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Phospholipid surfactant adsorption by respirable quartz and in vitro expression of cytotoxicity and DNA damage

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Abstract

Respirable-sized quartz was treated with a saline dispersion of dipalmitoyl phosphatidylcholine (DPPC), a primary component of pulmonary surfactant, to model the adsorption of phospholipid surfactant onto quartz dust following particle deposition in the bronchoalveolar region of the lung. Control and surfactant-treated dusts were used to challenge lavaged rat pulmonary macrophages in vitro over a 1-week period, to determine the effects of adsorbed surfactant on the expression of quartz cytotoxicity and genotoxicity. DNA damage was determined by the single cell gel electrophoresis 'comet' assay. Untreated quartz induced DNA damage, increasing with dose and with time of incubation of dust with macrophages over a 5 day period. DPPC treatment of quartz suppressed DNA damage through 1 day of macrophage challenge. DNA damage then increased over a 5 day period, to approximately half the positive control (untreated quartz) values. Cytotoxicity was measured by trypan blue dye exclusion and by the Live-Dead® fluorescence assay for cell viability. Cytotoxicity of surfactant-treated quartz measured one day after challenge of lavaged macrophages was suppressed to values near those of the negative controls, and then increased over a 1 week incubation period to levels near those expressed by native quartz positive controls. Quartz similarly treated with dioleoyl phosphatidylcholine mixed with DPPC substituted in one acyl group with a boron-containing fluorescent chromophore was used with confocal microscopy to measure particle-associated fluorescent surfactant in cells. Approximately half of the fluorescence intensity was lost over a 1 week period following challenge of lavaged macrophage. Results are discussed in terms of a model of restoration of quartz particle surface toxicity as prophylactic surfactant is removed from particle surface by cellular enzymatic digestion processes.

Keywords: Quartz; Phospholipid surfactant; Phospholipase; Alveolar macrophage; DNA damage; Cytotoxicity; Fluorescence microscopy

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1. Introduction

Upon deposition in a pulmonary alveolus, respired particles contact the surfactant-rich hypophase on the epithelial surface. Diacyl glycerophosphatidylcholines are phospholipids which are major components of pulmonary surfactant. Dipalmitoyl phosphatidylcholine (DPPC) in aqueous dispersion will adsorb onto the surface of respirable sized quartz particles and suppress their cytotoxicity (Marks, 1957; Wallace et al., 1985). Cell-free and in vitro cellular systems have been used to investigate the possibility of phospholipase enzymatic digestion of quartz-adsorbed DPPC resulting in surfactant removal and restoration of dust surface cytotoxicity (Wallace et al., 1992; Liu et al., 1997). This study investigated the time course over a 1 week period, at 2 day intervals, of restoration of cytotoxicity and genotoxicity of DPPC surfactant-treated quartz for rat pulmonary macrophages in vitro, and of the loss with time of the surfactant coating on quartz particles within macrophages in vitro. DNA damage was measured using the single cell gel electrophoresis assay. Cytotoxicity was measured using the trypan blue dye exclusion assay and the Live-Dead® fluorescent assay for cell viability. Quartz incubated with dioleyl phosphatidyl choline with 5–10% fluorescent chromophore-labelled phosphatidylcholine was used for fluorescent monitoring of particle loss of surfactant within cells.

2. Materials and methods

2.1. Quartz dust

Quartz used was commercially obtained as 'Min-U-Sil 5', which is a high-purity, small, crystalline silica containing 99% alpha quartz with 95% of particles smaller than 5 μm.

2.2. Surfactant

Dipalmitoyl phosphatidylcholine (DPPC; Calbiochem, San Diego, CA) was ultrasonically dispersed into 0.165 M NaCl physiologic concentration salt solution (PSS) as 5 mg DPPC/ml PSS. The mixture was sonicated at 72 W applied to 40 ml for 10 min, followed by centrifugation at 1500 *g* for 10 min to remove non-dispersed DPPC. Quartz was mixed with this dispersion at a ratio of 0.1 g surfactant/g of dust, a relative concentration providing surfactant bilayer coverage of the quartz (Wallace et al., 1992).

