

Insulin-induced p21ras Activation Does Not Require Protein Kinase C, but a Protein Sensitive to Phenylarsine Oxide*

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Insulin treatment of fibroblasts overexpressing the insulin receptor causes a rapid accumulation of the GTP-bound form of p21ras. We have studied the involvement of protein kinase C (PKC) in, and the effect of phenylarsine oxide (PAO), a putative inhibitor of tyrosine phosphatase activity on, this process. Activation of p21ras was not observed when the cells were stimulated with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and pretreatment with TPA for 16 h, sufficient to down-regulate PKC activity, did not abolish p21ras activation by insulin. These results show that PKC is not involved in the insulin-induced activation of p21ras.

Pretreatment of the cells with PAO for 5 min completely blocked insulin-induced p21ras activation. Addition of 2,3-dimercaptopropanol prevented this inhibition by PAO. Also, addition of PAO after insulin stimulation could reverse the activation of p21ras. Since PAO did not affect overall phosphorylation of the insulin receptor β -chain, we conclude that a PAO-sensitive protein is involved in the induction of p21ras activation by insulin.

The ras genes encode closely related 21-kDa proteins, p21ras, that can bind GTP and GDP and may function as signal transducing molecules (1, 2). ras proteins cycle between an active (GTP-bound) and an inactive (GDP-bound) conformation (3, 4). This ras-cycle is thought to be controlled by at least two different activities. First, activation of p21ras is facilitated by nucleotide exchange factors that promote the exchange of GDP for GTP (5–7). Second, p21ras can be inactivated by GTPase activating proteins (GAP,¹ NF-1) that stimulate the intrinsic GTPase activity of p21ras (8–12). Oncogenic mutations in the ras gene, found in a large number of human tumors (13), give rise to a ras protein that is constitutively in the GTP-bound form, and therefore active (14–17).

Using a variety of experimental approaches it has been shown that p21ras may function in several signal transduction pathways directed by membrane-associated tyrosine kinase

receptors, to mediate growth factor stimulated mitogenicity and gene expression (18–21). Indeed in several cases growth factor stimulation leads to an increase in the GTP-conformation of p21ras (22–26). In particular, stimulation of the T cell receptor (TCR) in T lymphocytes (25), and stimulation of the insulin receptor (IR) in fibroblasts expressing elevated levels of the human insulin receptor (24) resulted in a dramatic increase in the GTP-bound state of p21ras.

In T lymphocytes, p21ras activation is suggested to occur via the activation of protein kinase C (PKC), resulting in the inactivation of GAP (or NF-1) activity (25). Furthermore, proper TCR-signaling seems to require the function of two tyrosine kinases, lck (27) and fyn (28), and a tyrosine phosphatase, CD45 (29). The mechanism by which insulin triggers the activation of p21ras, however, is still elusive. Similarities between TCR and IR signaling suggest that the mechanism of p21ras activation by insulin may resemble that of TCR-induced p21ras activation. For instance, signal transduction initiated by either one of these receptors requires tyrosine kinase activity (lck and fyn for TCR, IR- β -chain for IR), and in both pathways tyrosine phosphatases are involved that are sensitive to phenylarsine oxide (CD45 for TCR (30), HA1 and HA2 for IR (31)). Also, like in TCR-signaling, in insulin-mediated signal transduction PKC may play an important role as well. First, insulin can stimulate the production of diacylglycerol from various sources, resulting in the subsequent activation of PKC (32, 33). Secondly, PKC-activating agents, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), mimic insulin action in some cases (34–36).

In this report we have investigated the involvement of PKC in, and the effect of PAO on insulin-induced p21ras activation. We found that in contrast to TCR-induced p21ras activation, PKC is not involved in insulin-induced activation. However, insulin-induced p21ras activation is sensitive to PAO.

