# Environmental Stressors in Health and Disease

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# Reactive Oxygen Species and Silica-Induced Carcinogenesis

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Epidemiological and pathological studies have established that occupational exposure to crystalline silica leads to the development of pulmonary fibrosis (1,2). Increasing evidence from epidemiological and animal studies has also implicated crystalline silica as a potential carcinogen (3,4) (e.g., inhalation of silica has been shown to be carcinogenic in rats) (5–9). Intrapleural administration of crystalline silica in rats leads to the induction of localized malignant histiocytic lymphomas. Epidemiological studies also show that there appears to be an increased lung cancer risk in many, but not all, human subjects with silicosis (4,9). Based on current evidence obtained from studies on laboratory animals and epidemiological studies on humans, the International Agency for Research on Cancer has classified crystalline silica as a human class 1 carcinogen (9).

Because silica is a newly established carcinogen, there have been few investigations concerning the mechanisms of silica-induced carcinogenesis. Studies have demonstrated that freshly fractured silica particles generate silicon-based free radicals such as $Si^{\bullet}$, $SiO^{\bullet}$, and $SiOO^{\bullet}$ (10). Upon reaction with aqueous medium, these particles generate hydrogen peroxide ($H_2O_2$), superoxide radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), and hydroxyl radical ($^{\bullet}OH$) (10). Oxygen free radicals, such as $O_2^{\bullet -}$ and $^{\bullet}OH$, and related oxygen reduction products, such as $H_2O_2$ and $^1O_2$, are collectively called reactive oxygen species (ROS). Because ROS are known to be involved in the carcinogenicity of a variety of substances, we hypothesize that silica-mediated free radical reactions may cause a persistent oxidative stress in the lung and play a key role in the mechanism of silica-induced carcinogenesis (10,11).

Most knowledge of the role of ROS in silica-induced carcinogenesis comes from in vitro studies. The generation of ROS represents one of the main mechanisms by which phagocytes kill invading organisms. ROS production increases in response to phagocyte activation by microorganisms, particulates, and chemicals, resulting in a sudden increase in oxygen consumption called the ''respiratory burst.'' Crystalline silica is a potent stimulant of respiratory burst. Respiratory burst (i.e., an increase in oxygen consumption) is associated with an elevated production of ROS (12–14). Silica particles are able to cause lipid peroxidation in vitro and in exposed workers (15). Through free radical reactions, silica particles are also able to cause DNA strand breaks, dG hydroxylation, and thymine glycol formation (11,16).

In the past one or two decades, chemical and cellular studies have contributed enormously to our understanding of the role of free radical reactions in the mechanism of diseases and cancer induced by silica. Recently, a subdiscipline of molecular toxicology and carcinogenesis has developed. New techniques are now available to understand the mechanism of silica-induced cellular injury and the role of free radical reactions in precise molecular terms. For example, what molecules are the mediators? What are the signal transduction pathways? This chapter will focus on silica-induced activation of activator protein (AP-1) and nuclear transcription factor (NF)-κB and the role of free radical reactions in these activation processes.

AP-1 was chosen due to its important role involved in a diversity of biological processes. This factor is a complex protein composed of homodimers and heterodimers of oncogene proteins of the Jun and Fos families. The genes encoding these proteins, *c-jun* and *c-fos*, can be induced by a variety of extracellular stimuli and function as intermediary transcriptional regulators in signal transduction processes leading to proliferation and transformation. This transcription factor interacts with regulatory DNA sequences known as TPA response elements or AP-1 sites. Such AP-1 binding to DNA results in the induction of mRNAs for a number of growth factors. The activity of AP-1 is modulated by several factors, including the redox state of the cell. The direct involvement of ROS in AP-1 activation was demonstrated by using defined ROS generating systems to challenge cultured cells. Both $H_2O_2$ and $O_2^{\bullet -}$ are capable of inducing the expression of several early-response genes including *c-jun* and *c-fos*. While the detailed mechanism of ROS-mediated AP-1 activation has not been elucidated, it has been suggested that AP-1 activation under oxidative conditions may be in part mediated by phosphorylation of jun proteins (17).

With regard to NF-κB, this transcription factor is found in many different cell types and is involved in the transmission of signals from the cytoplasm to the nucleus. It regulates a variety of genes involved in inflammatory or acute phase responses, such as expression of various cytokines and surface receptors (18–23). In cells that have inducible NF-κB activity, the active form of this factor is composed of two different subunits, p50 and p65 (24). In resting cells, NF-κB is retained in the cytoplasm in an inactive form and its DNA binding activity and nuclear/cytoplasmic distribution are controlled by binding to an inhibitory protein known as inhibitor-α (IκBα) and -β of NF-κB, p105 (precursor of p50), and p100 (precursor of p52). Upon activation with extracellular stimuli, these inhibitory proteins are proteolytically degraded or processed by proteasomes and certain proteases, which allows NF-κB to be released and then translocated into the nucleus in an activated form. This translocation initiates or regulates early response gene transcription by binding to a decameric motif GGGRNNYYCC (κB elements) found in promoter regions of cellular or viral genes.

This article summarizes our recent studies (25,26) on silica-induced activations of AP-1 and NF-κB, the role of ROS, and the implication in silica-induced carcinogenesis.

## I. FRESHLY FRACTURED SILICA CAUSES AP-1 ACTIVATION IN JB6 CELLS AND RLE/AP02 CELLS

To explore the effects of silica on the induction of AP-1 activity, $5 \times 10^4$ JB6/AP/κB cells were exposed to varying doses (10 ~ 300 μg/mL) of freshly fractured silica for 24 h. Freshly fractured silica caused a significant dose-dependent AP-1 activation in JB6 cells (Fig. 1). The AP-1 activation attained significance at a low concentration of 80 μg/mL of silica (≈40 μg/cm$^2$) and was maximal at 200 μg/mL (≈100 μg/cm$^2$). Based on this result, 200 μg/mL silica was selected as the concentration to be used for time course studies. At intervals from 12 to 72 h, relative AP-1 activity was tested using the luciferase assay. Induction of AP-1 activity was first observed after 12 h of incubation with silica. Thereafter, AP-1 activity increased to a maximal eightfold activation at 24 h (data not shown). Further incubation of cells with silica for 48 and 72 h resulted in a decrease of AP-1 activation.

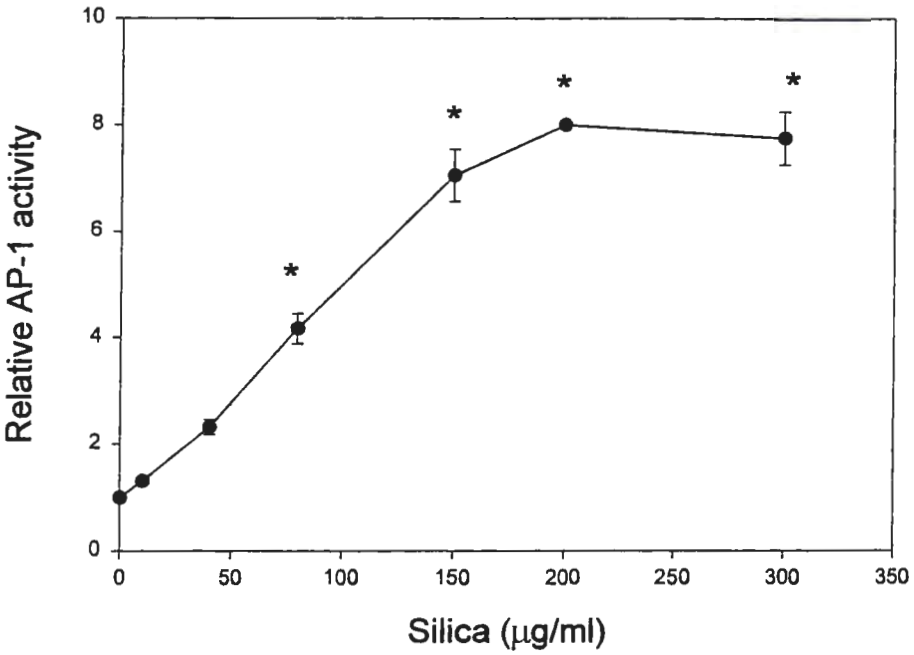


**Figure 1** Freshly fractured silica-induced AP-1 activation in JB6 P$^+$ cells. JB6/AP/κB cells ($5 \times 10^4$ cells in 1 mL of MEM medium with 5% fetal bovine serum) were seeded into each well of a 24-well plate. After overnight culture at 37°C, the cells were cultured in MEM plus 0.5% fetal bovine serum for 12 h. Then the cells were treated for 24 h with various concentrations of silica suspended in the same medium. The AP-1 activity was measured by the luciferase activity assay as described previously (25). Results, presented as relative AP-1 induction compared to the untreated control cells, are means and standard errors of nine assay wells from three independent experiments. *Indicates a significant increase from control ($p \leq 0.05$).

