Generation of Free Radicals from Freshly Fractured Silica Dust

Potential Role in Acute Silica-induced Lung Injury[1-3]

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SUMMARY Data presented here indicate that freshly fractured silica exhibits surface characteristics and biologic reactivity distinct from aged silica, and on this basis we propose that these surface features may lead to enhanced manifestations of lung injury. Grinding of silica produces ~10^{18} Si̇ and Si-Ȯ (silicon-based) radicals per gram of dust on the particulate surface which are characterized by an electron spin resonance (ESR) spectrum centered around $g = 2.0015$. These silicon-based radicals react with aqueous media to produce ȮH radicals, which are demonstrable using a DMPO spin trap. The concentration of silicon-based radicals in silica decreases with aging in air and exhibits a half-life of ~30 h, whereas its ability to generate ȮH radicals in aqueous solution decreases with a half-life of ~20 h. However, on storage in aqueous media, the concentration of silicon-based radicals and the dust's ability to generate ȮH radicals decrease significantly within a few minutes. Freshly ground silica is also more biologically reactive than aged silica, because freshly crushed silica activates a greater respiratory burst in alveolar macrophages than aged silica, i.e., storage of ground dust in air decreases silica-induced superoxide anion secretion, hydrogen peroxide release, and NBT reduction by 25%, 68%, and 43%, respectively. Furthermore, compared to aged silica, freshly ground silica exhibits a greater cytotoxic effect on cellular membrane integrity, i.e., a 1.5-fold increase in LDH release from macrophages, a 36-fold increase in hemolytic activity, and a three-fold increase in the ability to induce lipid peroxidation. Because acute silicosis is frequently associated with occupations in which freshly fractured crystalline silica of respirable size is generated, the present study suggests that fracture-generated silicon-based radicals may play a significant role in the pathogenesis of this disease. AM REV RESPIR DIS 1988; 138:1213-1219

Introduction

Occupational exposure to crystalline silica can be associated with either chronic or acute pulmonary disease. Chronic silicosis becomes manifest 20 to 40 years after first exposure and is characterized by development of concentric hyalinized nodular lesions in the lung with the development of dyspnea over a period of several decades. Acute silicosis, on the other hand, is manifested by a rapid onset after exposure and is characterized by the accumulation of an amorphous granular lipoprotein exudate in the airspaces and rapid development of respiratory disability within a few years (1-3).

Information is growing concerning the etiology of chronic silicosis. Studies suggest that several mechanisms may be involved in the development of fibrosis. Lung injury may result from silica-induced release of lysosomal enzymes from alveolar macrophages (4, 5). In addition, silica-induced activation of superoxide anion and hydrogen peroxide release from alveolar macrophages may result in oxidant-induced damage to lung parenchyma (6). Silica exposure can also result in the release of mediators from alveolar macrophages which enhance the proliferation of fibroblasts and the synthesis of collagen by these pneumocytes (7, 8).

In comparison with chronic silicosis, very little is known concerning the development of acute silicosis. Because the pulmonary responses to silica differ in the chronic and acute presentation of disease, it does not seem likely that acute silicosis can be explained simply as the response of the lung to high levels of silica. We propose that at least part of the acute response is due to some unique characteristic of the dust inhaled. Acute silicosis is commonly associated with sandblasting, rock drilling, tunnelling, and silica mill operations, i.e., operations in which silica particles are crushed or sheared (9). Therefore, it is possible that freshly sheared silica may have surface properties that make it more reactive with lung tissue than aged silica, and that it is this unique reactivity of freshly sheared silica that leads to manifestation of acute pulmonary disease.

Earlier studies have suggested that freshly fractured silica may exhibit surface reactivity not found in aged silica. Hochstrasser and Antinini (10) reported that silicon-based radicals could be generated upon cleavage of a quartz crystal under ultra-high vacuum (10^{-10} mm Hg). Karmanova and colleagues (11) reported release of singlet oxygen from silica dust upon heating, whereas Kolbanev and associates (12) reported generation of H_2O_2 from the reaction of freshly ground silica with water. In addition, Marasas and Harington (13) reported that silica exhibits oxidant properties that may be related to its pathogenicity. However, to date, a systematic evaluation of the generation of silicon-based radicals as a result of shearing or grinding under ambient air which mimics occupational conditions, as well as the decay of these surface radicals and its relevance to biologic reactivity, has not been conducted.

The objective of this study was to evaluate whether freshly ground silica was more surface reactive and/or cytotoxic than aged silica. First, the generation of short-lived silicon-based radicals on freshly ground silica and possible release of oxygenated radical species was evaluated using electron spin resonance (ESR) techniques. Secondly, the reactivity of freshly ground silica was monitored by comparing its ability to activate a respiratory burst in alveolar macrophages to that of aged silica. Lastly, cytotoxicity of fresh versus aged silica dust was

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compared by measuring their effects on red blood cell hemolysis, LDH release from alveolar macrophages, and lipid peroxidation.

