Critical role of GSH in silica-induced oxidative stress, cytotoxicity, and genotoxicity in alveolar macrophages

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1Pneumoconiosis Division, School of Medicine, Zhejiang University, Hangzhou 310013, People’s Republic of China; and 2Department of Community, Occupational, and Family Medicine, National University of Singapore, Singapore 119260, Republic of Singapore

Zhang, Zuoh, Han-Ming Shen, Qi-Feng Zhang, and Choon-Nam Ong. Critical role of GSH in silica-induced oxidative stress, cytotoxicity and genotoxicity in alveolar macrophages. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L743–L748, 1999.—The main objective of this study was to evaluate the critical role of glutathione (GSH) in silica-induced oxidative stress, cytotoxicity, and genotoxicity in rat alveolar macrophages (AMs). Silica-induced superoxide radical and hydrogen peroxide formation were determined with lucigenin-dependent chemiluminescence and 2',7'-dichlorofluorescin diacetate fluorescence test, respectively. The cytotoxicity of silica was estimated by lactate dehydrogenase leakage, and a comet assay was used for examining silica-induced DNA damage in AMs. The intracellular GSH content was modulated by N-acetylcysteine, a GSH precursor, and buthionine sulfoximine, a specific GSH synthesis inhibitor. It was found that silica led to a dose- and time-dependent decrease in GSH content in AMs. N-acetylcysteine increased intracellular GSH level and protected against silica-induced reactive oxygen species formation, lactate dehydrogenase leakage, and DNA strand breaks in AMs. In contrast, buthionine sulfoximine pretreatment depleted cellular GSH and enhanced the susceptibility of AMs to the cytotoxic and genotoxic effects of silica. It thus appears that GSH plays a critical role in protecting against silica-induced cell injury, most probably through its antioxidant activity.

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Glutathione (GSH) is among the most important antioxidants in organisms due to its potent antioxidant capacity, close involvement in many cellular functions, and abundance in tissues or cells (24, 25). It is well known that GSH plays an important role in the antioxidant mechanism in lungs. For instance, it has been noted that the epithelial lining fluid of normal lungs contains very high concentrations of this tripeptide, ~100 times higher than that found in the extracellular fluid of many other tissues (38). Some preliminary studies showed the involvement of GSH in silica-induced lung injury. An in vitro study by Boehme et al. (5) showed that silica treatment led to a decrease in intracellular GSH level in AMs and an increase in GSH level in culture medium, indicating the release of GSH by AMs. GSH content was decreased in silicotic lung tissues in rats in the early stage (15) and increased in the late stage (40). Epidemiologic data also showed that GSH in red blood cells was increased significantly in silicosis patients (6). However, so far there is little or no direct evidence showing the role of GSH in silica-induced cytotoxicity and genotoxicity in AMs. The main objective of the present study was to evaluate the role of GSH on silica-induced oxidative stress, cytotoxicity, and genotoxicity in AMs, the principal target cells of silica. In the present investigation, the intracellular GSH level in AMs was modulated by N-acetylcysteine (NAC), a GSH precursor, and buthionine sulfoximine (BSO), a specific GSH synthesis inhibitor. The data from these experiments provide direct evidence showing that GSH plays a critical role in silica-induced oxidative stress and cell injury.

EXPERIMENTAL PROCEDURES

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mide were all from Sigma (St. Louis, MO). RPMI 1640 medium, fetal bovine serum (FBS), low-melting- and normal-melting-point agarose were from GIBCO BRL (Life Technologies, Gaithersburg, MD).

Isolation and primary culture of AMs. Male Sprague-Dawley rats (body weight 220–250 g) were provided by the Animal Center, National University of Singapore. The rats were anesthetized with an intraperitoneal injection of a mixture of fentanyl citrate (0.063 mg/rat), fluoxisone (2 mg/rat; both from J assen), and midazolam (1 mg/rat; Hoffmann-La Roche). AMs were collected by lavaging isolated rat lungs with PBS after the animals were killed by bloodletting in the femoral artery. AMs were washed three times with PBS and cultured in RPMI 1640 medium containing 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin in a 5% CO2 incubator at 37°C.