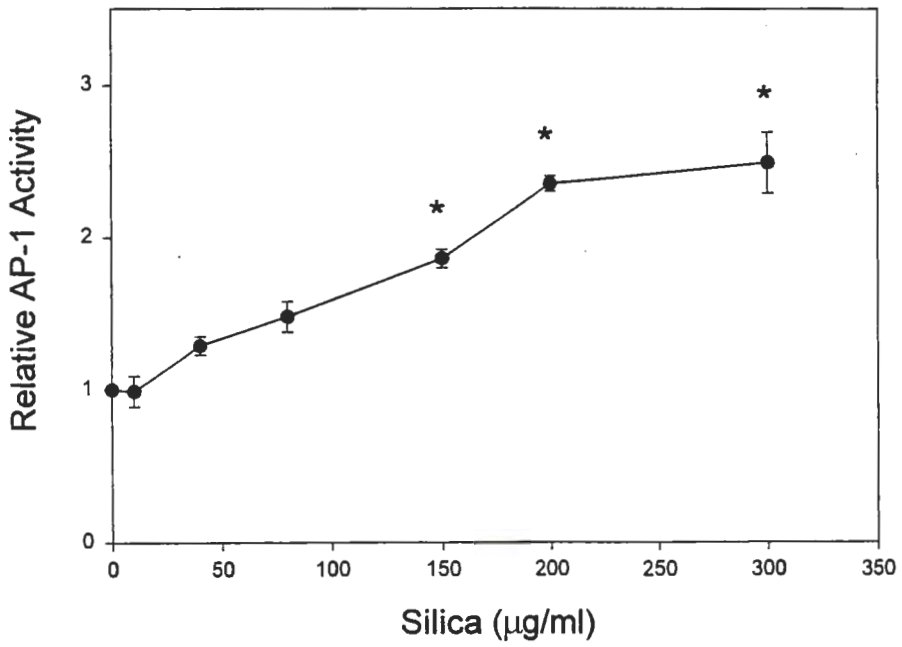


**Figure 2** Induction of AP-1 activity in RLE cells. RLE/AP02 cells ($5 \times 10^4$ cells in 1 mL of F12K medium containing 10% of fetal bovine serum), stably transfected with AP-1 luciferase reporter plasmid, were seeded into each well of a 24-well plate. After overnight culture at 37°C, cells were cultured in F12K medium plus 0.5% fetal bovine serum for 12 h. The cells were then exposed to various concentrations of silica suspended in the same medium for 72 h. Other experimental conditions were the same as those described in the legend to Figure 1. Results, presented as relative AP-1 induction compared to the untreated control cells, are means ± SEM of 12 assay wells from three independent experiments. * Indicates a significant increase from control ($p \leq 0.05$).

Since crystalline silica causes pulmonary epithelial hyperplasia and neoplastic lesions, we next asked whether freshly fractured silica would induce AP-1 activation in rat lung epithelial cells. Freshly fractured silica was incubated for 72 h with $5 \times 10^4$ RLE/AP02 cells stably transfected with AP-1-luciferase reporter plasmid. Freshly fractured silica causes a dose-dependent induction of AP-1 activation in RLE/AP02 cells (Fig. 2). At a silica concentration of 300 μg/mL (≈150 μg/cm$^2$), RLE cells exhibited AP-1 levels 2.5-fold greater than those observed in control cells. Time course studies in which a silica concentration of 200 μg/mL (≈100 μg/cm$^2$) was used indicated that significant induction of AP-1 activity was achieved after 24 h of exposure to silica (data not shown). The AP-1 induction in RLE/AP02 cells was different from that observed in JB6 cells. In RLE cells, the induction of AP-1 activity in response to silica occurred more slowly and persisted for at least 72 h. In addition, the maximal level of AP-1 induced by freshly fractured silica was lower in RLE cells (2.5-fold increase) than that in JB6 cells (eightfold increase). Because JB6 cells respond to a greater degree than RLE cells, JB6 cells were chosen for the further studies.

## II. TRANSACTIVATION OF AP-1 BY FRESHLY FRACTURED SILICA IN AP-1-LUCIFERASE REPORTER TRANSGENIC MICE

To investigate whether similar mechanisms exist in vivo, we used AP-1-luciferase reporter transgenic mice for these studies. The transgenic mice were exposed to freshly fractured silica (5 mg/mouse) by intratracheal aspiration of a silica suspension (70 mg/mL in 0.9% sterile NaCl). At the intervals of 1, 2, 3, and 4 days postexposure, animals were anesthetized with sodium pentobarbital and sacrificed by exsanguination. Lungs were removed and their luciferase activities were measured as described previously (25). Elevated AP-1 transactivation was not detected at 1-day postexposure (data not shown). However, AP-1 activation increased significantly at 2 and 3 days postexposure, and decreased toward control levels at 4 days postexposure (Fig. 3). At day 3 postexposure, the induction of AP-1 activation in lung tissue by freshly fractured silica was 22 times higher than that of the control group.

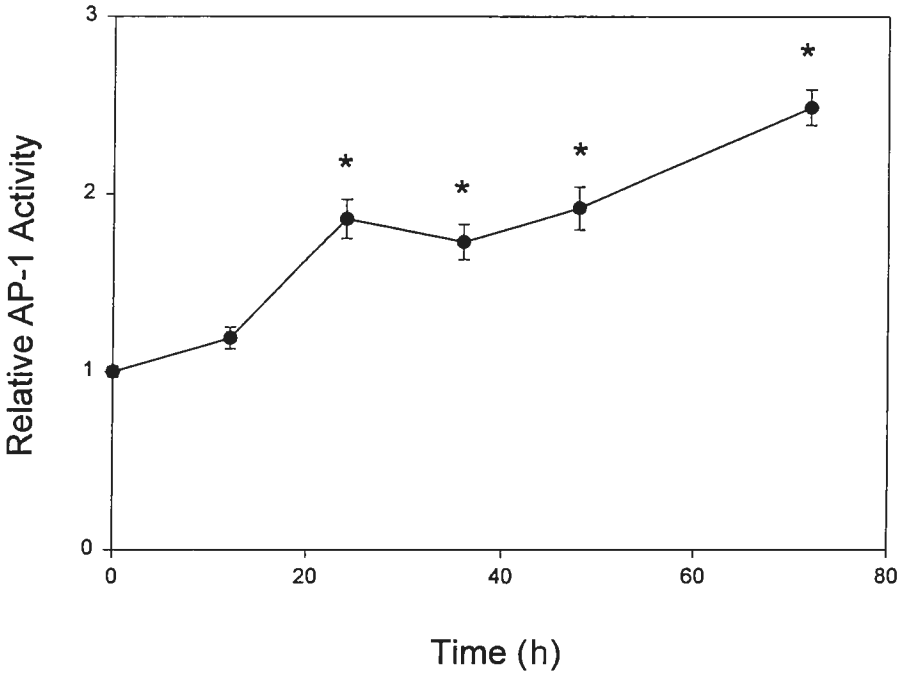


**Figure 3** Freshly fractured silica induces the transactivation of AP-1 in AP-1-luciferase reporter transgenic mice. The AP-1-luciferase transgenic mice were intratracheally instilled with 5 mg freshly fractured silica suspended in 0.07 mL of 0.9% sterile saline. At 2, 3, or 4 days postexposure, the mice were sacrificed and the lung tissue was removed. The luciferase activity of the tissue was measured as described previously (25). The results, presented relative to the level of luciferase activity of control groups, are means ± SEM of eight mice. * Indicates a significant increase from controls ($p \leq 0.05$).

