Amino-terminal and midmolecule parathyroid hormone-related protein, phosphatidylcholine, and type II cell proliferation in silica-injured lung

Randolph H. Hastings,1,2 Rick A. Quintana,1 Rebeca Sandoval,1 Douglas W. Burton,1,2 and Leonard J. Deftos1,2

1Research, Anesthesiology, and Medicine Services, Veterans Affairs San Diego Healthcare System, San Diego 92161; and 2Departments of Anesthesiology and Medicine, University of California San Diego, La Jolla, California 92093

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Am J Physiol Lung Cell Mol Physiol 285: L1312–L1322, 2003. First published August 15, 2003; 10.1152/ajplung.00314.2002.—Acute silica lung injury is marked by alveolar phospholipidosis and type II cell proliferation. Parathyroid hormone-related protein (PTHrP) 1–34 could have a regulatory role in this process because it stimulates phosphatidylcholine secretion and inhibits type II cell growth. Other regions of the PTHrP molecule may have biological activity and can also exert pulmonary effects. This study examined the temporal pattern for expression of several regions of PTHrP after silica lung injury and evaluated the effects of changes in expression on cell proliferation and lung phospholipids. Expression of all PTHrP regions fell at 4 days after injury. Reversing the decline in PTHrP 1–34 or PTHrP 67–86 with one intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 stimulates bronchoalveolar lavage disaturated phosphatidylcholine (DSPC) levels. Cell culture studies indicate that the peptides exerted direct effects on DSPC secretion by type II cells. Neither peptide affected type II cell proliferation with this dosing regimen, but addition of an additional intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 with one intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 stimulates bronchoalveolar lavage disaturated phosphatidylcholine (DSPC) levels. Cell culture studies indicate that the peptides exerted direct effects on DSPC secretion by type II cells. Neither peptide affected type II cell proliferation with this dosing regimen, but addition of an additional intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 with one intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 stimulates bronchoalveolar lavage disaturated phosphatidylcholine (DSPC) levels. Cell culture studies indicate that the peptides exerted direct effects on DSPC secretion by type II cells. Neither peptide affected type II cell proliferation with this dosing regimen, but addition of an additional intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 with one intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 stimulates bronchoalveolar lavage disaturated phosphatidylcholine (DSPC) levels. Cell culture studies indicate that the peptides exerted direct effects on DSPC secretion by type II cells. Neither peptide affected type II cell proliferation with this dosing regimen, but addition of an additional intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 with one intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 stimulates bronchoalveolar lavage disaturated phosphatidylcholine (DSPC) levels. Cell culture studies indicate that the peptides exerted direct effects on DSPC secretion by type II cells. Neither peptide affected type II cell proliferation with this dosing regimen, but addition of an additional intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 with one intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 stimulates bronchoalveolar lavage disaturated phosphatidylcholine (DSPC) levels. Cell culture studies indicate that the peptides exerted direct effects on DSPC secretion by type II cells. Neither peptide affected type II cell proliferation with this dosing regimen, but addition of an additional intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 with one intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 stimulates bronchoalveolar lavage disaturated phosphatidylcholine (DSPC) levels. Cell culture studies indicate that the peptides exerted direct effects on DSPC secretion by type II cells. Neither peptide affected type II cell proliferation with this dosing regimen, but addition of an additional intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 with one intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 stimulates bronchoalveolar lavage disaturated phosphatidylcholine (DSPC) levels. Cell culture studies indicate that the peptides exerted direct effects on DSPC secretion by type II cells. Another respiratory distress syndrome; cell surface receptors; pulmonary surfactant; phospholipidosis; type II pneumocyte

Address for reprint requests and other correspondence: R. H. Hastings, VA Medical Center (125), 3350 La Jolla Village Dr., San Diego, CA 92161-5085 (E-mail: rhhastings@ucsd.edu).
phospholipid secretion on a per type II cell basis. An additional factor contributes to the complexity: regions of the PTHrP molecule besides PTHrP 1–34 are known to exert biological effects. For example, we recently showed that PTHrP 38–64 stimulates type II cell proliferation during silica lung injury in rats (8). PTHrP 67–86, PTHrP 38–94, PTHrP 107–139, and PTHrP 140–173 have biological activities in some systems (7, 19, 31, 32, 34) but have not been studied extensively in the lung. Their effects on phosphatidylcholine levels and type II cell growth in normal and injured lung are unknown.

