Uptake and binding of radiolabelled phenylarsine oxide in 3T3-L1 adipocytes

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Phenylarsine oxide (PAO), a trivalent arsenical, has been shown to inhibit insulin-stimulated glucose transport in 3T3-L1 adipocytes, implicating vicinal dithiols in signal transmission [Frost & Lane (1985) J. Biol. Chem. 260, 2646-2652]. To assist in the direct identification of a PAO-binding protein which might be involved in this process, we have synthesized [3H]acetylamino phenylarsine oxide ([3H]APA0 from the amino derivative of phenylarsine oxide (NPAO). To assess the inhibitory effect of the product, a dual-labelling experiment was performed which showed that [3H]APA0 inhibited insulin-stimulated 2-deoxy[1-14C]glucose transport in 3T3-L1 adipocytes with a K_i of 21 μM, identical with that of the parent compound, NPAO. Further characterization revealed that over a wide concentration range, uptake of the labelled arsenic oxide was linear. Although the dithiol reagent 2,3-dimercaptopropanol (DMP) reversed PAO-induced inhibition of transport, it had no effect on the uptake of [3H]APA0. In a simple fractionation experiment approx. 50% of the radioactivity was associated with the cytosolic fraction and 50% with the total membrane fraction. Identification of radiolabelled proteins by non-reducing SDS/PAGE revealed fraction-specific binding, although many proteins were observed. Covalent modification was time-dependent and could be reversed by addition of DMP. These data further support a role for vicinal dithiols in insulin-stimulated glucose transport. Additionally, the probe described may offer a new means with which to identify the inhibitory protein or, more globally, to investigate mechanisms of action of vicinal dithiol-containing proteins.

INTRODUCTION

It has been known for many years that insulin stimulates the rate of glucose transport in specific target tissues. Both the insulin receptor (the β-subunit) (Ebina et al., 1985) and the glucose transporter (Mueckler et al., 1985) are integral proteins of the membrane. Although the signalling event between the two proteins is not known, it has been proposed that stimulation of transport activity by insulin may occur by recruitment of transporters to the cell surface (translocation) (Cushman & Wardzala, 1980; Suzuki & Kono, 1980) and/or by intrinsic activation (Baly & Horuk, 1987; Kahn & Cushman, 1987; Joost et al., 1988; Blok et al., 1988; Calderhead & Lienhard, 1988). It has been further suggested that autophosphorylation of the insulin receptor and subsequent activation of the tyrosine kinase play a role in signal transmission (Morgan et al., 1986; Ellis et al., 1986). Of potential importance is the observation that phenylarsine oxide (PAO) inhibits insulin-stimulated glucose transport (but not basal transport) in 3T3-L1 adipocytes (Frost & Lane, 1985). This inhibition could be reversed by 2,3-dimercaptopropanol (DMP) but not mercaptoethanol (MCE), suggesting the involvement of vicinal dithiols. Further experiments indicated that PAO interfered with post-receptor signalling, since neither insulin binding (Frost & Lane, 1985) nor receptor tyrosine kinase activity (Frost et al., 1987) were affected by the arsenical. However, PAO did inhibit certain insulin-stimulated serine-specific phosphorylation events in intact cells (Frost et al., 1987). One interpretation of this latter observation is that the activated insulin receptor stimulates the activity of a serine kinase. Other experiments have been presented which support the existence of such a cascade (Czech et al., 1988; Ray & Sturgill, 1988). However, other data also suggest that PAO may block a tyrosine-specific phosphatase (Bernier et al., 1987), perhaps inhibiting the turnover of a signal molecule.

With PAO covalently attached to a resin, preliminary data suggested that PAO must enter the cell to elicit inhibition (Saylor & Frost, 1989). Thus the goals of this study were to synthesize a labelled arsenical, to characterize its uptake into 3T3-L1 adipocytes, and to identify PAO-binding proteins. This information would provide the basis for future identification of the PAO-sensitive target in insulin-stimulated glucose transport, a step toward understanding how insulin functions inside the cell.

