Am J Physiol Lung Cell Mol Physiol 291: L257–L264, 2006.
First published February 17, 2006; doi:10.1152/ajplung.00007.2006.

Essential role of ROS-mediated NFAT activation in TNF-α induction by crystalline silica exposure

Qingdong Ke,[1] Jingxia Li,[1] Jin Ding,[1] Min Ding,[2] Liying Wang,[2] Bingci Liu,[3] Max Costa,[1] and Chuanshu Huang[1]

[1]*Nelson Institute of Environmental Medicine, New York University School of Medicine, Tuxedo, New York;* [2]*Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia; and* [3]*National Institute of Occupational Health and Poisons Control, Chinese Center for Diseases Control and Prevention, Beijing, China*

Submitted 4 January 2006; accepted in final form 15 February 2006

Ke, Qingdong, Jingxia Li, Jin Ding, Min Ding, Liying Wang, Bingci Liu, Max Costa, and Chuanshu Huang. Essential role of ROS-mediated NFAT activation in TNF-α induction by crystalline silica exposure. *Am J Physiol Lung Cell Mol Physiol* 291: L257–L264, 2006. First published February 17, 2006; doi:10.1152/ajplung.00007.2006.—Occupational exposure to crystalline silica has been associated with progressive pulmonary silicosis and lung cancer, but the underlying molecular mechanisms are not well understood. Previous studies have shown that crystalline silica exposure can generate reactive oxygen species (ROS) and induce the expression of the inflammatory cytokine tumor necrosis factor-α (TNF-α) in cells. TNF-α is believed to be critical in the development of silica-related diseases. Thus it will be of significance to understand the mechanisms of TNF-α induction by silica exposure. Given the fact that the transcription factor nuclear factor of activated T cells (NFAT) plays an important role in the regulation of TNF-α and can also be activated by ROS, in this study we investigated the potential role of ROS in silica-induced NFAT activity as well as TNF-α expression in Cl41 cells. The results showed that exposure of cells to silica led to NFAT transactivation and TNF-α induction, where superoxide anion radical ($O_2^-\cdot$), but not H_2O_2, was involved. The knockdown of NFAT3 by its specific small interfering RNA significantly attenuated the silica-induced TNF-α transcription. This study demonstrated that silica was able to activate NFAT in an $O_2^-\cdot$-dependent manner, which was required for TNF-α induction.

silica; nuclear factor of activated T cells; tumor necrosis factor-α; reactive oxygen species; signal transduction

EPIDEMIOLOGIC, CLINICAL, and pathological studies have shown that human exposure to silica is associated with the development of pulmonary diseases such as silicosis and chronic bronchitis as well as an increased risk of lung cancer (2, 27). The International Agency for Research on Cancer has classified crystalline silica as a class 1 human carcinogen (17). However, the molecular mechanisms by which silica causes such diseases are not well understood. Previous studies have shown that freshly fractured silica is able to generate reactive oxygen species (ROS) including superoxide anion radicals ($O_2^-\cdot$), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and silicon-based free radicals (Si·, SiO·, SiOO·) in cells (32, 33). These oxygen radicals have been shown to be associated with silica-induced lipid peroxidation, membrane damage, and DNA damage (6, 33). In addition, it has been shown that silica-induced oxidative stress can trigger phosphorylation of the mitogen-activated protein kinases (MAPKs) ERKs and p38, activate specific transcription factors including nuclear transcription factor κB (NF-κB) and activator protein-1 (AP-1), and, additionally, increase the expression of the inflammatory cytokine tumor necrosis factor-α (TNF-α) (7, 8, 11, 16, 29). TNF-α is expressed in multiple cell types and plays an important role in the pathogenesis of silicosis and other chronic inflammatory lung diseases. Therefore, it is of great importance to understand the mechanisms of the TNF-α induction following silica exposure. It is well known that the transcription factor nuclear factor of activated T cells (NFAT) plays a key role in the regulation of TNF-α, and the binding sites of NFAT have been found in the promoter region of TNF-α gene (34). However, the effect of silica on NFAT and its subsequent biological consequences have not been investigated.

Originally identified in T cells as an inducer of cytokine gene expression, NFAT was later found to exist in a variety of lymphoid and nonlymphoid cells, where it has been shown to play various roles in cells outside the immune system (i.e., cancer development) (4, 26). In the transcription factor NFAT family, which mainly includes NFAT1/NFATc2/NFATp, NFAT2/NFATc1/NFATc, NFAT3/NFATc4, NFAT4/NFATc3/NFATx, and NFAT5, NFAT3 is primarily expressed in nonimmune tissues and has been implicated in multiple biological processes (26). Nevertheless, all NFAT members contain three functional domains: the Rel similarity domain, which is responsible for DNA-binding activity and interaction with other transcription factors; the NFAT homology region, which regulates intracellular localization; and the transcriptional activation domain (26). In resting cells, NFAT proteins are phosphorylated as an inactive form and reside in the cytoplasm (26). Upon stimulation, NFAT proteins are dephosphorylated by calcineurin phosphatase, thereby leading to their translocation to the nucleus. Once in the nucleus, NFAT proteins become transcriptionally active and interact with other transcription factors to cause the expression of their target genes, including many immunologically important genes, such as TNF-α, interleukin (IL)-2, IL-4, and IL-5 (26). Cessation of the stimulation causes NFAT proteins to be rephosphorylated and translocated back to the cytoplasm, resulting in the termination of their activities. Cyclosporin A (CsA), a pharmacological inhibitor of calcineurin phosphatase, can inhibit NFAT activation by blocking its dephosphorylation, nuclear translocation, and DNA binding activity (21). On the other hand, recent

Address for reprint requests and other correspondence: C. Huang, Nelson Institute of Environmental Medicine, New York Univ. School of Medicine, 57 Old Forge Rd., Tuxedo, NY 10987 (e-mail: chuanshu@env.med.nyu.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

studies reported that environmental contaminants such as asbestos, vanadium, and nickel compounds were able to activate NFAT, with ROS working as the likely mediator of its activation (12, 13, 19).

Given the importance of NFAT on TNF-α expression, in this study we investigated the effects of silica on NFAT activation and the role of NFAT activation in silica-induced TNF-α transcription in mouse Cl41 cells. The role of ROS in silica-induced NFAT activation and TNF-α was also examined.