## III. ACTIVATION OF ERKs AND p38 KINASE BY FRESHLY FRACTURED SILICA IN JB6 CELLS

Since mitogen-activated protein kinases (MAP), including p38 kinase, ERKs, as well as JNKs, are the upstream kinases responsible for c-Jun phosphorylation and AP-1 activation (27–30), we tested which classes of MAPK were involved in the AP-1 activation by silica. Using antibodies specific for the above MAP kinase family and phospho-specific for the phosphorylated MAP kinases, we studied ERK1, ERK2, JNKs, and p38 kinase proteins and the protein phosphorylation of ERK1, ERK2, JNKs, and p38 kinase in JB6 $P^+$ cells. Exposure to freshly fractured silica significantly stimulated the phosphorylation of p38 kinase and ERKs. The time course of p38 kinase phosphorylation induced by silica (150 μg/mL) is shown in Figure 4A. Phosphorylation of p38 kinase was first apparent after 15 min of exposure to silica and its maximal activation occurred after 2 h of exposure. To examine the dose dependence of the p38 kinase response in cells exposed to silica, JB6 cells were treated for 2 h with various concentrations of silica. A dose-related increase in p38 kinase phosphorylation was observed in cells treated with increasing concentrations of silica with prominent increases at 100 μg/mL–200 μg/mL silica (Fig. 4B). Freshly fractured silica (150 μg/mL) also causes phosphorylation of ERK1 and ERK2 in a time-dependent manner (Fig. 5). In contrast, silica did not affect the phosphorylation levels of JNKs (Fig. 6). Similar results were obtained by using the RLE cell line (data not shown).

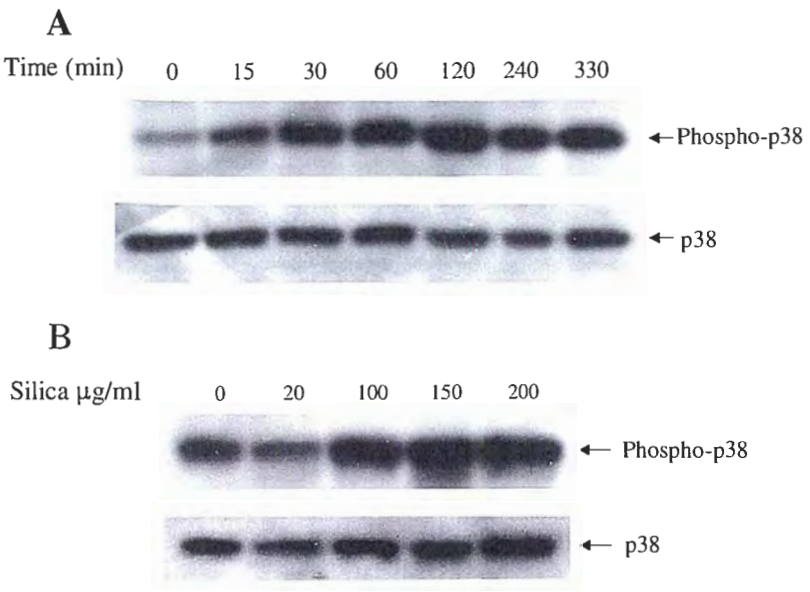


**Figure 4** Freshly fractured silica stimulates the phosphorylation of p38 MAP kinase. JB6 $P^+$ cells were cultured in 5% FBS MEM medium in 6-well (35-mm-diam) plates until 80% confluent and then cultured in 0.5% FBS MEM medium for 24 h. After this time, the cells were exposed to 150 μg/mL (47 μg/cm²) silica suspended in the same medium for different times as indicated (A) or to various concentrations of silica for 2 h (B). The cells were lysed and phosphorylated p38 kinase proteins and nonphosphorylated p38 kinase proteins were assayed using a PhosphoPlus MAPKs kit from New England Biolabs. The phosphorylated proteins and nonphosphorylated proteins were analyzed using the same transferred membrane blot.

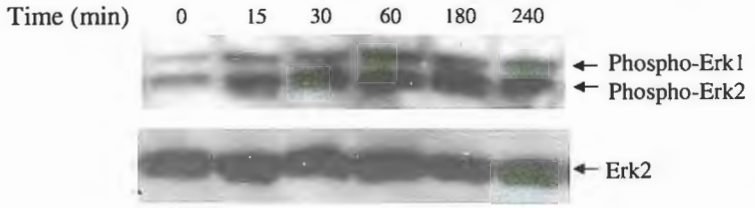


**Figure 5** Freshly fractured silica induces activation of Erk1 and Erk2. JB6 $P^+$ cells were cultured in 5% FBS MEM medium in 6-well (35-mm-diam) plates until 80% confluent. The cells were cultured in 0.5% FBS MEM medium for 24 h and then exposed to 150 μg/mL (47 μg/cm$^2$) silica suspended the same medium for different times as indicated. The cells were lysed and phosphorylated Erk1 and Erk2 proteins and nonphosphorylated Erk2 proteins were assayed using a PhosphoPlus MAPKs kit from New England Biolabs. The phosphorylated proteins and nonphosphorylated proteins were analyzed using the same transferred membrane blot.

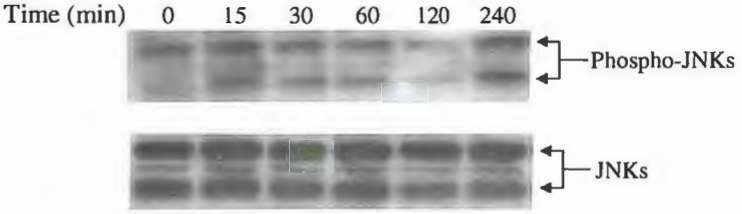


**Figure 6** Effect of freshly fractured silica on JNK activity. JB6 $P^+$ cells were cultured in 5% FBS MEM medium in 6-well (35-mm-diam) plates until 80% confluent. The cells were cultured in 0.5% FBS MEM medium for 24 h and then exposed to 150 μg/mL (47 μg/cm$^2$) silica suspended in the same medium for different times as indicated. The cells were lysed and phosphorylated JNK proteins and nonphosphorylated JNK proteins were assayed using a PhosphoPlus MAPKs kit from New England Biolabs. The phosphorylated and nonphosphorylated proteins were analyzed using the same transferred membrane blot.

These results suggested that ERKs and p38 kinase, but not JNK, may be involved in silica-induced AP-1 activation in JB6 cells as well as in RLE cells.

## IV. INHIBITION OF ERKs OR p38 KINASES BY SPECIFIC INHIBITORS BLOCKS FRESHLY FRACTURED SILICA-INDUCED AP-1 ACTIVATION

To further confirm that activation of AP-1 by silica is mediated through p38- and ERKs-dependent signal transduction pathways, we examined the effects of PD 98059 and SB 203580 on silica-induced AP-1 activation. PD 98059 has been shown to act as a highly selective inhibitor of MEK1 activation, while SB 203580 has been shown to be a specific inhibitor of p38 kinase. MEK1 is an upstream activator of ERKs. Silica-induced AP-1 activation was significantly inhibited by 20 to 50 μM PD 98059 or 2 μM SB 203580 (Fig. 7A and 7B).