EXPERIMENTAL

Acetylation of para-aminophenylarsine oxide

The synthesis of aminophenylarsine oxide (NPAO) has been described (Stevenson et al., 1978). The acetylation reaction was performed in a vented hood. The reaction vessel containing [3H]acetic anhydride (9.5Ci/mmol; 2.63 μmol/25 μCi) was capped with a septum stopper so that additions could be made through the septum. The bottom of the vessel was cooled in solid CO_2 to condense the acetic anhydride. The seal was broken and 2.5 μmol of NPAO, dissolved in 100 μl of anhydrous acetone, was injected into the reaction chamber. The vessel was brought room temperature and allowed to sit for 60 min, with mixing every 15 min. Unlabelled acetic anhydride (10 μmol, in 100 μl of anhydrous acetone) was added to the reaction and the entire sample was carefully dried under N_2. The vessel was washed with 8 x 1.0 ml of water. The washes were frozen and dried and a Speed Vac rotary evaporator (Savant). The dried precipitate was washed a second time, dried and resuspended in 4.0 ml of 95% ethanol. The solution was clarified by addition of 1 M HCl and then dried under N_2. The sample was finally suspended in 2 ml of 1:1

Abbreviations used: PAO, phenylarsine oxide; NPAO, para-aminophenylarsine oxide; APAO, acetylamino phenylarsine oxide; DMP, 2,3-dimercaptopropanol; MCE, mercaptoethanol; KRP, Krebs-Ringer phosphate buffer; PBS, phosphate-buffered saline.

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concentrations of PAO (0-80 nmol) or [3H]APAO, determined by modification of Ellman's procedure (Ellman, 1959). For each assay, 120 nmol of DMP was mixed with 3 ml of 20 mM-phosphate buffer, pH 8.0, just before analysis. Various concentrations of PAO (0-80 nmol) or [3H]APAO were then added to appropriate tubes, each containing 3 ml of the DMP solution. After 5 min, 250 nmol of DTNB [5,5'-dithiobis-(2-nitro)benzoic acid] was added to each tube and mixed immediately. The absorbance of the [3H]APAO samples was read at 412 nm and compared with the known values for PAO.

**Uptake and binding of [3H]APAO**

3T3-L1 adipocytes were incubated in serum-free medium for 2 h in a CO₂ incubator. The cells were then washed three times with 3.0 ml of Krebs-Ringer phosphate buffer, pH 7.4 (KRP). The cells were incubated in 1.0 ml of KRP with or without 1 μM-insulin for 10 min at 37°C. Following this, the cells were incubated with various concentrations of [3H]APAO in the presence or absence of 400 μM-DMP for the indicated times. The cells were then washed with 3 x 3.0 ml of ice-cold phosphate-buffered saline (PBS; 120 mM-NaCl/2.7 mM-KCl/10 mM-sodium phosphate, pH 7.4) and homogenized either in 0.5 ml of TES (Tris, 10 mM; EDTA, 1 mM; sucrose, 250 mM; pH, 7.3) for subcellular fractionation or in 0.5 ml of SDS (0.1%) for non-fractionated samples. The TES homogenate was centrifuged at 21,200 g for 70 min at 4°C. The supernatant (cytosolic fraction) was removed and the pellet (membrane fraction) was resuspended in 0.5 ml of TES. A portion of each fraction was assayed for radioactivity or was mixed with sample dilution buffer (Laemmli, 1970) and run on a 12.5% non-reducing SDS/polyacrylamide gel. After drying, the gels were incubated in 1 M-salicylic acid for 1 h. The dried gels were exposed to pre-flashed film for 7 days. When appropriate, lanes were analysed by laser densitometry.

**Labelling of proteins in vitro**

Cells were treated as described above with or without insulin for 10 min. The cells were then homogenized in 0.5 ml of TES and incubated with 50 μM-[3H]APAO at 37°C. The membranes were collected by centrifugation and resuspended in 0.5 ml of TES. Portions of the cytosolic and membrane fractions were counted for radioactivity or mixed with sample dilution buffer and run on a 12.5% non-reducing SDS/polyacrylamide gel as described above.