The stock suspension of silica was prepared in PBS, and the exposure concentration is expressed in micromolars per 106 cells.

The effects of NAC and BSO were examined by pretreating the cells for 12 h in FBS-free medium; silica was then directly added without washing the cells with PBS.

Determination of intracellular GSH content. The determination of intracellular GSH content was conducted according to Hiiss and Hill's (17) method with modifications. The stock solution of the fluorescent probe OPT (1 mg/ml) was freshly prepared in methanol. After various designated treatments, AMs were collected with cell scrapers and washed twice with PBS (800 rpm for 5 min at 4°C) and then resuspended in 0.1 M sodium phosphate-5 mM EDTA (pH 8.0). After the total cell number was counted and the cells were thoroughly disrupted by ultrasonication, the cell homogenate (0.75 ml) was mixed with 25% metaphosphoric acid (0.25 ml) to precipitate the proteins. After centrifugation (14,000 rpm for 10 min at 4°C), the supernatant (0.2 ml) was mixed with 0.1 M sodium phosphate-5 mM EDTA (1.7 ml) and OPT (0.1 ml of stock solution, final concentration 50 µg/ml). The fluorescence intensity of OPT was monitored with an excitation wavelength of 350 nm and an emission wavelength of 420 nm (Perkin-Elmer LS-5B luminescence spectrometer). A GSH calibration curve was established with standard GSH, and the GSH concentration is expressed in nanomoles per 106 cells.

Determination of lactate dehydrogenase leakage. Activity of lactate dehydrogenase (LDH) in the medium was measured with an Abbott (Chicago, IL) VP biochemical analyzer with a test kit as established earlier (27). Total LDH activity was also detected after the cells were disrupted by ultrasonication. LDH leakage (in percent) was calculated as (LDH activity in medium/total LDH activity) × 100.

Analysis of DNA damage. DNA damage was detected with single-cell gel electrophoresis (or comet assay) according to the method described earlier (41) with modifications. Briefly, fully frosted slides were covered with 0.7% normal-melting agarose as the first layer, a mixture of AMs and 0.7% low-melting agarose as the second layer, and 0.7% low-melting agarose as the third layer. After solidification at 4°C, the slides were immersed in lysing buffer (2.5 mM NaCl, 100 mM Na2EDTA, and 10 mM Tris, pH 10, with freshly added 1% Triton X-100 and 10% DMSO) at 4°C for 1 h. The slides were then placed in an electrophoresis tank filled with freshly prepared electrophoresis solution (300 mM NaOH and 1 mM Na2EDTA, pH 13) for 20 min. Electrophoresis was conducted at 4°C for 20 min (25 V and 0.3 A). The slides were then neutralized in neutralization buffer (0.4 M Tris-HCl, pH 7.5), stained with ethidium bromide, and examined under a fluorescence microscope (Nikon). Images of 100 randomly selected cells from each slide were analyzed. The degree of DNA damage was divided into 5 categories according to the percentage of DNA in the tail: grade 0, no damage, <5%; grade 1, low-level damage, 5–20%; grade 2, medium-level damage, 20–40%; grade 3, high-level damage, 40–95%; and grade 4, total damage, >95%.

Lucigenin-dependent chemiluminescence test. Lucigenin-dependent chemiluminescence (CL) in AMs was determined as described earlier (29). The CL assay mixture contained 1 × 106 cells and 100 µM lucigenin in 1 ml of PBS with and without the presence of silica. The CL reaction was initiated by the simultaneous addition of lucigenin and silica, and the CL level was monitored as relative light units with a luminometer (Lumi-One, Tampa, FL) for a total period of 10 min at room temperature.

