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Effect of Diesel Exhaust Particles (DEP) on the Activity of Phospholipase D (PLD) in RAW 264.7 Cells

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ABSTRACT. Diesel exhausted particles (DEP), a kind of fine particles with aerodynamic diameters less than 2.5 μm (PM2.5), is of great concern to human health because they remain in atmosphere for long periods, invade an indoor air environment, and can be breathed most deeply into lung and reached the alveoli because of their small size (0.1~0.4 μm in diameter). Epidemiological and experimental studies suggested that DEP may play an active role in the increased respiratory mortality and morbidity. In addition to their physical characteristics, the chemical components including polyaromatic hydrocarbon (PAH) are regarded as a carcinogen causing pulmonary tumors. PLD plays an important role in cell proliferation with various physiological phenomena and affects other enzymes by activating signal transduction pathway. We investigated the cytotoxic mechanism of DEP on RAW 264.7 cells focusing on the role in activation of PLD. Our results suggested DEP induced PLD activity through a specific signaling pathway involving phospholipase A_2, PLC, PKC and Ca^{2+} mobilization.

Keywords: Diesel exhausted particles (DEP), Phospholipase D (PLD), RAW 264.7 cells, Signaling pathway.

INTRODUCTION

Diesel exhaust particles (DEP) is one of the main air pollutants in urban area and they are increasing in quantity because of the increased requirements of the diesel-engine-powered cars and human activities. Potential sources from human activities include coal-fired power plants, industry and road transport. Recently, use of diesel-engine-powered cars has been increasing in the world because diesel engines offer better fuel efficiency and lower emissions of carbon dioxide than gasoline engines. Diesel engines, however, emit more nitrogen oxides and particles than gasoline engines (Harrison and Yin, 2000; McClellan, 1987).

Many epidemiological data show that DEP play an active role in the increased respiratory mortality and morbidity (Dockery and Pope, 1994; Dockery *et al.*, 1993; Fairley, 1990). Fine particles such as DEP, with aerodynamic diameters lower than 2.5 μm ($PM_{2.5}$), are of the greatest concern to human health because they remain in atmosphere for long periods, invade an indoor air environment, and can be breathed most deeply into lung where they are likely to be more toxic than coarse particles (Sloss and Smith, 2000; Yu and Xu, 1987). DEP has been proved to induce serious pulmonary injury, among which lethal pulmonary edema has been assumed to be mediated by RAW 264.7 cells (Bai *et al.*, 2001; Han *et al.*, 2001). Also DEP is known to be an important pathogen for fibrosis, edematous change, focal chronic alveolitis and allergic airway disorders, including asthma-like symptoms in experimental animals (Hyde *et al.*, 1985; Ichinose *et al.*, 1995; Sagai *et al.*, 1996).

Our experiments using RAW 264.7 cells showed that DEP augmented phospholipase D (PLD) activity *in vitro*. This mechanism is regulated by triggering the key steps of signaling pathway including phospholipase C (PLC), phospholipase A_2 (PLA_2) and protein kinase C (PKC) activation, and Ca^{2+} mobilization.

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MATERIALS AND METHODS

Materials

The materials used were purchased as follows: DEP from Kumho High-Tech, R&D Center (Seoul, Korea). Urban dust SRM #1649a from NIST (Geithersburg, MD, USA). DMEM from GIBCO-BRL (Bethesda, MD, USA). {1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino) hexyl]-1H-pyrrole-2,5-dione} (U73122), genistein, KN62 and sphingosine from Calbiochem (La Jolla, CA, USA). Ionomycin, ethylene glycol-bis(b-aminoethyl ether) N,N,N,N,-tetraacetic acid (EGTA), BAPTA/AM, mepacrine from Sigma (St. Louis, MO, USA). Silica gel 60 plates from Whatman (Clifton, NJ, USA). [^{3}H] myristic acid and [^{3}H] arachidonic acid (AA) from MEN Life Science Products (Boston, MA, USA).

Methods

Analysis of PLD assay. Confluent cells (2.0×10^6 cells/well) in 6-well plate were labeled with 5 Ci/ml [^{3}H] myristic acid in DMEM with 0.1% FBS for 16 hr and then labeled cells were stimulated with DEP for the indicated time. Incubations were performed at 37°C and terminated by washing the monolayers twice with ice-cold PBS and immediately adding 1 ml of ice-cold methanol. Cells were collected by scraping and were washed with an additional 1 ml 0.1 N HCl and 1 ml chloroform. The lower phase was dried under a stream of nitrogen and dissolved in a small volume of chloroform/methanol (2 : 1). [^{3}H]PtdOH was separated from other phospholipids by TLC using silica gel 60 on glass sheets with ethyl acetate/iso-octane/acetic acid/H_2O (11/5/2/10). Unlabeled PtdOH was run together with samples and the corresponding lipid spot was detected by exposure to iodine vapor, cut and placed into scintillation vials and radioactivity counted. The samples were added 0.5 ml methanol and 5 ml lipid scintillation cocktail to measure radioactivity (LS 6500, Beckman, USA).

