Silica Induces Macrophage Cytokines through Phosphatidylcholine-Specific Phospholipase C with Hydrogen Peroxide

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Silica particle-associated inflammation is implicated in the genesis of several pulmonary diseases, including silicosis and lung cancer. In this study we investigated the role of phosphatidylcholine-specific phospholipase C (PC-PLC) in silica-stimulated induction of TNF- α and IL-1 β and how PC-PLC activity is regulated by silica in a rat alveolar macrophage model. We demonstrated that inhibition of PC-PLC, which was achieved with tricychodecan-9-yl-xanthate (D609), blocked the silica-stimulated induction of TNF- α and IL-1 β in alveolar macrophage, suggesting that PC-PLC is involved in the silica-associated inflammatory response. PC-PLC activity was increased significantly by silica exposure, and this could be inhibited by MnTBAP, which catalyzes both the dismutation of O₂⁻ to O₂ and H₂O₂ and the dismutation of H₂O₂ to O₂ and H₂O, revealing that PC-PLC activity is regulated in a redox-dependent manner. This is further confirmed by the finding that PC-PLC activity was increased by exogenous H₂O₂. The intracellular calcium chelator BAPTA blocked the H₂O₂-increased PC-PLC activity, while the calcium ionophore, A23187, enhanced PC-PLC activity. The data indicate that PC-PLC plays critical roles in the silica-associated inflammatory response and that PC-PLC is regulated through redox- and calcium-dependent manners in alveolar macrophages.

Keywords: phosphatidylcholine-specific phospholipase C; redox signaling; calcium; cytokine; hydrogen peroxide

Silica pollution is both an occupational and environmental health concern. Exposure to silica particle has been associated with silicosis, lung cancers, and other pulmonary disorders (1). Although the underlying molecular mechanism of how silica causes these diseases is still elusive, it appears that inflammation caused by silica is one of the most common features and plays a critical role in the initiation and development of the pathogenesis of silica-associated diseases (1). The silica-associated inflammatory response is triggered by cytokines released from alveolar macrophages. For example, TNF- α (2, 3) and IL-1 β (4, 5), two cytokines that play important roles in the inflammatory response, are increased after silica particle exposure in alveolar macrophages (6). These inflammatory cytokines can in turn recruit more inflammatory cells into the alveolus and propagate the inflammatory response and injuries (7). It is now clear that the production of cytokines such as TNF- α and IL-1 β upon exposure to silica is dependent on NF- κ B signaling pathways in alveolar macrophages (3, 8). The upstream signaling event of how silica induces cytokines expression, however, remains unclear. Recently, it is found that reactive oxygen species (ROS) plays critical roles in

CLINICAL RELEVANCE

This study demonstrates that silica-induced production of hydrogen peroxide is essential to the activation of phosphatidylcholine specific phospholipase C in the mechanism of silica-induced cytokine induction in alveolar macrophages.

silica-caused inflammation response (9, 10). Crystalline silica is a stimulator of the respiratory burst in phagocytes beginning with superoxide (O₂⁻) that can then result in production of hydrogen peroxide (H₂O₂), hydroxyl radical ($\cdot$ OH), and peroxy-nitrite (ONOO⁻) (10). The surface of fresh ground silica particles also carries siloxyl radicals (Si $\cdot$ or SiO $\cdot$), which generate $\cdot$ OH in aqueous medium (11), but that is extremely short lived and would result in either production of H₂O₂ or oxidation of whatever was immediately adjacent to the site of $\cdot$ OH generation. On the other hand, the ROS, particularly H₂O₂, can also function as second messengers in signaling (12, 13). H₂O₂ can also participate in the activation of NF- κ B (14, 15) and the expression of inflammatory cytokines including TNF- α and IL-1 β (10). Nonetheless, the pathways through which silica signals in alveolar macrophages are not entirely resolved.