2.3. Cells

Pulmonary macrophages were obtained by lung lavage from male, $\approx$ 200 g, Sprague–Dawley rats. Animals were anesthetized with sodium pentabarbitol, exsanguinated at the renal artery, and lavaged with 80 ml of Ca^{2+}—and Mg^{2+}—free Hank's balanced salt solution (HBSS; Life Technologies, Baltimore, MD). Cell suspensions were centrifuged for 10 min at 600 *g*, the cell pellet was resuspended in 1 ml RPMI 1640 with 10% heat-denatured fetal calf serum (Sigma) and 2% penicillin-streptomycin solution (Life Technologies, Baltimore, MD). A 20 μl aliquot of the cells was mixed with 200 μl of trypan blue solution (Sigma) and 400 μl of PBS, and the cells counted using a hemacytometer The concentration of the cell suspension was adjusted to 5×10^6 cells/ml complete medium.

2.4. Single cell gel electrophoresis assay for DNA damage

Cells were plated with a density of 2.5×10^6/dish into 60 mm tissue culture dishes with 5 ml of RPMI 1640 medium with 25 mM HEPES buffer and L-glutamine (Life Technologies, Baltimore, MD), supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO), 100 units penicillin/ml, and 100 μg streptomycin/ml. Cells were cultured overnight before treatment. Macrophages were challenged with native and DPPC-coated quartz at the designed concentrations and incubated for 3 h, or for 1, 3, or 5 days. The medium was changed on the second day of treatment. Native quartz was used as a positive control. Cell suspensions were centrifuged for 10 min at 4°C, 300 *g*. Supernatants were discarded and cells were resuspended to an approximate density of 2×10^6/

Table 1
DNA migration for macrophages treated with native and DPPC-treated quartz for 3 h to 5 days

Treatment time	Concentration (μg/cm^2)	Tail length ($\bar{x} \pm$ SE μm)	
		Quartz	Quartz/DPPC
3 h	0	6.54 ± 1.55	3.90 ± 1.11
	20	12.37 ± 2.14*	5.34 ± 1.67
	40	19.77 ± 2.85**	5.39 ± 1.26
	80	30.99 ± 3.65**	4.61 ± 1.22
1 day	0		7.33 ± 1.21
	10		7.50 ± 1.12
	20		7.47 ± 0.95
	40	27.07 ± 2.94**	7.33 ± 1.12
3 days	0		7.82 ± 1.09
	10		12.50 ± 1.61*
	20		19.45 ± 2.36**
	40	43.22 ± 3.90**	27.51 ± 2.89**
5 days	0		9.33 ± 1.34
	10		14.82 ± 2.15*
	20		19.77 ± 2.45**
	40	66.40 ± 6.76**	37.83 ± 3.77**

* Compared with control $P<0.05$.
** Compared with control $P<0.01$.

ml, and kept at 4°C for the single cell gel electrophoresis (SCG) assay.