Measurement of the intracellular free calcium concentration ($[Ca^{2+}]_i$). RAW 264.7 cells were harvested after trypsinization 24 hr before the measurement of Ca^{2+} and seed onto 22 × 22 mm cover glasses at a concentration of 1×10^4 cells/dish. The cover glasses (2-cm diameter) were attached to the bottom of 35-mm plastic culture dishes. Cells were then washed with modified Hanks' buffer solution consisting of the following components: 127 mM NaCl, 0.8 mM $MgSO_4$, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM HEPES and 1 mM $CaCl_2$ (pH 7.4) and loaded with Fura-2/AM (10 mM) for 30 min at 37°C. Fluorescence in RAW 264.7 cells was measured at room temperature using the InCa™ Imaging System from Intracellular Imaging, Inc. (Cincinnati, OH, USA). $[Ca2=]_i$ was calculated from the standard curve generated *in situ* (Wahl *et al.*, 1992).

Effects of drugs on PLD activity. In order to characterize the signaling pathway, which increases the activity of PLD, we tested the effects of PLA2 inhibitor (mepacrine), PLC inhibitor (U73122), PKC inhibitor (sphingosine), CaM kinase inhibitor (KN62) and TPK inhibitor (genistein) on DEP-stimulated RAW 264.7 cells. The RAW 264.7 cells were pretreated with inhibitors for 40 min before the application of DEP. And the concentration of several inhibitors was followed by the previous reference (Lim *et al.*, 1997).

Statistics. The counting of [^{3}H]PtdOH was normalized to total dpm in phospholipid to correct the minor differences among experiments. [^{3}H]PtdOH formation was expressed either in index comparing to control or in percentage comparing to DEP. The results represented the means ± SE. Significant differences were determined by analysis of Student's *t*-test. A value of $P < 0.01$ was considered significant.

RESULT

The effect of DEP on PLD activity was investigated in RAW 264.7 cells labeled with [^{3}H] myristic acid. To obtain dose-response curve of DEP-induced PLD activity, RAW 264.7 cells were exposed with DEP at various concentrations (1, 5, 10 and 15 mg/ml). Concentration of DEP used in our study did not showed the change of cell viability for indicated time in MTT experiment (data not shown). DEP-stimulated formation of [^{3}H]PtdEt reached a peak at 5 mg/ml of DEP and didn't change with 10

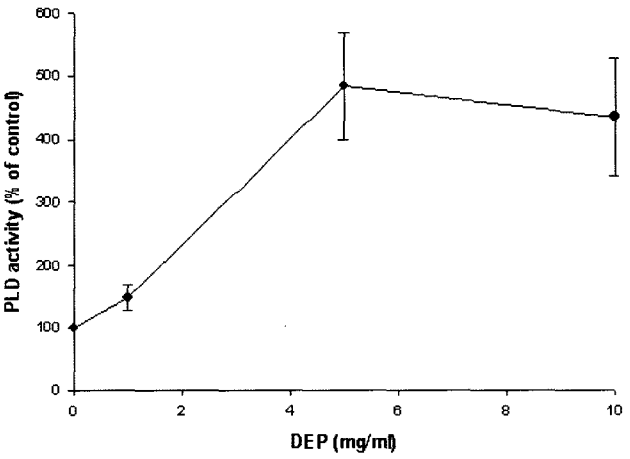

Fig. 1. Effect of dust particles on PLD activity released from RAW 264.7 cells at different concentrations (1, 5, 10 mg/ml). RAW 264.7 cells were incubated with dust particles for 2 hr. The results were presented as means ± SE of triplicates from three different experiments.

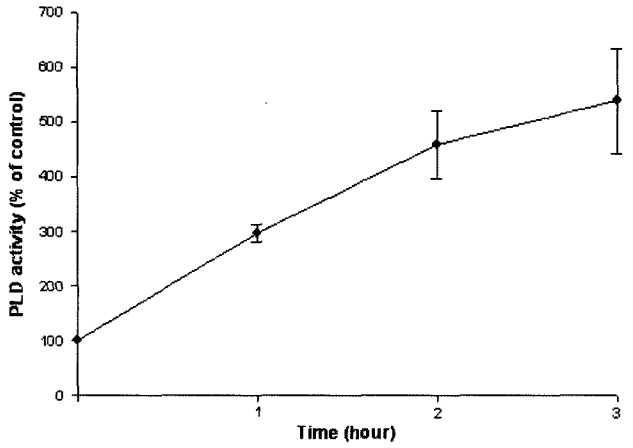

Fig. 2. Time-response curve of DEP on [^{3}H] PtdOH formation in the RAW 264.7 cells. RAW 264.7 cells were incubated with DEP of 5 mg/ml for the indicated times. The results were presented as means ± SE of triplicates from three different experiments.