Recent studies demonstrated that phosphatidylcholine-specific phospholipase C (PC-PLC) may be involved in the activation of NF- κ B signaling pathway and the release of inflammatory molecules (16, 17). PC-PLC hydrolyzes phosphatidylcholine to phosphorycholine and diacylglycerol (DAG), an activator of most protein kinase C (PKC) isoforms. It has been reported that PC-PLC plays significant roles in several signal transduction mechanisms, including MAPK and NF- κ B signaling pathways (18, 19). PC-PLC activity can be increased by many stimulators, including growth factors (20), LPS (16), cytokines (21, 22), and others (23, 24). We examined whether PC-PLC plays a role in the silica-induced inflammatory response in alveolar macrophages. Here we present findings that silica increased PC-PLC activity in a calcium- and redox-dependent manner, and that PC-PLC was involved in silica-induced expression of TNF- α and IL-1 β in rat alveolar macrophages.

MATERIALS AND METHODS

Reagents and Materials

Unless otherwise noted, chemicals were from Sigma (St. Louis, MO) and were at least analytical grade. BAPTA-AM was from Invitrogen (Molecular Probes, Eugene, OR). D609 was from BIOMOL International, L.P. (Plymouth Meeting, PA). [³H]choline was purchased from Amersham Biosciences (Pittsburgh, PA). MnTBAP was kindly provided by Dr. Brian J. Day of the National Jewish Medical and Research Center (Denver, CO). TRIzol reagent was from Life Technologies (Grand Island, NY). DNA-free was from Ambion (Austin, TX). Taq-Man reverse transcription reagent and SYBR Green PCR Master Mix

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were from Applied Biosystems (Foster City, CA). All chemicals used were at least analytical grade.

Silica Preparation

Ground silica was obtained from UNIMIN (Belvidere, IL). Four hundred grams of crushed silica was heated at 450°C for 52 h and then refluxed in 4N HNO₃ for 4 h to destroy organic contaminants, including LPS, and remove surface metals such as Fe and Al. The material was then rinsed with Milli-Q water (prepared with Millipore Milli-Q water system; Millipore, Billerica, MA) several times and refluxed again. Next, the silica sample was suspended in NaOH solution (pH 9.3), stirred for 1 h, and allowed to settle for 30 min. The supernatant was then collected and centrifuged at 15,000 × *g* for 10 min to collect < 2 μm particles. The silica was resuspended in Milli-Q water, and rinsed seven more times. After the eighth rinsing and centrifugation, the sample was resuspended in 0.1% HCl, swirled for 4 h, and collected by centrifugation. The sample was rinsed five times with Milli-Q water, collected by centrifugation, and dried.

Cell Culture

The NR8383 rat AM cell line (25) was a generous gift from Dr. G. H. Zhang, University of Texas Health Science Center at San Antonio. Cells were cultured in F-12K medium (Life Technologies, Grand Island, NY), supplemented with 15% heat-inactivated fetal bovine serum (Omega Sci., Tarzana, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂ at 37°C.

Determination of PC-PLC Activity

PC-PLC activity was determined using the method described by Zamorano and coworkers (22). Briefly, NR Cells were cultured for 48 h in the presence of [methyl-³H]choline chloride (1.0 μCi/ml). Medium was replaced with label-free Krebs Ringer Phosphate (KRP: 1 M PBS, 1.3 mM CaCl₂, 1 mM MgSO₄, 5 mM glucose, 1 mM HEPES, pH 7.4) 2 h before treatment. After treatment as indicated, cells were collected and the cell pellet was rinsed with 1× PBS before being resuspended in CH₃OH/CHCl₃/H₂O (2.5:1.25:1) to separate aqueous and organic phases. The water-soluble fraction containing choline metabolites was loaded on a LK6D silica gel Thin Layer Chromatography (TLC) plate (Whatman International Ltd, Maidstone, UK) in a solvent system of methanol/0.5%NaCl/28%NH₃ (50:50:1, vol/vol). Unlabeled phosphorylcholine was used as positive control. Spots were visualized by staining with iodine vapor and the bands matched to that of the unlabeled phosphorylcholine were scraped off. The radioactivity of each band was measured with a LS6500 Multi-Purpose Scintillation counter (Beckman Coulter, Fullerton, CA). The radioactivity of phosphorylcholine, the product of PC-PLC, reflects the corresponding PC-PLC activity.