The SCG assay was performed according to the procedure described by Tice et al. (1992) and Vijayalaxmi et al. (1992). Briefly, 0.5% of normal melting agarose (Sigma) and 0.5% of low melting point (LMP) agarose were melted in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS). Normal melting agarose (110 μl) was pipetted onto a fully frosted microscope slide, immediately covered with a 22×30 mm coverslip, and placed on ice for 10 min to solidify the agarose. Cell suspension (100 μl) was mixed with 900 μl of LMP agarose at 37°C, then 75 μl of the mixture ($\approx 1.5–2.0 \times 10^4$ cells) was added on the first agarose layer after the coverslip was removed carefully, covered with a coverslip again and kept on ice for 10 min. The coverslip was then removed and a top layer of 75 μl of LMP agarose was added before the coverslip was replaced and the slides were kept cold for another 10 min. The coverslip was removed and the slides were immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM Na_2 EDTA, 10 mM Tris, 1% sodium sarcosinate, pH 10.; 1% Triton X-100 and 10% DMSO v/v, mixed before use) for at least 1 h at 4°C. The following steps were carried out under red light. The slides were put on a horizontal electrophoretic tray, 1000 ml of freshly prepared alkaline-EDTA buffer (300 mM NaOH, 1 mM EDTA, pH > 13) was gently filled in to cover the slides to a height of about 2.5 cm above their surface for 40 min. Electrophoresis was run for 50 min at a voltage and current of 20 V (about 1 V/cm) and 300 mA by lowing the level of the buffer. The slides were placed horizontally in the trays and washed with Tris buffer (0.4 M Tris with HCl, pH 7.5) three times, then stained with 100 μl of 2 μg ethidium bromide/ml distilled water and covered with a coverslip. Cells were analyzed at 400 × magnification with a fluorescence microscope equipped with a 515–560 nm excitation filter and 590 nm barrier filter (Zeiss, D-7082 Oberkochen, Germany). The length of DNA migration was determined by measuring the tail length (distance between edge of head and end of tail) in microns. A minimum of 50 cells from each treatment were scored. All experiments were

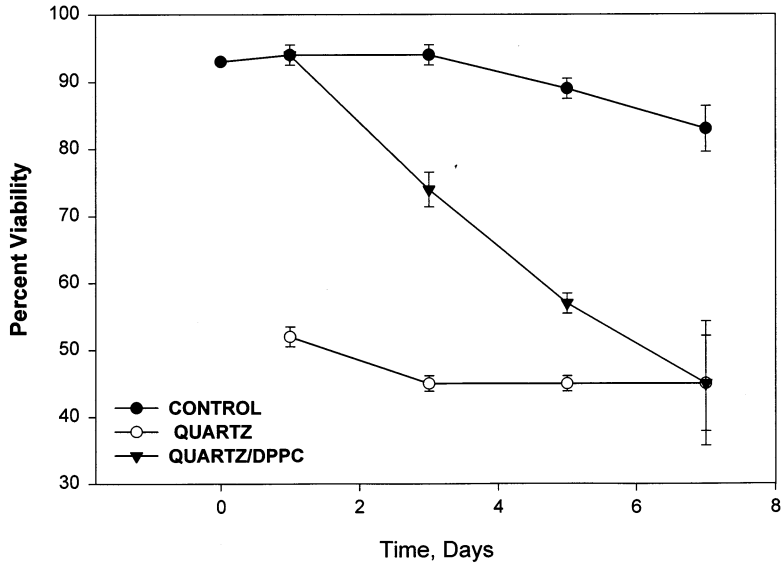

Fig. 1. Macrophage viability versus time after in vitro exposure to 1 mg/ml of native or surfactant-coated quartz particles (Trypan Blue Assay). Closed circles are data for non-exposed control cells; open circles are for native quartz-exposed cells; closed triangles are for DPPC-coated quartz-exposed cells.

repeated. Student's *t*-test was used for the statistical analysis. Significance was determined at an alpha level of 0.05.

2.5. Measuring cytotoxicity versus time

Two different cell viability assays were performed: the trypan dye blue exclusion and the Live-Dead® assays (Burghardt, 1994). For the trypan blue assay, 1×10^6 cells were seeded in a 100 mm Petri dish, and cells were incubated at 37°C and 5% CO_2 to permit cell adherence. The medium was removed, and suspensions of quartz in complete medium added to result in exposures of 80 μg quartz/cm^2 (1 mg/ml). After incubation of cells with dust for selected times of 1–7 days, the medium was transferred to a 15 ml centrifuge tube, the cells rinsed with 2 ml of PBS, the rinsings added to the tube, and 2 ml of trypsin solution (Sigma) added to the cell well, and 10 min allowed for cells to release from the plate. A 2 ml volume of complete medium were added and the wells rinsed repeatedly by pipetting the contents back and forth into the pipet. This rinsing also was added to the 15 ml tube. The tube was centrifuged for 10 min at 750 rpm, the supernatant discarded and the cells resuspended in 1 ml of PBS. Cells then were stained with trypan blue solution and counted. Live cells and dead cells were scored separately; non-staining cells were counted as viable.