MATERIALS AND METHODS

Plasmids and reagents. CMV-neo and NFAT-luciferase reporter plasmids were constructed as described in previous reports (15, 28). TNF-α-luciferase reporter plasmid containing human TNF-α promoter (−1260/+60) was a generous gift from Dr. Peter Johnson (National Cancer Institute, Frederick, MD) as described in previous studies (37). Fetal bovine serum (FBS) and Eagle's minimum essential medium (MEM) were purchased from BioWhittaker (Walkersville, MD). The substrate for the luciferase assay was purchased from Promega (Madison, WI). LipofectAMINE and G418 were obtained from GIBCO-BRL (Rockville, MD). CsA was purchased from Alexis Biochemicals (San Diego, CA); dihydroethidium was purchased from Molecular Probes (Eugene, OR). *N*-acetyl-L-cysteine (NAC), superoxide dismutase (SOD), catalase, and sodium formate were purchased from Sigma (St. Louis, MO); Specific antibodies against NFAT3, c-Jun, PKC-α, and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of freshly fractured silica. Crystalline silica was obtained from the Genetic Center, Pennsylvania State University (State College, PA). Detailed methods for preparation of freshly fractured silica were described in a previous study (7). Briefly, crystalline silica (0.2–10 mm in diameter) was ground for 30 min with a ball grinder equipped with agate mortar and balls. The ground silica was sieved through a 10-μm mesh filter for 20 min before use. Purity was checked with X-ray diffraction spectrometry, and diameter was determined by morphometric analyses, which indicated that fractured silica had a purity of 99.5% and a mean diameter of 3.7 μm.

Cell culture. The JB6 P^+ mouse Cl41 cell line and its transfectants Cl41 NFAT, Cl41 TNF-α, Cl41 small interfering RNA vector (si)NFAT TNF-α mass1, and Cl41 siNFAT TNF-α mass2 were cultured in monolayers at 37°C, 5% CO_2, with 5% FBS MEM containing 2 mM L-glutamine and 25 μg/ml gentamicin. The human lung bronchoepithelial A549 cell line and its NFAT-luciferase and TNF-α-luciferase reporter transfectants were cultured in Ham's F-12K medium supplemented with 10% FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin. The cultures were dissociated with trypsin and transferred to new 75-cm^2 culture flasks twice a week.

Generation of stable cotransfectants. Cl41 cells were cultured in a six-well plate until they reached 85–90% confluence. Five microliters of NFAT-luciferase or TNF-α-luciferase reporter plasmid DNA, one microgram of NFAT3/pSuppressor or empty pSuppressor vector, and ten microliters of LipofectAMINE reagent were used to transfect each well in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM without penicillin-streptomycin. Approximately 36–48 h after the beginning of the transfection, the medium was replaced with 5% FBS MEM containing 500 μg/ml G418. After selection for 28–45 days with G418, the stable transfectants were identified by measuring the basal level of luciferase activity. Stable transfectants Cl41 siNFAT TNF-α mass1 and Cl41 siNFAT TNF-α mass2 were established and cultured in G418-free 5% FBS MEM for at least 2 wk before each experiment.

NFAT activity assay. Confluent monolayers of stable Cl41 transfectants were trypsinized, and 8×10^3 viable cells suspended in 100 μl of MEM supplemented with 5% FBS were added to each well of 96-well plates. Plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. After cell density reached 80–90%, cells were exposed to silica at final concentrations as indicated in Figs 1 and 2 for NFAT induction. The cells were extracted with lysis buffer at selected time intervals after silica treatment, and their luciferase activity was determined with a luminometer (Wallac 1420 Victor 2 multilabel counter system) after the addition of 25 μl of lysis buffer for 30 min at 4°C. The results are expressed as NFAT activity relative to controls (relative NFAT activity).

TNF-α induction assay. Confluent monolayers of stable Cl41 TNF-α-luciferase reporter transfectants were trypsinized, and 8×10^3 viable cells suspended in 100 μl of MEM supplemented with 5% FBS were added to each well of 96-well plates. Plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. After cell density reached 80–90%, cells were exposed to silica at final concentrations as indicated in Figs. 3 and 4 for TNF-α induction. The cells were extracted with lysis buffer at selected time intervals after silica treatment, and their luciferase activity was determined with the luminometer after the addition of 25 μl of lysis buffer for 30 min at 4°C. The results are expressed as TNF-α activity relative to controls (relative TNF-α induction).

Transient transfection. Two million A549 cells were plated into a 10-cm dish and cultured until 90% confluence. Transfection was performed with a LipofectAMINE 2000 kit from Invitrogen (Grand Island, NY), and 20 μg of NFAT-luciferase or TNF-α-luciferase reporter plasmid DNA was used. Twenty-four hours after the transfection, 2×10^4 cells were plated into 48-well plates and cultured until ~80–90% confluence. The transfectants were then exposed to silica for time periods as indicated in Figs. 1 and 3 and extracted with 50 μl of lysis buffer for 30 min at 4°C, and their luciferase activity was determined with a luminometer. The results are presented as NFAT or TNF-α activity relative to controls (relative NFAT activity or TNF-α induction).

$O_2^-\cdot$ assay in intact cells. Dihydroethidium is a specific dye used for staining $O_2^-\cdot$ produced by intact cells. Cl41 cells (2×10^4/well) were seeded onto a glass coverslip in the bottom of a 12-well plate for 24 h. The cells were treated with silica in the presence of dihydroethidium (2 μM) for 30 min. The cells were then washed in PBS and fixed with 10% buffered formalin. The glass coverslip was mounted on a microscope slide and observed under a Sarastro 2000 (Molecular Dynamics, Sunnyvale, CA) laser scanning confocal microscope fitted with an argon ion laser.

Construction of siRNA NFAT expression vector. Specific siRNAs targeting mouse NFAT3/NFATc4 were designed with a siRNA converter and the website of Ambion (Austin, TX), according to the gene sequence in GenBank and the siRNA guideline and synthesized by Invitrogen. The target sequence was 5′-gccattgactctgcagatg-3′ (bases 1409–1427 of NM023699, mouse NFAT3 mRNA). The siRNA sequences were controlled via BLAST search and did not show any homology to other known human genes. The siRNAs were inserted into pSuppressor vector and verified by DNA sequencing. The siRNA vectors were designated as siNFAT3.

Western blot. Cl41 transfectants (2×10^5 cells) were cultured in a six-well plate to 90% confluence. The cells were then washed twice with ice-cold PBS and extracted with SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with rabbit-specific antibodies against NFAT3, c-Jun, PKC-α, and GAPDH. The protein band specifically bound to the primary antibody was detected with an anti-rabbit IgG-AP-linked ECF Western blotting system (Amersham Biosciences, Piscataway, NJ).

Statistical analysis. Student's *t*-test was used to determine the significance of differences of NFAT activities and TNF-α induction between silica-treated cells and medium control or various transfectants. Differences were considered significant at $P < 0.05$.

