Effect of Phenylarsine Oxide on Insulin-dependent Protein Phosphorylation and Glucose Transport in 3T3-L1 Adipocytes*  

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We have reported previously that phenylarsine oxide (PAO) blocks insulin-stimulated glucose transport in 3T3-L1 adipocytes (Frost, S. C., and Lane, M. D. (1985) J. Biol. Chem. 260, 2846–2852). As shown in the present study, the locus of inhibition is post-receptor. Insulin stimulated the extent of receptor autophosphorylation in solution and in the intact cell by approximately 4-fold. PAO had no effect on this activity. Using reduced and carboxamidomethylated lysozyme as a substrate for the tyrosine-specific receptor, insulin stimulated the rate of receptor kinase-catalyzed substrate phosphorylation by 2-fold; PAO had no effect on this stimulation. However, the insulin-stimulated, serine-specific phosphorylation of two endogenous phosphoproteins (pp24 and pp240) in the intact cell was blocked by 25 μM PAO. These complementary in vivo and in vitro studies demonstrate that the inhibition by PAO must be distal to the insulin receptor's protein tyrosine kinase activity.

The insulin receptor is a heterodimer composed of two identical insulin-binding subunits (the α-subunit, 135 kDa) covalently attached by disulfide bonds to two identical kinase subunits (the β-subunit, 92 kDa). It is known that insulin stimulates not only the extent of autophosphorylation of the β-subunit but also the catalytic activity toward artificial substrates (1–5). Thus the receptor, itself, could provide the initial steps in a cascade leading eventually to activation of the glucose transporter for glucose. Previous studies with 3T3-L1 adipocytes led to the discovery that a trivalent arsenical, phenylarsine oxide (PAO), blocked insulin-stimulated glucose transport but not basal transport (6). We suggested at that time that PAO was affecting a post-binding site because neither insulin association to cells at 37 °C nor insulin binding in solution were altered by the presence of PAO. In addition, the K₅₀ of the transporter for glucose was not affected by PAO, suggesting that the arsenical was not interfering with sugar transport, directly. We report, here, that PAO does not alter receptor phosphorylation or catalytic activity of the receptor kinase toward an artificial substrate. It does, however, perturb the ability of selective endogenous phosphoproteins to exhibit insulin sensitivity.

EXPERIMENTAL PROCEDURES

Cell Culture—After differentiation, 3T3-L1 adipocytes were maintained in culture as described previously (8).

1 The abbreviations used are: PAO, phenylarsine oxide; RCAM-lysozyme, reduced and carboxamidomethylated lysozyme; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, (ethylenebis(oxyethyl)enitrilo)tetraacetic acid.
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3-O-Methylglucose Uptake—3T3-L1 fibroblasts were grown and differentiated on 15-mm plastic coverslips set in 24-well Lindbro culture plates (5 x 10^5 cells/disc). Before experimentation, the cells were washed three times with 1.0 ml of Krebs-Ringer phosphate buffer, 37 °C. The coverslips were transferred to a vertical holder, made according to the design of Norton and Munck (9). Cells were preincubated at 37 °C for about 20 min in buffer containing 1 mM PAO or buffer without 200 nM PAO and insulin (1 µM) for 1 min. The holders were then lowered into a radioactive solution containing 50 µM 3-O-[methyl-3^2P]glucose (4.8 mCi/mmole) with hormone and/or inhibitor additions when appropriate for 1 min. The coverslips were then passed through a series of three washes of phosphate-buffered saline (25 °C) containing 0.3 mM phloretin to terminate and/or inhibitor additions when appropriate for 1 min. The coverslips were then washed several times with phosphate-free Dulbecco's modified Eagle's medium. The monolayers were then overlayed with this same medium (9). Identification of the β-Subunit of the Insulin Receptor and Insulin-sensitive Phosphoproteins in Situ—When the experimental protocol was complete, monolayers were washed three times with ice-cold phosphate-buffered saline over a 6-s period. Cells were extracted by addition of 2 ml of lysis buffer containing 25 mM Tris, 192 mM glycine, 25 mM EDTA, 5 mM EGTA, pH 8.3, with 0.1% SDS. The combined lysates were homogenized by hand in a glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 4 °C for 45 min at 40,000 x g in a Beckman 50.1 fixed angle rotor. Four ml was removed from under the lipid layer for exposure to 50 µl of WGA-Sepharose (10). An aliquot of the remaining 1 ml was run on an SDS-polyacrylamide gel for the identification of insulin-sensitive phosphoproteins (see below).

