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Phenylarsine Oxide Inhibits Acid Phosphatase-1

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Cells require intracellular communication systems through signal transductions for living. Most signal transductions in mammalian cells are regulated by protein phosphorylation and dephosphorylation, and these reversible reactions can turn on or off biological functions and thus regulate various cellular mechanisms such as growth, proliferation, differentiation.[1] Protein phosphorylation and dephosphorylation occur on serine, threonine, or tyrosine residues of target proteins by the action of protein kinases and phosphatases. Such phosphorylation and dephosphorylation induce conformational changes in target molecules and thus regulate their activity. Among phosphorylation-mediated signaling pathways, mitogen-activated protein kinase (MAPK) pathway is the most well-known signaling pathway that communicates a signal from the outside of cells to the DNA in the nucleus through phosphorylation of many proteins involved in those pathways. The major step for inactivation of MAPK pathway is dephosphorylation of phosphorylated signaling proteins by protein phosphatases that are members of protein tyrosine phosphatase (PTP) superfamily. PTP superfamily consists of over 100 enzymes. Based on the amino acid sequences of their catalytic domains, the PTPs can be grouped into four main families: (i) classical PTPs and dual-specificity protein tyrosine phosphatases (DUSPs), (ii) tyrosine-specific low molecular weight phosphatases, (iii) the cdc25 family, and (iv) Eyes absent (EyA) protein that was recently discovered to be tyrosine-, or dual serine- and tyrosine-specific protein phosphatases.[2] Many studies have shown that modulation of enzyme activities of PTPs are involved in regulating diverse cellular biological functions and diseases. Thus regulation of PTP activities and their chemical regulators are important in therapeutic studies as cancer, inflammation, and other diseases.[3]

Acid phosphatase-1 (ACP-1) is a genetically polymorphic enzyme member and belong to the low molecular weight protein tyrosine phosphatase family.[4] Its molecular weight is 18 kDa and its crystal structure has been reported.[5] ACP-1 has 3 isoforms due to alternative splicing of a single transcript. Isoform 1 and 2 have phosphatase activity but not isoform 3. Isoform 1 interacts with the SH3 domain of SPTAN1 while isoform 2 or 3 does not have apparent interaction with SPTAN1.[6] ACP-1 interacts with various receptor tyrosine kinases and docking proteins, including platelet-derived growth factor,[7] ephrinA2 receptor,[8] and β-catenin.[9] ACP-1 is involved in the early events of platelet-derived growth factor (PDGF) receptor signal transduction[7] and functions as a negative regulator of growth factor-induced cell proliferation, although in some cases it also acts as a positive regulator.[10-12]

Phenylarsine oxide (PAO), which is an organic trivalent arsenical and acts as an oxidant, is commonly used as an inhibitor of PTPs in biological research (Fig. 1(a)).[13] PAO crosslinks vicinal thiol groups of cysteinyl residues and thus inactivates PTPs that have conserved cysteinyl residues in the catalytic site.[14] Treatment of cells with PAO led to an increase in protein phosphorylation.[15,16] PAO has previously been shown to inhibit phosphatase activities of CD45, PTP HA1, PTP HA2, and PTPN11.[15,17,18] However, it is not clear whether PAO acts on other PTPs to inhibit their phosphatase activity.

In this study, we examined whether PAO inactivates phosphatase activities of several other PTPs. Seven recombinant PTPs were overexpressed in bacteria as 6 x His-tagged forms and purified by nickel-chelate column chromato-