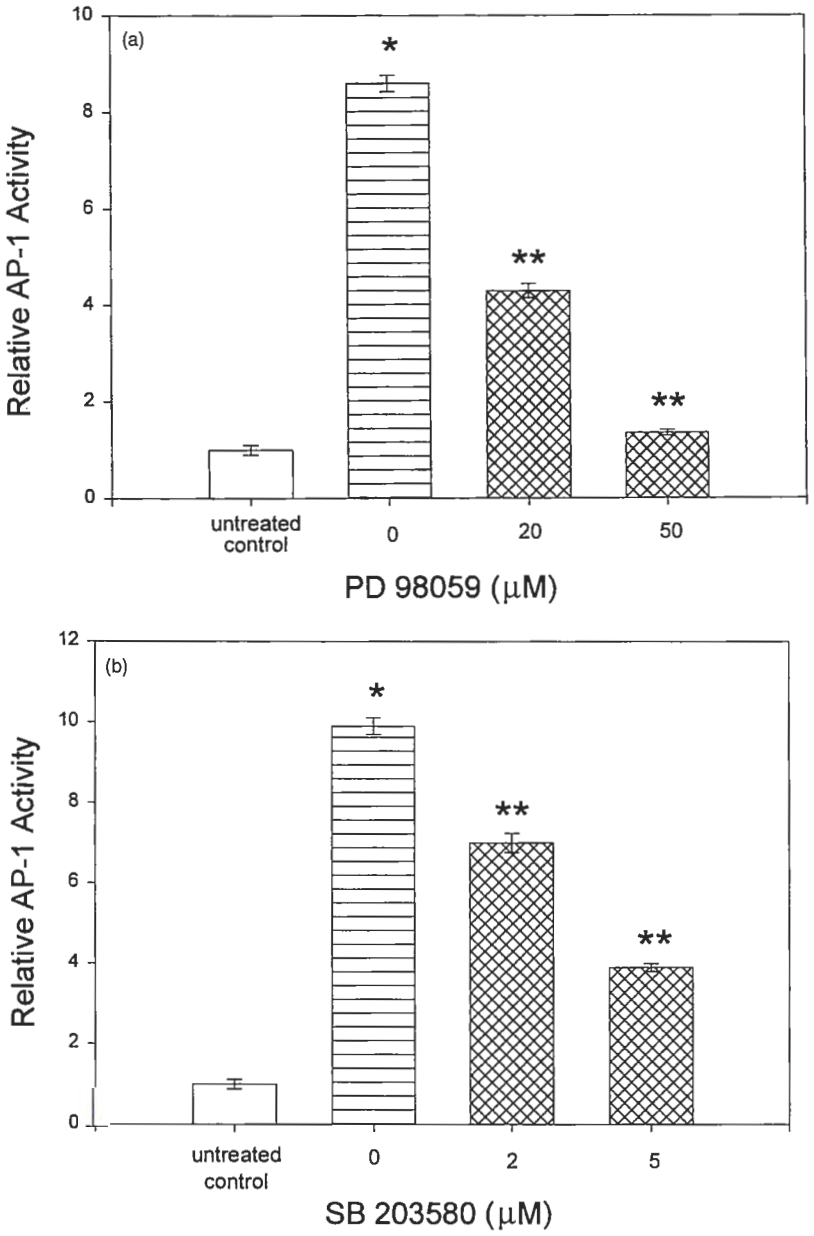


**Figure 7** Inhibition of silica-induced AP-1 activation by PD 98059 or SB 203580. JB6 cells ($5 \times 10^4$) were seeded into each well of a 24-well plate. After overnight culture at 37°C, the cells were cultured in MEM plus 0.5% fetal bovine serum for 12 h. Then the cells were pretreated with various concentrations of ERKs inhibitor, PD 98059 (a), or p38 inhibitor, SB 203580 (b) for 2 h and then exposed to 150 μg/mL silica in the presence of the inhibitors for 24 h. The AP-1 activity was measured by the luciferase activity assay as described in the "Materials and Methods." Results, presented as relative AP-1 induction compared to the control cells, are means and standard errors of twelve assay wells from two independent experiments. * Indicates a significant increase from untreated control and ** indicates significant decrease from silica alone ($p \leq 0.05$).

## V. PD 98059 AND SB 203580 INHIBITED FRESHLY FRACTURED SILICA-INDUCED AP-1 DNA BINDING ACTIVITY

To study the molecular basis of the induction on AP-1 activity by silica and further confirm the above findings, the AP-1 DNA binding activity was analyzed by the gel-shift assay. As shown in Figure 8, silica induces AP-1 DNA binding activity and PD 98059 or SB 203580 inhibited silica-induced AP-1 DNA binding activity. These data provide further support that ERKs and p38 kinase are involved in silica-induced AP-1 activation.

## VI. INDUCTION OF AP-1 ACTIVATION BY FRESHLY FRACTURED SILICA VERSUS AGED SILICA

Early studies have demonstrated that freshly ground silica generates more ROS than aged silica (10). To explore the differential effects of freshly fractured versus aged silica on the induction of AP-1 activity, $5 \times 10^4$ JB6 cells were exposed for 24 h to varying concentrations (10 ~ 300 μg/mL) of freshly fractured silica or fractured silica aged for 12 months before use. The AP-1 activation induced by freshly fractured silica was significantly higher than that of aged silica in JB6 cells (Fig. 9). The maximum AP-1 induction by freshly fractured silica was eightfold compared to controls, whereas the maximum AP-1 induction by aged silica was only twofold. This result indicated that freshly fractured silica exhibited a greater effect on AP-1 induction than aged silica.

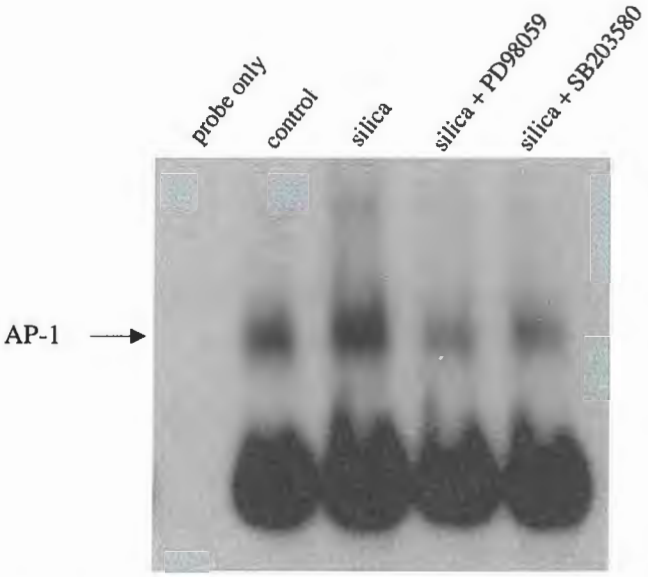


**Figure 8** Electrophoretic mobility shift assay. JB6 cells were seeded into each well of a 6-well plate until 80% confluent. Then the cells were cultured in MEM plus 0.5% fetal bovine serum for 24 h. The cells were pretreated with 20 μM of PD 98059 or 5 μM of SB 203580 for 2 h and then exposed to 150 μg/mL silica in the presence of the inhibitors for another 2 h. The AP-1 DNA binding activity was determined by gel-shift assay.