The initial goal of this study was to test the hypothesis that lung expression of PTHrP would fall early in the course of silica injury, as in hypoxic injury, for several regions of the molecule. Our two previous studies had employed only single immunoassays specific for PTHrP 1–34 or PTHrP 109–141 (8, 9). Because different regions could have independent biological activities, it was critical to know whether their expression changed in concert or independently. These studies demonstrate that measured values of PTHrP levels in several region-specific immunoassays all fell at 4 days after injury. Additional studies investigated whether changes in PTHrP levels at 4 days after silica injury would affect lung phospholipid levels, whether peptides other than PTHrP 1–34 would have effects, and whether PTHrP peptides had direct effects on type II cell phospholipid levels or effects through changes in type II cell proliferation. These experiments demonstrate complex effects of increasing and decreasing PTHrP 1–34 or PTHrP 67–86 levels on lung phospholipids. The results suggest that direct effects on type II cell DSPC secretion and effects on growth could interact in regulating lung phospholipid levels after silica injury.

METHODS

Specific Protocols

Time course for lung PTHrP expression in silica lung injury. Lung injury was induced by instilling rats with 5 μm silica particles, as described under General Methods below. Control animals received PBS in their lungs. Animals were killed at 0, 4, 7, 14, and 28 days after instillation for immunohistochemistry of lung PTHrP.

Effects of PTHrP on lavage phospholipid levels. Lung PTHrP levels were manipulated in rats instilled with silica or PBS by intratracheal treatment with PTHrP peptides or antibodies at the time of silica delivery and with daily subcutaneous injections of the same peptide or antibody. At the end of the 4-day experiment, phospholipids and DSPC were measured in BAL.

Effects of PTHrP peptides on phospholipid secretion by cultured type II cells. The purpose of this study was to investigate whether the effects of PTHrP 1–34 and PTHrP 67–86 on DSPC in silica-injured animals could be due to effects on type II cells. Normal alveolar type II cells were isolated as described below. DSPC secretion was assayed in the cultured cells treated with growth media alone, PTHrP peptides, PTHrP antibodies, or ATP as positive control.

Effect of PTHrP on proliferation markers and type II cell markers. This study evaluated whether phospholipid levels in the second set of experiments described above could be impacted by changes in type II cell numbers after PTHrP peptide or antibody treatments. Proliferating cell nuclear antigen (PCNA) expression was assessed as a measure of proliferation and alkaline phosphatase-positive cells were enumerated as an estimator of changes in type II cell numbers. In the silica-antibody treatment experiments, PCNA was validated as a proliferation marker by comparison with 5-bromo-2′-deoxyuridine (BrdU) incorporation. Animals were injected with 100 mg/kg BrdU ip 18 h before euthanasia.

Effect of repeat PTHrP instillation on proliferation. This study was performed to determine whether a second instillation of PTHrP peptide after the induction of injury had an augmented effect on type II cell proliferation. The protocol for administering PTHrP was similar to one used in a prior study (9) that showed that PTHrP 1–34 inhibited type II cell growth in hyperoxic lung injury. Animals were treated with silica and PTHrP 1–34 or PTHrP 67–86 as described in the second set of experiments above. In addition, they received an additional instillation of PTHrP peptide in BSA/PBS or BSA/PBS alone on the third day of injury 18 h before termination of the experiment. In addition, 100 mg/kg BrdU was given intraperitoneally 4 h before euthanasia. BrdU incorporation in corner alveolar cells was evaluated by immunohistochemistry as a measure of type II cell proliferation.

Effects of PTHrP peptides and antibodies on indexes of injury and inflammation. Lung injury and inflammation were evaluated in the 4-day silica PTHrP peptide or antibody experiments by measuring lung weight, BAL protein concentration, BAL cell, and differential counts.

General Methods

Animals. Male pathogen-free Sprague Dawley rats were obtained from Charles River (Hollister, CA). The Veterans Administration San Diego Healthcare System institutional animal care and use committee approved these studies.

Instillation. Anesthetized rats (250–350 g) were instilled with 10 mg of silica suspended in 1 ml of PBS as described previously (8). Control animals received PBS and the same peptide or antibody treatment as lung-injured animals, but no silica. Rats were killed at the end of the experimental period, and the lungs were removed and processed.