Cytokine mRNA Assay

NR Cells were cultured for 24 h before treatment. After treatment, cells were collected and washed with PBS. Total RNA was extracted using TRIzol reagent and treated with DNA-free reagent according to the manufacturers' protocols. DNA-free RNA samples were reverse transcribed using the TaqMan reverse transcription system (Applied Biosystems) and real-time PCRs were run with a Cepheid 1.2 real-time PCR machine. Briefly, 5 μl of reverse transcription reaction product was added to reaction tubes containing 12.5 μl SYBR Green PCR Master Mix and primer pair specific for cytokine mRNA; the total PCR sample was 25 μl. GAPDH was used as internal control. The primers were as follows: TNF-α, sense 5'-CCAGGAGAAAGTCAGCCTCCT-3', antisense 5'-TCATACCAGGGCTTGAGCTCA-3'; IL-1β, sense 5'-CACCTCTCAAGCAGAGCACAG-3', antisense 5'-GGGTTCATGGTGAAGTCAAC-3'; GAPDH, sense 5'-ACCCCAATGTATCGTTGT-3, antisense 5'-TACTCCTTGGAGGCCATGT-3.

Statistical Analysis

We used the comparative ΔΔC_T method for the relative mRNA quantitation. The relative quantification of target, normalized to an internal control (GAPDH), and an untreated sample is given by relative quantification = 2^{-ΔΔC_T} ΔΔC_T being defined as the difference of mean ΔC_T(HNE treated sample) and mean ΔC_T(untreated sample) and ΔC_T as the difference in mean C_T (cytokines) and C_T (GAPDH) as the

internal control. Threshold cycles (C_T) were selected in the line in which all samples were in logarithmic phase.

All data were expressed as the mean ± SE. Sigma Stat software was used for statistical analysis, and statistical significance was accepted when *P* < 0.05. The Tukey test was used for comparison of mRNA level, and one-way ANOVA was used for comparison of PC-PLC activity.

RESULTS

Involvement of PC-PLC in Silica-Induced Production of Cytokines in Alveolar Macrophages

Cytokine induction after silica particle exposure in alveolar macrophages has been well established (6). PC-PLC has been reported to be involved in NF-κB activation and cytokine production (16, 17). To study whether PC-PLC is involved in the silica-caused inflammatory response, we investigated the effect of D609, a purportedly specific inhibitor of PC-PLC, which we verified inhibited the catalytic activity, on the release of TNF-α and IL-1β, two critical cytokines involved in silica-associated inflammation. As shown in Figure 1, exposure of rat alveolar macrophages (NR8383 cells) to 50 μg/ml of silica particles increased the expression of both TNF-α (Figure 1A) and IL-1β (Figure 1B) at the mRNA level, significantly. Pretreatment with 40 μM D609 for 10 min abrogated silica-stimulated cytokine expression, suggesting that PC-PLC was required for silica-stimulated induction of inflammatory cytokines.

Silica Increases PC-PLC Activity in a Redox-Dependent Manner

PC-PLC activity can be increased by many reagents, but no prior data were available on the effect of silica exposure on PC-PLC activity. To determine if silica activates PC-PLC in alveolar macrophages, we analyzed the production of phosphorylcholine by

Figure 1. PC-PLC is involved in silica-induced production of cytokines at RNA level in alveolar macrophages. (A) TNF-α; (B) IL-1β. Rat alveolar macrophages (NR8383 cells) were treated with 50 μg/ml silica particle for 3 h, the total RNA was extracted, and then the relative mRNA levels of TNF-α and IL-1β were determined with real time RT-PCR assay. To confirm the involvement of PC-PLC in cytokine expression, cells were pretreated with 40 μM D609 for 10 min before silica treatment. *n* = 3, **P* < 0.01.

using a [^3H]choline and TCL method. Phosphorycholine is a hydrolysis product of phosphatidylcholine catalyzed by PC-PLC and its amount (shown as phosphorylcholine in TCL assay) reflects the activity of PC-PLC. As expected, 50 $\mu\text{g/ml}$ of silica increased PC-PLC activity significantly in the alveolar macrophages (Figure 2).

