# Involvement of Protein Kinase C, Phospholipase C, and Protein Tyrosine Kinase Pathways in Oxygen Radical Generation by Asbestos-stimulated Alveolar Macrophage

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Although asbestos stimulates oxygen radical generation in alveolar macrophages, the exact mechanism is still not clear. The purpose of this study was to compare the ability of three asbestos fibers (amosite, chrysotile, and crocidolite) to generate oxygen radicals in macrophages and examine the mechanism of this action. All asbestos fibers were able to induce chemiluminescence but chrysotile induced maximal chemiluminescence at higher concentrations than amosite and crocidolite. Protein kinase C (PKC) inhibitors (sphingosine and staurosporine) suppressed the ability of asbestos to induce oxygen radical generation. Phospholipase C (PLC) inhibitors (U73122 and neomycin) and protein tyrosine kinase (PTK) inhibitors (erbstatin and genistein) decreased oxygen radical generation of asbestos-stimulated alveolar macrophages. Oxygen radical generation was not suppressed by an adenylate cyclase activator (forskolin), a protein kinase A inhibitor (H-8), and a protein serine–threonine phosphatase inhibitor (okadaic acid). PLC and PTK inhibitors suppressed the increment of phosphoinositide turnover by amosite. These results suggest that asbestos fibers induce the generation of oxygen radicals through PTK, PLC, and PKC pathways in a dose–response pattern. — *Environ Health Perspect* 105(Suppl 5): 1325–1327 (1997)

Key words: macrophage, asbestos, protein kinase C, phospholipase C, protein tyrosine kinase

## Introduction

Asbestos, a fibrous particle, causes pulmonary fibrosis, bronchogenic carcinoma, and mesothelioma (*1,2*). Several reports suggest that the toxicity of asbestos is mediated by oxygen radicals in these diseases. Asbestos stimulates the release of oxygen radicals from alveolar macrophage(s) (AM) and the generation of oxygen radicals is an important primary event in asbestos-induced cell injury (*3–7*). Among asbestos fibers, chrysotile induces the generation of oxygen radicals in AM at noncytotoxic doses whereas crocidolite and amosite do not (*8*). One study reports no stimulatory effect of crocidolite in AM (*9*), but some authors note the generation of oxygen radicals in crocidolite-stimulated AM (*4,10*). These results suggest that asbestos fibers have different capabilities in the generation of oxygen radicals.

Although the exact mechanism for the release of oxygen radicals is not clear yet, it has been accepted that asbestos stimulates oxygen radical generation by interaction with membranes of AM; the signal transduction pathway for this phenomenon also involves phospholipase C (PLC) and protein kinase C (PKC) pathways (*8*). PLC hydrolyzes phosphatidyl inositol biphosphate ($PIP_2$) to diacylglycerol (DAG) and inositol triphosphate ($IP_3$). Whereas $IP_3$ increases intracellular calcium by releasing calcium from the endoplasmic reticulum, DAG activates PKC (*11*). PKC has been known to activate a reduced nicotinamide adenine dinucleotide phosphate oxidase on the plasma membrane, which produces superoxide anions from oxygen (*12*).

In human AM, silica increases the tyrosine phosphorylation of proteins at 46 and 50 kDa, which suggests the activation of a tyrosine kinase pathway (*13*). Currently there is no information about the role of the tyrosine kinase pathway in asbestos-stimulated AM. Chrysotile increases intracellular calcium by opening calcium channels. Intracellular calcium seems to prolong the production of chrysotile-stimulated superoxide anion, but it is not clear whether intracellular calcium may be involved in the release of oxygen radicals from amphiboles at an early stage (*1*).

The purpose of this study was to examine the hypothesis that three asbestos fibers (amosite, crocidolite, and chrysotile) induce the generation of oxygen radicals in rat AM, and to determine what kinds of signal transduction pathways are involved in oxygen radical generation.