Methods

Reagents

Horse heart ferricytochrome c, N-2-hydroxyethyl piperazine-N-2-ethane sulfonic acid (HEPES), horseradish peroxidase (Type IX), scopoletin, superoxide dismutase (SOD), catalase, cis-9-cis-12-octadecadienoic acid (linoleic acid), sodium benzoate, sodium dodecyl sulfate, diethylenetriaminepentaacetic acid (DETAPAC), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), and D-mannitol were obtained from Sigma Chemical Company, St. Louis, Missouri. The 5,5,-dimethyl-1-pyrroline-1-oxide (DMPO), 1,3 dimethyl-2-thiourea (DMTU), and 1,1,3,3-tetramethoxypropane were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin, and 1-butanol was obtained from Fisher Scientific Company, Pittsburgh, Pennsylvania.

Preparation of Silica

Crystalline silica (0.2 to 5.0 mm in diameter) was obtained from the Generic Respirable Dust Technology Center, Pennsylvania State University, State College, Pennsylvania. Silica was ground in an agate mortar with a pestle for 30 min and sieved through a 20-micron mesh filter before use. Representative samples of the ground silica were subjected to X-ray spectrometric analysis to confirm that the silica samples were mineralogically pure and contained no detectable trace elemental impurities. For measurement of decay of ESR signal or biologic effects, samples of a single stock of ground silica were taken at various times after grinding to assure uniformity of shearing and particle size.

Measurement of Electron Spin Resonance (ESR)

ESR spectra were obtained at X-band ($\sim$ 9.7 GHz) using a Bruker ER 200D ESR spectrometer at the Chemistry Department of West Virginia University. For accurate measurements of the g values and hyperfine splittings, the magnetic field was calibrated with a self-tracking NMR Gaussmeter (Bruker Model ER035M) and the microwave frequency measured with a Hewlett-Packard (Model 5340A) frequency counter. All the measurements were made at room temperature. Typical spectrometer settings are given in the figure legends.

Isolation of Alveolar Macrophages

Alveolar macrophages were harvested from male pathogen-free Sprague-Dawley rats by bronchopulmonary lavage using a calcium- and magnesium-free Hank's balanced salt solution (14). Macrophages from ten 8-ml lavages were sedimented by centrifugation at 500 g for 5 min at 2° C and suspended in HEPES-buffered medium containing 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, and 5 mM glucose (pH 7.4). Cell viability counts were made using the trypan blue dye exclusion procedure (15). Microscopic estimates of purity indicated that 90 to 95% of the lavaged cells were alveolar macrophages.

Activation of Alveolar Macrophages

Silica-induced activation of the respiratory burst in alveolar macrophages was monitored by measuring superoxide and hydrogen peroxide release. Superoxide anion release was monitored by measuring the superoxide-dependent (SOD inhibitable) reduction of cytochrome c spectrophotometrically at 550 nm using a Gilford Spectrophotometer (Model 300-N) (16). Briefly, alveolar macrophages (2×10^6 cells) were added to 2 ml of HEPES-buffered medium containing 0.12 mM cytochrome c either in the absence or presence of silica (1 mg/ml). At zero or 30 min, parallel cell suspensions were centrifuged at 2,000 g for 1 min and the absorbance of the supernatant was measured. Superoxide release was proportional to the difference between the absorbance values at 30 and zero min. Absorbance values were converted to nmoles of cytochrome c reduced using an extinction coefficient of 21 $mM^{-1}cm^{-1}$.

Hydrogen peroxide release was monitored by measuring the change in fluorescence of scopoletin in the presence of horseradish peroxidase (17). Fluorescence was monitored at an excitation wavelength of 350 nm and an emission wavelength of 460 nm using a Perkin-Elmer Fluorescence Spectrophotometer (Model MPG-36) equipped with a stirrer and temperature controlled at 37° C. Briefly, alveolar macrophages (5×10^6 cells) were added to 3 ml of HEPES-buffered medium containing 2.4 μM scopoletin and 6.6 units of horseradish peroxidase either in the absence or presence of silica (1 mg/ml). The decrease in fluorescence was converted to nanomoles of hydrogen peroxide released, using a standard curve constructed by adding known quantities of hydrogen peroxide to the assay cuvette.

Reduction of nitro blue tetrazolium (NBT) to formazan was also monitored to measure respiratory burst activity in alveolar macrophages, using a histochemical technique (18).

Measurement of Cytotoxicity

The cytotoxic potential of fresh or aged silica was monitored by determining the effects of these dusts on cellular membrane integrity, i.e., hemolysis of red blood cells and release of cytosolic LDH from alveolar macrophages, as well as the ability of silica to induce lipid peroxidation. Hemolytic activity of freshly ground or aged silica was measured in a 2% suspension of sheep erythrocytes as the amount of hemoglobin released after incubation in the presence or absence of silica (10 mg/ml for 1 h at 37° C). After treatment, the suspension was centrifuged and the absorbance of the supernates read at 540 nm using a Gilford Spectrophotometer (Model 300-N). Percentage of hemoglobin released was calculated as the ratio of the absorbance value for the supernatant sample from silica-treated red blood cells to that from cells lysed with 0.5% Triton X-100.