Little is known about how PC-PLC activity is regulated in alveolar macrophages. Based on the evidence that (1) PC-PLC is involved in H_2O_2 -primed alveolar macrophage stimulation (26) and (2) silica exposure is related to the generation of ROS (10), we hypothesize that PC-PLC activity is regulated in a redox-dependent manner. To test this, we first investigated whether an ROS scavenger could block silica-increased PC-PLC activity. Data in Figure 3 demonstrated that pretreatment with 100 μM MnTBAP for 10 min before exposure to 50 $\mu\text{g/ml}$ of silica particles abrogated the silica-increased PC-PLC activity. MnTBAP possesses both superoxide dismutase (SOD) and catalase activity, and can thus eliminate ROS (27). MnTBAP is also small enough to easily get into the interface of silica and the cell, which is important as illustrated by previous studies in which enzymes have been shown to be incapable of doing this unless added at very high concentrations (28). The data here revealed that ROS was involved in PC-PLC activation by silica.

To further confirm the hypothesis, we treated cells with exogenous H_2O_2 and then determined the PC-PLC activity. Fifty micromoles of exogenous H_2O_2 increased the production of [^3H]phosphorycholine significantly in 1 h, and this increase was completely blocked by D609 (Figure 4), suggesting that H_2O_2 can stimulate PC-PLC in alveolar macrophages.

Activation of PC-PLC Is Calcium-Dependent

It is now well established that ROS are involved in the activation of various signaling pathways via several mechanisms including protein thiol modification and changes in intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) (12). It has been known for many years that hydroperoxides are capable of elevating $[\text{Ca}^{2+}]_i$ through a variety of mechanisms, including influx from the media, release from the endoplasmic reticulum, and release of annexin VI from membranes (29). Previous reports suggested that calcium influx was likely involved in PC-PLC activation (30, 31). Thus, we determined if calcium elevation is also involved in PC-PLC activation by H_2O_2 . Pretreatment for 30 min with 5 mM BAPTA-AM (an agent that is hydrolyzed to produce the intracellular

Figure 2. Silica increases PC-PLC activity. [^3H]choline-labeled NR8383 cells were treated with 50 $\mu\text{g/ml}$ silica for 60 min, the [^3H]phosphorylcholine was extracted and separated with the TLC method as described in MATERIALS AND METHODS, and then the radioactivity was counted with a scintillation counter. To confirm the involvement of PC-PLC, cells were pretreated with 40 μM D609 for 10 min before silica treatment. $n = 3$, $*P < 0.01$.

Figure 3. MnTBAP blocked silica-increased PC-PLC activity. [^3H]choline-labeled cells were pretreated with 100 μM MnTBAP for 10 min, after which cells were incubated with or without 50 $\mu\text{g/ml}$ silica for an additional 60 min. Next the [^3H]phosphorylcholine was extracted and separated by TLC, and the radioactivity was counted with a scintillation counter. $n = 3$, $*P < 0.01$.

calcium chelator, BAPTA), which buffers hydroperoxide-induced increases in $[\text{Ca}^{2+}]_i$ in alveolar macrophages (32), inhibited H_2O_2 -increased PC-PLC activity (Figure 5A). In contrast, when cells were pretreated for 5 min with 250 nM A23187, a calcium ionophore that allows entry of calcium, which was 1.3 mM in the medium, the H_2O_2 -induced PC-PLC activation was significantly enhanced (Figure 5B). Thus, our data suggest that elevation of $[\text{Ca}^{2+}]_i$ is involved in H_2O_2 -increased PC-PLC activation in alveolar macrophages.

