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Acute Silica Toxicity: Attenuation by Amiodarone-induced Pulmonary Phospholipidosis

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Exposure to the toxic mineral dust silica has been shown to produce an acute inflammatory response in the lungs of both humans and laboratory animals. Coating silica with phospholipids reduces its toxicity when studied with *in vitro* systems. The drug amiodarone increases phospholipid within the cells, airways, and alveoli of the lungs. This increase in phospholipid is due to amiodarone's ability to inhibit phospholipase activity within alveolar macrophages (AMs) and whole lung. The purpose of this study was to determine whether the amiodarone-induced increase in pulmonary phospholipid would protect the lungs from acute damage caused by the intratracheal instillation of silica. Treatment of male Fischer 344 rats with amiodarone for 14 days caused an increase in phospholipid content in bronchoalveolar lavage fluid and AMs compared to vehicle-treated controls. The rats were then instilled with silica or saline vehicle. At both 1 and 14 days after silica exposure, pulmonary phospholipidosis was associated with a marked reduction in acute silica-induced pulmonary damage as assessed by biochemical parameters in bronchoalveolar lavage fluid, however; the influx of neutrophils into the airspaces was not reduced. Four times more phospholipid was bound to the silica recovered from amiodarone-treated rats compared to controls. The results of these *in vivo* experiments indicate that pulmonary phospholipidosis attenuates the acute damage associated with the intratracheal instillation of silica in rats. By using an *in vitro* cell culture system, we demonstrated that, in contrast to control AMs, phospholipidotic AMs were significantly more resistant to the cytotoxicity of surfactant-coated silica. We hypothesize that the attenuating effect of the phospholipidosis may be due to both an elevation in extracellular phospholipid in the airspaces as well as the ability of amiodarone to inhibit pulmonary phospholipases and thus prevent the enzymatic digestion of the phospholipid coating the silica. Through this inhibitory action, the retoxification of silica is inhibited and its cytotoxicity attenuated. **Key words:** acute lung damage, alveolar macrophages, amiodarone, bronchoalveolar lavage fluid, pulmonary phospholipidosis, silica. *Environ Health Perspect* 102: 372–378(1994)

Exposure of the lung to silica particles results in inflammation, damage to the respiratory epithelium and interstitial matrix, and fibrosis (1). Numerous studies in rats have shown that an inflammatory and pneumotoxic

response develops after acute intratracheal exposure to silica (2–4). This response is characterized as a rapidly progressive disease associated with extensive epithelial damage and abundant airway debris. Airspaces are filled with exudate composed of serum and lung proteins, surfactant lipids, and many inflammatory cells (5).

Amiodarone, an iodinated, benzofuran derivative with a cationic, amphiphilic structure, is approved for use in the treatment of life-threatening ventricular tachyarrhythmias. The administration of amiodarone to humans and laboratory animals has led to a pulmonary response characterized initially by the development of phospholipidosis (6–8). Morphologically, phospholipidosis appears as lysosomally derived lamellar inclusion bodies in many cells of the lungs, including AMs (9,10), endothelial and interstitial cells (6,11), bronchiolar epithelial cells (12), and alveolar type II cells (6,13). Of these cell types, the AMs appear to be the most susceptible to amiodarone treatment. As well as containing inclusions, these cells become hypertrophic, taking on a foamy appearance, and accumulating in elevated numbers in the alveoli. These altered cells have been referred to as "foamy AMs."

The pulmonary phospholipidosis induced by amiodarone is associated with impaired phospholipid catabolism. It has been demonstrated that this drug, and its principal metabolite desethylamiodarone, inhibit lysosomal phospholipase A₁ and A₂ activities (14–17). Due to this disruption in phospholipid breakdown, treatment of rats with amiodarone significantly increases the concentration of total phospholipid in whole lung and AMs (9,18,19). In our previous studies (9,19), it was shown that this development of phospholipidosis was both dose and time dependent as well as reversible.

Vallyathan et al. (20) have demonstrated that the surface properties of the silica particles may be responsible for the cytotoxicity caused by exposure to silica. A variety of substances such as phospholipid, organosilane, aluminum lactate, and polyvinylpyridine-*N*-oxide, have been used in a number of *in vitro* studies in an attempt to coat the silica and reduce its cytotoxicity (21–25). Based on these *in vitro* studies, our hypothe-

sis was that the increase in phospholipid levels within the lungs after the induction of phospholipidosis will protect them from damage caused by the exposure to cytotoxic particles.

In this study, we induced pulmonary phospholipidosis in rats using amiodarone and determined that this increase in intracellular or extracellular phospholipid reduced the acute toxic response in the lungs when challenged intratracheally with silica dust. In the measurement of the inflammatory response of silica for our study, a number of cellular and biochemical endpoints in the bronchoalveolar lavage fluids were measured. Lavage of the bronchoalveolar region collects epithelial lining fluid of the conducting airways and alveoli, the sites of pulmonary contact for instilled silica particles (4). Previous studies have indicated analysis of the bronchoalveolar lavage fluid is a sensitive means of characterizing acute inflammatory responses within the lungs (26–28).