PTHRP peptide and antibody treatment. Lung PTHrP levels were manipulated by systemic and intrapulmonary treatment with PTHrP peptides or antibodies. The peptides were PTHrP 1–34, PTHrP 38–64, PTHrP 67–86, or PTHrP 107–138 (Bachem, Torrance, CA). The intratracheal peptide dose was 15 μg in 0.05% BSA/PBS with or without the silica. In addition, rats received subcutaneous injections of 5 μg of the same peptide in 100 μl of PBS at the time of the first instillation and then daily for 3 days. Controls were treated with silica in BSA/PBS as the instillate and daily injections of BSA/PBS. Peptide was injected at repeated intervals to provide a systemic source for continued exposure to PTHrP over the 4-day experiment. Small peptides escape the alveolar space with a half-time <1 day (12). The intratracheal doses were quantities that were effective in altering type II cell proliferation in previous studies in normal and hyperoxic lung (8, 9, 16). The subcutaneous doses were taken from studies demonstrating changes in organ function and alterations of bone resorption rates with systemic PTHrP in rats (30). The antibodies were neutralizing mouse monoclonal antibodies IA5 directed against PTHrP 1–34 and 3B8 directed against PTHrP 67–86, both developed in our laboratory (10). Antibodies were purified by protein G affinity.
chromatography. The intratracheal and subcutaneous doses for antibodies were 75 and 37.5 μg of IgG, respectively. Control animals received 3H12 or SA4, irrelevant antibodies of the same mouse IgG isotype (IgG1). The systemic doses of PTHrP antibody were adjusted from studies in which we showed changes in growth of orthotopic PTHrP-expressing lung carcinomas in immunocompromised mice (10).

Lung preparation. The right lung was removed, weighed, and lavaged with four 4-ml aliquots of PBS and homogenized in lysis buffer with protease inhibitors. The homogenate was centrifuged at 16,000 g for 1 h at 4°C, and the supernatant was frozen at −70°C for subsequent assay (9). Bronchoalveolar lavage liquid (BAL) was centrifuged, and the supernatant was frozen at −70°C for subsequent assay. The cell pellet was resuspended in 1 ml PBS, stained with crystal violet, and counted in a hemocytometer. The left lung was fixed by tracheal instillation of 6 ml of 4% paraformaldehyde in 0.1 M phosphate buffer at 37°C, pH 7.4, and embedded in paraffin.

Immunostaining. PTHrP levels in BAL and lung homogenates were determined with previously described radiomunoassays based on antibodies to PTHrP 1–34, PTHrP 38–64, and PTHrP 109–141 (as described below) with reagents described below). The specificity of the assay was determined by immunoblotting (see below) since an immunostaining was lacking. Immunohistochemistry was also used to assess the purity of our PTHrP peptide preparations. PTHrP 1–34, PTHrP 38–64, and PTHrP 107–138 registered in only their specific assay, and the measured value agreed with the actual peptide concentration. PTHrP 67–86 contained no detectable PTHrP 1–34, PTHrP 38–64, or PTHrP 109–141 activity.

Immunohistochemistry. Lung sections (5 μm) were deparaffinized in xylene and hydrated through a graded ethanol series. Nonspecific protein binding was blocked with 10% goat serum. Sections were incubated overnight at 4°C with PC-10 (DAKO, Carpinteria, CA), a primary antibody for PCNA, or R759, our own rabbit polyclonal antibody directed against mouse IgG, respectively, in 0.5% BSA in PBS. Biotinylated goat anti-rabbit (Calbiochem, La Jolla, CA), diluted 1:33 with 10% goat serum in PBS, was applied for 25 min. Staining was completed by incubation with streptavidin-alkaline phosphatase or peroxidase (Vector Laboratories, Burlingame, CA). BrdU was stained with a kit from Zymed (South San Francisco, CA) following the manufacturer’s instructions. Lungs were incubated with a trypsin solution for 25 min and biotinylated mouse anti-BrdU antibody overnight at 4°C. Immunoreactivity was absent when primary antibody was omitted (not shown). Staining experiments were performed on lung sections from at least three animals per time point. Chromogens were Fast Blue and Vector Red alkaline phosphatase substrate, or 3',5'-diaminobenzidine hydrochloride for horseradish peroxidase development (Vector Laboratories). Staining was repeated at least once for each animal.