DISCUSSION

Inflammation underlies the pathogenesis of silica-induced lung diseases, though the signaling pathways through which silica particle stimulates the production of cytokines remains only partially resolved. In this study we showed that the induction of both $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ upon silica exposure of alveolar macrophages is through PC-PLC. We also demonstrated that PC-PLC activity was regulated via redox- and calcium-dependent signaling. Our data suggest that PC-PLC may be an upstream molecule involved in the inflammation response in alveolar macrophages.

Several studies have demonstrated that PC-PLC plays a role in cell signaling. PC-PLC has been shown to be involved in cell growth (33, 34), differentiation (35), and apoptosis (36). Schutze and colleagues first reported that PC-PLC was involved in the activation of $\text{NF-}\kappa\text{B}$ signaling (37), and this was confirmed by

Figure 4. Bolus H_2O_2 increases PC-PLC activity. [^3H]choline labeled cells were pretreated with 40 μM D609 for 30 min, and then were incubated with/without 50 μM H_2O_2 for an additional 15 min. [^3H]phosphorylcholine was extracted and separated by TLC. The radioactivity was counted with a scintillation counter. $n = 4$, $*P < 0.01$.

Figure 5. PC-PLC activity is regulated in a calcium-dependent manner. (A) BAPTA inhibits H₂O₂-increased PC-PLC activity. [³H]choline-labeled cells were pretreated with 5 mM BAPTA, a calcium chelator, for 30 min, and then were incubated with or without 50 μ M H₂O₂ for an additional 15 min. $n = 3$, $*P < 0.01$. (B) A23187 increases PC-PLC activity. [³H]choline-labeled cells were treated with 250 nM of A23187, a calcium ionophore, for 5 min, and the [³H]phosphorylcholine was extracted and separated by TLC. Radioactivity of the phosphorylcholine band was then counted with a scintillation counter. $n = 3$, $*P < 0.01$.

others (21, 38). Later, the requirement of PC-PLC activity for the induction of cytokines, such as TNF- α (39), IL-1 α (40), and IL-1 β (41), was reported. Our finding that PC-PLC is critical for the induction of TNF- α and IL-1 β by silica further confirms the role of PC-PLC in cytokine regulation. The hydrolysis of phosphatidylcholine by PC-PLC generates phosphorylcholine and DAG. The latter is a well-known second messenger that is involved in several signaling pathways, including activation of most PKC isoforms, while little is known about the role, if any, of phosphorylcholine in cell signaling.

An alternative potential mechanism of how PC-PLC is involved in NF- κ B signaling or TNF- α and IL-1 β regulation is through its involvement in signaling leading to the generation of H₂O₂. Although we have shown that H₂O₂ can activate PC-PLC, we and others have found that PC-PLC may play a critical role in regulation of respiratory burst, the major source of stimulated H₂O₂ production by macrophages (26). In other words, PC-PLC activation appears to be both upstream and downstream of H₂O₂ generation. Once produced, H₂O₂ can activate NF- κ B signaling (14, 42), which is involved in TNF- α and IL-1 β regulation.

Many reagents have been reported to increase PC-PLC activity, including growth factors such as insulin (20); cytokines such as TNF- α (21, 43), IFN- γ (44), and IL-4 (22); LPS (16); prostaglandin F₂ α (23); endothelin-1 (24); and others (17, 45, 46). In this study we found that PC-PLC was also activated by silica in alveolar macrophages (Figure 2). How PC-PLC is activated upon stimulation is currently not well characterized. Since many PC-PLC activators have specific receptors on cell membrane, it is