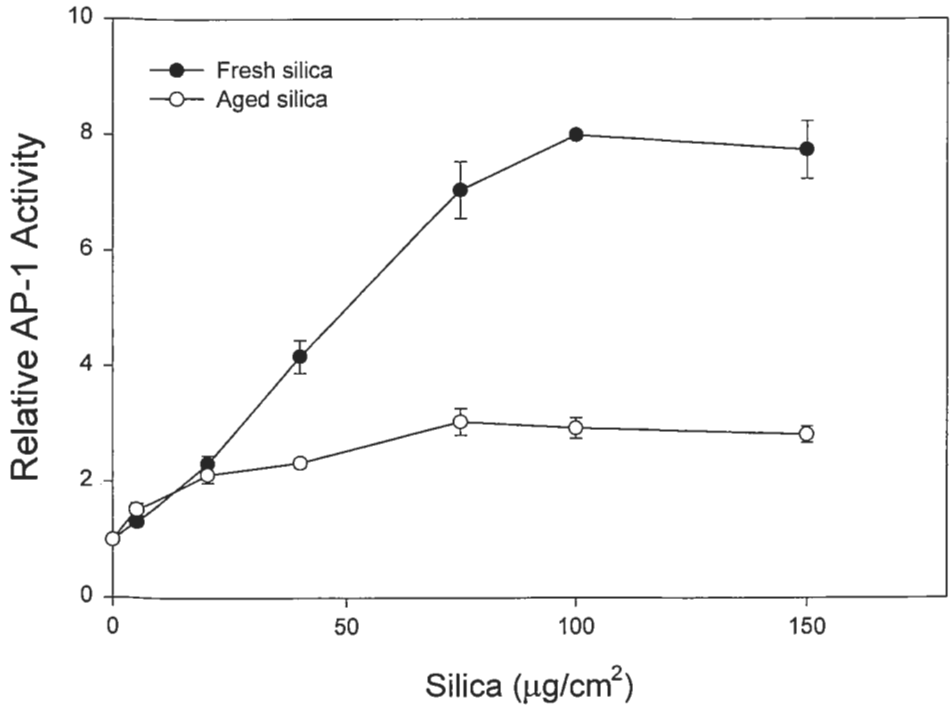


**Figure 9** Dose-dependent induction of AP-1 activation by freshly fractured silica versus aged silica. JB6 cells ($5 \times 10^4$ in 1 mL of MEM medium with 5% fetal bovine serum), stably transfected with AP-1 luciferase reporter plasmid, were seeded into each well of a 24-well plate. After overnight culture at 37°C, the cells were cultured in MEM plus 0.1% fetal bovine serum for 24 h. Then the cells were treated with 75 μg/cm² freshly fractured or 1-year-old silica for 24 h. The AP-1 activity was measured by the luciferase activity assay. Results, presented as relative AP-1 induction compared to the untreated control cells, are means and standard errors of eight assay wells from two independent experiments.

## VII. EFFECTS OF ANTIOXIDANTS AND CHELATING REAGENTS ON SILICA-INDUCED AP-1 ACTIVATION AND MAPKs PHOSPHORYLATION

The effects of antioxidant reagents on silica-induced AP-1 activation and MAPKs phosphorylation were investigated to further elucidate if ROS mediate AP-1 activation by silica. The effects of various antioxidants and chelating agents on silica-induced AP-1 activation are shown in Figure 10. Catalase, an enzyme for $H_2O_2$ decomposition, inhibited silica-induced AP-1 activation by 90%. Sodium formate, a scavenger for $^{\bullet}OH$ radical, had little or no effect. SOD, a $O_2^{\bullet -}$ radical scavenger, enhanced the AP-1 activation by 70%. Deferoxamine, a metal ion chelator that reduces the ability of metal ions to react with $H_2O_2$ and generate $^{\bullet}OH$, had no significant inhibitory effects. Both *N*-acetylcysteine, a nonspecific antioxidant, and PVPNO, a silanol blocker, significantly decreased AP-1 induction.

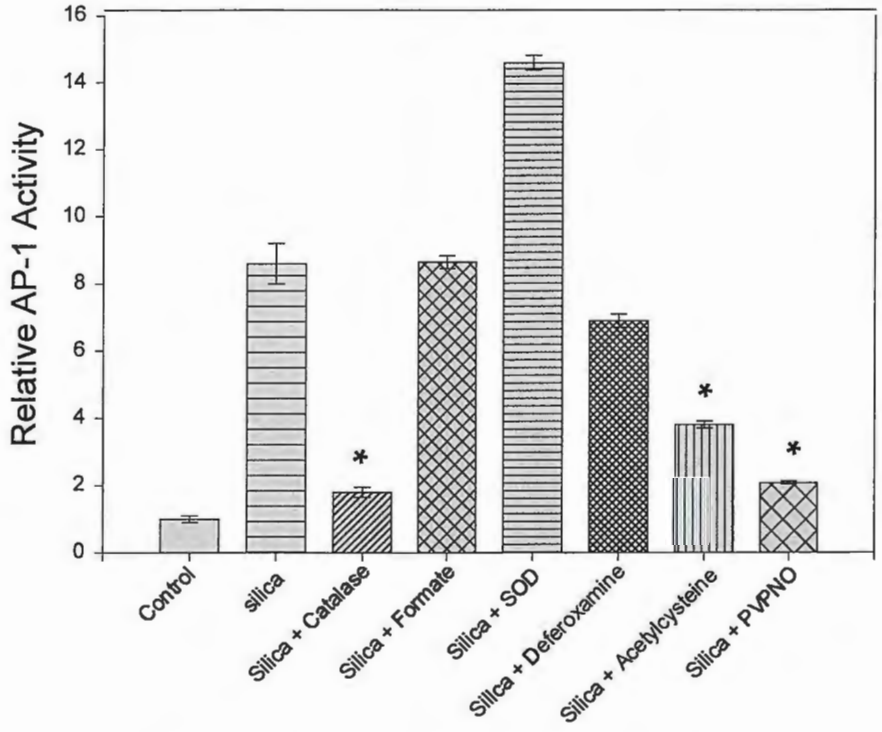


**Figure 10** Effect of antioxidant reagents on silica-induced AP-1 activation. JB6 cells ($5 \times 10^4$ cells in 1 mL of MEM medium containing 5% of fetal bovine serum) were seeded into each well of a 24-well plate. After overnight culture at 37°C, cells were cultured in the same medium plus 0.1% fetal bovine serum for 24 h. The cells were then exposed to 75 μg/cm$^2$ of freshly fractured silica and various reagents as indicated for 24 h. The concentrations of the reagent used were: catalase, 10,000 U/mL; sodium formate, 2 mM; SOD, 500 U/mL; deferoxamine, 1 mM; *N*-acetylcysteine, 1 mM; PVPNO, 50 mg/mL. Other experimental conditions were the same as those described in the legend to Figure 1. Results, presented as relative AP-1 induction compared to the untreated control cells, are means ± SEM of eight assay wells from two independent experiments. * Indicates a significant decrease from cells treated with freshly fractured silica ($p < 0.05$).

## VIII. ACTIVATION OF NF-κB

The mouse macrophage cell line, RAW 264.7 cells, was used to detect NF-κB activation by silica and other reagents. The cells were exposed for 6 h and NF-κB was analyzed in the nuclear extracts. As shown in Figure 11a, lane 1, the untreated cells did not exhibit any NF-κB activity. Upon treatment with LPS (5 μg/mL), the cells showed enhanced NF-κB binding activity (Fig. 11a, lane 2). Tetrandrine, a drug reported to decrease silica-stimulated oxidant production and cytokine release (31,32), inhibited LPS-induced NF-κB activation (Fig. 11a, lane 3). Figure 11b, lane 1 again showed the untreated cells as a control. Silica induced significant NF-κB activation (Fig. 11b, lane 2). Tetrandrine inhibited silica-induced NF-κB activation (Fig. 11b, lane 3). Similar experiments were carried out using PMA as a stimulant. As shown in Figure 11c, lanes 1–3, PMA also induced NF-κB activation and tetrandrine exhibited an inhibitory effect.