RESULTS

Induction of NFAT transactivation in CsA-sensitive manner in Cl41 cells by freshly fractured silica. In previous studies, it was shown that exposure of cells to freshly fractured silica (40–150 μg/cm^2) is able to induce AP-1 activation in Cl41 cells (7). To study the regulation of NFAT transcription activity in Cl41 cells after silica exposure, we generated a NFAT-luciferase reporter stable transfectant by cotransfecting the NFAT-luciferase reporter plasmid and the cytomegalovirus-neo plasmid into Cl41 cells. Exposure of stable transfectant Cl41 NFAT mass1 to various doses of silica ranging from 5 to 40 μg/cm^2 for 24 h resulted in a significant dose-dependent NFAT activation (Fig. 1*A*). The induction of NFAT activation by silica exposure was further confirmed in a transient transfection assay in human lung bronchoepithelial A549 cells (Fig. 1*B*). We noted that the silica dosage used did not show any observed cytotoxic effect on Cl41 or A549 cells (data not shown). These data indicate that silica exposure is able to cause NFAT activation in both mouse epidermal Cl41 cells and human lung bronchoepithelial A549 cells.

It has been demonstrated that in T cells, calcineurin, a calcium/calmodulin-dependent phosphatase, is the major NFAT activator (26). To test the role of calcineurin in silica-induced NFAT-dependent transcriptional activity in Cl41 cells, CsA, a widely used pharmacological inhibitor of calcineurin, was used. As shown in Fig. 1*C*, pretreatment of cells with CsA abrogated NFAT transactivation induced by silica, indicating that calcineurin is required for silica-induced NFAT activation. These data suggest that silica activates NFAT transcription activity in Cl41 cells through a pathway that is similar to that in T cells.

Silica-induced NFAT activation is dependent on ROS generation in cells. When Cl41 cells were exposed to silica, ROS were generated. Pretreatment of cells with SOD or catalase was shown to scavenge $O_2^-\cdot$ or H_2O_2 accumulation in cells, respectively, and to affect silica-induced AP-1 activation without changing cell viability (8). Additionally, our previous findings in the same cell line (12, 14) demonstrated similar effects of SOD and catalase when the cells were subjected to vanadate exposure or UV radiation, indicating that SOD and catalase are able to get into Cl41 cells. To test whether ROS generation by silica was implicated in NFAT activation, dihydroethidium, a specific fluorescent dye for $O_2^-\cdot$, was first applied to Cl41 cells treated with silica to monitor ROS generation. As shown in Fig. 2*A*, the fluorescent signal of $O_2^-\cdot$ was dramatically increased within 30 min after the addition of silica. However, addition of SOD (a specific $O_2^-\cdot$) to silica-exposed cells depleted the fluorescent signal back to the basal level. These results indicate that ROS (at least $O_2^-\cdot$) are generated during treatment of cells with silica.

ROS have been indicated to be involved in various silica-induced signaling pathways (32, 33). Therefore, it is reasonable to speculate that ROS generation may play a role in the NFAT activation caused by silica in cells. To examine this hypothesis, the effects of antioxidants on silica-induced NFAT activation were investigated. Cl41 NFAT cells cultured in 96-well plates were pretreated with antioxidants for 1 h, followed by silica exposure (10 μg/cm^2) in the presence of the same reagents for 24 h, and luciferase activity was determined. As shown in Fig. 2*B*, pretreatment of cells with NAC, a general antioxidant, blocked silica-induced NFAT activation, revealing that ROS are involved in silica-induced NFAT activation. To further delineate which ROS played a role in NFAT activation, several specific ROS scavengers such as SOD, catalase, and sodium formate were used. Figure 2*B* shows that SOD, a $O_2^-\cdot$ scavenger, strongly inhibited silica-induced NFAT activation. In contrast, catalase, a H_2O_2-scavenging enzyme, dramatically enhanced silica-induced NFAT activation. Sodium formate, a ·OH radical scavenger, had modest effects on NFAT activation. These data suggest that $O_2^-\cdot$ plays a major role in NFAT activation by silica exposure.

Induction of TNF-α in Cl41 cells by freshly fractured silica in an $O_2^-\cdot$-dependent manner. It has been shown that exposure of macrophages to silica results in increased TNF-α mRNA

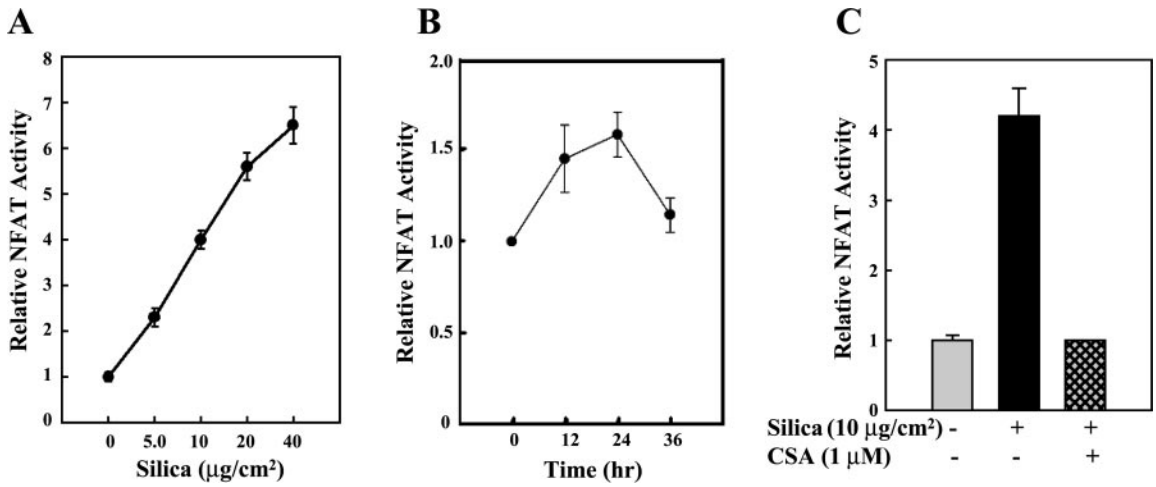

Fig. 1. Activation of nuclear factor of activated T cells (NFAT) in a cyclosporin A (CsA)-sensitive manner by silica. Cells of Cl41 NFAT mass1 (8×10^3) were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were exposed to different concentrations of silica as indicated (*A*) for 24 h in medium containing 0.1% fetal bovine serum (FBS); 2×10^4 NFAT-luciferase reporter transient-transfected A549 cells were seeded into each well of 48-well plates. After being cultured at 37°C overnight, the cells were exposed to silica in the medium containing 0.1% FBS for the time periods as indicated (*B*) or Cl41 NFAT cells were pretreated with 1 μM CsA for 1 h and sequentially exposed to 10 μg/cm^2 silica for 24 h (*C*). Luciferase activity was then measured. Bars indicate means and SDs of triplicate assay wells. The results are presented as relative NFAT activity.