For the Live-Dead® assay, $1.5–2 \times 10^6$ cells were seeded as above, and suspensions of quartz in minimal essential medium (MEM) added to result in exposures of 40 μg quartz/cm^2. After incubations from 1 to 5 days, cells were rinsed and resupended as above. Then 1–2 drops of the cell suspension were added to a cover-slip in a covered petri dish and allowed to settle for 15 min. The Live-Dead® assay reagents (Molecular Probes, Eugene, OR) were warmed and 1 μl of 2 mM EthD-1 and 0.25 μl of 4 mM calcein AM added to 3 ml PBS to make the staining solution. In the covered dish, 100 μl of the staining solution was added to the coverslip, and the mixture incubated for 30-45 min at room temperature. A 10 μl volume of staining solution was added to a clean microscope slide and the coverslip inverted and placed on the liquid drop. The cells were examined by fluorescence microscopy using an excitation wavelength of 450–490 nm. Live cells are bright green and dead cells are red. The percent

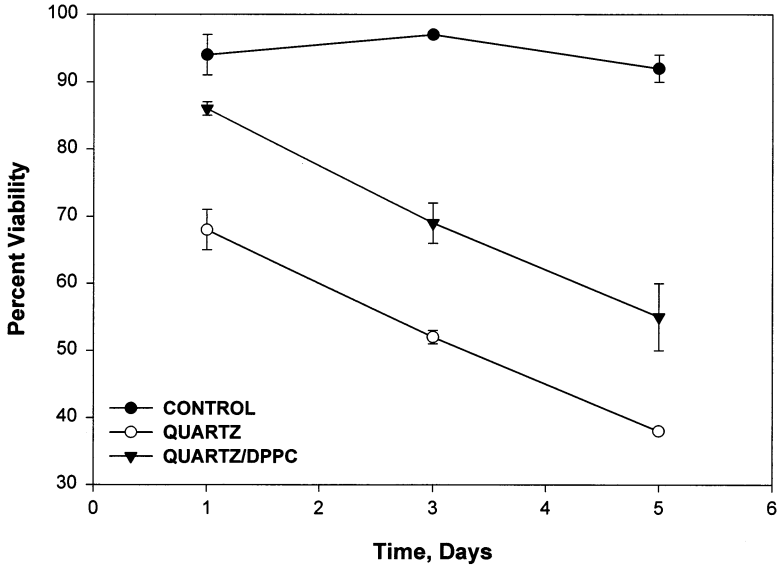

Fig. 2. Macrophage viability versus time after in vitro exposure to 0.5 mg/ml of native or surfactant-coated quartz particles (Live-Dead® Assay).

viability of each treatment was computed from 300 cells and duplicate samples were done for each treatment.

2.6. Measuring surfactant loss from particles in vitro versus time

Observation of the loss of surfactant from dusts used fluorescent-labelled surfactant consisting of a dipyrromethene boron difluoride fluorescent group substituted into one of the acyl chains of DPPC (BODIPY® FL, Molecular Probes, Eugene, OR). This was mixed with dioleoyl phosphatidylcholine (DOPC) at 1:20 labeled to unlabeled surfactant in chloroform to assure complete mixing. This was evaporated under N_2 to dryness. Saline dispersion of this labeled surfactant was prepared as described for DPPC above.