Labeling with [3^2P]Orthophosphate in Cell Culture—The labeling procedure for determining phosphorylated insulin receptor and phosphorylated cellular proteins has been presented in detail elsewhere (21). The monolayers were washed several times with phosphate-free Dulbecco's modified Eagle's medium. The monolayers were then overlayed with this same medium containing carrier-free ^32P orthophosphate (500 µCi/ml of medium). After 2 h in a 37 °C, CO₂ incubator, the nucleotide pool was fully equilibrated although the protein pool was not. For this reason, addition of effectors was made such that the final exposure to ^32P was identical with each experimental point.

Identification of the β-Subunit of the Insulin Receptor and Insulin-sensitive Phosphoproteins in Situ—When the experimental protocol was complete, monolayers were washed three times with ice-cold phosphate-buffered saline over a 6-s period. Cells were extracted by addition of 2 ml of lysis buffer containing 25 mM Tris, 192 mM glycine, 25 mM EDTA, 5 mM EGTA, pH 8.3, with 0.1% SDS. The plates were washed with 3 ml of this same buffer only containing 1.7% Triton X-100. The combined lysates were homogenized by hand in a glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 4 °C for 45 min at 40,000 x g in a Beckman 50.1 fixed angle rotor. Four ml was removed from under the lipid layer for exposure to 50 µl of WGA-Sepharose (10). An aliquot of the remaining 1 ml was run on an SDS-polyacrylamide gel for the identification of insulin-sensitive phosphoproteins (see below). Recovery of autophosphorylation of the insulin receptor was determined by centrifugation and washing five times with 1.0 ml of the lysis buffer containing 1.0% Triton X-100 and 0.04% SDS. Glycoproteins were eluted with the addition of Laemmli sample buffer containing 1.5 mM N-acetylglucosamine and 2% SDS for 30 min. A two-dimensional system was used to separate the receptor from other proteins, as described in detail earlier (10). Briefly, the entire sample was overlayed on a nonreducing tube gel (5% total acrylamide separating gel). The second dimension was performed on a reducing slab gel (8% total acrylamide separating gel). The second dimension was dried on Whatman No. 3MM paper, and the β-subunit of the insulin receptor was visualized by autoradiography after an 18-h exposure. The radioactive spot was cut from the gel, rehydrated with 0.1 ml of water, and counted in 10 ml of Fluorosol after heating for 2 h at 60 °C.

Identification of cellular phosphoproteins was performed in one of two ways. Fifty µl of extract was mixed with SDS sample buffer and run on a 12.5% SDS reducing gel. Molecular weight standards included myosin, M, 200,000; phosphorylase b, M, 97,000; bovine albumin, M, 68,000; ovalbumin, M, 45,000; a-chymotrypsin, M, 25,000; lysozyme, M, 14,400. The gel was dried on Whatman No. 3MM paper and phosphoproteins identified by autoradiography. Additionally, the segments were cut, rehydrated, and counted as above.

In Vitro Phosphorylation of the Insulin Receptor and Artificial Substrate—Insulin receptor from 3T3-L1 adipocytes was partially purified through the WGA-Sepharose chromatography step according to Kohanski and Lane (10). The receptor (approximately 0.1-0.5 pmol of insulin-binding sites) was incubated in the presence of absence of 1 µM insulin, 100 µM polyepitope substrate (reduced and carboxamidomethylated lysozyme), and 100 µM phenylarsine oxide in a buffer containing 20 µM [γ-3^2P]ATP (70 dpm/pmole), 50 nM Hepes, 5 mM Mg(CH3CO)2 at pH 6.9 containing 0.1% Triton X-100 in a total reaction volume of 220 µl. Reactions were initiated with the addition of labeled ATP, and an aliquot was removed and quenched with 0.3 mM phloretin to terminate sugar transport. With each new solution that the cells were exposed to, the holder was quickly raised and lowered five times, resulting in rapid equilibration of the buffer over the surface of the cells (9). The coverslips were transferred to 15 ml glass scintillation vials containing 1.0 ml of 0.1% sodium dodecyl sulfate. After the experiment was completed, 10 ml of scintillation fluid was added and vials were shaken until the solution cleared. Results were corrected for trapped min. The holder was then lowered into a radioactive solution containing 50 µM 3-O-[methyl-3^2P]glucose (4.8 mCi/mmole) with hormone and/or inhibitor additions when appropriate for 1 min. The coverslips were then washed several times with phosphate-buffered saline (25 °C) containing 0.3 mM phloretin to terminate and/or inhibitor additions when appropriate for 1 min. The coverslips were then washed several times with phosphate-free Dulbecco's modified Eagle's medium. The monolayers were then overlayed with this same medium (9). Identification of the β-Subunit of the Insulin Receptor and Insulin-sensitive Phosphoproteins in Situ—When the experimental protocol was complete, monolayers were washed three times with ice-cold phosphate-buffered saline over a 6-s period. Cells were extracted by addition of 2 ml of lysis buffer containing 25 mM Tris, 192 mM glycine, 25 mM EDTA, 5 mM EGTA, pH 8.3, with 0.1% SDS. The plates were washed with 3 ml of this same buffer only containing 1.7% Triton X-100. The combined lysates were homogenized by hand in a glass homogenizer with a Teflon pestle. The homogenates were centrifuged at 4 °C for 45 min at 40,000 x g in a Beckman 50.1 fixed angle rotor. Four ml was removed from under the lipid layer for exposure to 50 µl of WGA-Sepharose (10). An aliquot of the remaining 1 ml was run on an SDS-polyacrylamide gel for the identification of insulin-sensitive phosphoproteins (see below).