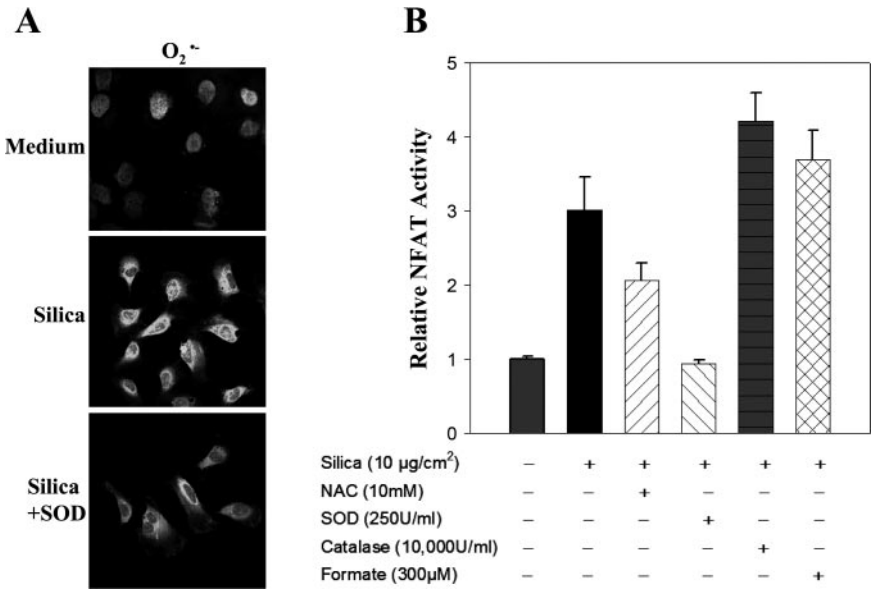

Fig. 2. Dependence of reactive oxygen species (ROS) in silica-induced NFAT activation in Cl41 cells. *A*: Cl41 cells were treated with 10 μg/cm^2 silica or pretreated with superoxide dismutase (SOD) for 30 min and then exposed to silica in the presence of 2 μM dihydroethidium for 30 min. The cells were washed once with PBS and fixed with 10% buffered formalin. The images were captured with a laser scanning confocal microscope. Bright spots represent oxidized dihydroethidium showing the intracellular localization of $O_2^{-}\cdot$. *B*: 8×10^3 cells of Cl41 NFAT mass were seeded into each well of 96-well plates. After being cultured at 37°C overnight, the cells were pretreated with indicated antioxidants for 1 h and then treated with 10 μg/cm^2 silica for 24 h in medium containing 0.1% FBS. Luciferase activity was then measured. Bars indicate means and SDs of triplicate assay wells. The results are presented as relative NFAT activity. NAC, *N*-acetyl-L-cysteine.

expression and protein secretion (29), but the induction of TNF-α at its transcription level by silica has not yet been investigated. To investigate whether silica can induce TNF-α transcription in Cl41 cells, we generated a TNF-α luciferase reporter stable transfectant by cotransfecting the TNF-α luciferase reporter plasmid and the cytomegalovirus-neo plasmid into the Cl41 cell line. As shown in Fig. 3*A*, exposure of Cl41 cells to silica resulted in a remarkable TNF-α induction at the transcription level. Maximum TNF-α induction by silica was shown at 10 μg/cm^2 for 24 h, according to dose and time course studies (Fig. 3, *B* and *C*). The results obtained from silica treatment in the TNF-α-luciferase reporter transient transfection assay also showed TNF-α induction by silica in A549 cells (Fig. 3*D*).

Previous studies have reported that ROS are involved in silica-induced TNF-α production in macrophages (11). To investigate whether silica-induced TNF-α is also mediated by ROS in Cl41 cells, Cl41 cells cultured in 96-well plates were pretreated with antioxidants for 1 h, followed by 24 h of exposure to silica (10 μg/cm^2) and measurement of luciferase activity. As was found in Cl41 NFAT cells, pretreatment of Cl41 TNF-α cells with NAC or SOD strongly inhibited silica-induced TNF-α, whereas catalase enhanced silica-induced TNF-α and sodium formate had minimal effects on the induction (Fig. 3*E*). These results are consistent with the effects of the antioxidants on silica-induced NFAT activation.

NFAT activation is required for silica-induced TNF-α transcription. Because NFAT is a transcription factor that can regulate TNF-α gene expression, the role of NFAT in silica-induced TNF-α was explored in Cl41 cells. We generated a stable transfectant with siRNA to NFAT3, which is primarily expressed in nonimmune tissues. As shown in Fig. 4*A*, introduction of NFAT3 siRNA dramatically reduced NFAT3 protein production compared with Cl41 TNF-α mass1 cells. Meanwhile, c-Jun, the major component of the transcription factor AP-1, and PKC-α were not affected, suggesting that the effect of NFAT3 siRNA was specific on NFAT. Specific knockdown of NFAT3 protein production by its siRNA resulted in a loss of silica-induced TNF-α transcription in the two siNFAT transfectants (Fig. 4*B*). These findings were further confirmed in time course and dose-response studies (Figs. 4, *C* and *D*). We noted that reduction of silica-induced TNF-α transcription in Cl41 siRNA TNF-α mass1 was greater than that in Cl41 siRNA TNF-α mass2 cells (Fig. 4, *B–D*), which was consistent with NFAT3 protein levels in the two mass cultures (Fig. 4*A*). These data demonstrated that NFAT activation is required for silica-induced TNF-α transcription.

DISCUSSION

Crystalline silica is a documented carcinogen, but the molecular and cellular mechanisms involved in silica-induced pathogenesis are not fully understood (2, 17, 27). In this study, we investigated the possible involvement of NFAT in silica-induced TNF-α transcription in mouse Cl41 cells. Exposure of Cl41 cells to silica resulted in a marked activation of NFAT as well as induction of TNF-α. These responses also occurred in human lung bronchoepithelial A549 cells. In addition, we demonstrated that ROS are the mediators of silica-induced NFAT activation and subsequent TNF-α transcription. By using specific antioxidant reagents, we identified $O_2^{-}\cdot$, formed during the interaction of silica with the cells, as a major reactive intermediate responsible for silica-induced NFAT activation and TNF-α induction. Conversely, another ROS, H_2O_2, appeared to play a negative role in the induction. Furthermore, by using NFAT3 siRNA, we showed that NFAT activation is required for silica-induced TNF-α transcription. These results demonstrate that silica is a specific inducer for NFAT transactivation and this NFAT activation regulates TNF-α transcription in Cl41 cells.