MATERIALS AND METHODS

Materials—Phenylarsine oxide (PAO), 2,3-dimercaptopropanol (DMP), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), insulin, protease inhibitors, and sodium vanadate were obtained from Sigma. Monoclonal antibody against the insulin receptor (RPN 538) and radiochemicals were from Amersham, and cell culture media were from GIBCO. Stock solutions for PAO and DMP were made in dimethyl sulfoxide (DMSO).

Cell Culture—A14 cells are NIH3T3 cells expressing 3×10^5 high affinity human insulin receptors ($K_d \leq 10^{-9}$ M) per cell (24). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 8% fetal calf serum (FCS, GIBCO). For serum starvation, subconfluent dishes were cultured in DMEM containing 0.5% FCS for 16 h.

[³²P]Orthophosphate Labeling of Cell Cultures—Cells were plated on 5-cm tissue culture dishes, at least 24 h prior to labeling, to exclude residual activation of the insulin receptor caused by trypsinization. After serum starvation for 16 h, cells were labeled for 3 h in phosphate-free/serum-free medium (GIBCO) supplemented with 200 μ Ci of [³²P]orthophosphate per 5-cm dish (24).

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¹ The abbreviations used are: GAP, GTPase-activating protein; TCR, T cell receptor; IR, insulin receptor; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PAO, phenylarsine oxide; DMP, 2,3-dimercaptopropanol; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Determination of GTP/GDP Ratio—³²P-Labeled cells were treated with different compounds as indicated, and lysates were made using a phase-split purification as described (37). Subsequently, p21ras was immunoprecipitated with monoclonal antibody Y13-259, and GTP/GDP nucleotides were eluted and separated by thin-layer chromatography as described (25). After autoradiography, GTP/GDP ratios were determined by counting the separated nucleotides in a scintillation counter.

80-kDa Phosphorylation—During serum starvation cells were either left untreated or treated with 100 ng/ml TPA for 16 h, and cells were labeled for an additional 3 h with [³²P]orthophosphate. Both untreated and TPA pretreated cells were either left unstimulated or stimulated with 100 ng/ml TPA for 5 min. Total protein lysates were separated on a 7.5% SDS-polyacrylamide gel. Gels were fixed and dried prior to autoradiography.

Insulin Receptor β -Chain Phosphorylation—After treatment with PAO, DMSO, and/or insulin, [³²P]orthophosphate-labeled cells were lysed in a buffer containing: 50 mM HEPES buffer, pH 7.4, 1% Triton X-100, 0.05% SDS, 0.5% deoxycholic acid, 100 mM NaCl, 5 mM MgCl₂, 1 mg/ml bovine serum albumin, 10 mM benzamidine, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate. The insulin receptor was immunoprecipitated using a monoclonal antibody coupled to protein A beads. Immunoprecipitates were analyzed on a 7.5% SDS-polyacrylamide gel.

RESULTS

PKC Is Not Involved in Insulin-induced Activation of p21ras—We have previously shown a rapid accumulation of the GTP form of p21ras induced by insulin in NIH3T3 fibroblasts overexpressing the human insulin receptor (A14 cells) (24). To analyze an upstream function for PKC activation in this process, we measured the effect of TPA treatment on the activation state of p21ras. As shown in Fig. 1, a 5-min

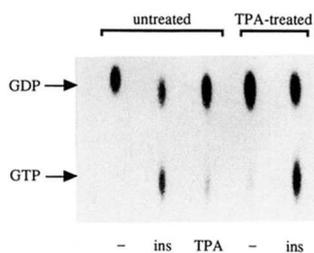


FIG. 1. The role of protein kinase C in p21ras activation. Cells were serum-starved for 16 h on 0.5% FCS, in the absence (untreated), or presence of 100 ng/ml TPA (TPA-treated). The cells were labeled with [³²P]orthophosphate for 3 h, and stimulated with growth factors for 5 min. Cells were lysed and p21ras was collected by immunoprecipitation with monoclonal Y13-259. Bound nucleotides were eluted and separated by thin-layer chromatography. A14 cells: unstimulated (-), stimulated insulin for 5 min (*ins*, 10 μ g/ml), or stimulated with TPA for 5 min (*TPA*, 100 ng/ml).