Results and Discussion

To define the steps in a metabolic cascade, an inhibitor of that process is particularly useful, as has been demonstrated multiple times in the various metabolic pathways. We have demonstrated that a trivalent arsenical, phenylarsine oxide (PAO), inhibits insulin-stimulated glucose transport (K, = 7 µM).

![FIG. 1. In vitro autophosphorylation of the insulin receptor.](image-url)
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Effect of PAO on the Tyrosine Kinase Activity of the Insulin Receptor in Vitro—The in vitro protein kinase assay allows us to examine the catalytic activity of the kinase, under defined conditions, both toward itself and artificial substrates in lieu of an endogenous substrate. The results are shown in Figs. 1 and 2. Insulin, at 1 μM, stimulated the incorporation of [γ-32P]ATP into the β-subunit of the soluble receptor, seen as an increase in the rate as well as in the extent of autophosphorylation (Fig. 1). PAO, at 100 μM, had no effect on either the basal or insulin-stimulated autophosphorylation rate. These studies were performed in the absence of added substrate so that the only protein phosphorylated in the assay mix was the β-subunit itself, shown in the inset of Fig. 1.

Since autophosphorylation did not appear to be altered by PAO, we tested the effect of PAO on the catalytic activity of the receptor toward an artificial substrate. RCAM-lysozyme has been shown to be an excellent substrate for the insulin receptor with a Km in the low micromolar range and phosphorylated strictly on tyrosine (4). As shown in Fig. 2, A and B, in the presence of partially purified insulin receptor, insulin pretreatment stimulated the rate of incorporation of 32P from [γ-32P]ATP into RCAM-lysozyme by about 2-fold. PAO, at 100 μM, did not affect the rate of phosphorylation by the receptor kinase either in the basal or insulin-stimulated state. Note that autophosphorylation of the receptor preincubated with insulin (in the presence or absence of PAO) was stimulated 2-fold during the first 2 min of incubation with RCAM-lysozyme (Fig. 2A); radioactivity incorporated into the β-subunit was basal (lanes 1–4), 275 ± 20 cpm; insulin-treated (lanes 5–8), 586 ± 22 cpm; PAO-treated (lanes 9–12), 280 ± 34 cpm; PAO- and insulin-treated (lanes 13–16), 582 ± 34 cpm. Further autophosphorylation was blocked by RCAM-lysozyme, consistent with results of Kohanski and Lane (4).

This fractional autophosphorylation correlates well with the 2-fold increase in the rate of substrate phosphorylation. Together, these results suggest that the protein kinase activity of the receptor in solution, be it toward itself or toward other tyrosine-containing substrates, is not altered by PAO.

Effect of PAO on Insulin-sensitive Phosphoproteins in Intact 3T3-L1 Adipocytes—The second approach for defining the

![Fig. 2. Effect of PAO on insulin receptor kinase-mediated substrate phosphorylation in solution. In A, partially purified insulin receptor from 3T3-L1 adipocytes was preincubated with or without 1 μM insulin and/or 100 μM PAO for 15 min. [γ-32P]ATP and RCAM lysozyme were added at time 0 and incubated as indicated. The reaction was terminated by addition of SDS sample buffer and an aliquot run on a reducing SDS-polyacrylamide gel to separate phosphorylated β-subunit from RCAM-lysozyme. For B, gel segments from the molecular size region of 14.4 kDa were excised, rehydrated, and counted by liquid scintillation spectrometry. The symbols depict the following preincubation experimental situation: O, no additions; △, plus insulin; ●, plus PAO; ■, plus PAO and insulin. In addition, the gel segments at 92 kDa were excised and counted (see text for quantitation).](image1)