The activation of NFAT includes dephosphorylation, nuclear translocation, and an increase in affinity for DNA binding (26). In response to stimuli, calcineurin becomes activated. Activated calcineurin subsequently dephosphorylates the cytoplasmic NFAT proteins, leading to NFAT nuclear translocation. NFAT may form a heteromeric transcriptional coactivator complex with some other transcription factors in the nucleus to activate gene expression. We found here that exposure of Cl41

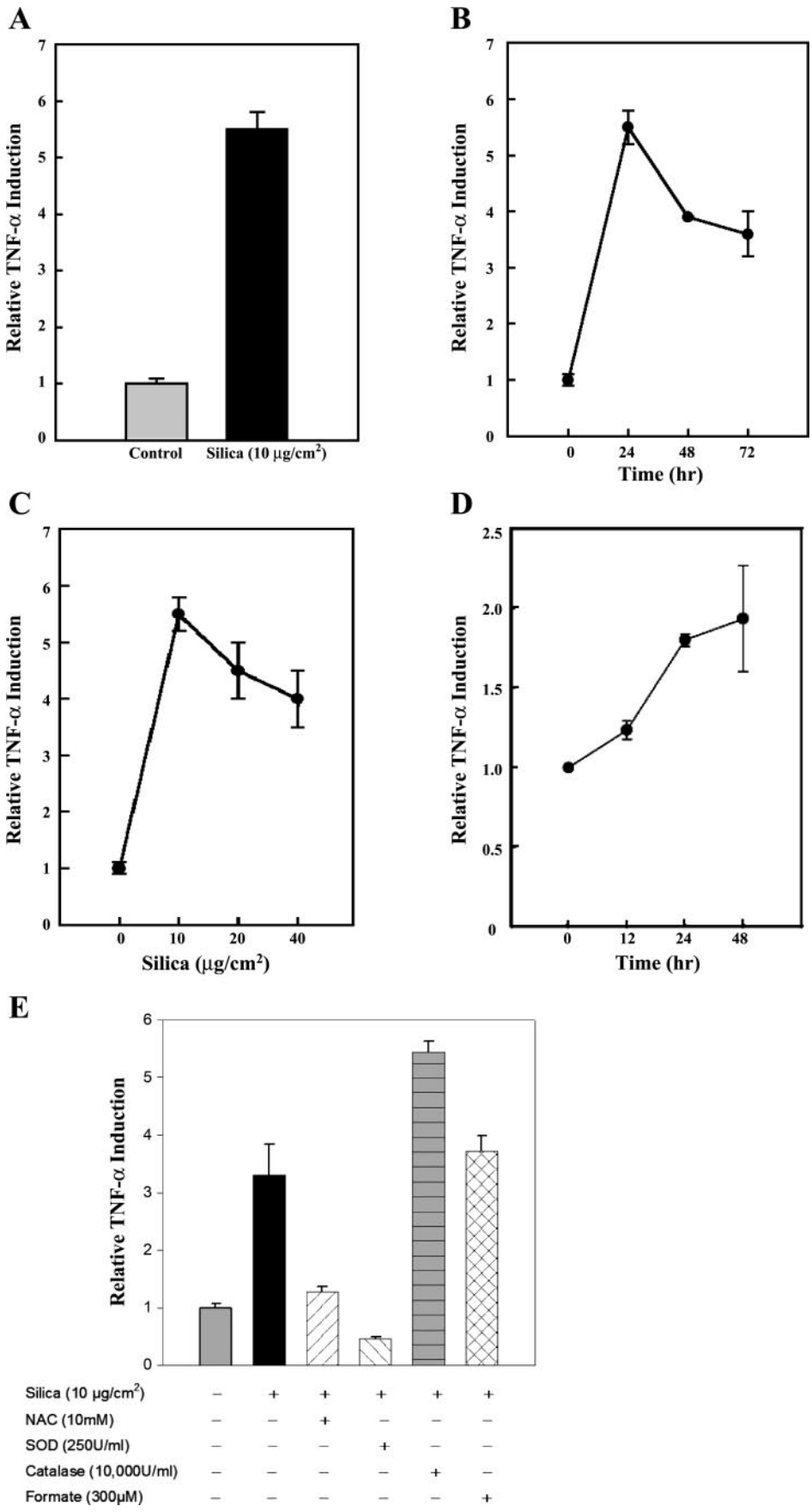

Fig. 3. Induction of tumor necrosis factor-α (TNF-α) by silica in Cl41 cells in a ROS-dependent manner. Cells of Cl41 TNF-α mass1 (8×10^3) were seeded into each well of 96-well plates. After being cultured at 37°C overnight, cells were treated with 10 μg/cm^2 silica for 24 h in medium containing 0.1% FBS (*A*). For a time course study, the cells were exposed to 10 μg/cm^2 silica for various time intervals as indicated (*B*). For a dose-response study, the cells were exposed to different concentrations of silica as indicated for 24 h (*C*). For the study of TNF-α induction in A549 cells, 2×10^4 TNF-α-luciferase reporter transient-transfected A549 cells were seeded into each well of 48-well plates. After being cultured at 37°C overnight, the cells were exposed to silica in medium containing 0.1% FBS (*D*). For a study on the effects of the antioxidants on silica-induced TNF-α, the cells were pretreated with indicated antioxidants for 1 h and then treated with 10 μg/cm^2 silica for 24 h (*E*). Luciferase activity was then measured. Bars indicate means and SDs of triplicate assay wells. The results are presented as relative TNF-α induction.