Analysis of DCFH-DA fluorescence in AMs. The level of hydrogen peroxide (H2O2) in AMs was measured with DCFH-DA as a fluorescence probe (28). The principle of this assay is that DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to nonfluorescent DCFH. In the presence of ROS (mainly H2O2), this compound is rapidly oxidized to highly fluorescent dichlorofluorescin (DCF) (4, 22). DCFH-DA was dissolved in absolute ethanol at a concentration of 5 mM as a stock solution and kept at −70°C in the dark. AMs were incubated in 24-well plates, each well containing 1.5 × 105 AMs and 2 µM DCFH-DA in 2 ml of culture medium. The reaction was initiated by the addition of DCFH-DA and incubated at 37°C for up to 4 h. The fluorescence intensity was measured with a plate reader (Tecan Spectrafluor-Plus), with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Analysis of data. All data were based on at least three independent experiments and analyzed with one-way ANOVA with Scheffé's test and are presented as means ± SD. A P value < 0.05 was considered as significant.

RESULTS

Effects of silica on intracellular GSH. Figure 1A shows the dose-dependent decrease in intracellular GSH when cells were treated with silica for 4 h. With the highest concentration of silica (100 µg/106 cells), the GSH level was only one-third of the control value. It was also noted that the silica-induced decline in intracellular GSH level was time dependent (Fig. 1B). The GSH concentration in the control cells remained at a constant level (~3 nM/106 cells), whereas the GSH level in the cells treated with silica (100 µg/106 cells) decreased dramatically from 1 h onward.

Effects of NAC and BSO on intracellular GSH content. In the present study, AMs were pretreated with 1 mM NAC and 1 mM BSO for 12 h before exposure to silica. Compared with the control cells, NAC significantly enhanced the intracellular GSH content of AMs, whereas BSO pretreatment markedly decreased the GSH level in AMs (Fig. 2). Although the subsequent silica exposure significantly reduced the GSH level, those cells with NAC pretreatment contained a higher level of GSH compared with the cells treated with silica only. On the other hand, BSO pretreatment further enhanced the extent of GSH depletion caused by silica exposure because the cells with both BSO and silica treatment displayed the lowest GSH level among all these groups (Fig. 2).
Effects of NAC and BSO on silica-induced cytotoxicity.

Figure 3 shows the different effects of NAC and BSO on silica-induced cytotoxicity as measured by the percentage of LDH leakage. After 16 h of incubation (12 h of pretreatment plus 4 h of silica incubation time), LDH leakage in the control, BSO-only, and NAC-only cells was 5.92 ± 1.70, 8.22 ± 0.66, and 6.82 ± 2.22%, respectively. These data suggest that BSO or NAC pretreatment alone did not cause any significant damage to the cell. In contrast, NAC pretreatment significantly reduced the LDH leakage induced by silica, whereas the cells pretreated with BSO showed a marked increase in LDH leakage compared with those cells exposed to silica only.

Effects of NAC and BSO on silica-induced genotoxicity. The effects of NAC and BSO on silica-induced DNA damage were examined with a comet assay. Figure 4 presents the data showing the different effects of NAC and BSO on the percentage of cells with grade 3 and 4 damage (cells with >40% DNA in the tail) caused by silica.
silica. NAC or BSO pretreatment alone did not cause significant DNA damage because >95% of the cells were grade 0 (data not shown). However, NAC was able to protect against silica-induced DNA strand breaks. The percentage of cells with grade 3 and 4 damage decreased from ~55% in silica-treated AMs to 26% in cells pretreated with NAC. In contrast, BSO pretreatment tended to enhance the extent of DNA damage induced by silica. The percentage of cells with grade 3 and 4 damage increased up to 67%. The above changes were generally consistent with the pattern observed in the cytotoxicity test (Fig. 3).

Effects of NAC and BSO on silica-induced O$_2^-$ and H$_2$O$_2$ formation in AMs. In this study, a lucigenin-dependent CL test was used for the determination of O$_2^-$ formation and a DCFH-DA fluorescence test was used for measurement of intracellular H$_2$O$_2$ formation. The results are summarized in Figs. 5 and 6, respectively. As shown in Figs. 5 and 6, NAC pretreatment did not change the O$_2^-$ level either in control cells or in silica-exposed AMs. In contrast, BSO pretreatment significantly enhanced the O$_2^-$ level in both control cells and silica-treated AMs (Fig. 5). The results in Fig. 6 show that NAC was able to significantly reduce the ROS level in both the control and silica-treated rat AMs. The DCF fluorescence intensity in NAC-pretreated cells decreased nearly 80% compared with that in the cells treated with silica alone. On the other hand, BSO pretreatment enhanced the ROS level in control cells as well as in silica-treated AMs, which is similar to its effect on O$_2^-$ formation (Fig. 5).