Cells were diluted in RPMI complete medium to 4×10^5/ml and 1 ml added to wells containing 12 mm glass cover slips in 24-well plates. Cells were allowed to adhere for 2 h in the incubator. Medium was removed and 1 ml of 0.1 mg/ml suspension of treated quartz was added to each well. Following incubation at 37°C under 5% CO_2 for desired times between 1 and 7 days, the cover slips were removed with forceps and rinsed twice by dipping in PBS for 5 s, and then fixing in 2% buffered formalin for 20 s. The excess liquid was removed by blotting the edge and rear surfaces of the slip; and the slip was placed cell side down into a 10 mm diameter nail polish well containing 4 μl of PBS on a microscope slide. The coverslip back was allowed to dry, and the edges sealed with nailpolish. Slides were viewed by confocal laser scanning microscopy (CLSM). Fields were selected by brightfield images. Confocal images were obtained for fluorescence ($\lambda_{em} > 520$ nm), reflectance, and transmitted polarized light. Slides were examined by fluorescence microscopy to observe the time-course of fluorescent surfactant associated with particles phagocytosed by cells, and by polarization and reflectance light microscopy to image cells and particulate material within cells. Collected images were digitally processed to overlay polarization and fluorescence images to localize fluorescence within cells. Quantitative average fluorescence values were obtained by using image analysis tools to estimate the fluorescence profile across a rectangle inside the boundaries of individual cells, and computing the average value over all cells for a given treatment. Fluorescence

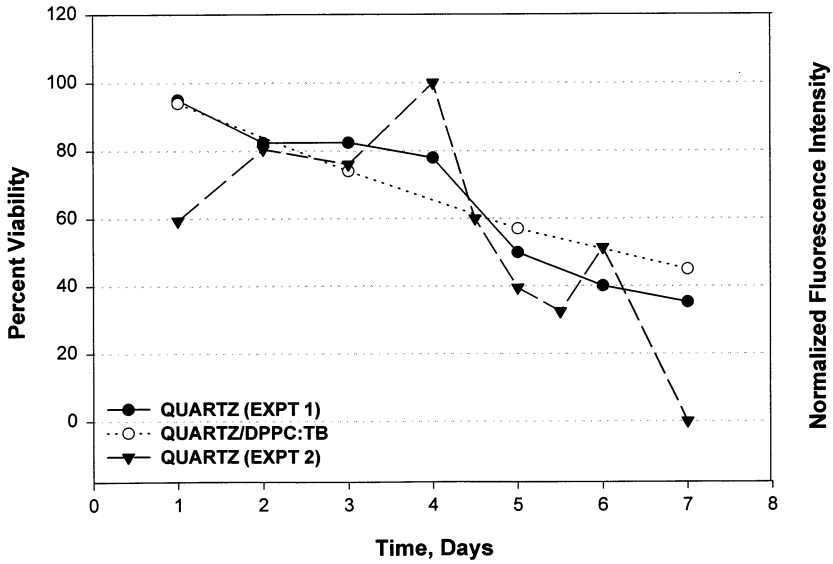

Fig. 3. Normalized fluorescence intensity averaged over macrophages in vitro versus time after exposure to 0.1 mg/ml fluorescent-labeled-surfactant-coated quartz. Closed circles connected by solid lines and closed triangles connected by dashed lines are surfactant fluorescence intensities measured in two separate tests. Open circles connected by dotted lines are trypan blue viability data for DPPC-treated quartz taken from Fig. 1.

values were corrected for background cell autofluorescence by measurements on non-quartz-challenged negative control cells.

3. Results

3.1. DNA damage

Table 1 lists migration tail lengths for DNA from macrophages treated with native and DPPC treated quartz for 3 h, 1, 3, and 5 days. After 3 h challenge, native quartz caused DNA damage in a dose-dependent manner, while DPPC coated quartz induced insignificant DNA migration. After 1 day challenge, DPPC-coated quartz did not increase DNA migration. However, DNA migrations were significantly elevated over the solvent controls after 3 and 5 days challenge with DPPC-treated quartz.