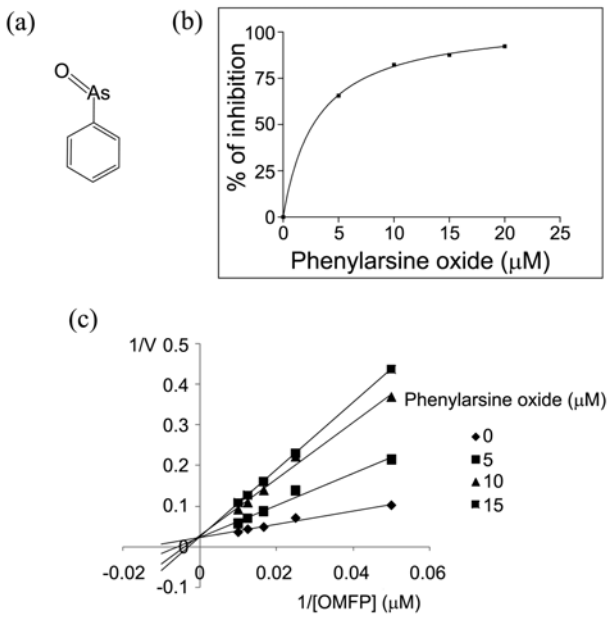

Figure 1. Inhibitory effect of PAO on ACP-1. (a) Chemical structure of phenylarsine oxide. (b) ACP-1 (100 nM) was incubated with various concentrations of PAO at 37 °C for 30 min. Fluorescence emission from the product was measured by a multiwell plate reader as described in Experimental Section. The graph is shown as the percentage of inhibitory effect by various concentrations of PAO. (c) Lineweaver-Burk plots of ACP-1 were generated from the reciprocal data.

Table 1. Inhibition of PTPs by phenylarsine oxide

Protein tyrosine phosphatase	IC_{50} (μM)
DUSP3	No inhibition
DUSP13	No inhibition
DUSP14	No inhibition
DUSP18	No inhibition
DUSP23	No inhibition
DUSP26	No inhibition
ACP-1	3.058 ± 0.66 (n = 3)

Inhibition of enzyme activity by PAO was measured with PTPs including DUSP3, DUSP13, DUSP14, DUSP18, DUSP23, DUSP26 and ACP-1. Each experiment was performed in triplicate. *In vitro* phosphatase assay was processed as in Experimental Section. Data are presented as mean ± S.E.M. IC_{50} of ACP-1 determined by this experiment is 3.058 ± 0.66 (n = 3) but other PTPs were not inhibited

graphy as described previously.[19] We measured the inhibitory effect of PAO on PTPs. Phosphatase activity assays were carried out *in vitro* using OMFP as a substrate. When PTPs were treated with PAO, only ACP-1 activity was clearly decreased by PAO (Table 1). Addition of dithiothreitol, a reducing agent, to the reaction abolished the inhibitory activity of PAO to ACP-1, suggesting that PAO oxidizes ACP-1 for inhibition (data not shown). When ACP-1was treated with various concentrations of PAO (0-20 μM), ACP-1 activity was decreased by PAO in a dose-dependent manner with an IC_{50} of 3.058 ± 0.66 μM (Fig. 1(b)). Kinetic studies with PAO and ACP-1 showed that PAO acts as a competitive inhibitor with the K_i of 3.85 μM, suggesting that PAO acts in the catalytic cleft of substrate binding site (Fig. 1(c)). Taken together, these results suggest that PAO induces the formation of an S-S bridge between the two catalytic site cysteines of ACP-1.

We also examined whether PAO could inhibit ACP-1 expressed in mammalian cells, since proteins expressed in

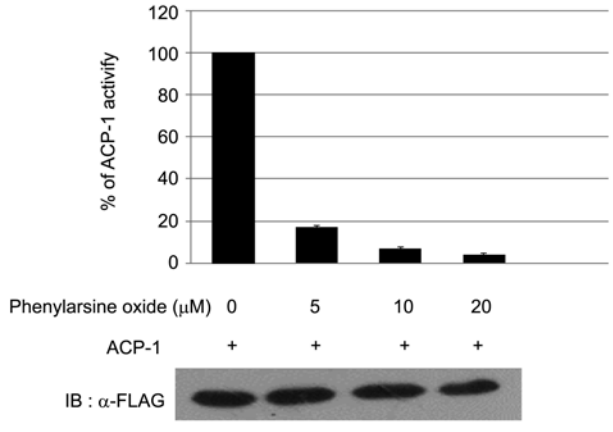

Figure 2. Inhibition of ACP-1 expressed in HepG2 cells by PAO *in vitro*. HepG2 cells were transfected with 3 μg of ACP-1 expression plasmid. After 48 h of transfection, HepG2 cell lysates were subjected to immunoprecipitation with anti-FLAG M2 agarose. Immunoprecipitated ACP-1 was incubated with various concentrations of PAO at 37 °C for 30 min. Fluorescence emission from the product was measured with a multiwell plate reader as described in Experimental Section.

mammalian cells undergo posttranslational modifications which might affect protein activity. HepG2 cells were transfected with FLAG-tagged ACP-1 expression plasmid. Transfected HepG2 cells were lysed and immunoprecipitation was carried out using anti FLAG M2-agarose. After immunoprecipitation, we measured phosphatase activity using OMFP as a substrate in the presence of PAO with incubation for 30 min at 37 °C (Fig. 2). The results showed that PAO could also inhibit ACP-1 expressed in mammalian cells.