Alkaline phosphatase staining. Endogenous alkaline phosphatase activity was evaluated as a marker for type II cells in silica-injured lungs as described by Miller and colleagues (22). Lung sections were deparaffinized, washed in Tris-buffered saline (TBS), and incubated for 30 min with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium in 100 mM Tris-HCl, pH 9.5, using the alkaline phosphatase substrate kit IV from Vector Laboratories. Sections were washed in TBS and deionized water, dehydrated, and sealed with coverslips. Sections from three random blocks were analyzed, and the results were combined for each animal. Alkaline phosphatase staining was quantified as a function of experimental manipulation with the same scoring system used for PTHrP immunoreactivity.

Phospholipid analyses. The phospholipid composition of cell-free BAL was determined in six to eight animals from each treatment group. Total lipid was extracted from BAL by the method of Bligh and Dyer (2), and DSPC was separated after reduction with osmium tetroxide on an alumina column (21). Phospholipid phosphorus was determined spectrophotometrically with the Bartlett assay. Individual phospholipids were separated by thin layer chromatography with activated silica plates (Whatman, Clifton, NJ) in a triethylamine-chloroform-ethanol-water (30:30:30:10 vol/vol/vol/vol). Plates were sprayed with molybdenum blue and scanned. The individual phospholipids were measured by densitometry of the scanned images using Chemilager version 5.1 software (Alpha Innotech, San Leandro, CA). Phospholipid levels were determined compared with a range of standards run on the same plate. Standards but not unknowns were quantified by phosphorus assay of spots scraped from the plate.

Type II cell preparation. Type II cells were isolated from uninjured 170- to 220-g rats by the method of Dobbs (6, 11). Briefly, the lungs were perfused and lavaged to remove airway inflammatory cells, digested with low concentrations of elastase, minced, filtered through 100-μm nylon filters, and washed free of proteases. We separated type II cells from the crude cell preparation by panning on IgG-coated plates. Cell counts were determined with a hemocytometer, and cell viability was assessed by trypan blue exclusion. Purity was determined from wet mounted cells stained with Phosphine 3R (Polysciences, Warrington, PA). Type II cell purity averaged 81 ± 5% (n = 9 isolations). Cells were plated at 10^5 cells/cm^2 in DMEM supplemented with 10% fetal bovine serum-gentamicin in an atmosphere of 10% CO2/90% air at 37°C.

Phosphatidylcholine secretion. Type II cells were plated in six-well plates and studied 24 h after isolation. Duplicate wells were studied for cells from each rat and experimental group. Cells were loaded for 24 h with 2.5 μCi [3H]choline (60 Ci/mmol, Amersham). The [3H]choline was removed, and the cells were washed and incubated for 3 h in serum-free media plus 0.1% albumin containing PTHrP 1–34, PTHrP 67–86, or ATP as the positive control. Additional experiments examined the effect of PTHrP antibodies compared with isotype control antibody (1 μg IgG/ml) on secretion stimulated by ATP. Media were aspirated, and cells were scraped into 1 ml of distilled water. Cells were sonicated, and aliquots were reserved for protein assay. Total lipid from cell sonicates was extracted (2). DSPC was isolated as described above (21) and measured by scintillation counting. DSPC was expressed as a percentage of total counts/min secreted into the media.

Data Analysis

Histochemical scoring. PTHrP, PCNA, and alkaline phosphatase reactivity were quantified as a function of experimental manipulation by a blinded observer who evaluated the percentage of alveoli containing stained corner cells. This number ranged from <100% (signifying that not all alveoli had stained cells) to almost 200% (signifying two positive cells, on average, per alveolus). The percentage of immunoreactive cells/alveolus was scored on an ordinal scale, with >2, 2–5, 5–20, 25–50, 51–75, 76–100, 101–125, 126–150, 151–175, and 176–200% corresponding to scores of 0–8, respectively. We evaluated BrdU incorporation by counting the number of positive nuclei per field at ×200 magnification in 15 fields per section. The fields were systematically spaced to cover the entire lung section, and the first field was selected at random using a table of random numbers. The average of the 15 values was reported.
Statistics. Lung alkaline phosphatase staining and PTHrP immunoreactivity were compared among groups by nonparametric analysis of variance. PTHrP levels, phospholipid contents, phospholipid secretion, and BrdU incorporation were compared among groups by analysis of variance. The Tukey and Dunn tests were used for post hoc pair-wise parametric and nonparametric comparisons, respectively (33). Data are reported as means ± SE. Significance was accepted if the probability of a type I error was < 0.05.