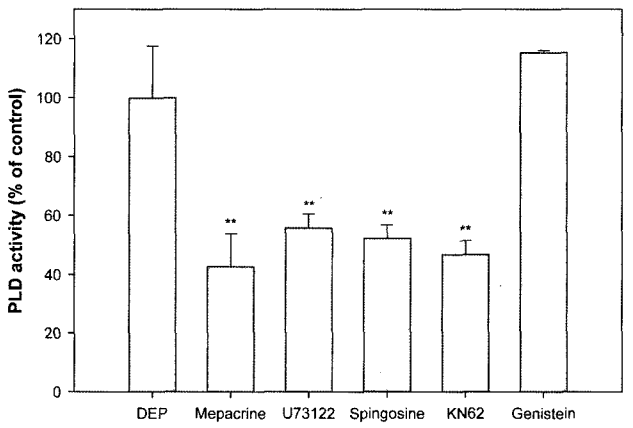

Fig. 3. Effects of several inhibitors on DEP-induced PLD activity in the RAW 264.7 cells. RAW 264.7 cells were pretreated with inhibitors for 40 min followed by DEP of 5 mg/ml for 2 hr. The results were presented as means ± SE of triplicates from three different experiments. **, $P < 0.01$.

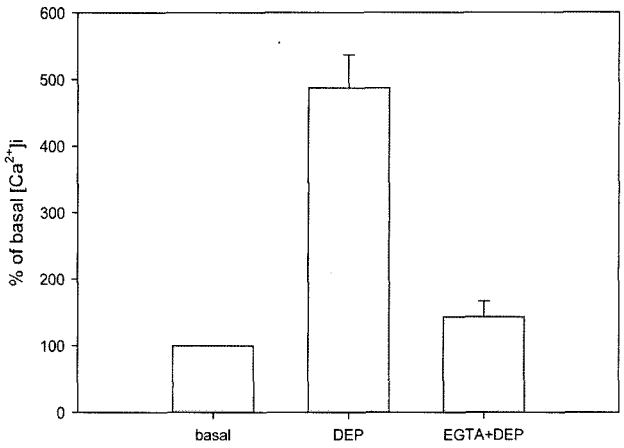

Fig. 4. Effects of the extracellular calcium-free condition on DEP (1 mg/ml)-induced [Ca^{2+}]$_i$ increase. RAW 264.7 cells were pretreated with calcium chelator, EGTA for 30 min followed by DEP of 5 mg/ml for 2 hr. The results were presented as means ± SE of triplicates from three different experiments.

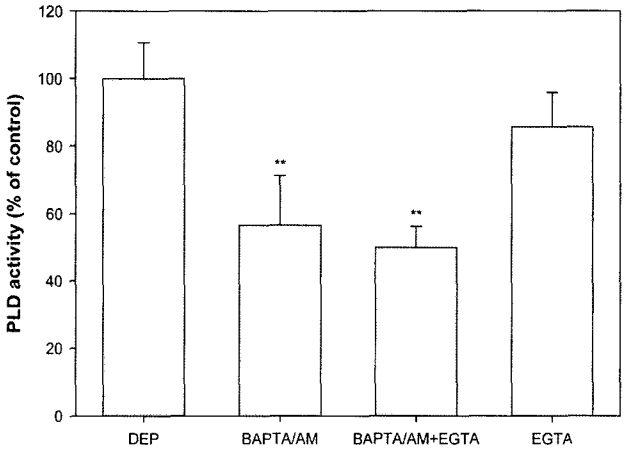

Fig. 5. Effects of BAPTA/AM or/and EGTA on DEP (5 mg/ml)-induced PLD activation. RAW 264.7 cells were pretreated with calcium chelators for 30 min followed by DEP of 5 mg/ml for 2 hr. The results were presented as means ± SE of triplicates from three different experiments. **, $P < 0.01$.

mg/ml of DEP (Fig. 1).

DEP-induced PLD activity was increased with time-dependent manner. DEP-induced increase of PtdEt was maintained until the time point of 3 hr (Fig. 2).

It is well known that PLD is released from membrane phospholipids through the agonist-induced activation of several pathways, such as PLA_2, PLC, PKC and Ca^{2+} mobilization. The RAW 264.7 cells were pretreated with inhibitors for 40 min, and then incubated with 5 mg/ml DEP for 2 hr. As shown in Fig. 3, DEP-induced PLD activity was not affected by the tyrosine protein kinase-inhibitor (genistein), whereas 50 μM mepacrine (PLA_2 inhibitor), 10 μM U73122 (PLA_2, PLC inhibitor), 5 μM spingosine (PKC inhibitor) and 40 μM KN62 (CaM kinase II inhibitor) inhibited PLD activity, respectively. These data suggested that PLD activation was the prevailing pathway stimulated by DEP.