The effects of freshly ground or aged silica on membrane integrity were also monitored by measuring cytosolic lactate dehydrogenase (LDH) release from alveolar macrophages. Alveolar macrophages (2×10^6) were incubated for different time intervals in a shaking water bath at 37° C in the presence or absence of silica (1 mg/ml). After incubation, cell suspensions were centrifuged and LDH released from the macrophages was estimated in the supernate (19). The reaction mixture in a total volume of 3 ml contained phosphate buffer (pH 7.4), 0.1 ml of enzyme supernatant, 0.07 mg/ml NADPH, and 0.0007 M sodium pyruvate. The reaction was initiated by the addition of sodium pyruvate to preincubated reaction mixture. LDH secretion was expressed as percent total enzyme released by Triton X-100 lysis of cells. One unit of LDH activity equals the amount of enzyme that catalyzes the reduction of a μmole of reduced nicotinamide adenine dinucleotide in 1 min at 37° C as measured spectrophotometrically by a decrease in absorbance at 340 nm.

Peroxidation of the polyunsaturated lipid linoleic acid (cis-9-cis-12-octadecadienoic acid) by freshly ground or aged silica was monitored using a fluorescence method (20) with some modifications. The reaction mixture in a total volume of 0.5 ml contained freshly ground or aged silica and 20 μl of 0.52 mM linoleic acid emulsion in 95% ethanol in HEPES buffer (pH 7.4) without calcium and glucose. After 1 h of incubation in a shaking water bath at 37° C, the reaction was terminated by the addition and mixing of 0.5 ml of 3% sodium dodecyl sulfate followed by 2.0 ml of 0.1 N HCl, 0.3 ml 10% phosphotungstic acid, and 1.0 ml 0.7% 2-thiobarbituric acid, respectively. The mixture was then heated for 30 min at 95 to 100° C, and the thiobarbuturic acid reactive substance formed was extracted with 5 ml 1-butanol after cooling. The test tubes were then centrifuged at 3,000 rpm for 1 min, and the fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission using a Perkin-Elmer fluorospectrophotometer (Model MPG-36). Malondialdehyde standards were prepared from 1,1,3,3,-tetramethoxypropane, and the malondialdehyde produced was calculated from the standard graph.

Effect of Scavengers

SOD, catalase, sodium benzoate, mannitol, DMSO, and DMTU were added to the cytotoxicity assays of hemolysis, H_2O_2 release, and lipid peroxidation in different concentrations to evaluate the most effective dose response. SOD and catalase were added to the test in final concentrations of 85, 170, and 340 units/ml and 312, 625, and 1250 units/ml, respectively. Sodium benzoate, DMSO, and DMTU were added to the test bioassays in 0.05, 0.1, and 0.5 mM, and 0.1 M final concentrations. All the scavengers were added immediately prior to the addition of test silica

and evaluated with positive and negative controls. In few experiments, SOD and catalase were denatured by placing in a boiling water bath for 3 min and then used in the test.

Statistical Analysis

Data presented are means ± standard deviations except where indicated. Measurements concerning the time-dependent decay of silicon-based radicals by ESR or loss of biologic activity were made on the same preparation of ground silica by a one-way analysis of variance. In other experiments, comparisons of data were made by a two-tailed Student's *t* test. In all data comparisons, a probability value of less than 0.05 was considered significant.

Results

The presence of reactive free radical sites on the surface of silica particles was monitored using ESR spectroscopy. No ESR signal was observed with unground crystalline silica particles. However, upon grinding of these silica particles in air, an ESR spectrum centered around g = 2.0015 ± 0.0003 was observed (figure 1A). Such a signal is characteristic of silicon-based radicals (Si-Ȯ and Ṡi), the so-called E-center (10, 21). Comparing the peak-to-peak height of the ESR signal from freshly ground silica to that of diphenyl picrylhydrazyl (DPPH), a standard of known radical concentration, we estimate that approximately 10^{18} silicon-based radicals were generated per gram of silica after 30 min of grinding in air. As discussed elsewhere (22), these radicals are localized on the dust surface and the decay in air was noted by the decrease in signal height, Ipp, with a half-life of about 30 h in air (figure 1B) and an approximate half-life of few minutes in PBS buffer (data not shown). It should be noted that the ESR signal of freshly ground silica decreased in air by about 80% after approximately first-order kinetics. However, 20% of the ESR signal was detectable even after 4 wk of storage in air.

ESR data also suggest that freshly ground silica can react with water to release short-lived, oxygenated free radicals. The generation of such radicals was monitored by ESR after the addition of a spin trap, DMPO, to an aqueous suspension of freshly ground silica. The ESR signal observed from freshly ground silica in aqueous solution in the presence of DMPO is shown in figure 2A. The ESR spectrum was centered around g = 2.0059 ± 0.0003 and exhibited a 1:2:2:1 quartet pattern with a splitting of 14.9 G, quite characteristic of a DMPO-ȮH adduct (23–26). Therefore, the results indicate ȮH radicals can be produced during the reaction of freshly ground silica in an aqueous environment.