RESULTS

Time Course for Lung PTHrP Expression in Silica Lung Injury

PTHrP was measured in lung homogenates with three immunoassays based on different epitopes spanning the PTHrP molecule, PTHrP 1–34, PTHrP 38–64, and PTHrP 109–141. Lung PTHrP levels were stable in control animals from 0 to 14 days after instillation of PBS in all three immunoassays (Fig. 1, solid bars). However, levels of all three portions of the PTHrP molecule were significantly decreased at 4 days after silica instillation (*P < 0.01 vs. uninjured PBS controls, **P < 0.05). At 14 days after silica, PTHrP 38–64 levels decreased further, while PTHrP 1–34 and PTHrP 109–141 levels increased. PTHrP 1–34 levels exceeded baseline values at 14 days. Average lung homogenate concentrations in uninjured controls were 104,000 ± 10,000, 650 ± 60, and 41,000 ± 4,000 pg/ml. Data represent immunoassay values from 4–13 animals per group and time point. Similar results were obtained when PTHrP was expressed as pg/gm lung (not shown). The data for PTHrP 109–141 were presented in a modified format in a previous article (8).

Cytochemical stain. PTHrP 67–86 immunoreactivity was present in corner alveolar cells at all time points (Fig. 2). Immunoreactivity reached a nadir at 4 and 7 days after silica exposure and rose thereafter (Table 1). At the 14- and 28-day time points, lungs contained large PTHrP-immunoreactive cells that were not present earlier.

Statistics. Lung alkaline phosphatase staining and PTHrP immunoreactivity were compared among groups by nonparametric analysis of variance. PTHrP levels, phospholipid contents, phospholipid secretion, and BrdU incorporation were compared among groups by analysis of variance. The Tukey and Dunn tests were used for post hoc pair-wise parametric and nonparametric comparisons, respectively (33). Data are reported as means ± SE. Significance was accepted if the probability of a type I error was < 0.05.
**Effects of PTHrP on Lavage Phospholipid Levels**

**Effects of PTHrP peptides.** Silica lung injury by itself increased total BAL phospholipids close to twofold compared with PBS control animals, from 1,028 ± 103 to 1,863 ± 218 nmol/lavage for total phospholipids and from 510 ± 47 to 878 ± 166 nmol/lavage (P < 0.05, n = 7), respectively. DSPC represented 53.2 ± 5.8% (n = 12) and 41.9 ± 4.3% (n = 5) of the total phospholipids in uninjured and injured animals, respectively (P = not significant). Treatment with PTHrP 1–34 and PTHrP 67–86 increased BAL DSPC content in animals with silica lung injury more than twofold above levels in untreated injured animals (P < 0.05, n = 5–7 rats/group) (Fig. 3). DSPC content did not differ between animals treated with PTHrP 1–34 and those treated with PTHrP 67–86. BAL total phospholipid levels were also greater than untreated injured controls after treatment with the two peptides, but the difference was statistically significant only for PTHrP 1–34 and not PTHrP 67–86 (P = 0.058). The other PTHrP peptides did not have a significant effect on BAL phospholipids or DSPC. None of the peptides altered BAL phospholipids significantly in uninjured lung. Silica injury altered the distribution of BAL phospholipids among seven different species (Table 2). Phosphatidylcholine represented a significantly lower fraction of the total phospholipids in silica-injured compared with uninjured lungs. Sphingomyelin and phosphatidylinositol made up a significantly greater fraction of the phospholipids in the injured state. The ratio of phosphatidylcholine to sphingomyelin decreased from 62.8 ± 14.9 (n = 6) in control animals to 21.4 ± 2.6 (n = 6) in silica-injured animals treated with PBS (P < 0.01). PTHrP 1–34 and PTHrP 67–86 treatment did not affect the distribution of phospholipid species in injured lung.