assumed that PC-PLC is activated through receptor-mediated signaling pathways. It has been reported that PKC (20) and Ras (47) can affect PC-PLC activity. In contrast, Murthy and Makhoul found that PC-PLC was not PKC sensitive (30). Our data suggest that PC-PLC activity is regulated in a redox-dependent manner in alveolar macrophages, as shown by the inhibition of silica-stimulated PC-PLC activity by MnTBAP, an SOD and catalase mimic (Figure 3), and by the finding that bolus H₂O₂ increased PC-PLC activity (Figure 4). Here the source of ROS upon the exposure of silica may be either from siloxyl radicals (Si $\cdot$ or SiO $\cdot$) on the surface of silica particles (11), and/or from the respiratory burst initiated by phagocytosis of the particles. Many PC-PLC stimulators, if not all, seem to use ROS as a signaling messenger. For example, TNF- α (48), LPS (49, 50), and insulin (51) have been found to activate signaling by generating ROS. Thus, our results suggesting that PC-PLC activation by silica is regulated via a redox-dependent manner is an important (although perhaps not entirely surprising) finding.

In this study we also demonstrated that the intracellular calcium chelator blocked PC-PLC activation (Figure 5A), while the calcium ionophore enhanced the PC-PLC activation (Figure 5B), suggesting that calcium elevation is an upstream event for PC-PLC activation. This result is consistent with findings from other research groups (30, 31), who found that calcium chelators could inhibit PC-PLC activity. On the other hand, Nofer and coworkers reported that PC-PLC could facilitate the thapsigargin-induced loss of intracellular calcium, and the authors suggested that there existed a physiologic feedback mechanism activated by calcium influx that acted through consecutive activation of PC-PLC to limit the rise of [Ca²⁺]_i (52). It is currently not clear how silica elevated the [Ca²⁺]_i. Several studies suggest that elevated [Ca²⁺]_i is from influx from the extracellular environment (53, 54). However, Chen and colleagues observed that chelation of extracellular calcium had no effect on silica-elevated [Ca²⁺]_i and concluded that silica increased [Ca²⁺]_i via mobilization of intracellular calcium pool (55). It seems that ROS is involved in silica-elevated [Ca²⁺]_i elevation, since antioxidant treatment blocked the [Ca²⁺]_i increase (56). Indeed, several studies (including studies from our lab) have reported the elevation of [Ca²⁺]_i after ROS stimulation (32, 57). The exact mechanisms by which silica increases [Ca²⁺]_i and by which calcium activates PC-PLC remain to be determined.

Based on the numerous reports of the involvement of PC-PLC in inflammatory responses, further studies are warranted, including the mechanisms by which PC-PLC is activated by various agents and how PC-PLC interacts with other signaling pathways. It must be mentioned here that currently the major tool to identify the involvement of PC-PLC in pathophysiologic processes in the mammalian system remains the use of its pharmacologic inhibitor, D609. Although the DNA sequence of PC-PLC from bacteria is established (58), the gene for PC-PLC has not been identified in any eukaryote. Indeed, there is no statistically significant homology between bacterial PC-PLCs and that of any mammalian protein. As such, reports that antibodies to bacterial PC-PLC react with the mammalian enzyme must be viewed with skepticism. Until mammalian PC-PLC can be isolated or cloned, D609 and the activity assay for PC-PLC remain the only tools for investigation of this important enzyme.

Cytokines, such as IL-1 β and TNF- α , released from alveolar macrophages after silica particle exposure play critical roles in silica-associated pulmonary pathogenesis. As shown by the present study and others (4, 5), both IL-1 β and TNF- α were increased by silica exposure. Interleukin-1 β has been implicated in the deposition of collagen and pulmonary fibrosis elicited by silica and bleomycin (59). TNF- α is also required for the development of silica-induced fibrosis and silicosis is absent in TNF- α knockout

mice (60). We showed that PC-PLC is involved in TNF- α and IL- β induction by silica. Furthermore, we demonstrated that PC-PLC regulation is redox- and calcium-dependent. Our findings about the role of PC-PLC in silica-induced cytokine expression and the regulatory mechanism of PC-PLC should provide new insight into the inflammatory response.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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