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Silica Induced Phospholipase D (PLD) Activation in Rat2 Fibroblasts

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ABSTRACT. To define the effect of silica on the stimulator of signaling pathway, we studied the phospholipase D (PLD) activity in the Rat2 fibroblasts. Silica stimulated the accumulation of labeled [^{3}H] phosphatidylethanol([^{3}H] PEt) in a time- and concentration-dependent manner. This Silica-induced PLD activity was partially attenuated by the pretreatment with U73122 (phospholipase C inhibitor), genistein (protein tyrosine kinase inhibitor), PD 98056 (MEK inhibitor) and mepacrine (phospholipase A_2 inhibitor). But, sphingosine (protein kinase C inhibitor) and DPI (NADPH reductase inhibitor) had not effect the PLD activity. Silica also increased the PLA_2 activity about four fold, which imply that the PLD activity is more influenced by the mobilization of PLA_2 than other signaling mediators. The PLD activity also partially inhibited calcium chelator EGTA or/and BAPTA/AM compared to silica. Finally, we concluded that a silica-stimulated phospholipase D activity is present in the Rat2 fibroblasts and is modulated by combination of various signaling mediators.

Keywords: PLD, Silica, Rat2 fibroblast, Signaling mediator, PLA_2, Silicosis.

INTRODUCTION

Silica is a well-known occupational fibrogenic agent and its primary target cell is an alveolar macrophage (Kumar *et al.*, 1992). Particle-stimulated macrophages are believed to release various mediators which can regulate the inflammation as well as pulmonary fibrosis (Lugano *et al.*, 1984). Among these mediators, oxygen radicals were generated in silica-stimulated alveolar macrophages through several signal transduction pathways. In these pathways the activations of phospholipase C (PLC), protein kinase C (PKC), protein tyrosine kinase (PTK), and the increment of intracellular calcium level have been known to be involved (Lim *et al.*, 1997).

Several investigators have suggested a close relationship between phospholipase C (PLC) and PLD activation, since many agonists stimulate not only PLC but also PLD (Singer *et al.*, 1997; Freeman *et al.*, 1995; Billah and Annthes, 1990). Receptor-linked activation of PLD may occur via several distinct mechanisms and may involve multiple factors including calcium, protein kinase C (PKC) and G-proteins (Exton, 1997a, b; Gargett *et al.*, 1996; Olson *et al.*, 1991). A large number of agonists that increase PLC activity in mammalian cells also stimulate phospholipase A_2 (PLA_2) activity (Nishizuka, 1995). Therefore, it appears that receptor-mediated activation of PLD and cross-talk between these phospholipases are important signal transduction pathways in functional responses in many types of cells (Boarder, 1994; Nishizuka, 1992). Although phospholipases are important in the signal transduction pathways in silica-stimulated alveolar macrophages, the exact roles of the phospholipases are still not clear.

Besides alveolar macrophages, fibroblasts in interstitium can interact with silica particles passing through alveolar epithelium. Silica stimulates fibroblasts leading to cell proliferation as well as morphological and func-

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tional changes (Driscoll *et al.*, 1996; Lasky *et al.*, 1995). It has been observed that silica induces the generation of oxygen radicals in the Rat2 fibroblasts resulting in the *phosphorylation of extracellular signal-regulated* protein kinase (ERK) and mitogen-activated protein kinase kinase (MEK) (Cho *et al.*, 1999).

There are very few investigations studied on the relationship between the silica and the PLD-mediated signal transduction pathway. Moreover, there is no report yet about the activation of PLD in silica-stimulated fibroblast. Considering the importance of PLD, it is needed to clarify the role of PLD in the silica-stimulated fibroblast.

The purpose of this study is to characterize and investigate the signaling pathways related to the activation of PLD in silica-stimulated Rat2 fibroblasts.

MATERIALS AND METHODS

Materials

The materials used were purchased as follows: Silica, ionomycin, mepacrine, PD98059, ethylene glycol-bis(b-aminoethyl ether) N,N,N,N,-tetraacetic acid (EGTA) and BAPTA/AM from Sigma (St. Louis, MO, USA). {1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione} (U73122), genistein, Diphenyleneiodonium Chloride (DPI) and sphingosine from Calbiochem (La Jolla, CA, USA). DMEM from GIBCO-BRL (Bethesda, MD, USA). Silica gel 60 plates from Whatman (Clifton, NJ, USA). Phosphatidylethanol (PEt) from Avanti Polar Lipids (Grand Island, NY, USA). [^{3}H] palmitic acid and [^{3}H] arachidonic acid (AA) from MEN Life Science Products (Boston, MA, USA).