![Fig. 3. Effect of PAO on insulin-sensitive glycoproteins. 3T3-L1 adipocytes equilibrated with 32P, for 2 h were exposed to or not to 25 μM PAO for 10 min followed by incubation in the presence or absence of 1 μM insulin for an additional 10 min. After harvesting, glycoproteins were collected by chromatography with WGA-Sepharose, eluted with N-acetylglucosamine, and separated by two-dimensional SDS-polyacrylamide gel electrophoresis (2). The open arrow indicates the position of the β-subunit of the insulin receptor. The closed arrow indicates the pp240, an insulin-sensitive phosphoprotein. Experimental conditions represented in panel A, control; panel B, plus insulin; panel C, plus insulin and PAO. Duplicate experiments were run, only one of which is illustrated.](image2)
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Table 1

Effect of phenylarsine oxide on insulin-sensitive phosphoproteins

Intact 3T3-L1 monolayers were pre-equilibrated with [32P]orthophosphate for 2 h and then exposed or not to 25 μM PAO for 10 min. Monolayers were then stimulated or not with 1 μM insulin for 10 min. Following detergent extraction of the monolayers, the [32P]-labeled proteins were resolved by nonreducing/reducing two-dimensional SDS-PAGE of the WGA-Sepharose-adsorbed proteins (insulin receptor β-subunit and pp240; Fig. 3) or by one-dimensional reducing SDS-PAGE of non-adsorbed proteins (pp18 and pp24; Fig. 4). Quantitation of radio-label in the rehydrated gel segments was by liquid scintillation counting; data were normalized to total radioactivity per 4 × 10^6 cells according to the aliquot size as a fraction of the total extract. The predominant phosphoamino acids were phosphotyrosine (β-subunit) and phosphoserine (pp240, pp24, and pp18). Details of 3-O-methylglucose uptake are given in the text.

| Treatment           | β-Subunit | pp240 | pp24 | pp18 | 3-O-Methylglucose uptake |
|---------------------|-----------|-------|------|------|--------------------------|
|                     | cpm/μg × 10^6 cells | pmol/min/10^6 cells |      |      |                          |
| No additions        | 124 ± 12  | 45 ± 7 | 38,800 | 14,800 | 13.6 ± 1.5               |
| +Insulin            | 517 ± 78  | 332 ± 93 | 64,960 | 7,520  | 52.3 ± 2.7               |
| +PAO + insulin      | 532 ± 86  | 40 ± 7  | 37,360 | 8,000  | 13.9 ± 1.6               |

Fig. 4. Effect of PAO on insulin-sensitive phosphoproteins. An aliquot of extract not adsorbed to WGA-Sepharose from Fig. 3 was analyzed by SDS-polyacrylamide gel electrophoresis. Thus, when present, 25 μM PAO was incubated with cells for 10 min after [32P] equilibration followed by incubation with 1 μM insulin for 10 min. In A, the proteins were visualized by autoradiography and the gel slices rehydrated and counted for radioactivity (quantitation in text). Shown in A is the autoradiogram of extracts from control cells, insulin-treated cells, and PAO and insulin-treated cells. To determine the time frame over which pp24 and pp18 were phosphorylated, cells were labeled for 2 h followed by addition of 1 μM insulin for varying times (B). After washing in phosphate-buffered saline, the cells were extracted as described under Experimental Procedures and an aliquot was analyzed by SDS-polyacrylamide gel electrophoresis. The closed circles represent the radioactivity from the higher molecular weight protein (pp24), the closed triangles represent the radioactivity from the lower molecular weight protein (pp18). The inset shows the radioactive bands of pp24 and pp18 as visualized by autoradiography.

The site of PAO inhibition was to determine the effect of PAO on receptor phosphorylation in situ. 3T3-L1 adipocytes were equilibrated with [32P], and then preincubated with or without PAO at 25 μM for 10 min. Following a 10-min incubation with or without insulin, the cells were extracted and assayed for [32P] incorporated into the β-subunit. The autoradiogram of a two-dimensional SDS-PAGE is presented in Fig. 3 and the data are summarized in Table I. As described earlier (2), insulin increased the extent of phosphorylated receptor by about 4-fold (Fig. 3, panels A and B) specifically on tyrosine. The only form of the receptor observed in the nonreducing first dimension exhibited an apparent Mr, of 350,000 from which the 95-kD β-subunit was generated in the reducing second dimension. In two separate experiments, PAO had no effect in the incorporation of label into the β-subunit in either the basal or insulin-stimulated receptor (PAO-treated basal receptor not shown; insulin-stimulated, or PAO- and insulin-treated receptor shown in Fig. 3, panels B and C, respectively). Note also that PAO did not affect the mobility of the receptor in the first dimension and thus does not appear to alter the structural integrity of the holoreceptor.