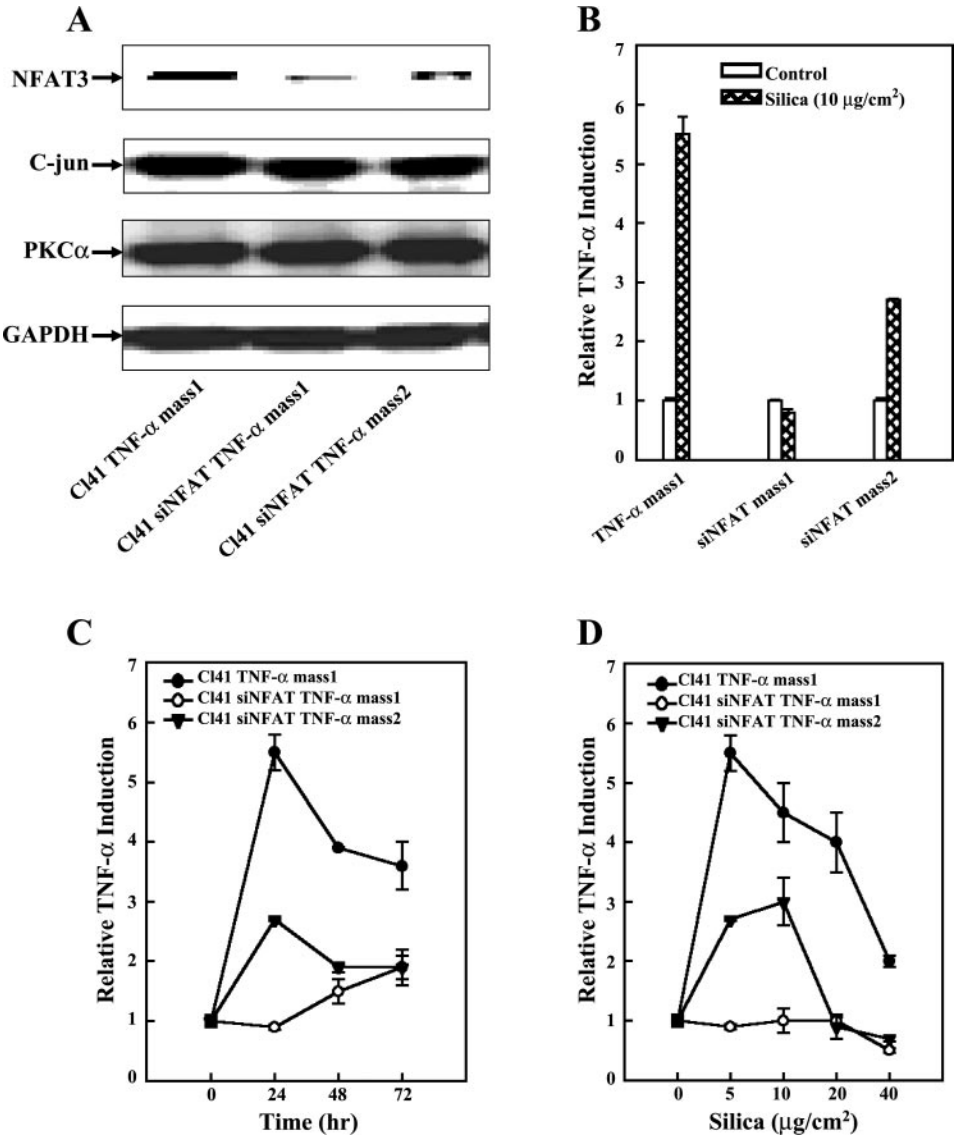

Fig. 4. Requirement of NFAT activation for silica-induced TNF-α transcription. *A*: Cl41 vector control and its small interfering RNA (si)NFAT transfectants were cultured in 5% FBS MEM in 6-well plates until 90% confluent. The cells were lysed, and Western blots were conducted with antibody against NFAT3. The same blot was blotted with antibodies against c-Jun, PKC-α, and GAPDH after a stripping procedure. GAPDH served as the loading control. *B–D*: 8×10^3 cells of Cl41 TNF-α mass1, Cl41 siNFAT mass1, and Cl41 siNFAT mass2 were seeded into each well of 96-well plates. After being cultured at 37°C overnight, cells were treated with 10 μg/cm^2 silica for 24 h in medium containing 0.1% FBS (*B*). For a time course study, the cells were exposed to 10 μg/cm^2 silica for various time intervals as indicated (*C*). For a dose-response study, the cells were exposed to different concentrations of silica as indicated for 24 h (*D*). Luciferase activity was then measured. Bars indicate means and SDs of 3 repeat assay wells. The results are presented as TNF-α-dependent transcription activity relative to control.

cells to silica results in NFAT activation. Pretreatment of cells with CsA abolished this NFAT transactivation, suggesting that calcineurin activation is required for the NFAT activation induced by silica.

ROS such as H_2O_2, $O_2^-\cdot$, and OH play various roles in living cells as second messengers to elicit physiological responses or as a toxic intermediate leading to cellular damage (31). In the mitochondria of eukaryotes, molecular oxygen is converted into $O_2^-\cdot$, a moderately reactive species capable of generating H_2O_2, which in turn can produce highly reactive hydroxyl radicals (·OH) via metal-dependent breakdown (5). Cells possess effective mechanisms to control ROS. Among these is the synthesis of enzymes such as SOD, which converts $O_2^-\cdot$ to O_2 and H_2O_2; catalase, which converts H_2O_2 to H_2O and O_2; and glutathione peroxidases (GPXs), which are responsible for the removal of H_2O_2 and other organic hydroperoxides (5, 9). In addition, chemical reagents such as sodium formate can scavenge ·OH radical effectively. Previous studies have suggested that ROS could act as regulators of signal transduction and may have unique roles (18). Accumulating evidence has suggested a vital role of ROS in mediating cellular responses to various extracellular stimuli (18). It has been demonstrated that crystalline silica is a potent stimulant of ROS production (32, 33). Interestingly, silica-induced oxidative stress can activate spe-

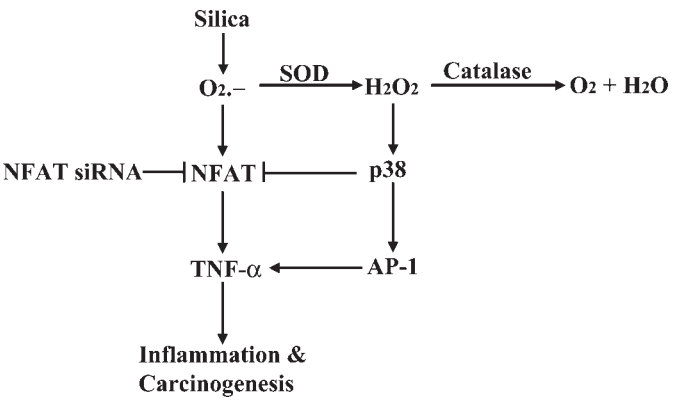

Fig. 5. Model of ROS-mediated NFAT activation in silica-induced TNF-α.