In the present study, we showed that PAO inhibits activity of ACP-1 in a dose-dependent manner and is a potent competitive-inhibitor of ACP-1. A role for ACP-1 in tumorigenesis has recently been suggested. *In vivo* studies demonstrated that ACP-1 is a positive regulator of both tumor onset and growth.[11] It has also been reported that overexpression of ACP-1 is sufficient to confer transformation upon nontransformed epithelial cells.[8] Therefore, this study for PAO against ACP-1 will have a beneficial effect in ACP-1-related diseases such as tumor.

Experimental Section

Purification of 6 x His-tagged Phosphatase Proteins. PTP expression plasmids were constructed in pET28a (+) (Novagen, Darmstadt, Germany) and transformed into BL21 (DE3)-RIL *E. coli*. Recombinant proteins were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside at 37 °C, 30 °C or 22 °C for 3-16 h. Cells were harvested and then lysed by sonication in 50 mM Tris-HCl (pH 8), 300 mM NaCl, 1% Tergitol-type NP-40, 1 mM phenylmethylsulphonyl fluoride (PMSF). The lysates were clarified at 11000 rpm for 30 min at 4 °C. the supernatants were applied by gravity flow to a column of Ni-NTA resin (PEPTRON, Daejon, Korea). The resin was washed with 20 mM Tris-HCl (pH 8), 500 mM NaCl, 50 mM imidazole and eluted with 20 mM Tris-HCl (pH 8), 500 mM NaCl, 200-300 mM imidazole. Eluted protein was dialyzed overnight against 20 mM Tris-HCl (pH 8), 100 mM NaCl, 30% glycerol, 0.5 mM PMSF before storage at −80 °C.

***In vitro* Phosphatase Assays and Kinetic Analysis.** Activities of PTPs were measured using the substrate 3-*O*-methylfluorescein phosphate (OMFP; Sigma, St. Louis, MO) in a 96-well microtiter plate assay based on methods described previously. PAO (Sigma) and OMFP were solubilized in H_2O and DMSO, respectively. All reactions were performed at a final concentration of 1% DMSO. The final incubation mixture (100 μL) were optimized for enzyme activity and composed of 30 mM Tris-HCl (pH 7), 75 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.33% bovine serum albumin (BSA), 100 nM of PTP, and PAO. Reactions were initiated by addition of OMFP and incubated for 30 min at 37 °C. Fluorescence emission from product was measured with a multiwell plate reader (synergy H1 (BioTek); excitation filter, 485 nm; emission filter, 535 nm). The reaction was linear over the time period of the experiment and was directly proportional to both enzyme and substrate concentrations. Half-maximal inhibition con-

stant (IC_{50}) was defined as the concentration of an inhibitor that caused a 50% decrease in the PTP activities. Half-maximal inhibition constants and best curve fit for Lineweaver-Burk plots were determined by using the curve fitting program Prism 3.0 (GraphPad Software). All experiments were performed in triplicate and were repeated at least three times.

Inhibition Study. The inhibition constant (K_i) to ACP-1 phosphatase for the inhibitor was determined by measuring the initial rates at several OMFP concentrations for each fixed concentration of the inhibitor. The data were fitted to the following equation to obtain the inhibition constant of reversible competitive inhibitors. The slopes obtained were replotted against the inhibitor concentrations. The K_i value was obtained from the slopes of these plots.

$$1/V = K_m (1 + [I]/K_i) V_{max} [S] + 1/V_{max}$$

Effect of PAO on ACP-1 Purified from HepG2 Cells. HepG2 cells were transfected with FLAG-ACP-1 expression plasmid. After 48 h of transfection, cells were washed twice with phosphate buffered saline (PBS) buffer and lysed in PTP lysis buffer (0.5% NP-40, 0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1% glycerol, 1 mM PMSF, and 1 μg/mL aprotinin) for 30 min at 4 °C. Cleared cell lysates from centrifugation were mixed with washed FLAG M2-agarose (Sigma-Aldrich, St. Louis, MO) and incubated for 16 h at 4 °C using rotation device. After incubation, FLAG M2-agarose was washed three times with PTP lysis buffer and measured their phosphatase activities.

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