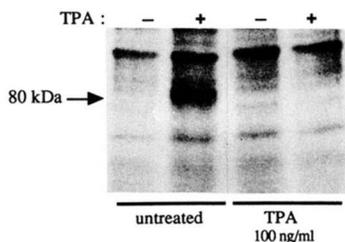


FIG. 2. PKC depletion by prolonged exposure to TPA. Cells were serum-starved for 16 h on 0.5% FCS, in the absence or presence of TPA (100 ng/ml). Cells were labeled with [³²P]orthophosphate for 3 h, and stimulated with TPA for 5 min (100 ng/ml). Cells were lysed and proteins were separated on polyacrylamide gel electrophoresis. Gels were fixed and dried before autoradiography. A14 cells: untreated (untreated), pretreated with TPA (TPA, 100 ng/ml); either unstimulated (-), or stimulated with TPA (+) for 5 min. The arrow indicates the position of the 80-kDa protein.

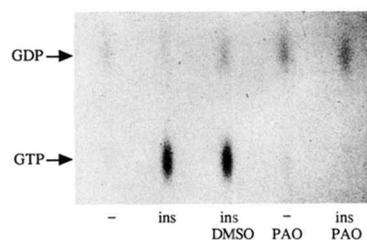


FIG. 3. Inhibition of insulin-induced p21ras activation by PAO. Cells were serum-starved for 16 h on 0.5% FCS, and labeled for 3 h with [³²P]orthophosphate. Cells were preincubated with, or without PAO, or DMSO for 5 min. After preincubation, insulin was added to the cells for 5 min, and GTP/GDP ratios were determined as described in Fig. 1. A14 cells: unstimulated (-), or stimulated with insulin (*ins*, 10 μ g/ml); without pretreatment, pretreated with DMSO (*DMSO*, 1 μ l/ml), or pretreated with PAO (*PAO*, 25 μ M).

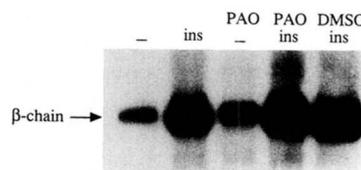


FIG. 4. Effect of PAO on autophosphorylation of the insulin receptor β -chain. Cells were serum-starved and labeled with [³²P]orthophosphate as described in the legend of Fig. 1. After pretreatment with PAO, or DMSO for 5 min, cells were stimulated with insulin (5 min, 10 μ g/ml). Cells were lysed and the insulin-receptor was precipitated using monoclonal antibody RPN538. The precipitates were analyzed on a 7.5% SDS-polyacrylamide gel. A14 cells: unstimulated (-) or stimulated with insulin (*ins*); without pretreatment, pretreated with PAO (*PAO*, 25 μ M), or pretreated with DMSO. The arrow indicates the position of the insulin receptor β -chain.

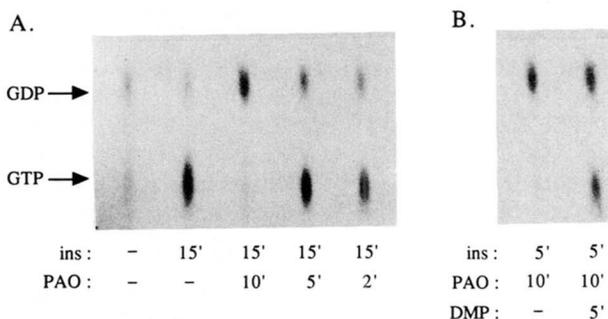


FIG. 5. Reversion of p21ras activation and PAO inhibition. Cells were serum-starved and labeled with [³²P]orthophosphate as described in the legend of Fig. 1. The cells were then treated with insulin (10 μ g/ml), PAO (25 μ M), and/or DMP (50 μ M) for various lengths of time prior to lysis as indicated. Cell lysates were made and GTP/GDP ratios on p21ras determined as described under "Materials and Methods." **A**, A14 cells: untreated (-), or treated with insulin for 15 min; without addition of PAO (-), or with addition of PAO for 10, 5, or 2 min prior to lysis. **B**, A14 cells: pretreated with PAO for 5 min (25 μ M), and stimulated with insulin (10 μ g/ml) in the absence (-) or presence of DMP for 5 min prior to lysis.