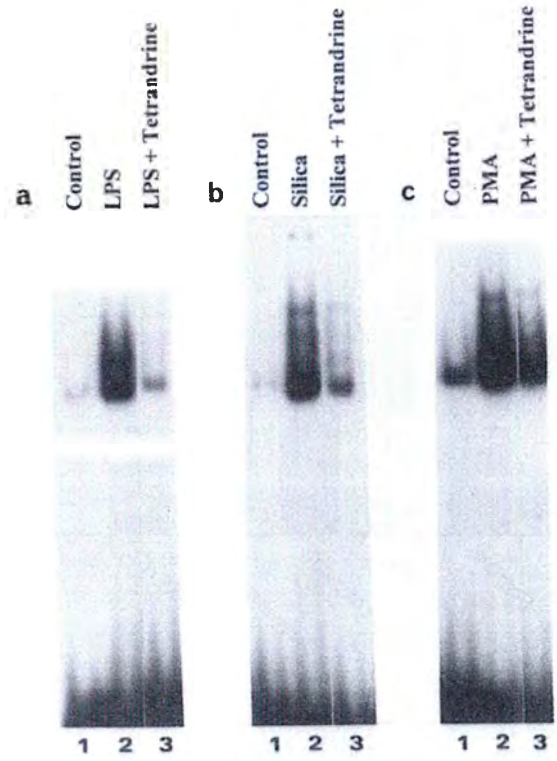


**Figure 11** Induction of DNA binding activity of NF-κB protein by LPS, silica, and/or PMA and the effect of tetrandrine. RAW 264.7 cells were adjusted to a density of $5 \times 10^6$/mL and treated for 6 h with different stimuli, then subjected to extraction of the nuclear proteins as described previously (26). DNA binding activity of the NF-κB protein was detected with a probe of $^{32}$P-labeled double-stranded NF-κB binding oligonucleotide by EMSA. (a) Lane 1, untreated cells; lane 2, cells + 5 μg/mL LPS; lane 3, cells + 5 μg/mL LPS + 150 μM tetrandrine. (b) Lane 1, untreated cells; lanes 2, cells + 100 μg/mL silica; lanes 3, cells + 100 μg/mL silica + 150 μM tetrandrine. (c) Lane 1, untreated cells; lane 2, cells + 10 nM PMA; lane 3, cells + 10 nM PMA + 150 μM tetrandrine.

## IX. EFFECT OF ANTIOXIDANTS ON NF-κB ACTIVATION

The effect of catalase on silica-induced NF-κB activation was evaluated to study the role of $H_2O_2$. As shown in Figure 12a, catalase caused a dose-dependent inhibition of silica-induced NF-κB activation. In contrast, SOD enhanced silica-induced NF-κB activation (Fig. 12b). A stronger enhancement effect was observed at a higher SOD concentration. Inactivated catalase or SOD did not exhibit any observable effects (data not shown). The metal chelator, deferoxamine, also inhibited silica-induced NF-κB activation (Fig. 12c, lanes 1–3). Deferoxamine itself did not cause any NF-κB activation (Fig. 12c, lane 4).

The involvement of iron-catalyzed reactions in the NF-κB activation is further implicated by the ability of Fe(II) but not Fe(III) to induce NF-κB activation (Fig. 12d).

The effects of the antioxidant, ascorbate, and the ·OH radical scavenger, formate, were also tested for their effect on NF-κB activation induced by LPS or silica. Both ascorbate and formate significantly inhibited silica-induced NF-κB activation (data not shown). PVPNO, a silanol (SiOH) blocker, was used to test for a possible role of SiOH in the mechanism of silica-induced NF-κB activation. As shown in Figure 13, PVPNO caused a dramatic inhibition of NF-κB activity.

Similar to the results using a peritoneal monocyte cell line outlined above, silica was able to cause NF-κB activation in rat primary alveolar macrophage cells. Formate, deferoxamine, and PVPNO inhibited this activation (data not shown).

Occupational exposure to silica is associated with the development of silicosis and lung cancer (3–9). The molecular mechanisms involved in silica-induced carcinogenesis are unclear. We hypothesize that activation of nuclear transcription factors induced by silica is a primary event in the initiation of signal transduction cascades at the cell membrane level leading to the induction of early response genes that are critical in carcinogenesis. We have studied the effect of silica on the activation of AP-1 and the signal transduction pathways involved in AP-1 activation in cell culture models and in transgenic mice, and the silica-induced NF-κB activation. We have demonstrated that silica stimulates AP-1 DNA binding activity as well as AP-1 transactivation activity. Silica induced an eightfold increase of AP-1 activity in JB6 epidermal cells and a 2.5-fold increase in rat lung epithelial cells. Silica also stimulated AP-1 transactivation in pulmonary tissues of transgenic mice. At 3 days after intratracheal aspiration of silica, the AP-1 activity was elevated 23-fold as compared to the controls. Most importantly, we have found that phosphorylation of ERK1, ERK2, and p38 kinases was induced by freshly fractured silica. Phosphorylation is involved in silica-induced AP-1 activation. These data demonstrate that freshly fractured silica induces AP-1 activation through MAPK signal transduction pathways.

Previous studies using different model systems have suggested an important role of AP-1 activation in preneoplastic-to-neoplastic transformation in cell culture and animal models (33–36). AP-1 is a critical mediator of tumor promotion and is involved in a diversity of processes. This transcription factor is able to alter gene expression in response to a number of stimuli, including the tumor promoter (TPA), EGF, TNF-α, IL-1, and UV irradiation (33). Some of the genes regulated by AP-1 are involved in immune and inflammatory responses, tumor promotion, and tumor progression. These include cytokines, such as IL-1, TNF-α, GM-CSF, collagenase IV, and stromelysin (37–39). Overexpression of *c-jun* in JB6 $P^+$ cells causes neoplastic transformation. Inhibition of AP-1 activity by either pharmaceutical agents, such as fluocinolone acetonide or retinoic acid, or molecular biological inhibitors, such as dominant negative *c-jun* and dominant negative phosphatidylinositol-3 kinase, was found to block tumor promoter–induced neoplastic transformation (34,35,40–43).

AP-1 is a complex protein composed of homodimers and heterodimers of oncogene proteins of the Jun and Fos families. The genes encoding these proteins, *c-jun* and *c-fos*, are inducible in response to a variety of extracellular stimuli and function as intermediary transcriptional regulators in signal transduction processes leading to proliferation and transformation. The activation of AP-1 may trigger downstream signal cascades, such as *jun*, *fos*, and other target genes. The members of the Jun and Fos protein families may couple cell signaling events at the cell surface to changes in gene expression which modu-