**Precipitation of radiolabelled proteins with trichloroacetic acid**

After incubation with [3H]APAO in the absence or presence of 200 μM-DMP, cells were washed with 3 x 3.0 ml of PBS and extracted in 0.1% SDS. To 100 μl of the extract, 10 μl of 100% trichloroacetic acid was added and incubated on ice for 30 min. After centrifugation for 5 min at 4°C, the pellet was resuspended in 100 μl of sample dilution buffer containing 10 μl of 10-mM-NaOH. Portions were sampled for radioactivity.

**Materials**

[3H]Acetate anhydride was purchased from Amersham Corp. (Arlington Heights, IN, U.S.A.). Protein standards were purchased from Bio-Rad (Rockville Center, NY, U.S.A.). Insulin was a gift from Dr. Ronald Chance, Eli Lilly, Co., Indianapolis, IN. U.S.A. NPAO was a gift from Dr. Kenneth Stevenson, University of Calgary, Calgary, Canada. All other supplies were of the highest quality available.

**RESULTS**

**Effect of arsenicals on insulin-stimulated deoxyglucose transport**

As shown earlier (Frost & Lane, 1985) and verified here, PAO effectively blocked insulin-stimulated glucose transport (K, 9 μM) (Fig. 1a). Since a derivative of this arsenical was to be used for radiolabelling and subsequent identification of binding protein(s), it was necessary to demonstrate its effectiveness in the transport assay. Accordingly, we show in Fig. 1(a) that NPAO inhibited insulin-stimulated transport in a manner similar to PAO, although the curve was shifted to the right (K, 21 μM). Unlabelled PAO (synthesized as described in the Experimental section but using unlabelled acetate anhydride) gave similar results. Using a dual-labelling procedure we then monitored the effect of [3H]APAO on deoxy[1-14C]glucose transport. With increasing labelled arsenical concentration, increasing inhibition of glucose transport was observed (Fig. 1b). An inhibition constant of 21 μM was calculated, identical to that of the unlabelled PAO derivatives.

**Uptake of [3H]APAO**

Since the labelled arsenical inhibited insulin-stimulated transport as did the parent compound, we then determined cell-dependent uptake (association) of the radiolabelled PAO. Over a wide range of concentrations the uptake of the labelled arsenical was linear (Fig. 1c). At each concentration, approx. 8% of the added radioactivity was taken up during the 30 min incubation. Approx. 90% of the cell-associated label could be precipitated with trichloroacetic acid.

When cells were preincubated with insulin to first activate transport followed by NPAO addition, we were able to determine the minimum time required for arsenical action. As shown in Fig. 2, inhibition of insulin-stimulated transport could be seen within 2.5 min of NPAO addition, although the half-time was 10 min (inset). The association of labelled arsenical also appeared to be time-dependent, ultimately reaching a steady-state plateau (Fig. 3a). The half-time for association was 10 min, identical to that for inhibition from the stimulated state. Because the association data suggested exchange between the cell and extracellular buffer, we also measured the efflux of [3H]APAO (Fig. 3b). As can be seen, cell-associated radioactivity fell to a steady level as [3H]APAO was released from 'pre-loaded' cells into the buffer. Thus there was a cell-associated pool or compartment (about 30% of the associated label) that allowed rapid exchange of [3H]APAO. It should be pointed out that in a comparable glucose transport experiment, simple washing as was done in the above release experiment was not sufficient to reverse PAO-induced inhibition of insulin-stimulated transport (results not shown). Thus the exchangeable [3H]APAO did not contribute to the inhibitory process. As shown below, most of the radioactivity was complexed covalently to cellular proteins.

**Effect of thiol reagents on [3H]APAO uptake**

It has been shown previously that the dithiol reagent DMP
Uptake of phenylarsine oxide

1. Fig. Vol. 269 3T3-L1 (a) presence or absence of various concentrations of arsenical (PAO, prevented and/or reversed the inhibition of insulin-stimulated glucose transport by PAO (Frost & Lane, 1985). This was also true for NPAO-induced inhibition, as shown in Fig. 4(a). Despite its ability to reverse inhibition, DMP had no effect on the association of radiolabelled arsenical to whole cells (Fig. 4b and Table 1). This suggests that the hydrophobic character of the arsenical rather than its interaction with dithiols dictates physical location. Unlike DMP, MCE was ineffective in reversing inhibition (Fig. 4a). However, MCE enhanced association by 4-fold (Fig. 4b) presumably because of non-specific interaction of the labelled arsenical with free thiols which are exposed upon reduction of oligomeric proteins.