In this investigation, we also attempted to determine the mechanism by which pulmonary phospholipidosis protects the lungs from silica damage. To simulate our *in vivo* experiments in this study, we used an *in vitro* system where viability of amiodarone-induced foamy AMs and normal, control AMs was measured after incubation with native silica or silica treated with Survanta, a commercially available pulmonary surfactant. Survanta is a natural bovine lung extract containing phospholipids, neutral lipids, fatty acids, and surfactant-associated proteins (29).

Materials and Methods

Amiodarone was a gift from Wyeth-Ayerst Research (Princeton, New Jersey). Crystalline Min-U-Sil silica (U. S. Silica Corp., Berkeley Springs, West Virginia) was a gift from Val Vallyathan, National

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Institute for Occupational Safety and Health. Purity of the silica was determined by automated X-ray diffractometry and was 99.5% alpha quartz. Size fraction of <5 µm in diameter was made by a centrifugal airflow particle classifier. Of this fraction, 98% was <5 µm with a median area equivalent diameter of 3.5 µm as estimated by automated scanning electron microscopic image analysis. Survanta (Ross Laboratories, Columbus, Ohio) samples were supplied by the West Virginia University Department of Pharmaceutical Services. It is the policy of the pharmacy that, after use in human patients, any remaining reconstituted samples of Survanta in opened vials is discarded. The Survanta samples used in this investigation represented unused drug that was ready to be discarded. After use by the pharmacy, the unused surfactant samples were frozen at -20°C and stored until ready for experimentation. Enzyme reagents were purchased from Sigma Chemical Company (St. Louis, Missouri). Other chemicals used in this study were from Fisher Chemical Company (Pittsburgh, Pennsylvania).

The experimental design will be described briefly, then detailed in subsequent sections. Male Fischer 344 rats were treated by oral gavage with amiodarone or water (pair-fed controls) for 14 days. We intratracheally instilled silica suspended in saline into one-half of each of the two treatment groups. The remaining animals received an intratracheal instillation of the saline vehicle. Thus, for these experiments, four study groups were used. The groups and the designations used in this study are as follows: 1) amiodarone-treated + silica: A-SI; 2) water-treated + silica: W-SI; 3) amiodarone-treated + saline: A-SA; and 4) water-treated + saline: W-SA. Bronchoalveolar lavage was performed on the animals 1 and 14 days after the instillation exposures. We assessed three basic indicators of pulmonary damage: a total and differential cell count to characterize the cellular aspects of inflammation; total protein to quantify increased permeability of the bronchoalveolar-capillary barrier; and the activity of the lysosomal enzyme β-glucuronidase to detect activated or lysed phagocytes. To ensure that a phospholipidotic condition was induced by the amiodarone treatment, the total phospholipid content of the cells and lavage fluid recovered from the lungs was also measured.

We obtained Male Fischer 344 rats weighing 200–250 g from Hilltop Laboratories (Scottsdale, Pennsylvania). Rats were given a conventional laboratory diet (Purina Chow Pellets) and tap water *ad libitum* during a 5-day acclimation period. We then treated the rats for 14 days with 150 mg amiodarone/kg of body weight, *per os*, suspended in water (2 ml/kg body

weight). Along with treated animals, an equal number of control rats were dosed with water in equivalent volumes for 14 days. Food was restricted from the control rats to maintain comparable weights between the groups because amiodarone treatment causes a reduction in weight gain. At the time the animals were lavaged, the weights of all the animals in the study were within 30 g of each other. To maintain the phospholipidotic condition, the amiodarone (and water-control) treatments continued after the intratracheal instillations of silica or saline vehicle until the animals were lavaged.

We measured drug levels in the AMs recovered from the animals treated for 14 days with amiodarone using HPLC as described by Reasor et al. (9). For the phospholipid assay, samples of cell-free bronchoalveolar lavage fluid and alveolar macrophages recovered from the animals treated for 14 days with amiodarone were extracted with chloroform/methanol (2/1, v/v) as described by Folch et al. (30). Total lipid phosphorous in the cells and the bronchoalveolar lavage fluid was quantified by the method of Ames and Dubin (31) following ashing in 10% MgNO₃ in 70% ethanol to liberate inorganic phosphorous from the lipid.

Before silica was instilled in the animals, it was cleaned by boiling in 1.0 M HCl for 60 min. The silica was suspended and sonicated for 15 min in 0.9% sterile saline at a concentration such that each rat received a constant dose volume of 0.5 ml per rat. One-half of all rats used in this study were dosed intratracheally with a single instillation of 2.5 mg or 10 mg/100 g body weight of silica with a mean particle size of <5 µm. In some preliminary studies, these doses were shown to cause significant acute toxicity. The remaining animals were instilled with an equal volume of sterile saline (vehicle control). We lightly anesthetized the rats by intraperitoneal injection of sodium methohexital, and using a modified laryngoscope to illuminate the larynx, intratracheally instilled each rat with either the saline containing silica or the saline vehicle.