3.2. Cytotoxicity assays

Fig. 1 compares the cytotoxicity of native quartz and DPPC-coated quartz on alveloar macrophages in vitro. Viability was measured by trypan blue dye exclusion. Data points are average and standard error values for five replicate experiments for percent macrophage viability versus time for 1, 3, 5, and 7 days following cell challenge. The control cells, not challenged with quartz, show a slow decline in measured viability over the 7 day period from 93 to 83%. At day 1 the viability of native quartz-challenged cells had decreased to 52%. The viability of the DPPC-quartz challenged cells was equal to the non-challenged control cells at day 1. On day 3 the viability of DPPC-quartz challenged cells had decreased to 75%; and by day 7 this had further decreased to 45%, the value for the native quartz positive control.

Fig. 2 shows in vitro cytotoxicity measured by the Live-Dead® fluorescence assay, as the averages and standard errors for two experiments. Non-challenged control cell viability changed little, from 94% on day 1 to 92% on day 5. Viability of native quartz-challenged cells was 68% at day 1, and declined to 38% on day 5. Again, DPPC-treated quartz manifested lower cytotoxicity than native quartz at day 1, 86% viability. That viability decreased with time to 55% at day 5.

3.3. Fluorescent surfactant loss

Qualitative observation indicated a major fraction of fluorescent surfactant on quartz particles was lost over a 7 day period after phagocytosis by macrophage. Quantitative average fluorescence values, shown in Fig. 3, were obtained in two experiments. In each experiment 50 cells were measured at each time point. In the first data set, fluorescence is seen to decrease smoothly from day 1 to approximately half of initial intensity by day 7. The second data set shows a similar general diminution over a 1 week period, but with considerable scatter in the data, particularly for the day 1 and day 4 values. Particles not phagocytosed by cells were observed to retain their fluorescence intensity over the time course of the experiment. Fig. 3 also shows an overlay of the time course of cytotoxicity restitution for DPPC-treated quartz, taken from the data of Fig. 1.

4. Discussion

Native quartz dust expressed both prompt cytotoxic and DNA damaging activities. Incubation in DPPC surfactant adequate to provide at least a bilayer of lipid coating promptly suppressed these activities through 1 day incubation with cells in vitro. Cytotoxic and genotoxic activity were restored to near positive control values over a 1 week period.The comparable time course of expression of cytotoxicity and of DNA damage does not clarify the question of the independence of quartz genotoxicity activity from its other cytotoxic activities. A prior study of micronucleus induction in Chinese hamster lung fibroblasts (V79 cells) found suppression of genotoxic activity over a 1-week time period for DPPC surfactant-treated quartz; but proliferation of V79 cells prevented parallel measurement of the time course of cytotoxicity (Liu et al., 1996). Fluorescence activity, indicative of retention of adsorbed surfactant, decayed to near background levels in the 1-week period. This time-course is consistent with measures of digestion with time of radiolabeled DPPC from quartz particles by the P388D1 cell line (Hill et al., 1995); and it is similar to the time course of cytotoxicity to rat macrophage expressed by quartz treated with Survanta® commercial mixed surfactant (Antonini et al., 1994). The relationship of the expression of DPPC-treated quartz cytotoxicity with surfactant digestion by phospholipase A2 (PLA2) in a cell-free system has been detailed in parallel tests using wet-chemical analysis of surfactant digestion and hemolysis assay for restored surface toxicity (Wallace et al., 1992). The PLA2 system has been used to validate the fluorescent analogue used here as a tracer for digestive removal of the unlabeled surfactant (Das, 1993). Surfactant components of the pulmonary alveolar hypophase can adsorb onto silica particle surface and suppress prompt membranolytic toxicity; subsequent secondary lysosomal or extracellular enzymatic processes can digest the particle-adsorbed surfactant. Fig. 3 indicates a similar time course for restoration of DPPC-treated quartz cytotoxicity and loss of particle-adsorbed surfactant for this in vitro macrophage system, suggesting that the prophylactic effect of adsorbed surfactant on quartz toxic activity diminishes with time as phagolysosomal digestion of quartz-adsorbed surfactant proceeds.

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