Compartmentation of \(^{3}H\)APA0
To identify the subcellular compartment to which the labelled arsenical associates, a simple fractionation was performed. After incubation with \(^{3}H\)APA0 for 30 min in the absence or presence of insulin, the cells were washed, homogenized and separated by centrifugation into membrane and cytosolic fractions. Assay of these fractions revealed that 52% of the radioactivity was associated with the membranes, while a surprising 48% was associated with the cytosol (Table 1). The fractions were analysed by non-reducing SDS/PAGE. The cytosolic fraction contained at least ten labelled proteins, ranging in molecular mass from 13 kDa to 95 kDa, several of which were unique to this fraction (including 13.9 kDa, 15.4 kDa, 36 kDa) (Fig. 5). The membrane fraction also showed unique proteins (30 kDa and 64 kDa). For comparison, a second experiment is shown in which intact cells were treated with insulin or not, homogenized and secondarily incubated with \(^{3}H\)APA0. The incubation in vitro labelled proteins of identical migration as in the intact cells but labelling was more intense, presumably because of greater accessibility to \(^{3}H\)APA0. Insulin had no significant effect on uptake (see Table 1) or binding of \(^{3}H\)APA0 in any fraction or under any experimental condition.

Time-dependent binding of \(^{3}H\)APA0 and its reversal
When cells were preincubated for 10 min with NPAO and then assayed in the presence of insulin and DMP, inhibition of transport was quickly reversed (Fig. 6a). A corresponding experiment was performed with \(^{3}H\)APA0 to follow protein-specific binding and its reversal by DMP. Figure 6(b) shows the time-dependent binding of \(^{3}H\)APA0 to proteins extracted from intact cells at various time points during a 10 min preincubation.

\(\Delta\): NPAO, ■: unlabelled APA0, ◆: This was followed by incubation with 1 \(\mu\)M-insulin for 10 min and subsequently with 200 \(\mu\)M-2-deoxy[\(^{3}H\)]glucose for 10 min. The cells were then washed with 3 x 3.0 ml of ice-cold PBS and air-dried. The cells were extracted with 1.0 ml of 0.1% SDS and a portion was counted for radioactivity. The data are expressed as uptake of deoxyglucose in nmol/min per 10\(^6\) cells, representing the averages of experiments performed on four different days (\(n = 8\)) with a standard deviation of less than 8%. (b) 3T3-L1 adipocytes were preincubated for 10 min at 37 °C in the presence of various concentrations of \(^{3}H\)APA0, determined from the specific radioactivity of the arsenical, for 10 min. This was followed by incubation with insulin for 10 min and 200 \(\mu\)M-2-deoxy[\(^{14}C\)]glucose for an additional 10 min. Uptake was terminated by washing, as described above, and extraction to determine transport of deoxyglucose and uptake of labelled PAO (see c). The data represent the averages of duplicate points from four different experiments (\(n = 8\)), with standard deviations of less than 10%. The values for insulin-stimulated and basal glucose transport were 1.57 ± 0.05 and 0.091 ± 0.008 nmol/min per 10\(^6\) cells respectively. (c) Uptake of \(^{3}H\)APA0 from the experiment described in (b). The data are shown as the average uptake of arsenical in mmol/10\(^6\) cells, with a standard deviation of less than 10% (\(n = 8\)). The duration of the uptake experiment was 30 min.