**DISCUSSION**

The involvement of ROS and oxidative damage in silica-induced pulmonary inflammation, fibrosis, and carcinogenesis has been extensively studied (30, 36). It is well known that GSH is the major intracellular antioxidant with multiple biological functions. One of its most important functions is to protect against oxidative damage caused by ROS through enzymatic and nonenzymatic reactions (24, 25). Therefore, it will be of interest to evaluate the effect of GSH on silica toxicity. The present study was thus undertaken to assess the role of intracellular GSH on silica-induced oxidative stress, cytotoxicity, and genotoxicity in primary cultured rat AMs. The intracellular GSH level was modulated with NAC, a GSH precursor, and BSO, a specific GSH synthesis inhibitor. The results obtained show that NAC was able to increase the intracellular GSH content in AMs and suppress silica-induced ROS formation, LDH leakage, and DNA damage. In contrast, BSO pretreatment led to intracellular GSH depletion and enhanced the susceptibility of AMs to silica-induced oxidative stress and cell injury. Therefore, the present study provides convincing evidence supporting the notion that GSH plays an important role in silica-induced oxidative stress, cytotoxicity, and genotoxicity in AMs.

The present study demonstrates that the intracellular GSH level decreased after silica exposure in a dose- and time-dependent manner. Compared with that in the untreated control cells, the GSH content was reduced >70% when AMs were treated with 100 µg silica/10^6 cells for 4 h (Fig. 1B). This finding is essentially consistent with an earlier report by Boehme et al. (5) that silica exposure leads to GSH release from AMs. An in vivo study also showed that the concentrations of antioxidant molecules, including GSH, decreased in lung tissues after intratracheal instillation of silica dust (15). On the other hand, it is noted that there was a maximal decrease in intracellular GSH in AMs 2 and 4 h after silica exposure (Fig. 1B), which coincides with the dramatic increase in LDH leakage at the same time points after silica treatment. The percentage of LDH leakage in silica-treated cells at 1, 2, and 4 h was 14.6, 30.1, and 75.4%, respectively, whereas the percentage of LDH leakage in the control cells was consistently <12% (Z. Zhang, Shen, Q.-F. Zhang, and Ong, unpublished data). It is believed that there are three possible mechanisms accounting for the decline in cellular GSH
in response to silica exposure: 1) via GSH peroxidase (GPx) reaction, 2) via the GSH transferase reaction, and 3) via GSH efflux (5, 14). GPx mainly catalyzes the direct reaction of GSH with ROS such as H$_2$O$_2$ and hydroxyl radical, resulting in the formation of GSSG. Based on our preliminary investigations, the concentration of GSSG in AMs did not change significantly compared with that in the control cells (data not shown), indicating that GPx may not play a major role in silica-induced GSH depletion. Moreover, the close resemblance of the time course of GSH with that of LDH leakage tends to suggest that GSH efflux is an important mechanism accounting for silica-induced intracellular GSH depletion in AMs.