In order to verify further the presence of the ȮH radicals, 30% ethanol was added as a secondary ȮH radical trap (27). Under these conditions, the intensity of the DMPO-ȮH adduct signal decreased because ethanol served as a scavenger for the ȮH radicals (figure 2B). The reaction of ȮH radicals with ethanol results in the production of ethanolyl radicals that in turn react with DMPO to give the spin adduct, DMPO-$CHOCH_3$. The characteristic ESR signal of this DMPO-$CHOCH_3$ adduct was observed as indicated by arrows that became the dominant peaks in excess ethanol (figures 2B and 2C). DETAPAC or EDTA, metal chelators, had little effect on the DMPO-

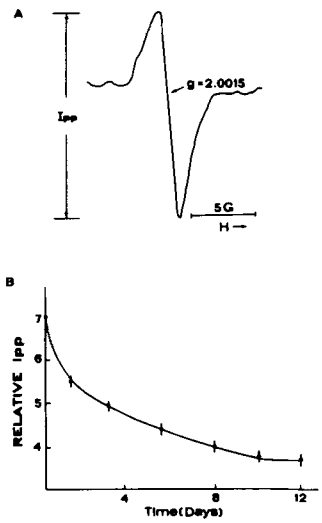

Fig. 1. A. A typical ESR spectrum of silicon-based radicals in silica dust ground in air for 30 min. The signal height, Ipp, is proportional to the radical concentration. B. A plot of the silicon-based radical concentration as a function of storage time in air. The spectrometer settings were: receiver gain, 3.2 × 10^5; modulation amplitude, 2 G; scan time, 100 s; field, 3,470 ± 50 G.

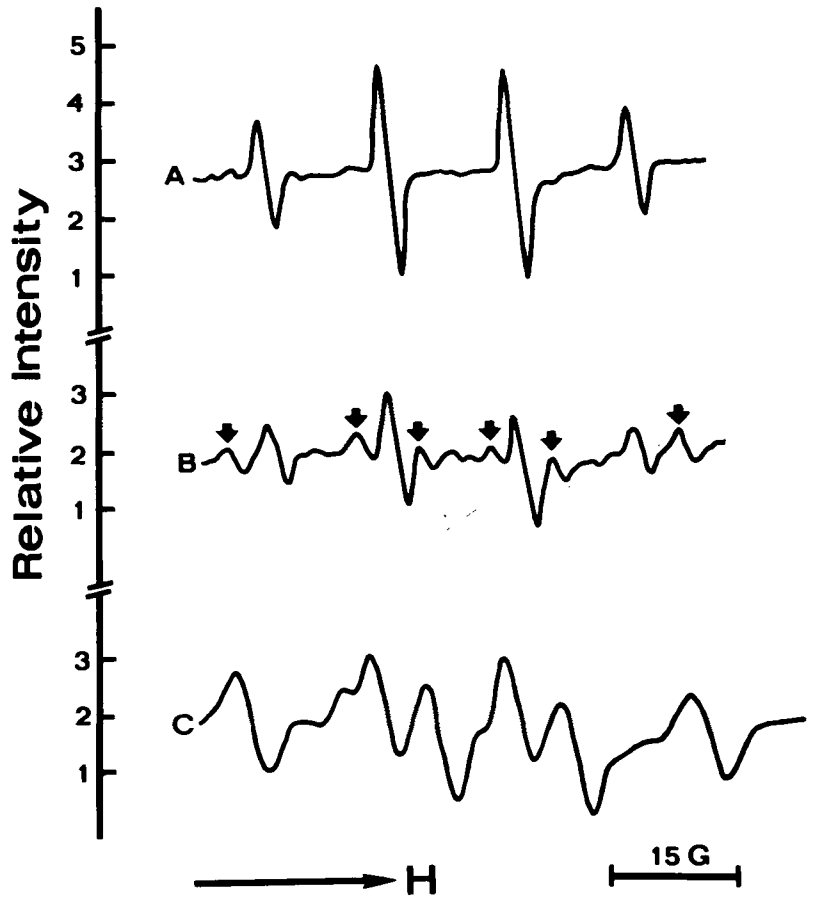

Fig. 2. ESR spectra observed from freshly ground silica in aqueous solution in the presence of either a spin trap, 100 mM DMPO (A), or 400 mM DMPO plus an ȮH radical scavenger, 30% ethanol (B). The arrows in (B) indicate signals from trapped ethanolyl radicals. Figure 2C shows the spectrum in 95% ethanol where the peaks marked by arrows in (B) become dominant. The spectrometer settings were: receiver gain, 5 × 10^5; modulation amplitude, 2 G; scan time, 100 s; field, 3,460 ± 75 G. Normally, no background ESR signal was detectable in samples of DMPO without silica. If a signal was detectable with DMPO alone, this stock was discarded and new DMPO stock was used.

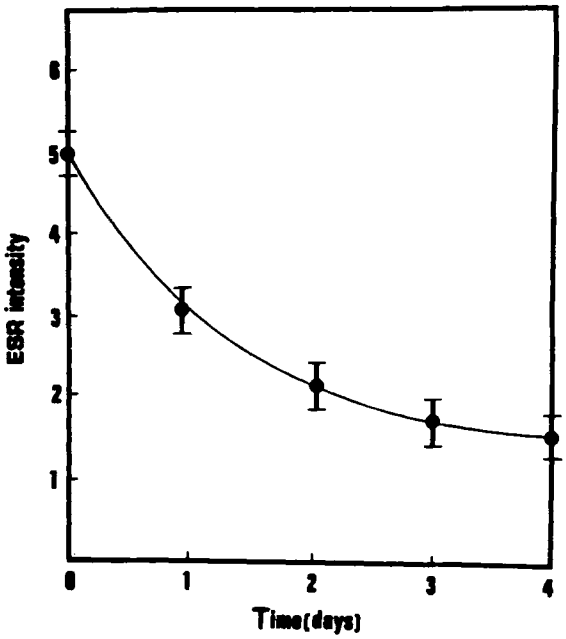

Fig. 3. ESR intensity of the DMPO-ȮH adduct as a function of the freshness of ground silica. Dust samples for ESR measurements were taken from the same stock of ground silica stored in air at various times after crushing. The concentration of DMPO was 100 mM in aqueous solution. Spectrometer settings were the same as those given in figure 2.