## Methods

Crocidolite, amosite, and chrysotile (Union Internationale Contre le Cancer reference standard sample) were obtained from the Japan Industrial Safety and Health Association (Tokyo, Japan). Ketamine, staurosporine, verapamil, 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride, forskolin, okadaic acid, and *Escherichia coli* lipopolysaccharide (LPS) were obtained from Sigma Chemical (St. Louis, MO). Sphingosine, neomycin, U-73122, H-8, and genistein were obtained from Calbiochem (La Jolla, CA). Erbstatin analog was obtained from Research Biochemicals International (Natick, MA). LPS, ketamine, and neomycin were stored at –20°C in isotonic saline. Other drugs were stored at –20°C in dimethyl sulfoxide (DMSO). The stock solutions were diluted 1000-fold into the mixing buffer such that

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Abbreviations used: AM, alveolar macrophage(s); DAG, diacylglycerol; DMSO, dimethyl sulfoxide; HBSS, Hanks balanced salt solution; $IP_3$, inositol triphosphate; LPS, lipopolysaccharide; PI, phosphoinositide; $PIP_2$, phosphatidyl inositol biphosphate; PKC, protein kinase C; PLC, phospholipase C; PTK, protein tyrosine kinase.

the final DMSO concentration was no higher than 0.1%.

All experiments were performed with male Sprague-Dawley rats (250–300 g). We used seven rats for chemiluminescence and two rats for PLC activity. LPS was administered at 0.4 mg/kg. Rats were given LPS via trachea under ketamine anesthesia 20 hr before AM isolation. The animals were anesthetized with sodium phenobarbitone (50 mg, ip). AM were harvested by bronchoalveolar lavage. Bronchoalveolar lavage was performed by cannulating the right and left main bronchi with a sterilized 19-gauge polypropylene tube. Saline solution (10 ml) was injected into the lung via a polypropylene tube and then slowly aspirated into a collection trap using a syringe of negative pressure. This procedure was repeated 10 times. Thereafter, cell suspension was centrifuged at $300 \times g$ for 10 min at 4°C. The pellet was treated to hypotonic shock if erythrocyte contamination was observed, and the cells were centrifuged again. The pellet was resuspended in HEPES buffer at 4°C. Total cell number was measured by hemocytometer and cell viability was evaluated by trypan blue exclusion (> 95%). AM were cultured for 2 hr at the concentration of $1 \times 10^6$ cells after elimination of nonadherent inflammatory cells (*14*).

Oxygen radical generation was measured with chemiluminescence. The reaction mixture consisted of 0.2 ml AM cell suspension ($1 \times 10^6$ cells), and 0.2 mM luminol in HEPES–Hanks buffer. Macrophages were allowed to equilibrate in the luminometer for 5 min prior to stimulation with asbestos. After the pretreatment of cells with each drug for 30 min, asbestos was added to the incubation medium. Chemiluminescence was measured with a luminometer (1250, Bio-orbit, Turk, Finland) for 30 min. For the quantitative analysis of chemiluminescence, the highest emission was assessed as the peak height compared with that of controls (*15*).

AM cultures were metabolically labeled for 20 hr in inositol-free medium containing 0.5% heat-inactivated fetal calf serum and 1 µCi [$^3$H]myo-inositol (NEN, Dupont, Wilmington, DE). Cells were washed with Hanks balanced salt solution (HBSS) and incubated in HBSS containing 10 mM LiCl and 0.2% bovine serum albumin with or without the specified additives. Inositol phosphates and free inositol were extracted into cold 10 % perchloric acid. The extraction solution was neutralized by 10% KOH and loaded onto AG 1-X8 (formate form) columns. The [$^3$H]inositol phosphates were eluted sequentially using 0.2 M ammonium formate/0.1 M formic acid (for $InsP_1$), 0.4 M ammonium formate/0.1 M formic acid (for $InsP_2$), 1 M ammonium formate/0.1 M formic acid (for $InsP_3$)(*16*).