Although an endogenous substrate (tyrosine-specific) for the insulin receptor has not yet been conclusively identified, several candidate proteins have been detected (13, 14). In addition, various proteins have been noted whose insulin-stimulated phosphorylation occurs on serine (15–17). We have identified several such proteins in 3T3-L1 adipocytes, as shown in Figs. 3 and 4 and the results summarized in Table I. One such protein is a glycoprotein (pp240) observed in the WGA-Sepharose eluate along with the insulin receptor. Shown in Fig. 3 is the migration of this phosphoprotein, in relation to the β-subunit of the receptor, in the second dimension of a reducing SDS-PAGE (see Experimental Procedures). Insulin stimulated the extent of phosphorylation exclusively on serine residues (data not shown) by about 7-

As shown by Frost and Lane (8), 25 μM PAO is the minimal concentration required to elicit maximal inhibition of insulin-stimulated glucose transport.
fold (Fig. 3, panels A and B). Pretreatment of the cells with 25 μM PAO blocked this insulin-stimulated event (Fig. 3, panel C).

A smaller molecular weight protein (pp24) has also been identified which exhibits insulin-sensitivity (Fig. 4 and Table I). This protein is not adsorbed to WGA-Sepharose. As shown, insulin stimulated the incorporation of 32P by about 2-fold, specifically on serine. PAO inhibited the insulin-stimulated phosphorylation of this protein as well (Fig. 4A). Interestingly, the insulin-stimulated phosphorylation of this protein is time-dependent (τa = 1.7 min) (Fig. 4B), occurring in the "window" between receptor phosphorylation (τa = 8 s) and transport activation (τa = 2.5 min) (2). This suggests that pp24 may be an intermediate in an insulin-sensitive cascade. A similar insulin-sensitive phosphoprotein has been identified by Blackshear et al. (17), although the time course of activation is extended, suggesting parallel activation with transport. Like the inhibition of insulin-sensitive phosphorylation of pp240 and pp24, insulin-stimulated glucose transport is inhibited by PAO (Table I), confirming earlier results. It should be emphasized that PAO reduced the insulin-sensitive component of pp240, pp24, and glucose transport to basal values; the basal values, themselves, were not affected by PAO (data not shown). Unlike pp240 and pp24, the phosphorylation state of other proteins, altered by the presence of insulin, is not changed due to the presence of PAO. For example, the phosphorylation state of a smaller molecular weight protein (pp18) is reduced in the presence of insulin and not reversed by PAO (Fig. 4). This suggests that PAO does not generally and nonspecifically disrupt the response pathways of insulin in whole cells and indicates a divergence in the signaling system at some point along the (hypothetical) cascade since PAO blocks only one branch of the path.

Independent studies by Cushman and Wardzala (18) and Suzuki and Kono (19) provide evidence that the glucose transporter is recruited from an intracellular storage site to the plasma membrane in the presence of insulin. It would be attractive if phosphorylation of the transporter were the signal that initiated this recruitment. However, recent studies by Gibbs et al. (20) show that insulin does not alter the extremely low phosphorylation state of the transporter. So even though the insulin-stimulated kinase activity seems to be important for signaling the transport system, the transporter itself does not appear to be phosphorylated.

Thus, a variety of experiments show positive correlation between the receptor kinase and acceleration of the glucose transport rate. Our results suggest that PAO, which inhibits the activation of glucose transport by insulin, does not affect the function of the insulin receptor, itself, as determined by insulin binding and phosphorylation. PAO appears to interact at a post-receptor point, but not at the transporter, uncoupling the activated receptor from the glucose transporter system and several insulin-sensitive phosphoproteins. Interestingly, it has been shown recently in 3T3-L1 adipocytes, that a phosphotyrosyl-containing protein (pp15) accumulates in the presence of insulin and PAO (21). The accumulation of this phosphoprotein and the decrease in the phosphoseryl proteins pp240 and pp24 are consistent with PAO inhibition at an intervening step.

These data, then, demonstrate two important facts. First, the receptor does not appear to communicate directly with the transporter; at least one additional step must exist between the receptor and the glucose transport system. Second, by localizing the site of PAO action we may be able to identify some of the components of the insulin "cascade." Thus, PAO is not just a pharmacological agent but may itself be a useful tool for the examination of the mechanism of some of insulin's actions inside the cell.

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