On days 1 or 14 after instillation exposure, bronchoalveolar lavage was performed on the animals. The rats were deeply anesthetized with an overdose of sodium pentobarbital and exsanguinated by severing the abdominal aorta. The lungs of each rat were first lavaged with one separate aliquot of warm, calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS), pH 7.4, which was left in the lungs for 30 sec, aspirated, reinstalled for an additional 30 sec and then withdrawn. We used a volume of 2.0 ml/100 g of animal body weight for this lavage to take

into account any variations in body weights of the rats lavaged. This lavage sample was centrifuged at 500g for 7 min, and the resultant cell-free supernatant was analyzed for various biochemical parameters. Additionally, the lungs were further lavaged 10 times with 5-ml aliquots of HBSS. These samples were also centrifuged for 7 min at 500g and the cell-free lavage fluid discarded. The cell pellets from all washes for each rat were combined, washed, and resuspended in 2 ml of HBSS buffer. We then counted and differentiated the cells.

Total cell numbers were determined using a hemacytometer. Using a Shandon cytopsin centrifuge, 1.5×10^5 cells were spun for 4 min at 400 rpm and pelleted onto a slide. Cells (200/rat) were differentiated on the cytocentrifuge-prepared slides after staining with Wright Giemsa Sure Stain (Fisher Scientific, Pittsburgh, Pennsylvania). AMs, neutrophils, and lymphocytes were counted.

We determined the protein content of cell-free bronchoalveolar lavage fluid samples by the method of Hartree (32) using bovine serum albumin as the standard. We assayed β-glucuronidase activity in the cell-free bronchoalveolar lavage fluid by the method of Lockard and Kennedy (33).

To determine the amount of phospholipid bound to silica in the lungs of control and phospholipidotic rats, control and amiodarone-treated rats were intratracheally instilled with a single 10 mg/100 g body weight dose of silica as described above. Bronchoalveolar lavage was performed on the animals 1 day after the silica instillations. We used HBSS to lavage cells and silica from the lungs of the animals. A total of 80 ml of lavage fluid was recovered from each animal and centrifuged at 100g for 3 min. The resulting pellet for each rat was washed and resuspended in 0.5 ml HBSS. The cell/silica suspension was placed on top of a 1.0 ml dibutyl phthalate (d = 1.080 g/ml) cushion in a microfuge tube. To separate the silica from the cells, we spun the suspensions in a Beckman Microfuge (Beckman Instruments, Palo Alto, California) at a setting of 2 for 30 sec. The lavage cells remained on top of the cushion, while the silica particles formed a pellet on the bottom of the tube. The cushion was drawn off by a Pasteur pipette. The pellet was washed with saline twice. A 2:1 volume/volume mixture of chloroform and methanol was added to the pellet and vortexed for 30 sec to extract the phospholipid from the silica. We included a blank which contained 20 mg silica not instilled in the animals. The samples were then spun in the microfuge for 5 min at a setting of 10. For analysis of total phospholipid associated with the silica, 100-µl

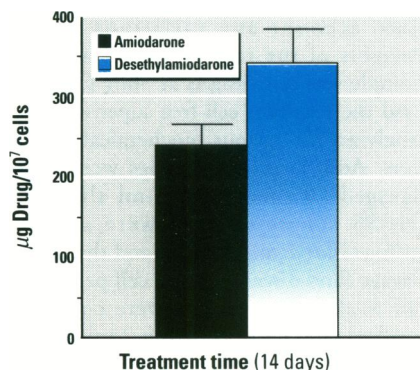


Figure 1. The accumulation of amiodarone and its principal metabolite, desethylamiodarone, from cells recovered from the lungs of rats. Animals were treated for 14 days with an oral 150 mg/kg daily dose of amiodarone. Values are means \pm SE ($n = 4$).

aliquots of the supernatant were added to glass tubes in duplicate and dried overnight. We quantified total lipid phosphorous by the method of Ames and Dubin (31) as described above. An empty microfuge tube was weighed before separating the silica on the phthalate cushion. The phthalate was removed, the lipid was extracted from the silica, and the silica was dried. We then weighed the microfuge tube with the silica peller. The previous weight of the empty microfuge tube was subtracted from this value for the measurement of the amount of silica recovered from the lungs of the animals. The phospholipid content of each sample was determined as $\mu\text{mol/mg silica}$.

In Vitro Cell Viability Study

The rats were treated daily for 14 days with 150 mg amiodarone or water/kg of body weight, *per os* (pair-fed controls), as described above. AMs were recovered from control and amiodarone-treated animals by pulmonary lavage. The cells were resuspended in sterile culture medium (34) and plated at a concentration of 1.0×10^6 cells/tissue culture well. We incubated AMs from amiodarone-treated and control animals with a 0.5 mg/ml concentration of native silica or silica coated with the Surfactant. The surfactant-treated silica was prepared by suspending the silica in Surfactant and incubating for 1 hr at 37°C. The excess surfactant was removed from the silica by centrifugation at 500g for 10 min according to the method of Wallace et al. (35). The supernatant was removed, the silica was washed with HBSS by centrifugation, and then resuspended in HBSS. The amount of surfactant phospholipid bound from this procedure was $0.012 \pm 0.002 \mu\text{mol/mg silica}$ ($n = 4$).