ȮH signal (data not shown). This suggests that ȮH radicals are generated directly from the reaction of silicon-based radicals with water (see DISCUSSION).

The ability of silica to react with water and generate ȮH radicals decreased with time after crushing, as shown by the decrease in the intensity of the DMPO-ȮH adduct ESR signal as a function of time after grinding and storage of silica in air (figure 3). The half-time for this decay was approximately 20 h.

In order to determine if freshly ground silica was a more potent stimulant of the respiratory burst in alveolar macrophages, superoxide anion release, hydrogen peroxide secretion, and NBT reduction were monitored after *in vitro* exposure of alveolar macrophages to either freshly crushed or aged silica. Freshly crushed silica activated alveolar macrophages to a greater extent than did silica after storage, i.e., silica-induced superoxide release decreased by 16% and 27% after storage of silica in phosphate buffer for 24 or 96 h, respectively (figure 4), whereas silica-induced hydrogen peroxide secretion decreased by 65% after 24 h of storage (figure 5). Furthermore, NBT staining was 69 ± 11% with freshly ground silica compared to 39 ± 8% after 48 h of storage. The half-time for the decrease in the ability of ground silica to activate alveolar macrophages was approximately 22 h, which was comparable with half-life for the production of the ȮH radicals from freshly ground silica (figure 3).

The next series of experiments compared the cytotoxicity of freshly crushed silica with that of aged silica dust. The results indicate that freshly ground silica exhibited a greater effect on membrane integrity than did silica dust after storage. Indeed, compared to aged silica, freshly ground silica was significantly more potent in inducing hemolysis of red blood cells (figure 6). The half-time for decay of the hemolytic potential of crushed silica was approximately 10 h. Although there was a tendency for the silica-induced release of cytosolic enzyme LDH to decline as silica aged, the differences were minimal (figure 7) compared to hemolytic changes.

Cytotoxicity of freshly ground and aged silica was also determined by monitoring the ability of these dusts to induce peroxidation of lipids (table 1). It is evident from the data presented that freshly ground silica induced lipid peroxidation in a dose-dependent fashion. This is clearly evident for dust freshly ground (zero to 5 min). The ability of ground silica to peroxidize lipids decreased with storage at all doses, i.e., the rate of silica-induced lipid peroxidation declined markedly over the first 48 h after grinding and remained relatively constant thereafter.

Effects of scavengers on the prevention of silica-induced cytotoxicity are presented in table 2. Addition of exogenous SOD and catalase inhibited the cytotoxicity of freshly ground silica in various bioassays at different levels. The results indicate that in bioassays of hemolysis, SOD, catalase, and sodium benzoate provided a partial protection, whereas DMSO, DMTU, and mannitol were totally noneffective in preventing

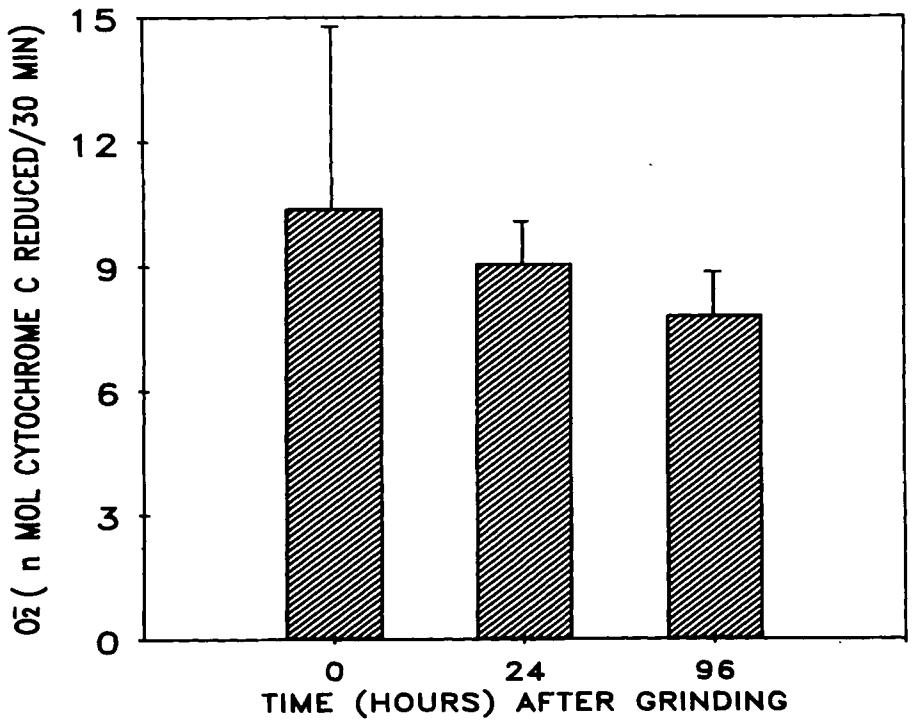

Fig. 4. The effect of freshly ground or aged silica on superoxide anion secretion from alveolar macrophages. Superoxide secretion was measured spectrophotometrically by monitoring the reduction of cytochrome c. The data presented are the means ± SD of five experiments.