cific transcription factors including NF-κB and AP-1 (7, 8, 29). Moreover, it was also reported that silica triggers phosphorylation of MAPKs including ERKs and p38 through ROS-mediated reactions (7). In those studies, H_2O_2 was shown to be specifically responsible for silica-induced NF-κB and phosphorylation of MAPKs. Involvement of ROS in NFAT activation in T cells was first demonstrated by the observation that treatment of T cells with dithiocarbamate resulted in the inhibition of NFAT activation and NFAT-mediated cytokine gene expression (20, 30). More recent studies also showed that intracellular H_2O_2 levels were associated with vanadium- or nickel-induced NFAT activation (12, 13). In the present study, we demonstrated that silica exposure generated ROS in Cl41 cells and ROS regulated NFAT activation induced by silica. However, we showed that $O_2^-\cdot$ was involved in silica-induced NFAT activation, whereas H_2O_2 seemed to play a negative role. The evidence comes from the observations that *1*) silica exposure caused increase of $O_2^-\cdot$ production in cells, and addition of SOD depleted $O_2^-\cdot$ generation; *2*) NAC (a general antioxidant) blocked silica-induced NFAT activation; *3*) SOD (converts $O_2^-\cdot$ to H_2O_2) inhibited silica-induced NFAT activation; and *4*) catalase (converts H_2O_2 to H_2O) dramatically increased silica-induced NFAT activation. Recent studies have reported that multiple protein kinases, including MAPKs (ERKs, JNKs, and p38), glycogen synthase kinase-3β, and PKA were able to phosphorylate NFAT (1, 3, 10, 25, 26, 35). Phosphorylation of NFAT by these kinases opposed nuclear localization of NFAT either by promoting nuclear export or by impeding nuclear import that is a key step for NFAT inactivation. Specifically, it has been shown that the p38 can phosphorylate NFAT3 and contribute to the inactivation of NFAT3 (36). Silica has been shown to induce H_2O_2-mediated p38 phosphorylation in 15 min, and its maximal activation was obtained at 2 h after silica exposure (7). Therefore, it is plausible that when cells were treated with silica alone silica-generated $O_2^-\cdot$ resulted in NFAT activation. However, in the presence of SOD, the rapid conversion of $O_2^-\cdot$ to H_2O_2 by SOD led to the accumulation of H_2O_2 in silica-exposed cells. Immediately, H_2O_2 may activate p38, which subsequently phosphorylates NFAT, leading to its inactivation. On the other hand, the depletion of H_2O_2 by catalase in the silica-exposed cells caused a significant enhancement of NFAT activation. Additionally, scavenging of ·OH by sodium formate may result in a modest effect on cellular H_2O_2 and contribute to slight NFAT activation. Thus it appeared that H_2O_2 negatively regulated the signaling pathway(s) for NFAT activation.

TNF-α is a critical mediator in the pathogenesis of silicosis, is associated with effective immune and inflammatory responses, and may be related to cancer development. It has been shown that silicosis is absent in TNF-α knockout mice and anti-TNF antibody can effectively prevent the occurrence of silicosis (22–24). In macrophages, silica has been shown to induce TNF-α mRNA expression and protein release, in which H_2O_2 and NF-κB were involved (29). In this study, we showed that exposure of Cl41 cells to silica resulted in strong induction of TNF-α at its transcription level. Interestingly, as with NFAT activation in silica-exposed cells, $O_2^-\cdot$, but not H_2O_2, was responsible for the mediation of silica-induced TNF-α transcription. It is known that NFAT plays an essential role in TNF-α gene expression, and four NFAT-binding sites in the TNF-α gene promoter region have been identified (34). The data obtained with NFAT3 siRNA in this study demonstrated that the knockdown of NFAT3 by its specific siRNA significantly attenuated the TNF-α transcription induced by silica, strongly suggesting that NFAT activation is required for silica-induced TNF-α transcription. It should be noted that the increase of NFAT activity by silica is dose dependent within a dosage range from 5 to 40 $\mu g/cm^2$, whereas the induction of TNF-α decreases at higher doses of silica (>20 $\mu g/cm^2$). The explanation for this could be that higher doses of silica exposure also induce other inhibitory signaling pathways, which may cause the downregulation of TNF-α induction. On the basis of the evidence provided from this study as well as previous studies in the literature, we propose the model for silica-induced NFAT activation and TNF-α shown in Fig. 5.

In conclusion, this study showed that silica is able to induce NFAT activation in Cl41 cells and $O_2^-\cdot$ generation by silica was critical for its activation, which mediated silica-induced TNF-α transcription. These results will help us to further understand the molecular mechanisms of silica-induced diseases.

GRANTS

This work was supported in part by National Institutes of Health Grants R01-CA94964 (C. Huang), R01-CA-112557 (C. Huang), R01-CA-103180 (C. Huang), ES-012451 (C. Huang), and ES-000260 (M. Costa), National Natural Science Foundation of China Grant 30440420593 (C. Huang, B. Liu), and 973 National Key Basic Research and Development Program Grant 2002 CB 512905 (B. Liu).

REFERENCES

1. **Beals CR, Sheridan CM, Turck CW, Gardner P, and Crabtree GR.** Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* 275: 1930–1934, 1997.
2. **Castranova V, Vallyathan V, and Wallace WE.** *Silica and Silica-Induced Lung Diseases*. Boca Raton, FL: CRC, 1996.
3. **Chow CW, Rincon M, Cavanagh J, Dickens M, and Davis RJ.** Nuclear accumulation of NFAT4 opposed by the JNK signal transduction pathway. *Science* 278: 1638–1641, 1997.
4. **Crabtree GR and Olson EN.** NFAT signaling: choreographing the social lives of cells. *Cell* 109, *Suppl*: S67–S79, 2002.
5. **Curtin JF, Donovan M, and Cotter TG.** Regulation and measurement of oxidative stress in apoptosis. *J Immunol Methods* 265: 49–72, 2002.
6. **Ding M, Chen F, Shi X, Yucesoy B, Mossman B, and Vallyathan V.** Diseases caused by silica: mechanisms of injury and disease development. *Int Immunopharmacol* 2: 173–182, 2002.
7. **Ding M, Shi X, Dong Z, Chen F, Lu Y, Castranova V, and Vallyathan V.** Freshly fractured crystalline silica induces activator protein-1 activation through ERKs and p38 MAPK. *J Biol Chem* 274: 30611–30616, 1999.
8. **Ding M, Shi X, Lu Y, Huang C, Leonard S, Roberts J, Antonini J, Castranova V, and Vallyathan V.** Induction of activator protein-1 through reactive oxygen species by crystalline silica in JB6 cells. *J Biol Chem* 276: 9108–9114, 2001.
9. **Fleury C, Mignotte B, and Vayssiere JL.** Mitochondrial reactive oxygen species in cell death signaling. *Biochimie* 84: 131–141, 2002.
10. **Gomez del Arco P, Martinez-Martinez S, Maldonado JL, Ortega-Perez I, and Redondo JM.** A role for the p38 MAP kinase pathway in the nuclear shuttling of NFATp. *J Biol Chem* 275: 13872–13878, 2000.
11. **Gossart S, Cambon C, Orfila C, Seguelas MH, Lepert JC, Rami J, Carre P, and Pipy B.** Reactive oxygen intermediates as regulators of TNF-α production in rat lung inflammation induced by silica. *J Immunol* 156: 1540–1548, 1996.
12. **Huang C, Ding M, Li J, Leonard SS, Rojanasakul Y, Castranova V, Vallyathan V, Ju G, and Shi X.** Vanadium-induced nuclear factor of activated T cells activation through hydrogen peroxide. *J Biol Chem* 276: 22397–22403, 2001.
13. **Huang C, Li J, Costa M, Zhang Z, Leonard SS, Castranova V, Vallyathan V, Ju G, and Shi X.** Hydrogen peroxide mediates activation

of nuclear factor of activated T cells (NFAT) by nickel subsulfide. *Cancer Res* 61: 8051–8057, 2001.