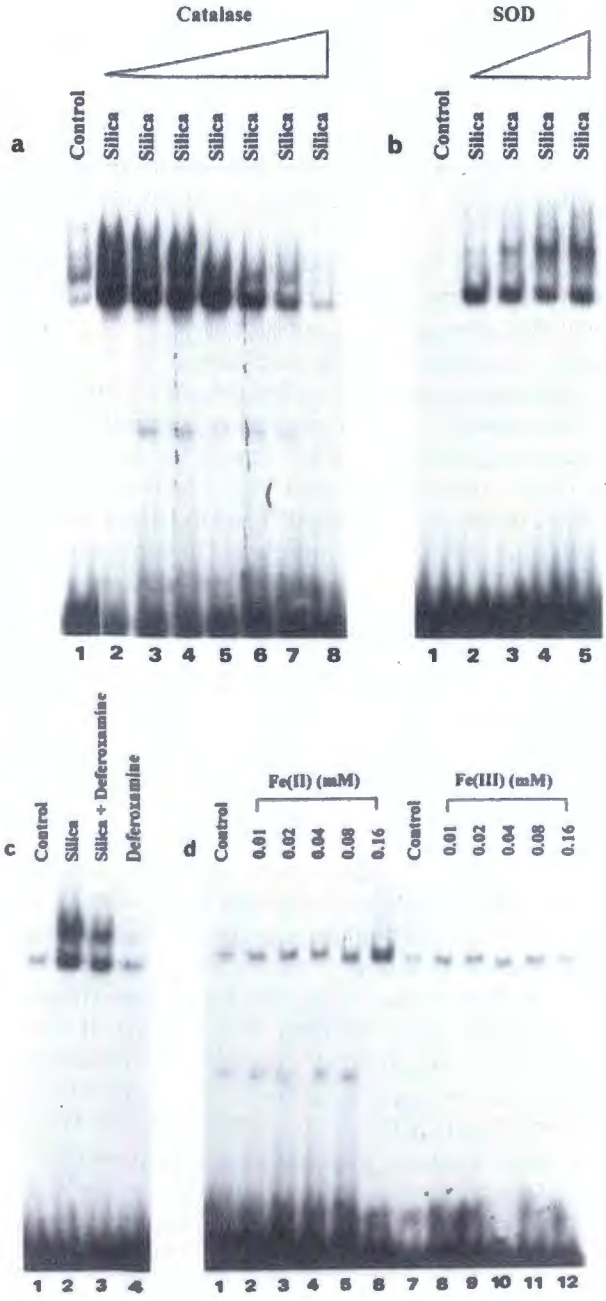


**Figure 12** Effect of antioxidants on silica-induced NF-κB activation in macrophages. (a) Effect of catalase on silica-induced NF-κB activation. Lane 1, untreated cells ($5 \times 10^6$/mL); lane 2, cells + 100 μg/mL silica; lane 3, cells + 100 μg/mL silica + 1250 units/mL catalase; lane 4, cells + 100 μg/mL silica + 2500 units/mL catalase; lane 5, cells + 100 μg/mL silica + 5000 units/mL catalase; lane 6, cells + 100 μg/mL silica + 10,000 units/mL catalase; lane 7, cells + 100 μg/mL silica + 20,000 units/mL catalase; lane 8, cells + 100 μg/mL silica + 40,000 units/mL catalase. (b) Effect

late cell responses, including proliferation and changes in phenotype. The important element of this study is that freshly fractured silica stimulates AP-1 activation, which may be one of the critical mechanisms for silica-induced carcinogenesis. The level of AP-1 induction by silica was lower in rat lung epithelial cells than that in JB6 epidermal cells. This may be due to the following differences between these two cell lines: (1) the rate of luciferase gene expression; (2) the half-life of luciferase; (3) the basal MAPK or AP-1 activities; or (4) the antistress enzyme activity.

The signal transduction pathways leading to transcription factor activation have been extensively studied in the last several years. It is believed that stress-related signals, such as UV light or ROS, induce the activation of MAP kinase pathways (ERKs, JNKs, and p38). AP-1 is a downstream target of these three MAP kinase members (44). We have studied the possible role of the MAPK family, including p38 kinase, ERKs, and JNKs, in silica-induced AP-1 activation. We have found that freshly fractured silica induced phosphorylation of ERKs and p38 kinase, but not JNKs. Pretreatment of cells with p38 and ERK inhibitors, PD 98059 or SB 203580, inhibited AP-1 transactivation as well as AP-1 DNA binding activity induced by silica. Thus, these results suggest that silica-induced AP-1 activation may be mediated through MAPK p38 and ERK pathways.

The development of AP-1 luciferase transgenic mice makes it possible to study the role of AP-1 activation in tumor promotion in vivo (45). We have shown that freshly fractured silica is able to cause AP-1 activation in transgenic mice. Maximal AP-1 activation was increased by 23-fold in pulmonary tissues at 3 days after intratracheal aspiration of silica. However, the cell types involved in this AP-1 activation response have not yet been identified. Additional studies are required to answer this question.

Our studies also show that freshly fractured silica particles are much more potent in inducing AP-1 activation than aged silica. As demonstrated earlier, freshly fractured silica particles generate more ROS upon reaction with cells or aqueous medium than aged silica (46). Due to the enhanced ROS generation, freshly fractured silica is more potent in causing lipid peroxidation, DNA damage, and other cell injuries. It appears that silica-induced AP-1 activation involves ROS-mediated reactions. Among ROS, $H_2O_2$ plays a major role in silica-induced AP-1 activation as supported by the following results: (1) freshly fractured silica is able to generate $H_2O_2$ upon reaction with aqueous medium as reported earlier (14); (2) catalase, whose function is to remove $H_2O_2$, blocked the AP-1 activation; (3) sodium formate, a scavenger of $^{\bullet}OH$ radical, did not exhibit any effect; and (4) SOD, whose function is to remove $O_2^{\bullet -}$ and generate $H_2O_2$, enhanced the AP-1 activation.

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of SOD on silica-induced NF-κB activation. Lane 1, untreated RAW 264.7 cells ($5 \times 10^6$/mL); lane 2, cells + 100 μg/mL silica; lane 3, cells + 100 μg/mL silica + 160 units/mL SOD; lane 4, cells + 100 μg/mL silica + 640 units/mL SOD; lane 5, cells + 100 μg/mL silica + 1280 units/mL SOD. (c) Effect of deferoxamine on silica-induced NF-κB activation. Lane 1, untreated RAW 264.7 cells ($5 \times 10^6$/mL); lane 2, cells + 100 μg/mL silica; lane 3, cells + 100 μg/mL silica + 1.6 mM deferoxamine; lane 4, cells + 1.6 mM deferoxamine. (d) NF-κB activation by Fe(II) and Fe(III). Lane 1, untreated 264.7 cells ($5 \times 10^6$/mL); lane 2, cells + 0.01 mM Fe(II); lane 3, cells + 0.02 mM Fe(II); lane 4, cells + 0.04 mM Fe(II); lane 5, cells + 0.08 mM Fe(II); lane 6, cells + 0.16 mM Fe(II), lane 7, untreated cells; lane 8, cells + 0.01 mM Fe(III); lane 9, cells + 0.02 mM Fe(III); lane 10, cells + 0.04 mM Fe(III); lane 11, cells + 0.08 mM Fe(III); lane 12, cells + 0.16 mM Fe(III).


**Figure 13** Effect of PVPNO on silica-induced NF-κB activation. Lanes 1, untreated RAW 264.7 cells ($5 \times 10^6$/mL); lane 2, cells + 100 μg/mL silica; lane 3, cells + 100 μg/mL silica + 20 μg/mL PVPNO; lane 4, cells + 100 μg/mL silica + 50 μg/mL PVPNO.

We have demonstrated that silica particles are also able to activate NF-κB and that $^{\bullet}OH$ radicals may play a key role in silica-induced NF-κB activation. The following experimental observations support this conclusion.

1. Silica is able to generate $^{\bullet}OH$ radical in the presence and absence of $H_2O_2$ as demonstrated by spin trapping measurements in earlier (14,46,47) and present studies.
2. Catalase blocked the NF-κB activation.
3. SOD exhibited an opposite effect. It may be noted that earlier studies have shown that molecular oxygen was consumed in the generation of $O_2^{\bullet-}$ by silica suspensions (12,48). Silica-induced DNA damage was inhibited in an argon atmosphere, indicating that the oxygen radicals responsible for DNA damage were generated from $O_2$ via $O_2^{\bullet-}$ and $H_2O_2$ as intermediates [Eq. (1)] (12,48).

$$O_2 \rightarrow O_2^{\bullet-} \rightarrow H_2O_2 \rightarrow {}^{\bullet}OH. \quad (1)$$

4. Metal ions, Fe(II) but not Fe(III), enhanced the NF-κB activation. It is known that Fe(II) generates $^{\bullet}OH$ from $H_2O_2$ via the Fenton reaction [Eq. (2)].