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Fig. 1. Characterization of effects of APA0 in 3T3-L1 adipocytes
(a) 3T3-L1 adipocytes were preincubated for 10 min at 37 °C in the presence or absence of various concentrations of arsenical (PAO,
Fig. 2. Time-dependent inhibition of insulin-stimulated glucose transport

Cells were preincubated for 10 min with 1 μM insulin. NPAO (50 μM) was added and transport was measured for 2.5 min intervals at various times thereafter. The inset shows the same data plotted on a log scale for half-time determination. ◊, Basal; ●, insulin-stimulated; ▲, insulin plus NPAO.

analogous to the experiment with NPAO described in Fig. 6(a). If DMP was added at the same time as [3H]APAO, no labelled proteins were observed (results not shown). If DMP was added after 10 min (again analogous to Fig. 6a), DMP immediately caused a decrease in the amount of label bound to proteins (Fig. 6c). Denitometric analysis of the autoradiogram was performed to compare labelled proteins and quantify binding. The actual profiles for lane 4 in Fig. 6(b) (10 min uptake of [3H]APAO) and lane 2 in Fig. 6(c) (10 min uptake of [3H]APAO followed by 5 min incubation with DMP) are presented in Fig. 7(a). The upper trace reveals that ten major proteins were labelled by the arsenical after 10 min of preincubation (see legend for molecular masses). At 5 min after addition of DMP (lower trace), label remained only in proteins 1, 6, 9 and 10, which may be resistant to DMP because of inaccessibility, by virtue of either structure or compartmentation. Fig. 7 also confirms the time-dependent binding (b) and further shows that within 2.5 min of DMP

Fig. 3. Time-dependent uptake of [3H]APAO

(a) 3T3-L1 adipocytes were incubated with 4 μM (▲), 10 μM (●), 25 μM (●) or 50 μM (■) [3H]APAO for various times at 37 °C. As indicated, the cells were washed and extracted with 0.1 % SDS and analysed for radioactivity. The data are expressed in nmol/10⁶ cells. The standard error in duplicate determinations was less than 10 %.

(b) Cells were pre-loaded with 50 μM [3H]APAO for 30 min at 37 °C. After six washes with PBS over 30 s, the cells were incubated for up to 120 min in 1.0 ml of KRP containing 5 mM glucose. Radioactivity was measured in both the buffer (●) and in SDS-solubilized cells (◊). The total radioactivity (▽) (cell-associated plus released) did not change over the course of the experiment. The data represent the average of duplicate determinations, with an error of less than 10 %.

Fig. 4. Effect of thiol reagents on transport inhibition and [3H]APAO uptake

(a) Cells were incubated in KR P with 50 μM-NPAO for 10 min. This was followed by the concurrent addition of thiol reagent (▲, DMP; ●, MCE) and 1 μM-insulin. After 10 min, transport was measured during a 10 min interval. The data represent the averages of three separate experiments. The insulin-stimulated and basal values for transport were 1.50 ± 0.08 and 0.21 ± 0.01 nmol/min per 10⁶ cells respectively. (b) Cells were incubated for 10 min in the presence of 50 μM [3H]APAO. At that point, dithiol reagent (▲, DMP; ●, MCE) was added and the incubation was continued for an additional 20 min. Cells were washed and extracted in 0.1 % SDS for counting of radioactivity. As in (a), total exposure to the arsenical was 30 min. The data represent the averages of three separate experiments.
Table 1. Uptake of [H]APAO

Cells or homogenate was incubated with 25 μM [H]APAO for 30 min followed by fractionation as described in the Experimental section. The values represent duplicate experiments with standard error of less than 7%. The corresponding values for glucose transport in similar cells were as follows (in nmol/min per 10⁶ cells): basal, 0.14 ± 0.01; 1 μM-insulin, 1.78 ± 0.02; 25 μM-NPAO + insulin, 0.72 ± 0.03; NPAO + insulin + 200 μM-DMP, 1.77 ± 0.05.

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addition, more than 90% of the protein-associated radioactivity was lost (Fig. 7c), which correlates with the immediate recovery of transport stimulation in the presence of DMP (Fig. 6a). From these data, we conclude that binding of [H]APAO (and thus inhibition) was dependent on the interaction of the arsenical with

![Graph](image_url)