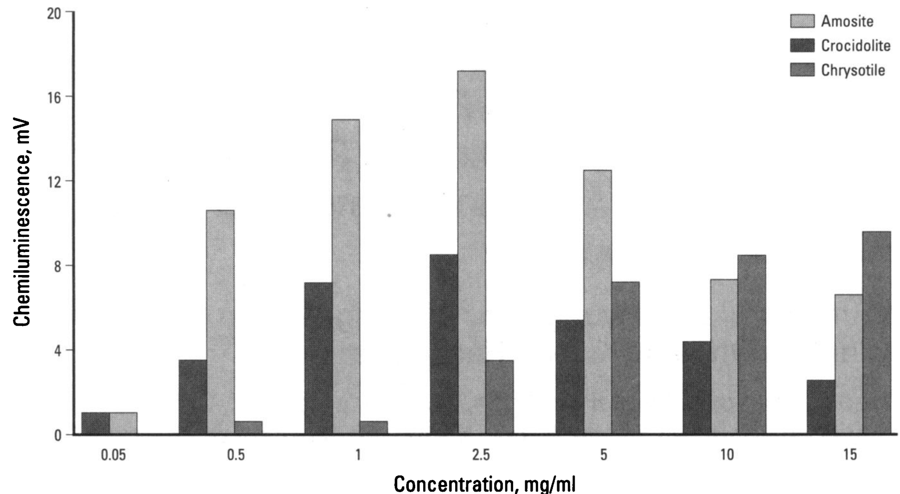


**Figure 1.** Concentration–response effect of asbestos fibers on chemiluminescence of lipopolysaccharide-treated alveolar macrophages ($1 \times 10^6$ cells) from one rat. Experiments were repeated six times with consistent results (SD < 10%).

## Results and Discussion

Three asbestos fibers (amosite, chrysotile, and crocidolite) were examined to evaluate their ability to generate oxygen radicals in AM. All asbestos fibers induced chemiluminescence in a dose–response pattern although effective doses were different. Among the three fibers, amosite showed the highest level of chemiluminescence at a lower dose. Dose–response patterns in crocidolite and amosite were similar, but they differed from the chrysotile. Chemiluminescence appeared within 1 min; peak level was observed within several minutes (Figure 1). Our data demonstrated that amphiboles were stronger in oxygen radical generation at lower doses than serpentine fibers.

To investigate the role of calcium in asbestos-stimulated AM, two drugs that decrease intracellular calcium levels were given as pretreatments. In all asbestos fibers, the calcium channel blocker verapamil inhibited chemiluminescence to approximately 40% of control values (Table 1). These results support other observations that verapamil inhibits chrysotile-induced stimulation of AM (*1*). Also, 3,4,5-trimethoxybenzoate hydrochloride, which inhibits the release of calcium from endoplasmic reticulum, decreased the effect of asbestos on chemiluminescence (Table 1). These results support the fact that calcium is an important factor in the

**Table 1.** Effect of various drugs on chemiluminescence of asbestos-stimulated alveolar macrophages ($1 \times 10^6$ cells).

| | Chemiluminescence, mV[a] | | |
|---|---|---|---|
| Treatment group | Amosite, 5 mg/ml | Crocidolite, 5 mg/ml | Chrysotile, 10 mg/ml |
| Control | 12 ± 1.5 | 6 ± 1 | 9 ± 0.8 |
| Verapamil (50µM) | 4.92 ± 1.2* | 1.92 ± 0.2* | 3.96 ± 0.3* |
| TMB-8 (50 µM) | 8.4 ± 0.5* | 3.96 ± 0.2* | 5.49 ± 0.4* |
| Staurosporine (2 µM) | 0.3 ± 0* | 0.3 ± 0* | 0.3 ± 0* |
| Sphingosine (50 µM) | 0.3 ± 0* | 0.3 ± 0* | 0.3 ± 0* |
| Neomycin (2 mM) | 1.68 ± 0.2* | 0.9 ± 0* | 3.24 ± 0.1* |
| U73122 (10 µM) | 4.68 ± 0.2* | 1.44 ± 0.2* | 2.7 ± 0.1* |
| Genistein (20 µM) | 2.64 ± 0.1* | 1.92 ± 0.2* | 2.97 ± 0.2* |
| Erbstatin (10 µM) | 0.3 ± 0* | 0.3 ± 0* | 0.3 ± 0* |
| Okadaic acid (0.5 µM) | 14 ± 1 | 4.8 ± 2.3 | 13 ± 0.7 |
| H-8 (0.2 µM) | 12 ± 2 | 4.8 ± 3.2 | 9 ± 0.5 |
| Forskolin (1 µM) | 12 ± 0 | 4.5 ± 2.2 | 8 ± 0.7 |