We incubated cells with the native or surfactant-coated silicas for 24, 48, and 72 hr at 37°C in a tissue incubator containing

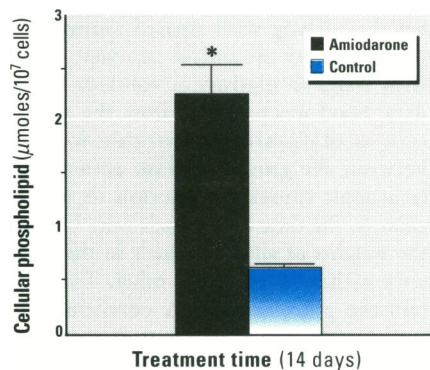


Figure 2. Total phospholipid content of the cells recovered from the lungs of control and amiodarone-treated rats. Animals were treated for 14 days with an oral 150 mg/kg daily dose of amiodarone. The pair-fed control animals received water. Values are means \pm SE ($n = 6$). Mean value of the phospholipid content of the amiodarone-treated animals was significantly greater than control value (* $p < 0.05$).

air and 5% carbon dioxide. After incubation, a 0.10% final concentration of trypsin was added to each well, and the plates were incubated for 5 min to remove attached alveolar macrophages. Cell viability was measured on aliquots from each well by trypan blue exclusion.

Statistical Analysis

Comparisons between the phospholipid levels of the two treatment groups (Figs. 2, 3, 8) were made by the Student's *t*-test analysis. For Figures 4–7 and Table 1, comparisons were made among the different treatment groups used. The significance of the interaction among the treatment groups was analyzed by using two-way analysis of variance for each time point at each dose. If significance was observed, the significance between each of the individual groups was analyzed by one-way analysis of variance using a Duncan's

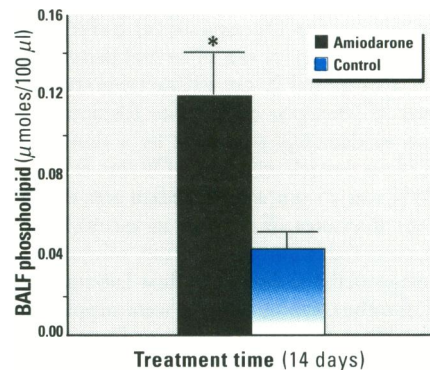


Figure 3. Total phospholipid content of the acellular bronchoalveolar lavage fluid (BALF) recovered from the lungs of control and amiodarone-treated rats. Animals were treated for 14 days with an oral 150 mg/kg daily dose of amiodarone. The pair-fed control received water. Values are means \pm SE ($n = 6$). Mean value of the phospholipid content of the amiodarone-treated animals was significantly greater than control value (* $p < 0.05$).

Multiple Range Test. The criterion for significance was $p < 0.05$.

Results

The levels of amiodarone and its principal metabolite desethylamiodarone, were measured in the AMs recovered from the lungs of animals treated for 14 days with 150 mg/kg/day of amiodarone (Fig. 1). The 14-day amiodarone treatment led to substantial accumulation of both amiodarone and desethylamiodarone in AMs. Treatment for 14 days with amiodarone resulted in a fourfold increase in total phospholipid in the cells (Fig. 2) and a threefold increase in total phospholipid in the extracellular lavage fluid (Fig. 3) recovered from the lungs when compared with control animals that received water vehicle.

Treatment with silica alone (W-SI) at the two doses used in this study caused a significant increase in the total protein lev-

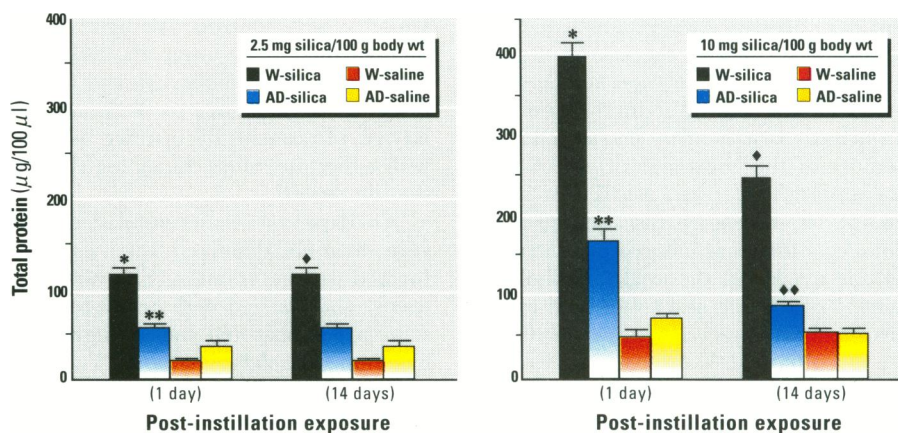


Figure 4. Total protein content of the cell-free bronchoalveolar lavage fluid from the lungs of rats 1 and 14 days after silica instillation (2.5 mg or 10 mg/100 g body weight). Half of the animals were pretreated for 14 days with an oral 150 mg/kg daily dose of amiodarone (AD). The control animals received water (W). Silica or saline vehicle then were instilled intratracheally and AD administration continued. Values are means \pm SE ($n = 4-8$). At each time point for each silica dose used, groups with different symbols are significantly different ($p < 0.05$).