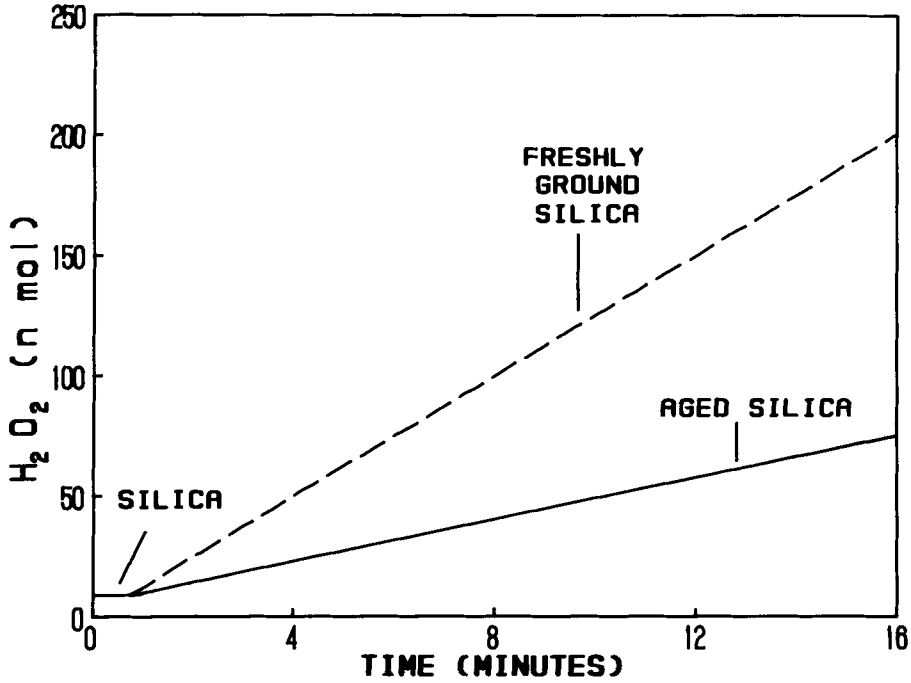

Fig. 5. The effect of freshly ground of aged silica on the secretion of hydrogen peroxide from alveolar macrophages. Hydrogen peroxide secretion was measured fluorometrically by monitoring the oxidation of scopoletin in the presence of horseradish peroxidase.

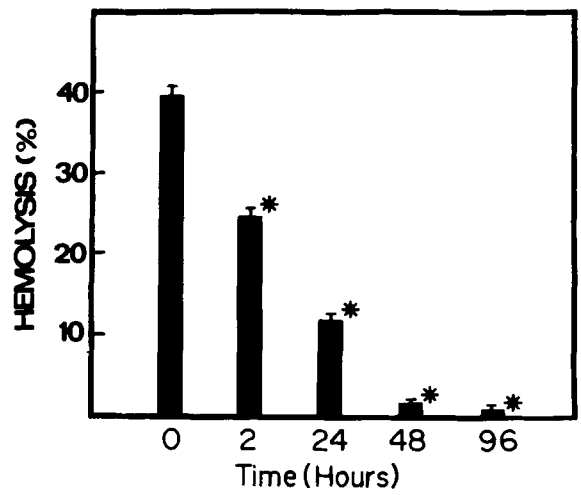

Fig. 6. Hemolytic potential of freshly ground or aged silica (stored in phosphate buffer). Hemolysis of red blood cells was measured spectrophotometrically and expressed as the percent lysis compared to that with 0.5% Triton X-100. The data presented are the means ± SD of a minimum of four experiments. Asterisk indicates values significantly different than time zero at $p < 0.05$.

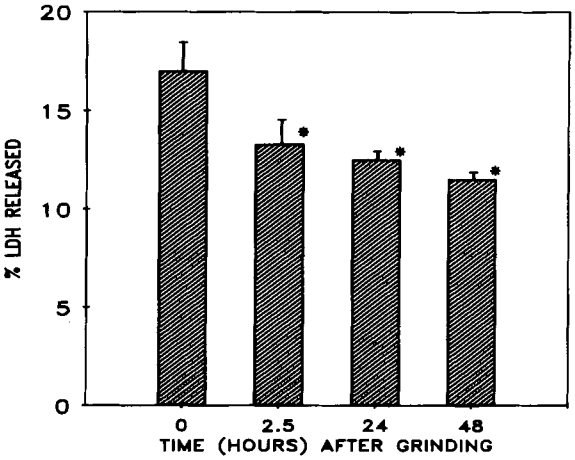

Fig. 7. Release of cytosolic lactate dehydrogenase, LDH, from alveolar macrophages exposed to either freshly ground or aged silica. LDH release was measured by the method of Wroblewski and LaDue (19). The data presented are the means ± SD of seven experiments. Asterisk indicates values significantly different than time zero at $p < 0.05$.