**Effects of PTHrP antibodies.** We treated silica-injured animals with neutralizing PTHrP antibodies to decrease lung PTHrP levels. The effects on phospholipids differed with antibody specificity. BAL phospholipids were 25% lower in silica-injured lungs treated with anti-PTHrP 67–86 antibody 3B8 than in lungs treated with the isotype control antibody (Fig. 4). In contrast, phospholipids were not significantly different from control after treatment with 1A5, the antibody to PTHrP 1–34. A similar decrease in phospholipids after 3B8 alone was observed in uninjured lungs instilled with PBS on day 0. We used immunoassays and immunohistochemistry to investigate whether the antibody treatments were effective in reducing PTHrP levels. IA5 significantly lowered PTHrP 1–34 and PTHrP 38–64 levels in silica-injured lung by 35%–50% compared with isotype control (Fig. 5, P < 0.05) but left PTHrP 109–141 levels unchanged. PTHrP 1–34 and PTHrP 38–64 levels decreased to a similar extent in

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**Table 1. PTHrP immunoreactivity in alveolar corner cells of normal silica-injured rats**

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<tr>
<th>Experimental Group Days After Silica Treatment</th>
<th>Staining Score by Rat</th>
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Scores of 0–4 signify that <2, 2–25, 26–50, 51–75, and >76% of the alveolar corner cells were immunoreactive for parathyroid hormone-related protein (PTHrP), respectively. Each number represents a separate animal. *P < 0.05 vs. 0- and 7-day group.

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**Fig. 3. Effect of PTHrP peptides on phospholipids in normal and silica-injured lungs.** Control PBS instillation (A) and silica instillation (B) were performed as described in previous figures. Rats were treated with 15 µg of PTHrP 1–34, PTHrP 38–64, PTHrP 67–86, or PTHrP 107–138 in the intratracheal instillate and 5 µg of subcutaneous injections of the same peptide daily on days 0–3. The untreated groups received no PTHrP peptide in the instillate and were injected subcutaneously with PBS. Bronchoalveolar lavage liquid (BAL) was extracted with chloroform/methanol to measure total lung phospholipids and disaturated phosphatidylcholine (DSPC) after 4 days. None of the PTHrP peptides altered BAL phospholipids in uninjured PBS lungs. Silica injury augmented BAL DSPC and total phospholipid levels by approximately twofold at 4 days (*P < 0.05, comparing uninjured and injured no-treatment groups). In silica-injured lungs, PTHrP 1–34 and PTHrP 67–86 treatment increased BAL total phospholipid and DSPC levels by over twofold compared with injured lungs receiving PBS treatment (**P < 0.05 vs. control). The effects of PTHrP 1–34 did not differ significantly from the effect of PTHrP 67–86. Data represent results from 4–6 rats per group.
uninjured lungs treated with PTHrP 1–34 antibody (data not shown). We are unable to measure PTHrP 67–86 directly by immunoassay, but it might have an effect on assays for other portions of the molecule if PTHrP 67–86 in the lung were present as a larger molecule. However, none of the levels differed between lungs treated with isotype control or 3B8, the PTHrP molecule. However, none of the levels differed between lungs treated with isotype control or 3B8, the PTHrP 67–86 antibody. PTHrP 67–86 immunoreactivity was uniformly low after 4 days of silica injury with or without PTHrP antibody treatment (data not shown).

Effects of PTHrP Peptides on Phospholipid Secretion by Cultured Type II Cells

We studied cultured cells to investigate whether the peptides had a direct effect on type II cells. PTHrP 1–34 and PTHrP 67–86 both stimulated DSPC secretion to an extent that nearly matched the effect of ATP, a potent secretagogue for type II cells (Fig. 6). The 3B8 antibody, directed against PTHrP 67–86, significantly reduced the stimulatory effect of ATP on phosphatidylcholine secretion. ATP-stimulated phosphatidylcholine secretion was 100 ± 3.3, 94.2 ± 4.7, and 81.6 ± 2.2% of control ATP-stimulated secretion in cells treated with 8A4 (isotype control), 1A5 (anti-PTHrP 1–34), and 3B8 (anti-PTHrP 67–86), respectively (*P < 0.05 vs. 8A4).