14. **Huang C, Li J, Ke Q, Leonard SS, Jiang BH, Zhong XS, Costa M, Castranova V, and Shi X.** Ultraviolet-induced phosphorylation of $p70^{S6K}$ at Thr^{389} and Thr^{421}/Ser^{424} involves hydrogen peroxide and mammalian target of rapamycin but not Akt and atypical protein kinase C. *Cancer Res* 62: 5689–5697, 2002.
15. **Huang C, Mattjus P, Ma WY, Rincon M, Chen NY, Brown RE, and Dong Z.** Involvement of nuclear factor of activated T cells activation in UV response. Evidence from cell culture and transgenic mice. *J Biol Chem* 275: 9143–9149, 2000.
16. **Hubbard AK, Timblin CR, Shukla A, Rincon M, and Mossman BT.** Activation of NF-κB-dependent gene expression by silica in lungs of luciferase reporter mice. *Am J Physiol Lung Cell Mol Physiol* 282: L968–L975, 2002.
17. **International Agency for Research on Cancer.** *Silica, Some Silicates, Coal Dust and para-Aramid Fibrils. IARC Monographs on the Evaluation of Carcinogenic Risks Humans,* vol. 68. Lyon: IARC, 1997.
18. **Leonard SS, Bower JJ, and Shi X.** Metal-induced toxicity, carcinogenesis, mechanisms and cellular responses. *Mol Cell Biochem* 255: 3–10, 2004.
19. **Li J, Huang B, Shi X, Castranova V, Vallyathan V, and Huang C.** Involvement of hydrogen peroxide in asbestos-induced NFAT activation. *Mol Cell Biochem* 234–235: 161–168, 2002.
20. **Martinez-Martinez S, Gomez del Arco P, Armesilla AL, Aramburu J, Luo C, Rao A, and Redondo JM.** Blockade of T-cell activation by dithiocarbamates involves novel mechanisms of inhibition of nuclear factor of activated T cells. *Mol Cell Biol* 17: 6437–6447, 1997.
21. **Park J, Yaseen NR, Hogan PG, Rao A, and Sharma S.** Phosphorylation of the transcription factor NFATp inhibits its DNA binding activity in cyclosporin A-treated human B and T cells. *J Biol Chem* 270: 20653–20659, 1995.
22. **Piguet PF, Collart MA, Grau GE, Sappino AP, and Vassalli P.** Requirement of tumour necrosis factor for development of silica-induced pulmonary fibrosis. *Nature* 344: 245–247, 1990.
23. **Piguet PF, Grau GE, and Vassalli P.** Subcutaneous perfusion of tumor necrosis factor induces local proliferation of fibroblasts, capillaries, and epidermal cells, or massive tissue necrosis. *Am J Pathol* 136: 103–110, 1990.
24. **Piguet PF, Vesin C, Grau GE, and Thompson RC.** Interleukin 1 receptor antagonist (IL-1ra) prevents or cures pulmonary fibrosis elicited in mice by bleomycin or silica. *Cytokine* 5: 57–61, 1993.
25. **Porter CM, Havens MA, and Clipstone NA.** Identification of amino acid residues and protein kinases involved in the regulation of NFATc subcellular localization. *J Biol Chem* 275: 3543–3551, 2000.
26. **Rao A, Luo C, and Hogan PG.** Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 15: 707–747, 1997.
27. **Reiser KM and Last JA.** Silicosis and fibrogenesis: fact and artifact. *Toxicology* 13: 51–72, 1979.
28. **Rincon M and Flavell RA.** Transcription mediated by NFAT is highly inducible in effector CD4+ T helper 2 (Th2) cells but not in Th1 cells. *Mol Cell Biol* 17: 1522–1534, 1997.
29. **Rojanasakul Y, Ye J, Chen F, Wang L, Cheng N, Castranova V, Vallyathan V, and Shi X.** Dependence of NF-κB activation and free radical generation on silica-induced TNF-α production in macrophages. *Mol Cell Biochem* 200: 119–125, 1999.
30. **Saccani S, Saccani A, Varesio L, Ghosh P, Young HA, and Sica A.** Divergent effects of dithiocarbamates on AP-1-containing and AP-1-less NFAT sites. *Eur J Immunol* 29: 1194–1201, 1999.
31. **Sen CK and Packer L.** Antioxidant and redox regulation of gene transcription. *FASEB J* 10: 709–720, 1996.
32. **Shi X, Castranova V, Halliwell B, and Vallyathan V.** Reactive oxygen species and silica-induced carcinogenesis. *J Toxicol Environ Health B Crit Rev* 1: 181–197, 1998.
33. **Shi X, Ding M, Chen F, Wang L, Rojanasakul Y, Vallyathan V, and Castranova V.** Reactive oxygen species and molecular mechanism of silica-induced lung injury. *J Environ Pathol Toxicol Oncol* 20, *Suppl* 1: 85–93, 2001.
34. **Tsai EY, Yie J, Thanos D, and Goldfeld AE.** Cell-type-specific regulation of the human tumor necrosis factor alpha gene in B cells and T cells by NFATp and ATF-2/JUN. *Mol Cell Biol* 16: 5232–5244, 1996.
35. **Tsatsanis C, Patriotis C, and Tsichlis PN.** Tpl-2 induces IL-2 expression in T-cell lines by triggering multiple signaling pathways that activate NFAT and NF-κB. *Oncogene* 17: 2609–2618, 1998.
36. **Yang TT, Xiong Q, Enslen H, Davis RJ, and Chow CW.** Phosphorylation of NFATc4 by p38 mitogen-activated protein kinases. *Mol Cell Biol* 22: 3892–3904, 2002.
37. **Zhang X, Ye J, Wang L, Manosroi J, Shi X, and Rojanasakul Y.** Rapid and sensitive assay of tumor necrosis factor-α gene transcription. *Pharm Res* 18: 408–411, 2001.