hemolysis. Among the various scavengers, catalase provided the maximal protective effect in hemolysis. On the other hand, in bioassays of lipid peroxidation and H_2O_2 release, many hydroxyl radical scavengers such as DMSO, DMTU, mannitol, sodium benzoate, and catalase provided a significant inhibitory effect in one or both the bioassays. Catalase provided a complete abrogation of fresh silica-induced secretion of H_2O_2 even at an enzyme concentration of 312 units/ml, whereas it was effective in preventing lipid peroxidation to a 52 ± 11% level only at a higher concentration (1,250 units/ml). DMTU and sodium benzoate were less effective in preventing H_2O_2 release. A potential for DMTU and sodium benzoate to react with H_2O_2 could not be ruled out.

Discussion

Our results provide evidence that mechanical crushing of crystalline silica for 30 min in air at room temperature produces a significant concentration ($\sim 10^{18}$ radicals/g of silica) of silicon-based radicals and that these radicals decay with time. Although the generation of silicon-based radicals upon cleavage of silicon crystals under ultra-high vacuum (10^{-10} mm Hg) has been reported earlier (10), recent studies from our laboratories and by Fubini (28) have shown the generation of free radicals from silica crushed in air. This report further indicates that these silicon-based radicals exhibit a half-life in the order of 30 h in air and have the ability to generate ȮH radicals in aqueous medium, making freshly ground silica biologically more reactive than aged silica. Our data also demonstrate that even after 4 wk of storage in air after grinding, as much as 20% of the original ESR signal remained detectable. This suggests the existence of at least two modes for the decay of the silicon-based radicals, i.e., a fast decay with an "average" half-life of about 30 h and a much slower decay with a much longer half-life. The decay mechanism is probably related to their reactions with a variety of chemical species in the atmosphere, including trace amounts of water vapor. We propose the following reaction sequence:

$$\text{Si-}\dot{\text{O}} + H_2O \rightarrow \text{SiOH} + \dot{\text{O}}\text{H} \quad (1)$$
$$\text{Si-}\dot{\text{O}} + \dot{\text{O}}\text{H} \rightarrow \text{SiOOH} \quad (2)$$

Equation (1) indicates that the silicon-based radicals could react with water in biologic solutions to generate ȮH radicals. Results shown in figure 2 confirmed

TABLE 1

EFFECT OF GRINDING CRYSTALLINE SILICA ON THE RATE OF LIPID PEROXIDATION AND THE TIME-DEPENDENT LOSS OF LIPID PEROXIDATION POTENTIAL ON STORAGE IN AIR*

Silica (mg/ml)	Malondialdehyde Formation (μ mol) Time After Grinding 0–5 min	24 h	48 h	96 h
1.25	5.71 ± 0.70	4.72 ± 0.55 ($p < 0.03$)	1.95 ± 0.45 ($p < 0.01$)	2.03 ± 0.49 ($p < 0.01$)
2.5	6.48 ± 0.11	4.58 ± 0.36 ($p < 0.01$)	1.66 ± 0.26 ($p < 0.01$)	1.43 ± 0.14 ($p < 0.01$)
5.0	7.50 ± 0.63	4.34 ± 0.32 ($p < 0.01$)	1.96 ± 0.26 ($p < 0.01$)	1.54 ± 0.36 ($p < 0.01$)

* Data presented are the means ± SD of a minimum of four sets of experiments in duplicate. p values given are for differences compared to 0–5 min after grinding silica. Each experimental set used the same stock of freshly ground silica at various times after grinding.

TABLE 2

COMPARATIVE EFFECT OF SCAVENGERS IN THE PREVENTION OF CYTOTOXICITY INDUCED BY FRESHLY FRACTURED SILICA*

Scavenger	Percent Decrease in Comparison to Controls† Hemolysis	H_2O_2 Release	Lipid Peroxidation
SOD, 340 units/ml	30 ± 6	21 ± 1	49 ± 23
Catalase, 1,250 units/ml	80 ± 16	100 ± 0	52 ± 11
Sodium benzoate, 0.1 M	31 ± 10	26 ± 2	75 ± 8
DMSO, 0.1 M	1 ± 0.5	100 ± 0	100 ± 0
DMTU, 0.1 M	3 ± 2	37 ± 5	100 ± 0
Mannitol, 0.1 M	1 ± 0	100 ± 0	100 ± 0

* Data presented are the means ± SD of a minimum of two experiments in each assay.
† Assays were carried out in the presence of 10, 1, and 5 mg/ml for hemolysis, H_2O_2 release, and lipid peroxidation, respectively.

the formation of ȮH radicals when freshly crushed silica was suspended in aqueous solution. Because ȮH radicals are highly reactive toward biologic tissue (21, 29, 30), the generation of such radicals by fresh dust could have important implications regarding the effects of inhaled silica.