Fig. 6. Effect of DMP on arsenical binding
(a) Cells were preincubated with (●, ▲) or without (○) 50 μM-NPAO for 10 min. At zero time, 1 μM-insulin with (●) or without (○, ▲) 400 μM-DMP was added to the cells and transport was examined at various times. (b) Cells were incubated for various times with 28 μM-[H]APAO (620 c.p.m./pmol). Cells were washed with PBS and lysed with 0.1% SDS. An aliquot was mixed with sample dilution buffer and run on a non-reducing SDS gel. Lanes 1-4 represent preincubation times with [H]APAO of 2.5, 5.0, 7.5 and 10 min respectively, and show an increase in labelling intensity with time. (c) Cells were preincubated with [H]APAO for 10 min as in (b), at which point 400 μM-DMP was added. At the appropriate time thereafter cells were homogenized in 0.1% SDS and an aliquot was mixed with sample dilution buffer for SDS/PAGE. Lanes 1-7 represent incubation times with DMP of 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 and 30.0 min respectively, and show a decrease in labelling intensity with time.

dithiol-containing proteins. This was supported by trichloroacetic acid precipitation experiments, which demonstrated that the amount of precipitated radiolabelled proteins was decreased from 91% in the absence of DMP to 19% in the presence of DMP.

DISCUSSION

The data presented here demonstrate the use of a new labelled derivative of PAO to identify dithiol-containing proteins. We
The monothiol reduces disulphide bonds which are distant from one another and through this action can dissociate subunits of oligomeric proteins. The labelled arsenical can then interact non-specifically with the exposed sulphydryl groups. It had been our initial hope that we could utilize MCE to reduce non-specific interactions, but the results proved the opposite to be true. On the other hand, the dithiol reagent does not function to dissociate proteins but reduces redox vicinal sulphydryls (Webb, 1966). Since DMP reverses PAO inhibition of transport and [3H]PAO binding, this strongly suggests that PAO binds to proteins which contain vicinal dithiols. It is apparent that insulin action requires the activation of a protein(s) which contains a redox dithiol.

One could envisage a variety of processes with which the arsenical might interfere. Perhaps the most simple would be intercalation into membranes, reducing fluidity and thus affecting membrane function. However, uptake of the arsenical was not sufficient for inhibition: covalent modification of neighbouring sulphydryls was required. Although unlikely, based on the chemical specificity of the arsenical, sulphydryl modification could cause extensive cross-linking of proteins in either the membrane or other compartment. However, we have found no evidence that such cross-linking occurs, as no residual protein was observed at the top of the non-reducing gels. Since neither hormone binding nor kinase activity of the insulin receptor are affected by PAO (Frost et al., 1987), one could suggest that the arsenicals interfere with a secondary signal. This could be accomplished by either direct interaction with that signal leading to reduction of an endogenous activity (a kinase or phosphatase), or indirectly by limiting access to the signalling pathway (interfering with receptor internalization). It has been shown previously that internalization of several hormone–receptor complexes is blocked by PAO (Wiley & Cunningham, 1982; Hertel et al., 1985), including the insulin receptor in 3T3-C2 fibroblasts (Knutson et al., 1983) which serve as the control cell line for 3T3-L1 adipocytes. However, it is unclear whether insulin receptor internalization is required for activation of intracellular events.

Of potential interest is the cytosolic protein which migrates at 15.4 kDa and can be reversibly labelled with [3H]PAO. During differentiation of the 3T3-L1 cell line, translation of adipocyte-specific proteins occurs (Mackall et al., 1976), one of which is a fatty-acid-binding protein which migrates as a 15 kDa species (Bernlohr et al., 1984). Although there are two cysteine residues in the amino acid sequence of this protein, they are distant from each other (Bernlohr et al., 1984), which would not provide the most stable arsenical–dithiol configuration (which is a five- or six-membered ring as occurs with PAO–lipoic acid; Webb, 1966). Attempts at positive identification of the labelled 15 kDa protein by immunoprecipitation with an antibody specific for the fatty-acid-binding protein have proved unsuccessful. Thus the 15 kDa protein that binds the labelled arsenical may merely co-migrate with the fatty-acid-binding protein.

In summary, we have been able to synthesise a labelled trivalent arsenical which has inhibitory properties that are similar to those of the parent compound. We have used this analogue to quantify uptake and demonstrate binding to dithiol-specific proteins. These results could be useful in identifying the factor whose interaction with the dithiol-modifying arsenical prevents insulin-stimulated glucose transport. In a more general sense, this labelled arsenical may be useful in analysing the mechanism of action of dithiol-containing proteins.

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