Methods

Cell culture and treatment. Rat2 fibroblasts were grown in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum and 1% (W/V) penicillin–streptomycin mixture. These cells were maintained at 37°C under 95% room air and 5% CO_2 in a humidified chamber. The Rat2 fibroblasts were cultured in 6-well tissue culture plates at the density of 3.0×10^6 cells/well, and labeled with [^{3}H] palmitic acid (1 μCi/well) for 18 hours at 37°C.

The silica suspension was prepared just prior to use. Stock solution of particles was dispersed in PBS at a concentration of 10 mg/ml and was then diluted with culture media.

Analysis of PLD activity. The cells were scraped off the culture plates using a rubber policeman, placed in polypropylene tubes and centrifuged at 300 g for 10 min. The supernatant was carefully collected. 0.25 ml of cold methanol was added and mixed thoroughly. The suspension was incubated on ice for 10 min. And then 0.175 ml of chloroform and 0.25 ml of H_2O were added and mixed thoroughly. After centrifugation at 300 g for 10 min, the $CHCl_3$ layer was evaporated in a Speed Vac Concentrator (Savant Instr,. NY, USA). The residues were dissolved in developing solution [ethyl acetate/iso-octane/acetic acid (11 : 5 : 2), by vol.] and aliquots applied to silica gel plates. The radioactivity of isolated [^{3}H] PEt was measured by liquid scintillation spectrophotometer (LS 6500, Beckman, USA). Pure PEt was used for the standard substance.

Measurement of PLA_2 activity. The Rat2 fibroblasts were seeded as the same of PLD activity measurements. The cells were incubated with starvation medium (DMEM containing 0.1% FBS) for 2 hours followed by [^{3}H] arachidonic acid (0.5 Ci/ml) at 37°C and treated without or with silica (2.5 mg/ml) for 30 min. The results were presented as means ± SE of triplication from three different experiments. The cells were scraped off and placed in polypropylene tubes and centrifuged at 300 g for 10 min. The supernatant was measured for the radioactivity by liquid-scintillation spectrophotometer (LS 6500, Beckman, USA).

Effects of drugs on PLD activity. In order to characterize the signaling pathway for the increment of PLD activity, we tested the effects of PKC activator (PMA), PKC inhibitor (sphingosine), PTK inhibitor (genistein), MEK inhibitor (PD98059), PLA_2 inhibitor (mepacrine), NADPH reductease inhibitor (DPI) and PLC inhibitor (U73122) on silica-induced PLD activation. The Rat2 fibroblasts were pretreated with activators or inhibitors for 30 min before the application of silica. And the concentrations of many drugs were followed by the previous references (Lim *et al.*, 1997).

Effects of Ca^{2+} on PLD activity. To determine the involvement of Ca^{2+} for PLD activation by silica (2.5 mg/ml) fibroblasts were exposed to the starvation media containing 1 μM or 10 μM ionomycin for 30 min. The Rat2 fibroblasts without any treatment were used as control. Then, we examined the effects of Ca^{2+} chelators of both extracellular and intracellular on silica-mediated PLD activation. Before the stimulation with silica, the Rat2 fibroblasts were exposed either to the starvation media containing 0.1 mM EGTA for 5 min or 1 mM BAPTA/AM for 20 min.

Statistics

After counting of [^{3}H]-cpm in PEt, it was normalized for total cpm in phospholipid to correct minor differences in labeling. [^{3}H] PEt formation was expressed either index (compare to control) or percentage (com-

pare to silica). The results were represented as means ± SE. Significances of the differences betweeen the data were determined by analysis of Students *t*-test. A value of $P < 0.05$ and $P < 0.01$ was considered as significant.

RESULT

Activity of PLD

To observe the dose-response curve of silica-induced PLD activity, the Rat2 fibroblasts were exposed to silica particles at various concentrations (0.5, 1.0, 2.5 and 5.0 mg/ml) of silica for 30 min at 37°C. The results were presented as means ± SE of triplicates from three different experiments. Silica-stimulated formation of [^{3}H] PEt represented in a dose dependent manner and it reached peak at the concentration of 2.5 mg/ml of silica and a plateau up to 5.0 mg/ml of silica (Fig. 1).