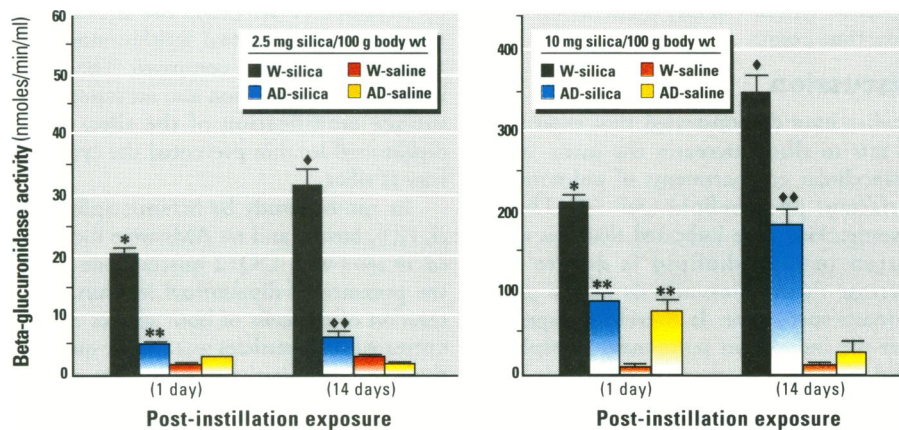


Figure 5. β -Glucuronidase activity of the cell-free bronchoalveolar lavage fluid from the lungs of rats 1 and 14 days after silica instillation (2.5 mg or 10 mg/100 g body weight). Half the animals were pretreated for 14 days with an oral 150 mg/kg daily dose of amiodarone (AD). The control animals received water (W). Silica or saline vehicle then were instilled intratracheally and AD administration continued. Values are means \pm SE ($n = 4-8$). At each time point for each silica dose used, groups with different symbols are significantly different ($p < 0.05$).

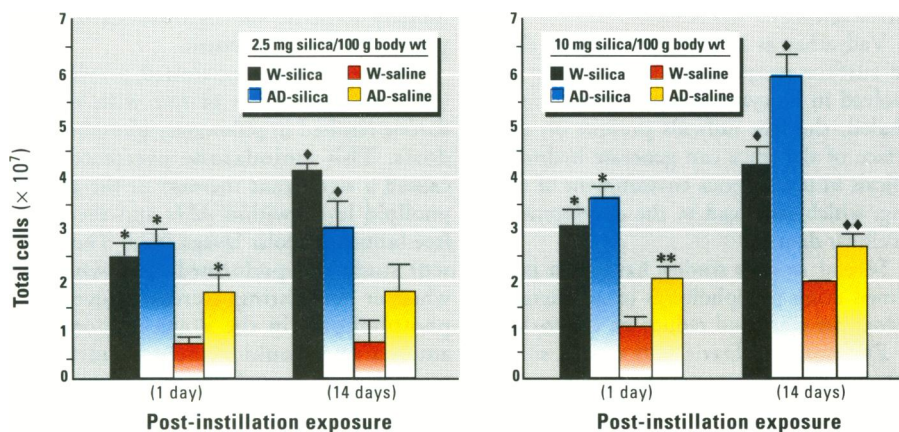


Figure 6. Total number of cells recovered from the lungs of rats 1 and 14 days after silica instillation (2.5 mg or 10 mg/100 g body weight). Half the animals were pretreated for 14 days with an oral 150 mg/kg daily dose of amiodarone (AD). The control animals received water (W). Silica or saline vehicle then were instilled intratracheally and AD administration continued. Values are means \pm SE ($n = 4-8$). At each time point for each silica dose used, groups with different symbols are significantly different ($p < 0.05$).