TMB-8, 3,4,5-trimethoxybenzoate hydrochloride. [a]Data represent the mean ± SD of six experiments. *, significantly different from control group ($p < 0.0001$).

signal transduction mechanism for the generation of oxygen radicals in asbestos-stimulated AM.

Sphingosine and staurosporine, which are PKC inhibitors, suppressed asbestos-induced chemiluminescence. Pretreatment of sphingosine (50 µM) completely suppressed the effects of asbestos fibers on the generation of oxygen radicals in AM. Staurosporine also showed nearly identical effects at 2 µM (Table 1). The data support the hypothesis that oxygen radical generation is mediated through PKC (*12*).

To examine the role of PLC, we pretreated AM with PLC inhibitors, neomycin, and U73122. U73122 inhibited chemiluminescence to approximately 40% of control values at the concentration of 1 µM. Asbestos-induced chemiluminescence was decreased by pretreatment with neomycin at 2 µM, and its level was between 20 and 40% of control values (Table 1). Although PLC inhibitors could not suppress asbestos-induced chemiluminescence completely, our results showed that asbestos-induced oxygen radical generation in AM was mediated through the PLC pathway. These results support other observations that demonstrate the involvement of the PLC pathway in oxygen radical generation by asbestos (*8*).

In this experiment, we observed the effects of protein tyrosine kinase (PTK) inhibitors on asbestos-induced chemiluminescence. Genistein and erbstatin suppressed asbestos-induced chemiluminescence (Table 1). Our results suggest the involvement of a PTK pathway in the oxygen radical generation in asbestos-stimulated AM.

Pretreatment with okadaic acid (serine–threonine phosphatase inhibitor) did not change asbestos-induced chemiluminescence. Also, H-8 (protein kinase A inhibitor) and foskolin (adenylate cyclase activator) did not change the effect of asbestos (Table 1).

To further clarify relationships in the signal transduction pathway, we measured phosphoinositide (PI) turnover in asbestos-stimulated AM after the pretreatment of PTK and PLC inhibitors. Amosite stimulated PI turnover in AM, whereas U73122 and neomycin completely suppressed PI turnover in amosite-stimulated AM. Two PTK inhibitors showed different inhibition levels in PI turnover similar to those in chemiluminescence. Genistein suppressed amosite-stimulated PI turnover, and erbstatin inhibited the action of amosite on PI turnover (Table 2). From these results, we suggest that the activation of PTK and PLC is involved in asbestos-induced PI turnover.

**Table 2.** Effect of various drugs on phospholipase C activity of amosite-stimulated alveolar macrophages ($1 \times 10^6$ cells).

| Drug | PLC activity (CPM)[a] |
|---|---|
| Control | 241 ± 26 |
| Neomycin (2 mM) | 101 ± 8** |
| U73122 (10 µM) | 99 ± 22** |
| Genistein (20 µM) | 197 ± 8* |
| Erbstatin (10 µM) | 121 ± 6* |

CPM, counts per minute. [a]Concentration of amosite: 20 mg/ml. *, significantly different from control group ($p<0.05$). **, significantly different from control group ($p<0.01$).

In summary, different asbestos fibers induced the generation of oxygen radicals with different dose–response patterns. Our results also show that asbestos stimulated oxygen radical generation in AM through PTK, PLC, and PKC pathways. Moreover, intracellular calcium might play an important role in oxygen radical generation.

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