TPA stimulation of A14 cells did not result in a shift of the p21ras GTP/GDP ratio. Next, we pretreated the A14 cells with 100 ng/ml TPA for a period of 16 h, to deplete the cells of PKC activity (38), and measured the p21ras GTP/GDP ratio after insulin stimulation. This TPA pretreatment did not influence the insulin-induced shift in the GTP/GDP ratio on p21ras. As a control for PKC down-modulation, we analyzed the phosphorylation of the 80-kDa MARCKS protein, a major substrate of PKC often used as a marker for PKC activity in intact cells (38-40). A 5-min stimulation with TPA was sufficient to stimulate 80-kDa phosphorylation (Fig. 2).

Induction of 80-kDa phosphorylation by a short stimulation with TPA was abolished after pretreatment with 100 ng/ml TPA for 16 h, indicating that cells are depleted of TPA-sensitive PKC activity using this protocol. From these results we conclude that PKC is not involved in the activation of p21ras by insulin.

PAO Inhibits the Activation of p21ras by Insulin—Phenylarsine oxide has been found to interfere with signal transduction from the insulin receptor (41, 42). To test its effect on the shift in the p21ras GTP/GDP ratio induced by insulin, we added PAO 5 min prior to insulin stimulation. PAO completely blocked activation of p21ras at a concentration of 25 μ M (Fig. 3). Addition of solvent alone (DMSO) had no effect on the insulin-induced activation of p21ras. To ascertain that addition of PAO does not influence insulin-induced phosphorylation of the β -chain of the insulin receptor, thereby inhibiting p21ras activation, we immunoprecipitated the insulin receptor from 32 P-labeled cell lysates. Consistent with previous findings (42), PAO treatment resulted in a small increase in phosphorylation rather than an inhibition of insulin-induced β -chain phosphorylation (Fig. 4). This suggests that PAO inhibition does not affect the insulin receptor itself, but interacts with a protein that functions somewhere between the activated receptor and p21ras.

Reversion of p21ras Activation and PAO Inhibition—To gain further insight in the process affected by PAO, we investigated the kinetics of inhibition. For this, we stimulated the cells 15 min prior to lysis with insulin. Full activation of p21ras (~70% GTP-bound) is achieved within 2 min, and remains at this level for at least 15 min (24). PAO was added at different time points during a 15-min insulin stimulation, starting at 10 min prior to lysis. We did no longer observe an insulin-induced shift in the GTP/GDP ratio on p21ras when PAO was added for 10 min, but activation was hardly affected when PAO treatment lasted only 5 min (Fig. 5A).

Inhibition of insulin-induced responses by PAO can be prevented by addition of DMP, a compound containing vicinal sulfhydryls, thus capable of competing for PAO binding. We observed complete reversion of PAO inhibition when 50 μ M DMP was added simultaneously with insulin (Fig. 5B), similar to findings with insulin-induced glucose uptake (41).

DISCUSSION

We have studied the role of PKC in the insulin-induced activation of p21ras, since PKC mediates p21ras activation in T lymphocytes (25). Depletion of PKC by prolonged TPA treatment in A14 cells did not abolish the ability of insulin to induce a shift in the p21ras GTP/GDP ratio. Such a pretreatment did result in the loss of TPA-sensitive PKC activity as judged by the ability of TPA to induce phosphorylation of the 80-kDa substrate (MARCKS protein) of PKC. Likewise, PKC activation by a short incubation with TPA did not mimic the effect of insulin on the activation state of p21ras. Although not all PKC isozymes are sensitive to TPA (43–45), the PKC isozyme that mediates activation of p21ras in T cells is activated by TPA (25). Furthermore, in NIH3T3 cells the PKC- α isozyme seems to be the major form that is expressed, and this PKC isozyme is sensitive to TPA down-modulation (46–48). We conclude that (a TPA-sensitive) PKC is not involved in insulin-induced activation of p21ras, and that the mechanism of p21ras activation by insulin in A14 cells differs from the activation of p21ras by TCR stimulation.