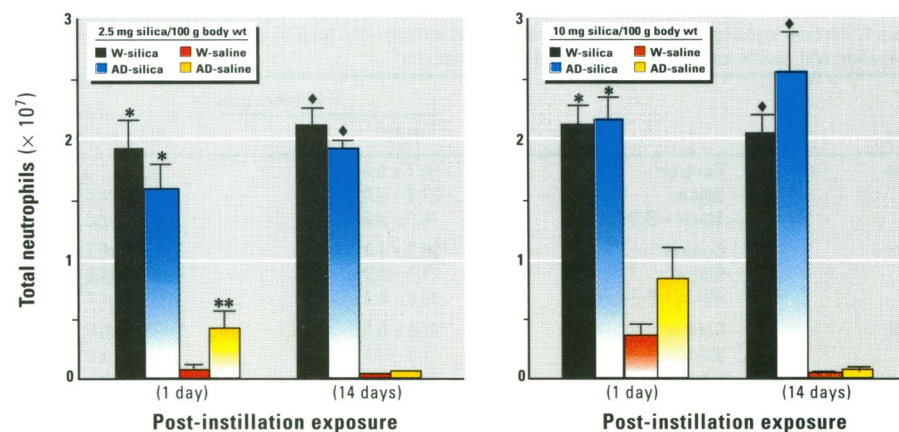


Figure 7. Total number of neutrophils recovered from the lungs of rats 1 and 14 days after silica instillation (2.5 mg or 10 mg/100 g body weight). Half the animals were pretreated for 14 days with an oral 150 mg/kg daily dose of amiodarone (AD). The control animals received water (W). Silica or saline vehicle then were instilled intratracheally and AD administration continued. Values are means \pm SE ($n = 4-8$). At each time point for each silica dose, groups with different symbols are significantly different ($p < 0.05$).

els of the cell-free bronchoalveolar lavage fluid 1 and 14 days after exposure (Fig. 4). This increase was reduced significantly at both time points in the A-SI group of animals, which were pretreated with amiodarone and were then instilled with silica. The protein levels of the group that received both amiodarone and the high dose of silica were, however, still elevated significantly above the two control groups (A-SA and W-SA). In contrast, when using a 2.5 mg/100 g body weight dose of silica and measuring the response 14 days after exposure, the amiodarone-induced phospholipidosis completely prevented the increase in protein in bronchoalveolar lavage fluid caused by the silica.

The W-SI groups, which received silica alone at both doses, had significantly elevated levels of β -glucuronidase activity within the cell-free bronchoalveolar lavage fluid 1 and 14 days after exposure compared to the W-SA control group (Fig. 5). The presence of the amiodarone-induced phospholipidosis (A-SI group) attenuated the silica-induced rise in β -glucuronidase activity from both doses. This reduction in β -glucuronidase activity was greatest at the low silica dose group, although in neither group was it complete. One day after the instillation exposure, the A-SA group that received both amiodarone and saline had a significantly elevated level of β -glucuronidase activity.

Mean total cell counts for all treatment groups are presented in Figure 6. Compared to the W-SA group, significant increases in total cell numbers were observed for all time periods and dose regimens in the A-SI and W-SI treatment groups. Increases were also seen in the A-SA group of animals except at 14 days in the low silica dose experiment. These findings indicate that both the silica and amiodarone treatment can individually increase the number of cells in the lavage fluid of rats. The cell number from combined amiodarone and silica treatments (A-SI group) was not different from the W-SI group of animals.

Compared to the W-SA groups, neutrophils were significantly increased in the AD-SI and W-SI groups of animals at both time points and silica doses (Fig. 7). This influx of neutrophils in the W-SI and A-SI groups accounted for nearly all the increases in total cells. A significant increase was also seen in the A-SA group 1 day after the instillation exposure when compared to the W-SA group for the small dose silica group.

In analysis of other cell types, the W-SI group also had significantly increased numbers of lymphocytes in the lavage fluid compared to the W-SA control (data not shown). Amiodarone pretreatment did not

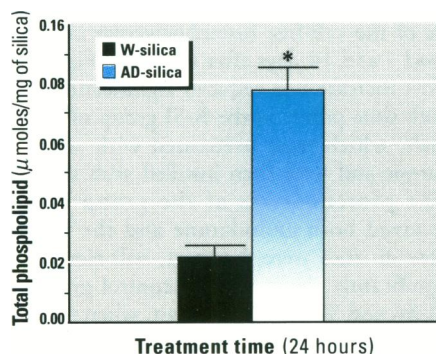


Figure 8. Total phospholipid bound to silica particles recovered from the lungs of control and amiodarone-treated rats. Animals were treated for 14 days with an oral 150 mg/kg daily dose of amiodarone (AD). The control animals received water (W). Silica (10 mg/100 g body weight) then was intratracheally instilled into each animal of both groups. Values are means \pm SE ($n = 6$). The mean value of the phospholipid bound to silica from the AD-silica group was significantly greater than the value of the W-silica group (* $p < 0.05$).

protect against this response. No consistent response pattern was observed when evaluating the change in the number of AMs (data not shown).

To measure the amount of phospholipid bound to the silica, control and amiodarone-treated animals were intratracheally instilled with silica (10 mg/100 g body weight). One day after the instillation, there was nearly a four-fold greater amount of phospholipid associated with the silica recovered from the animals that had developed amiodarone-induced pulmonary phospholipidosis when compared with control animals (Fig. 8).

In Vitro Cell Viability Study

In an attempt to determine the mechanism by which amiodarone-induced pulmonary phospholipidosis protects the lungs from the damage caused by silica, an *in vitro* experiment was performed. AMs from amiodarone-treated and control animals were incubated for 24, 48, and 72 hr with native silica or silica treated with Surfactant (Table 1). When incubating the AMs from both treatment groups without silica for all three time points, cell viability was greater than 97%, and no significant differences were observed between the cells from the two treatment groups. When the cells were exposed to native silica, a dramatic and equal loss of viability was demonstrated in the control and phospholipidotic AMs at each of the three time points. In the cells from the control group, the silica treated with surfactant caused an exposure time-dependent reduction in cell viability of 21%, 51%, and 68% at 24, 48, and 72 hr, respectively. The silica treated with surfactant caused significantly less cytotoxicity to the phospholipidotic AMs. A 7–10%

reduction in cell viability occurred for the three time points.

