, R., Kozka, I. J., and Holman, G. D. (1992) *Biochem. J.* **281,** 809–817.
47. Durden, D. L., Rosen, H., Michel, B. R., and Cooper, J. A. (1994) *Exp. Cell Res.* **211,** 150–162.
48. Bianchini, L., Nanda, A., Wasan, S., and Grinstein, S. (1994) *Biochem. J.* **301,** 539–544.