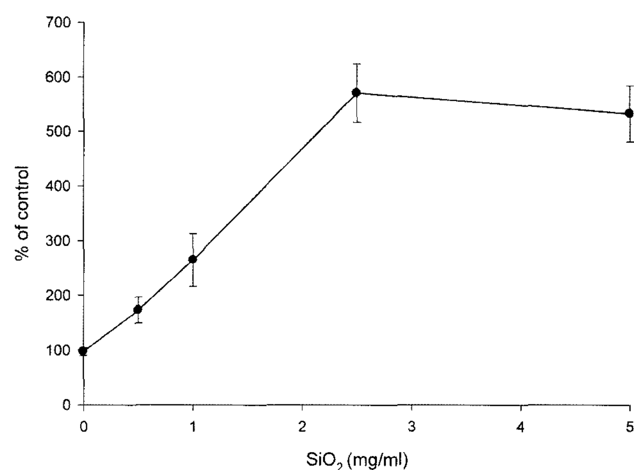

Fig. 1. Dose-response curve of silica on [^{3}H] PEt formation in the Rat2 fibroblasts. Control; non silica-treated group.

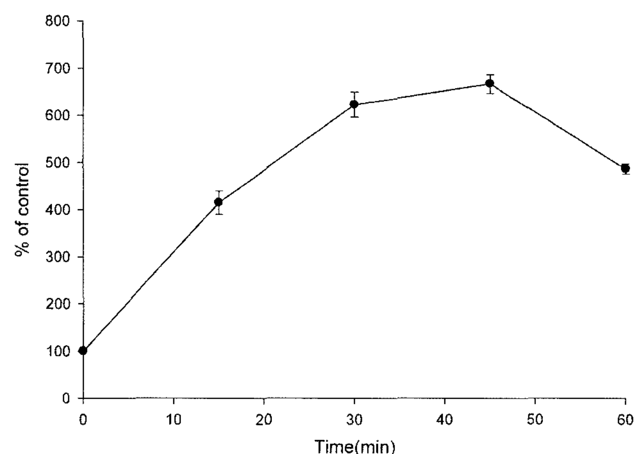

Fig. 2. Relationship between incubation time and silica (2.5 mg/ml)-induced [^{3}H] PEt formation in the Rat2 fibroblasts. Control; non silica-treated group.

The Rat2 fibroblasts were labeled for 18 hours with 1 μCi of [^{3}H] palmitic acid at 37°C. At indicated time points, radioactivity of [^{3}H] PEt was determined as described in materials and methods. When the Rat2 fibroblasts were incubated with silica in the presence of ethanol, [^{3}H] PEt formation increased for 45 min. But [^{3}H] PEt formation decreased after 45 min (Fig. 2).

Inhibitor assay

The Rat2 fibroblasts were pretreated with activator or inhibitors for 30 min before application of silica (2.5 mg/ml). As shown in Table 1, PLC inhibitor, PTK inhibitor, PLA_2 inhibitor and MEK inhibitor inhibited the silica-induced PLD activity significantly ($P < 0.05$). But NADPH reductase inhibitor and PKC inhibitor did not inhibit the silica-induced PLD activity.

Activity of PLA_2

As shown in Table 1, the silica-induced PLD activity was more inhibited by mepacrine, PLA_2 inhibitor ($P <$ 0.01) than other treatments ($P < 0.05$). And PLA_2 activ-

Table 1. Effects of activator and various inhibitors on silica-induced PLD activity in the Rat2 fibroblasts

Activator or inhibitors (μM)	Mean ± SE
silica only	100.00
silica + PMA (1.0)	157.67 ± 27.17
silica + U73122 (10.0)	70.93 ± 6.65*
silica + sphingosine (5.0)	106.60 ± 7.30
silica + genistein (40.0)	72.17 ± 6.29*
silica + mepacrine (50.0)	62.00 ± 6.72**
silica + PD98059 (20.0)	73.43 ± 3.55*
silica + DPI (10.0)	88.97 ± 8.90

*; $P < 0.05$ *vs* silica only group.
**; $P < 0.01$ *vs* silica only group.

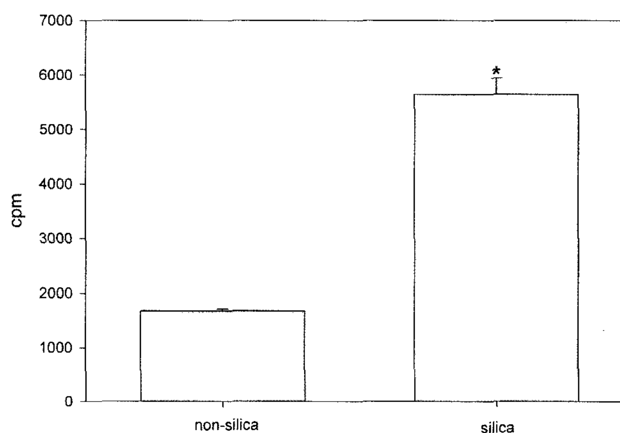

Fig. 3. Silica-induced PLA_2 activity in the Rat2 fibroblasts. *; $P < 0.05$ *vs* non-silica group.