Discussion

Studies have demonstrated that exposure of rats to silica increases the intra- and extracellular compartments of pulmonary surfactant phospholipid (36–38). These investigations have indicated that this elevation in phospholipid is due to an increase in the biosynthesis of the pulmonary surfactant. It therefore appears that the increase in pulmonary phospholipid may be a mechanism by which the lungs protect themselves when challenged with silica. Clearly, an elevation in surfactant alone is inadequate to protect against toxicity, as massive damage occurs while phospholipid levels are elevated (4,38); however, by increasing the phospholipids within the cells and airways within the lungs before the insult, it may be possible to reduce the damage caused by the exposure to silica.

Vallyathan et al. (20) hypothesized that the surface properties of silica may be involved in its cytotoxicity. When silica is inhaled, the free radicals present on the surface of the silica can generate hydroxyl radicals in the aqueous environment of the lung, which may lead to the development of cellular damage.

Several *in vitro* studies have been performed using phospholipids in an attempt to coat the silica and reduce its cytotoxicity. Emerson and Davis (21) coated silica with alveolar lining material and compared the cytotoxicity produced with uncoated silica. They demonstrated that the coated silica was effectively phagocytized by the AMs but was less cytotoxic than uncoated silica. Consequently, they concluded that inhaled silica particles that become coated by surfactant lipids may reduce or delay the AM toxicity. In another *in vitro* study

by Wallace et al. (22), the biological responses of dipalmitoyl lecithin-modified and native silica were compared. The findings of this investigation also indicated that surface modification of the silica with dipalmitoyl lecithin prevented the cytotoxicity of silica.

In another study by Schimmelpfeng et al. (25), bovine and rat AMs were incubated *in vitro* with DQ12 quartz alone or in the presence of dipalmitoyl lecithin. The reaction of the cells of both species to the untreated dust particles was similar qualitatively and quantitatively, with a loss of viability and release of lactate dehydrogenase after incubation. In the presence of dipalmitoyl lecithin, the toxicity of quartz to the bovine AMs disappeared completely, while the rat AMs were also protected, but to a lesser degree. While such *in vitro* experiments are consistent with the hypothesis that surfactant may play a protective role against silica-induced pulmonary toxicity, the significance of this process *in vivo* is unknown.

As we have demonstrated previously (9,19,39), treatment of rats with amiodarone resulted in pulmonary phospholipidosis. This amiodarone pretreatment caused a significant increase in the phospholipid levels within AMs and the cell-free bronchoalveolar lavage fluid. The present study was performed to investigate whether preexisting increased levels of phospholipids in the lungs induced by amiodarone would protect them from acute damage caused by the intratracheal instillation of silica.

Within the bronchoalveolar lavage fluid, a variety of cellular and biochemical parameters were measured to characterize the acute inflammatory response caused by silica. Markers of pulmonary damage within the acellular component of the lavage fluid were assayed. Total protein and β -glu-

Table 1. Percent viability of alveolar macrophages from amiodarone-treated and water-treated rats after incubation with native silica and silica treated with surfactant^a

Time		Treatment groups	
		H ₂ O ^b	Amiodarone ^b
24 hr	Control	98.7 \pm 0.6	98.5 \pm 1.0
	Silica	21.2 \pm 2.8*	28.2 \pm 4.6*
	Silica + Surfactant	78.9 \pm 2.9**	92.7 \pm 2.6***†
48 hr	Control	98.3 \pm 1.1	98.8 \pm 0.8
	Silica	21.5 \pm 3.7*	23.4 \pm 4.8*
	Silica + Surfactant	48.8 \pm 4.4**	92.3 \pm 1.4***†
72 hr	Control	97.5 \pm 0.5	99.3 \pm 0.5
	Silica	7.9 \pm 3.1*	12.3 \pm 2.8*
	Silica + Surfactant	32.6 \pm 3.9**	90.3 \pm 2.3***†

^aSee text for methods.

^bValues are means \pm SE ($n = 4-5$). At each time point for the cells from both treatment groups, the mean values of the percent viability for the two silica groups are significantly different than the values of their respective controls and the silica + Surfactant groups (* $p < 0.05$); the mean values of the percent viability of the two silica + Surfactant groups are significantly different from their respective controls (** $p < 0.05$); and the mean values of the percent viability of the two silica + Surfactant groups are significantly different from each other († $p < 0.05$).

curonidase activity were analyzed. Total and differential cell counts were also evaluated on the cells recovered from the lungs. The pneumotoxic response we demonstrated with the silica instillation was consistent with the study of Lindenschmidt et al. (4). As in that study, pulmonary damage, as measured biochemically, was still present 14 days after the silica instillations. When using silica doses of 2.5 mg and 10 mg/100 g body weight, the alveolar phospholipidosis produced by amiodarone pretreatment markedly attenuated the elevations of the acellular lavage parameters indicative of damage after silica instillation. This protection, which was virtually complete with the low silica dose, was consistent 1 and 14 days after the silica exposure for total protein and β -glucuronidase activity.