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + {}^{\bullet}OH. \quad (2)$$

Fe(III), on the other hand, is unable to generate $^{\bullet}OH$ radical without being first reduced to Fe(II).

5. Metal chelator, deferoxamine, also reduced the NF-κB activation. Deferoxamine chelates metal ions, such as Fe(II) or Fe(III), to make them less reactive toward $H_2O_2$ and thus attenuated the generation of $^{\bullet}OH$ radicals.
6. The antioxidant, ascorbate, and an $^{\bullet}OH$ radical scavenger, formate, inhibited NF-κB activation.
7. Tetrandrine, which has been reported to inhibit $H_2O_2$ release from macrophages (49), and also function as an $^{\bullet}OH$ radical scavenger (13), inhibited NF-κB activation.

It may be noted that silica is a fibrogenic agent due to its ability to elicit resident macrophages to release inflammatory mediators and cytokines that can promote fibroblast proliferation and collagen deposition. It has been suggested that NF-κB activation is crucial in cytoplasmic/nuclear signaling that occurs when cells are exposed to injury-producing conditions (50). NF-κB serves as a second messenger to induce a series of cellular genes in response to an environmental perturbation. Among cellular genes regulated by NF-κB are several proinflammatory or fibrogenic cytokines, including IL-2, IL-6, and TNF-α (22). NF-κB activates these genes by acting as a transcriptional factor and binding to the NF-κB consensus sequence in their promoters. Reactive oxygen intermediates have been suggested to be mediators of NF-κB activation in response to a variety of initiators such as Cr(VI) and phorbal esters (51–54). Since it is possible that silica particles cause NF-κB activation via free radical reactions, it is proposed that signals for a variety of silica-induced responses are due to a common signaling component, which is regulated by reactive oxygen species. It may be noted that a recent study has shown that *N*-acetylcysteine did not block silica-induced NF-κB activation (18). Although *N*-acetylcysteine is considered an antioxidant, it is not an efficient $^{\bullet}OH$ scavenger and thus may not inhibit silica-induced NF-κB activation via $^{\bullet}OH$ initiated reactions.

The results obtained from our studies have shown that tetrandrine is able to inhibit silica-induced NF-κB activation. While tetrandrine has been reported to retard and reverse the fibrotic lesions of silicosis in humans and in rats, its mechanism of action is unclear. As mentioned earlier, tetrandrine is an effective inhibitor of silica-induced $H_2O_2$ production by macrophages (31). In addition, recent studies have shown that tetrandrine is capable of scavenging $^{\bullet}OH$ radicals and inhibiting silica-induced lipid peroxidation (13). Tetrandrine has been shown to be an effective inhibitor of IL-1 secretion from alveolar macrophages activated by silica or LPS (32). We have demonstrated that tetrandrine inhibited LPS-induced NF-κB activation. It is possible that silica or LPS causes increased secretion of IL-1 or other cytokines via activation of NF-κB. It appears that the mechanism of NF-κB activation induced by LPS, although it can be inhibited by tetrandrine, is different from that for silica. For silica, both ascorbate and formate exhibited inhibitory effects. For LPS, the effect of these antioxidants is weak. While it is likely that one of the steps involves antioxidant activity of tetrandrine toward $^{\bullet}OH$ radical, other mechanisms may also exist. For example, tetrandrine decreases the stimulant-induced increase in intracellular calcium concentration due to its channel blocker effect and inhibits the production of NO in activated macrophages (55). The latter is especially important, since the production of NO has a direct effect on silica-induced NF-κB activation (19). It may be noted that

although other transcription factors, such as AP-1, are also important, NF-κB may be particularly relevant in the inflammatory, immune, and acute phase responses after silica exposure.

Our studies have also shown that deferoxamine reduces silica-mediated ·OH radical generation and attenuates silica-induced NF-κB activation. Deferoxamine is widely used for the prevention and treatment of iron overload (56,57). It inhibits ·OH radical generation from $H_2O_2$ by transition metals, including Fe, Cr, and V (58,59). A large dose of deferoxamine (50 mg/kg/day) can be safely injected into humans (57). Thus, further investigation on the use of deferoxamine or other metal chelators may offer a possible preventative strategy against silica-induced fibrosis and carcinogenesis.

Another important result obtained from our recent studies is that PVPNO inhibited silica-induced NF-κB activation. SiOH groups on the silica surface have been considered to be involved in silica-induced cellular damage (4,10,60,61). Chemical modification of the silica surface can be used to reduce toxicity in vitro and fibrosis in vivo. It is known that when silica particles are exposed to water, surface silicon-oxygen bonds (Si–O) are hydrated, resulting in the formation of SiOH groups. PVPNO is able to bind to SiOH groups. It has been reported to inhibit silica-induced toxicity (62), to decrease and delay the development of silicosis in experimental animals and in humans (63,64), and to block the interaction of the silica surface with the phosphate groups of DNA in vitro (16). It has also been reported that PVPNO inhibits silica-induced production of oxygen radicals in cells (65,66). The ESR spin trapping measurements in the present study have demonstrated the inhibitory effect of PVPNO on ·OH radical generation by silica plus $H_2O_2$,

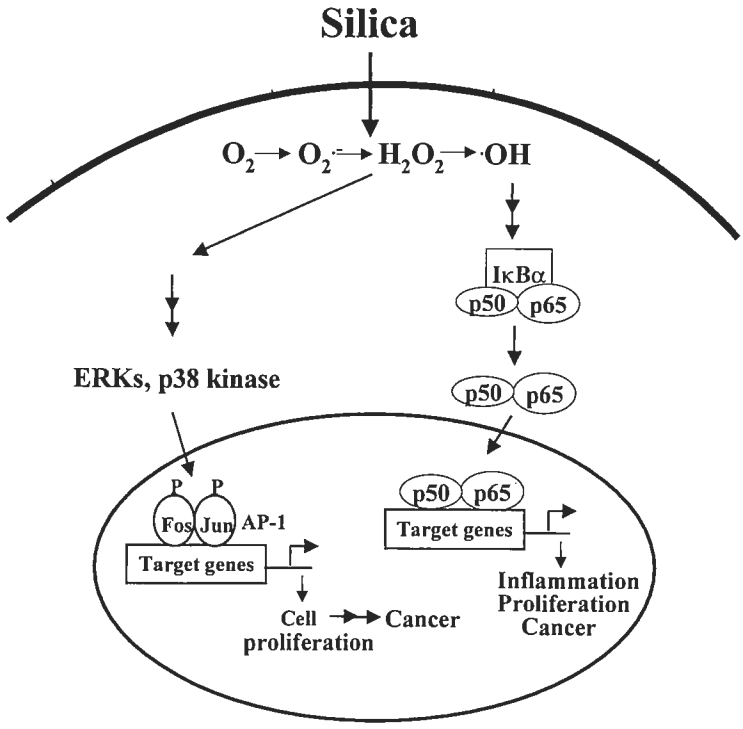


**Figure 14** Schematic representation of silica-induced generation of ROS and mechanistic scheme for silica-induced carcinogenesis.

implying the involvement of SiOH group in the generation of $^{\bullet}OH$ radical from $H_2O_2$ by silica.

Our studies present a molecular model for the elucidation of events involved in cell proliferation and carcinogenesis by crystalline silica (Fig. 14). By activating transcription factors, such as AP-1 and NF-κB, silica may induce chronic cell proliferation, which subsequently contributes to silicosis and carcinogenesis in the lung. It is possible that activation of AP-1 is a crucial event that initiates cell proliferation and progression through the cell cycle. Biopersistent silica particles may provide prolonged redox signals and growth stimulus during the long latency periods of tumorigenesis and, thereby, contribute to the eventual fixation of genetic changes caused by silica itself or other agents. Furthermore, the induction of AP-1 and NF-κB may affect changes in cell phenotype, which contribute to neoplastic transformation.

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