Table 2. Effects of silica and ionomycin on [^{3}H] PEt formation in the Rat2 fibroblasts

	Mean ± SE
Control	100.00
SiO_2	735.67 ± 59.48*
ionomycin 1 μM	455.67 ± 82.00*
ionomycin 10 μM	614.00 ± 28.74*

*; $P < 0.05$ *vs* control group.
Control; non silica-treated group.

Table 3. Effects of EGTA or/and BAPTA/AM on silica (2.5 mg/ml)-induced PLD activation

	Mean ± SE
silica only	100.00
silica + EGTA (0.1 mM)	87.67 ± 8.33
silica + BAPTA/AM (1 mM)	73.00 ± 7.15
silica+ BAPTA/AM + EGTA	66.00 ± 4.93*

*; $P < 0.05$ *vs* silica only group.

ity was measured to evaluate further effect of PLA_2 activity. The activity of PLA_2 was 4 times higher in the silica treated group than non-treated group (Fig. 3).

Ca^{2+} effect

As shown in Table 2, silica-induced [^{3}H] PEt formation was stimulated by ionomycin through dose-dependent manner. Ionomycin at 1 μM increased [^{3}H] PEt formation 5 folds, and 6 folds at 10 μM concentration. Regarding the source of Ca^{2+}, EGTA showed the decreased stimulation about 13%, BAPTA-AM decreased the effect of silica about 27% and the combination of EGTA with BAPTA/AM represents significant reduction about 34% of silica-induced formation of [^{3}H] PEt (Table 3).

DISCUSSION

Silicosis has the characteristics of chronic inflammation caused by the toxicity of silica. Proliferated fibroblasts are the typical phenomenon and the accelerated proliferation may trigger stereotypic pathophysiologic response in silicosis. It has been also reported that the cytotoxicity is believed to have some correlation with the extent of fibrogenecity and the fibroblasts may play the important role in silicosis as well as alveolar macrophage.

It is generally accepted that PLD is activated through four pathways; PKC, calcium, GTP-binding proteins and protein tyrosine kinases (Thompson *et al.*, 1991). But, the cross-talk of each mediator involved in the activation of PLD is not still clear. Because the unique feature of PLD to catalyze the transfer of the phosphatidyl moiety of phospholipids to alcohol (trans-phosphatidylation), the formation of phosphatidylalcohol is a useful indicator in estimation of the PLD activity (Yu and Liu, 1996). In order to investigate the activation of PLD by silica, we studied the formation of [^{3}H] PEt in the silica stimulated Rat2 fibroblasts.

In this study (Fig. 1 and 2), the silica-induced PLD activity showed the same pattern in the dose- and time-dependent manners similar to the previous result reported by Cha *et al.* (1998). But the involved signaling mediators were very different because they used alveolar macrophage as the effector cell. As shown in Table 1, silica induced PLD activation in the Rat2 fibroblasts was triggered by cross-talk of various signaling mediators. Among the mediators, PLA_2 was most effective on the activation of PLD.

PLD activity has been reported to be at least dependent on the mobility of extracellular Ca^{2+} in a variety of cell types in response to various agonists such as FMLP in human peritoneal neutrophils (Kanaho *et al.*, 1992) and angiotensin in vascular smooth muscle (Lassegue *et al.*, 1991; Freeman *et al.*, 1994, 1995). In human amnion cells, PLD is activated by calcium ionophore A23187 (Mizunuma *et al.*, 1993). Also, Ca^{2+} ionophores increased activity of PLD (Kanaho *et al.*, 1993). However, little has been known about the contribution of the specific pools of Ca^{2+} (i.e., intracellular *vs* extracellular) to the activation of PLD in the Rat2 fibroblasts or the route of entry for extracellular Ca^{2+} in response to silica stimulation observed silica-stimulated PLD activity through intra- and/or extra-calcium in alveolar macrophage. Also Lim *et al.* (1997) reported that silica-induced cytosolic calcium mobilization was composed of intracellular stores of Ca^{2+} mediated by PLC activation and influx from extracellular Ca^{2+} through the plasma membrane in the Rat2 fibroblasts. As shown in Table 2, calcium ionophores markedly increased the PLD activity. But the proportion of influence to the activity of PLD was very little (Table 3), which imply that the intracellular Ca^{2+} mediated by PLC has less effects on silica-induced PLD activation in the Rat2 fibroblasts comparing to Ca^{2+} influxed from the extracellular source.

Based on these results, I suggest that silica-stimulated activation of PLA_2 leads mainly to the stimulation of PLD, which is coordinated by various signaling mediators such as PLC, PTK and MEK in the Rat2 fibroblasts.

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