The finding that PAO can inhibit both insulin-induced, as well as TCR-induced signal transduction, prompted us to evaluate the effect of PAO on insulin-induced p21ras activation. A pretreatment of 5 min with PAO resulted in a complete

inhibition of p21ras activation, although the overall insulin receptor phosphorylation remained unaffected. Addition of 2,3-dimercaptopropanol to cells preincubated with PAO resulted in restoration of the insulin-induction of p21ras activation. Thus, PAO seems to exhibit its inhibitory effect on p21ras activation through binding of vicinal sulfhydryls (41). The kinetics and characteristics of inhibition by PAO are similar to that found for the inhibition of insulin-induced glucose uptake by PAO (42). This may indicate that the same PAO-sensitive protein is involved in the generation of both effects. Inhibition of insulin-induced glucose uptake by PAO is thought to occur through inactivation of a tyrosine phosphatase, since several proteins, phosphorylated in response to insulin, were shown to accumulate in the presence of PAO (49). PAO had no effect on proteins phosphorylated in response to serum or platelet-derived growth factor, indicating that there is some specificity in the inhibiting effect of PAO. One of the proteins that could be detected after insulin stimulation in the presence of PAO is a 15-kDa phosphoprotein (50). This protein was shown to be the fatty acid-binding protein 422(aP2) that can be phosphorylated by the insulin receptor *in vitro*, in a fatty acid-dependent manner (51). Recently, it was shown that the dephosphorylation of this protein can be mediated by two different tyrosine phosphatases, HA1 and HA2, both associated with the cell membrane (31). These two phosphatases were purified from 3T3 adipocytes, and shown to be inactive in the presence of PAO. In T lymphocytes PAO inhibits CD45 phosphatase activity, having no effect on the kinase activity of both *lck* and *fyn*, again indicating that PAO may be a specific tyrosine phosphatase inhibitor (30). However, PAO binds to vicinal sulfhydryl groups (52) and may interact with other proteins as well, so clearly a more detailed analysis is needed before a definite conclusion concerning the function of the PAO-sensitive component in this mechanism can be drawn.

Accumulation of the GTP-bound form of p21ras in A14 cells is maximal within 2 min after insulin addition, and remains at this elevated level (~70% GTP bound) for at least 15 min (24). Thus we were able to study whether PAO could not only prevent, but also reverse the activation of p21ras induced by insulin. When cells were stimulated with insulin for 15 min and PAO was present during the last 10 min, we observed complete reversion of insulin-induced p21ras activation, whereas addition of PAO 5 min prior to lysis had almost no effect. The implications of these results are several-fold. First, the fact that we indeed observe reversion points to a mechanism in which constitutive activation of upstream elements is necessary for sustained p21ras activation. Secondly, while a 5-min treatment with PAO is still without effect on the insulin stimulation, 10 min after addition of PAO all p21ras is converted to the GDP form. Thus, complete inactivation takes place in 5 min. The fact that we find no effect of PAO during the first 5 min of treatment is probably due to the time PAO needs to cross the cellular membrane (53). Since hydrolysis of GTP bound to p21ras *in vitro* is rather slow (54, 55), this implies that the activity of a GTPase activating protein (GAP or NF-1) is considerable in these cells, at least in the absence of an insulin stimulus.

Thus far, the nature of the PAO-sensitive protein is still unknown. It could be one of the tyrosine phosphatases as discussed above. This would imply that tyrosine dephosphorylation is an essential step in insulin induction of p21ras activation, alternatively, PAO might inhibit another protein activity. In both cases, PAO can be used as a tool for deciphering the pathway between insulin stimulation and p21ras activation.

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