In the assessment of the cellular parameters of the lavage fluid, the silica instillation, at both doses, caused an increase in the number of cells recovered from the lungs at the two time points after the exposure. This elevation in cell number was due largely to the influx of neutrophils into the lungs. Although the increased phospholipids within the lungs reduced the biochemical indices of damage caused by silica, it had no effect in preventing this infiltration of neutrophils. In the study by Lindenschmidt et al. (4), aluminum oxide and titanium dioxide increased the number of neutrophils in the lungs, but caused a significantly lower degree of pneumotoxicity when compared with silica. As there were no differences in the cellular response of the two silica groups used in this study, it appears the silica is reaching the same areas within the airspaces of the lung. This indicates that the protective effect of the phospholipidosis is probably not due to a difference in distribution of silica in the lungs as a result of this condition.

Neutrophils appear within the pulmonary interstitium and the bronchoalveolar lavage fluid within a day after the intratracheal instillation of silica. Chemotactic substances released from AMs are a likely explanation for the attraction of neutrophils to the lungs. The presence of neutrophils in the lungs is important because of their potent ability to cause lung tissue injury (5). Thus, it appears that the chemotactic signal which recruits the neutrophils into the lungs was unaffected by phospholipidosis. Given that neutrophils have been implicated in silica-induced lung injury (40), it is conceivable that treatment with amiodarone either directly or indirectly renders them less active.

Pulmonary surfactant, which is 90–95% phospholipid, forms an insoluble film on the surface of the alveoli of the lungs (41). It is possible that an increase in the amount of phospholipid coating the silica

particles in the phospholipidotic lungs may be involved in the protection against acute silica damage. To further investigate this idea, silica was intratracheally instilled into the lungs of either control rats or amiodarone-treated rats that had developed pulmonary phospholipidosis. We found that there was a fourfold greater amount of phospholipid associated with the silica removed from the lungs that had developed phospholipidosis when compared with normal lungs. However, there was still a measurable amount of phospholipid bound to the silica recovered from the control lungs. Therefore, the presence of a phospholipid coating alone on silica is inadequate to protect against damage.

It may be that a silica particle that has adsorbed surfactant is phagocytized by the AMs without an immediate cytotoxic effect. The coated particle then may be subjected to phospholipase activity associated with the AMs. These enzymes have been shown to be active on components of surfactant. Hostetler et al. (42) indicated that phospholipases can hydrolyze phospholipids, such as dipalmitoyl lecithin, present in surfactant. Thus, the removal of the phospholipid coating of the silica by phospholipases may retoxify the silica, resulting in damage to the AMs.

When AMs were incubated for 24, 48, and 72 hr with native silica, the control and phospholipidotic AMs were equally sensitive to the cytotoxic action of the particles. Therefore, the presence of elevated phospholipid within the phospholipidotic cells provided no protection against the cellular injury caused by silica. The phospholipidotic cells were, however, more resistant to the cytotoxic action of the surfactant-coated silica than were the control AMs. When exposed to surfactant-coated silica, control cells lost viability in a time-dependent manner, while the phospholipidotic cells were virtually unaffected over the 3-day period. It may be that over the exposure period, control alveolar macrophages digest a portion of the surfactant from the surface of the silica, in effect retoxifying the particles, resulting in loss of cellular viability. Thus, the presence of a surfactant coating on the silica particles would delay, but not prevent, the cytotoxicity of silica. Within the phospholipidotic cells, the inhibition of lysosomal phospholipases by amiodarone and desethylamiodarone effectively prevents digestion of adsorbed surfactant from the silica, thus preventing retoxification from occurring. This scenario would be consistent with the reduced cytotoxicity of surfactant-coated silica toward the phospholipidotic cells and offers an explanation for the protective effect of the phospholipidosis against the acute toxicity of silica *in vivo*.

The significance of phospholipase inhibition is further illustrated by the fact that the amount of surfactant bound to the silica in this experimental *in vitro* condition was less than we measured on silica recovered from control animals where damage was the greatest. This would suggest that the increased quantity of phospholipid bound to the silica in the phospholipidotic rats may be of less significance than the cellular phospholipase inhibition in protecting against acute silica toxicity in this model.

In a recent editorial, Hook (43) speculated whether pulmonary surfactant plays a role in the defense of the lungs. The results of this study would support the idea that a sustained elevation in acellular phospholipid in the airspaces and alveoli occurring before silica exposure may delay the acute toxicity of this dust.

The results of this study, however, are only applicable for silica's acute toxic response. The influence of the reduction in the initial pneumotoxic response to silica by the phospholipidosis on the eventual development of fibrosis is unknown.

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