The effect of DEP on the increase of Ca^{2+} was abolished by the addition of EGTA, calcium chelator (Fig. 4). RAW 264.7 cells were exposed to a serum-free DMEM containing 500 μM EGTA or 1 mM BAPTA/AM prior to stimulation with DEP. PLD activities were inhibited 43% with BAPTA/AM, 50% with BAPTA/AM+EGTA and 14% with EGTA, respectively (Fig. 5).

DISCUSSION

DEP is easily reached the alveoli during inhalation because of their small size (Harrison and Yin, 2000; Sloss *et al.*, 2000), and inhaled DEP may cause pulmonary tumors (McClellan *et al.*, 1987; White *et al.*, 1983), fibrosis, edematous change, focal chronic alveolitis and allergic airway disorders, including asthma-like symptoms in experimental animals (Hyde *et al.*, 1985; Sagai *et al.*, 1996; Ichinose *et al.*, 1995). Although the underlying mechanisms on these adverse health effects are largely unknown, it has been suggested that DEP induce oxidative lung damage and inflammation (Takizawa *et al.*, 2000; Bai *et al.*, 2001; Ito *et al.*, 2000). Thus, DEP may be a risk factor in environmental lung disease although the toxicological mechanism of DEP remains unclear.

Several results suggested that mouse lung macrophage produce PtdEt and these species play an important role in particles-induced pulmonary injury (Lim *et al.*, 1997; Lennartz, 1999) Although mechanism producing PLD is still not clear, several signal transduction pathway have been suggested (Berridge and Irvine, 1984, 1989; Nishizuka, 1989; Singer *et al.*, 1997). It is generally accepted that PLD is activated through four pathway; PKC, calcium, GTP-binding proteins and tyrosine protein kinase.

In order to investigate the effect of DEP on the PLD activation, we studied the formation of [^{3}H] PtdEt in the DEP-stimulated RAW 264.7 cells.

PLD activity was reported as the least dependent pattern on the mobility of extracellular Ca^{2+} in a variety of cell types in response to agonists such as FMLP in human perioneal neutrophils (Kanoho *et al.*, 1992) and angiotensin in vascular smooth muscle (Lassegue *et al.*, 1991; Freeman and Tallant, 1994; Freeman *et al.*, 1995). 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 RAW 264.7 cells or the route of entry for extracellular Ca^{2+} in response to DEP stimulation. Based on these reports, we hypothesized that the elevation in intracellular Ca^{2++} in response to DEP stimulation induced PLD activation. Furthermore, an efflux of intracellular (ER) Ca^{2+} may be more import source in regulating PLD stimulation, since DEP-induced PLD activity was more inhibited in the presence of BAPTA/AM compared to EGTA. However, it appears that both influx of extracellular calcium and efflux of intracellular calcium store contribute to the maximal activation of PLD induced by DEP in RAW 264.7 cells. These results indicated that PLD might be activated via calcium-mediated pathway.

Because a unique feature of PLD is the ability to catalyze the transfer of the phosphatidyl moiety of phospholipids to alcohol (transphophatidylation), the formation of phosphatidylalcohol is a useful indicator of PLD activity (Exton, 1999).

It is reported the silica-induced cytosolic calcium mobilization was composed of releasing from intracellular stores of Ca^{2+} mediated by PLC activation and influx of Ca^{2+} from extracellular space through the plasma membrane in rat alveolar macrophage (Lim *et al.*, 1997). In this study, we examined that DEP-induced cytosolic calcium mobilization was composed of releasing from intracellular stores of Ca^{2+} by PLC activation in RAW 264.7 cells.

Intracellular signalling by Ca^{2+}-mobilizing agonists is believed to be initiated by receptor-mediated hydrolysis of phosphatidyl-iositol 4,5-bisphosphate by specific phospholipase C. This hydrolysis leads to the generation of two signalling substances, inositol 1,4,5-triphosphate and diacylglycerol (DAG). Inositol 1,4,5-trisphosphate mobilizes Ca^{2+} from intracellular store (Berridge and Irvine, 1984; 1989). DAG, concern with Ca^{2+}, activates a phospholipid-dependent protein kinase, protein kinase C (PKC) (Nishizuka, 1989).

This study suggests the first evidence for DEP-induced PLD activity and demonstrates that this activation occurs through a specific signaling pathway involving phospholipase A_2, PLC, PKC and Ca^{2+} mobilization.

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