We note that SiOOH could be formed during the decay of silicon-based radicals (equation 2) and that SiOOH could be hydrolyzed to produce H_2O_2 according to the following reaction (12):

$$\text{SiOOH} + H_2O \rightarrow \text{SiOH} + H_2O_2 \quad (3)$$

This H_2O_2 could react with Fe^{2+} possibly present as a trace impurity in aqueous solution or on the silica particles themselves to generate ȮH radicals by the Fenton reaction as follows:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \dot{O}H \quad (4)$$

Our results show that DETAPAC, a strong metal chelator, had little effect on the DMPO-ȮH adduct ESR signal generated by freshly crushed silica in aqueous solution, i.e., there was little or no variation in the g value or the splitting pattern of the signal, with less than a 20% decrease in signal intensity even at an extremely high (3 mM) concentration of chelator. Because an iron-DETAPAC complex is unable to catalyze ȮH radical formation by equation 4 (24–27), these results indicate that the ȮH radicals detected were generated directly from the reaction of silicon-based radicals with water possibly according to reaction:

$$\text{Si} + H_2O_2 \rightarrow \text{SiOH} + \dot{O}H \quad (5)$$

This hypothesis is supported by our finding that addition of catalytic amounts of catalase, which disproportionates H_2O_2 and $\dot{O}_2^-$, suppressed the formation of ȮH from freshly ground silica.

Other investigators have shown that silica could peroxidize the lipids in biologic membranes (31–33). Our investigation confirms these results and, in addition, indicates that the ability of silica to peroxidize lipids was enhanced by grinding of silica (table 1). The parallelism of the time dependence of lipid peroxidation with decay behavior of the ȮH radical production by freshly ground silica (figure 3) leads us to propose that this enhanced ability may be related to the generation of ȮH radicals by the reaction of freshly ground silica with water. Because lipid peroxidation has been proposed as a mechanism for cellular damage (34–37), it is possible that some of the harmful effects of silica on membrane integrity, such as hemolysis (figure 6) and LDH release (figure 7), could be mediated by this mechanism.

Data presented in this study indicate that freshly ground silica is more biologically reactive than aged silica, i.e., freshly crushed silica induces a greater respiratory burst in alveolar macrophages (figures 4 and 5) and greater cytotoxicity (figures 6 and 7, table 1). The generation of silicon-based radicals on the silica surface and the formation of ȮH radicals in solution may partially explain the enhanced reactivity of freshly fractured silica. Another possibility is that silanol (SiOH) groups on the fractured surface are formed by the hydrolysis of silicon-based radicals (equation 1) and that these groups increase the interaction between dust and chemical sites on the cell membrane. The presence of the surface silanol groups has been verified by infrared spectroscopy (38). Cell membranes contain several sites, i.e., oxygen or nitrogen groups that have electron pairs that can form hydrogen bonds with silanols. For example, bonding between a secondary amide and silanol has been reported (39). The formation of such hydrogen bonds may bring the silica particle and cell membrane closer together and, thus, provide more favorable conditions for the intiation of lipid peroxidation or activation of the cell surface.

It should be noted that the silicon-based radicals generated by grinding (figure 1) and the resultant ȮH radicals formed in water (figure 3) decayed over the course of days. Although the reactivity (figures 4 and 5) and cytotoxicity (figures 6 and 7, table 1) of freshly ground silica also decreased with time, a substantial potency remained even after 4 days. In addition, silica dust aged for years still retained the ability to stimulate alveolar macrophages (40), decrease membrane integrity (33, 41), and cause lipid peroxidation (31–33). Therefore, silicon-based reactive oxygen species can only partly explain the biologic reactivity of silica. Indeed, we have found that superoxide dismutase, catalase, and sodium benzoate were only partially effective in decreasing lysis, in contrast to a significant or complete abrogation of H_2O_2 release and lipid peroxidation by several hydroxyl scavengers (table 2). It is in-

teresting to note that all the potent ȮH scavengers provided a significant protective effect in lipid peroxidation and H_2O_2 release except for DMTU, sodium benzoate, and SOD. This ability to ameliorate the fresh silica-induced lipid peroxidation and H_2O_2 release by several hydroxyl scavengers provides direct correlation between the hydroxyl radical generation and cellular injury. Therefore, it is our hypothesis that although aged silica is biologically reactive and cytotoxic, freshly fractured silica is more potent due to newly generated silicon-based radicals as well as the propagation of other oxygenated radicals in aqueous environment. These reactive radicals plus the enhanced levels of reactive forms of oxygen secreted from alveolar macrophages exposed to freshly crushed silica may result in oxidant loads that exceed the capacity of the defense mechanisms of lung tissue.

In conclusion, this study documents that respirable-size freshly ground silica contains silicon-based radicals that react with aqueous environments to produce ȮH radicals. The free-radical concentration and the biologic reactivity of freshly ground silica are higher than those of aged silica as measured by ESR and silica-induced $\dot{O}_2^-$ and H_2O_2 release from macrophages and by lipid peroxidation. We, therefore, propose that silicon-based radicals on silica and the resultant generation of ȮH radicals may play a significant role in cell membrane damage by initiation of lipid peroxidation through a chain reaction. This mechanism should be particularly relevant to the pathogenesis of acute silicosis where inhalation of fresh silica occurs, as in sandblasting, rock drilling, tunnelling, and silica flour mill operations.

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