The availability of cysteine is one of the speed-limiting factors in GSH synthesis, and NAC acts as a precursor of GSH to facilitate GSH synthesis (11). In this study, NAC pretreatment was able to increase the intracellular GSH level and inhibit silica-induced LDH leakage and DNA strand breaks (Figs. 2-4), which is consistent with an earlier study (39) showing that the addition of NAC was responsible for a decrease in silica-induced cytotoxicity in AMs. Furthermore, the present study also examined the effects of NAC on silica-induced ROS formation. It is interesting to note that NAC pretreatment significantly suppressed H$_2$O$_2$ formation (Fig. 6), whereas no observable inhibitory effect was found on the production of O$_{2}^{•-}$ in silica-treated cells (Fig. 5). The differential effects of NAC on different species of ROS were also observed in an earlier study by Aruoma et al. (2). They demonstrated that NAC is a potent scavenger of H$_2$O$_2$ and hydroxyl radicals, whereas the scavenging activity of NAC against O$_{2}^{•-}$ was found to be rather weak. At present, the antioxidant property of NAC has been well characterized, and it is known that NAC acts as a potent antioxidant through the following two mechanisms: 1) as a precursor of GSH to facilitate intracellular GSH synthesis and 2) as a direct ROS scavenger (2, 11). Therefore, the inhibitory effects of NAC against silica-induced ROS formation, LDH leakage, and DNA strand breaks clearly indicate the important role of GSH in protection against silica toxicity in AMs. Some earlier studies (7, 8) in animals and humans used aerosolized GSH to enhance pulmonary GSH content and to counteract the imbalance of oxidant-antioxidant in idiopathic pulmonary fibrosis. Based on the in vitro studies presented here, NAC appears to be more suitable for the augmentation of pulmonary GSH level in silicosis and other lung diseases to prevent pulmonary injury and fibrosis.

BSO is a specific inhibitor of $\gamma$-glutamylcysteine synthetase, the key enzyme in intracellular GSH synthesis. It has been widely used for depleting intracellular GSH in various cells and tissues (1, 34). In the present study, BSO pretreatment led to a 70% reduction in GSH content in control cells and a further decline in GSH level in silica-treated AMs (Fig. 2). Accordingly, the cells pretreated with BSO became more susceptible to the toxic effects of silica as shown by the significantly increased LDH leakage and DNA strand breaks (Figs. 3 and 4). A previous study (23) evaluated the effect of nonprotein sulfhydryl moieties (including GSH) depletion caused by BSO on silica-induced inflammation and fibrosis in the mouse lung, and the results suggested that GSH is able to lessen the potential of silica in eliciting acute lung injury. Results from the present study also showed that BSO-pretreated AMs generated a higher level of ROS in both the control and silica-exposed cells (Figs. 5 and 6), indicating that GSH mainly acts as an ROS scavenger to protect against silica-induced cell injury. The aggravating effect of BSO on silica-induced oxidative stress, cytotoxicity, and genotoxicity further supports the notion that GSH plays a critical role in the toxicity of silica. It seems that silica-induced ROS formation led to the depletion of GSH and impairment of the antioxidant system, which, in turn, exacerbates the oxidative damage in silica-exposed cells.

Silica has recently been classified as a confirmed human carcinogen (20). Silica-elicited DNA damage and changes in cell proliferation are the two key factors related to its carcinogenicity (30). It has been proposed that the silica-induced DNA damage is caused by two major mechanisms: free radical mediation and direct binding with DNA (30). An early study showed that asbestos-induced oxidative DNA damage (8-hydroxydeoxyguanosine formation) was augmented by BSO and ameliorated by NAC, indicating that GSH modulates the genotoxic effects of asbestos (18). In the present study, silica-induced DNA strand breaks were evaluated with a comet assay, and the inhibitory effect of NAC and the aggravating effect of BSO on silica-induced DNA damage provide the first evidence showing the protective role of GSH in the genotoxicity of silica. On the other hand, changes in cell proliferation, apoptosis, activation of transcription factors, and induction of oncogene expression are believed to be closely related to the carcinogenic effect of silica (30). For instance, it has been found that silica is a potent inducer of nuclear transcription factor nuclear factor-$\kappa$B, most probably through the production of ROS (9, 10). Nuclear factor-$\kappa$B is one of the key molecules controlling many important functions such as immunity, inflammation, and apoptosis (3). GSH has been suggested to play an inhibitory role in its activation (16). Therefore, further studies on the involvement of GSH in silica-induced DNA damage and changes in cell proliferation will certainly help to obtain a better understanding of the carcinogenicity of silica.

In summary, the present study evaluated the critical role of GSH in silica-induced oxidative stress, cytotoxicity, and genotoxicity in cultured rat AMs. It is believed that the understanding of the importance of GSH will be beneficial to the development of preventive or therapeutic agents in control of silica-induced pulmonary fibrosis and carcinogenesis.

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