Effects of PTHrP on Proliferation Markers and Type II Cell Markers

Effects of PTHrP peptides. Silica-injured lungs were stained for PCNA as a proliferation marker and alkaline phosphatase as a marker for type II cells. Lung PCNA expression was similar in silica-injured lungs treated with PBS, PTHrP 1–34, or PTHrP 67–86 (Fig. 7) (8). Neither peptide caused significant changes in the number of alkaline phosphatase-positive cells in silica-injured lungs compared with PBS treatment (Fig. 8). Staining scores were 4 (3.5, 4), 4 (3.3, 4), and 4 (3.5, 5) [median (interquartile gap)] for PBS-, PTHrP 1–34-, and PTHrP 67–86-treated silica-injured lungs, respectively. Many alkaline phosphatase-positive cells stained for PCNA (Fig. 7D), indicating that type II cells were proliferating. PCNA-positive cells that did not stain for alkaline phosphatase were also present.

Effects of PTHrP antibodies. The two PTHrP antibodies differed in effects on phospholipids and also differed with respect to effects on PCNA expression in silica-injured lung. PCNA scores were not different in isotype control animals and animals treated with 3B8, 2 (2, 2) and 2 (1, 4), respectively, but increased to 7 (5, 7.5) in animals receiving 1A5 (*P < 0.05, Fig. 9, A–C). BrdU staining was also increased in silica-injured animals treated with 1A5 compared with isotype control antibody but was unchanged after 3B8 treatment (Fig. 9, D–F). Thus the PTHrP 67–86 antibody reduced BAL phospholipids and had no effect on proliferation, whereas the PTHrP 1–34 antibody stimulated proliferation and did not effect phospholipids.

Effect of Repeat PTHrP Instillation on Proliferation

Administering a second intratracheal dose of PTHrP 1–34 or PTHrP 67–86 near the end of the 4-day period resulted in growth effects that were not observed with the single initial instillation and subcutaneous dosing.
alone. Both PTHrP 1–34 and PTHrP 67–86 inhibited BrdU incorporation in corner cells of silica-injured lungs compared with untreated injured lungs (Fig. 10). The number of BrdU-positive cells per field was reduced from 13 ± 1 in untreated lungs to 3 ± 2 and 6 ± 1 cells/field in lungs treated with PTHrP 1–34 and PTHrP 67–86, respectively (P < 0.05, n = 3–4 animals/group).

**Effects of PTHrP Peptides and Antibodies on Indexes of Injury and Inflammation**

Lung weights did not vary among any of the antibody-treated groups in silica-injured lung (Table 3). BAL total protein was somewhat higher in the two
injured groups treated with PTHrP antibody compared with isotype, but the $P$ value was not significant ($P = 0.075$). In injured lungs, PTHrP 67–86 antibody treatment resulted in higher BAL total cell, macrophage, and neutrophil counts than treatment with isotype control antibody. However, in uninjured lungs, the same antibody decreased cell counts compared with isotype control.

DISCUSSION

This study first tested and confirmed the hypothesis that PTHrP expression would decrease early in silica lung injury as assessed by immunoassay measurements of multiple regions of the molecule. Lung levels of PTHrP 1–34, PTHrP 38–64, PTHrP 67–86, and PTHrP 109–141 decreased 4 days after exposure to silica. PTHrP 1–34, PTHrP 67–86, and PTHrP 109–141 expression then returned toward baseline by 14 days after injury. A biphasic pattern of changes in PTHrP expression also occurs in hyperoxic lung injury (9, 14) and may be a general response to injury of various types. PTHrP 38–64 levels showed a somewhat different time course than observed for the other peptides, suggesting that lung injury may alter PTHrP processing.

![Fig. 9. Effect of PTHrP antibody treatment on proliferation markers in silica-injured lung. Rats with silica lung injury were treated with isotype control antibody 8A4, PTHrP 1–34 antibody 1A5, or PTHrP 67–86 antibody 3B8 as described in Fig. 4. At 3.5 days of injury, animals were injected with 100 mg/kg 5-bromo-2′-deoxyuridine (BrdU) ip. Lungs were fixed, embedded, and processed for immunohistochemical localization of PCNA (A–C) or BrdU (D–F) at 4 days. Treatment with the PTHrP 1–34 antibody (B, E) significantly increased expression of PCNA (pink reaction product) and incorporation of BrdU (brown reaction product) into alveolar corner cells compared with isotype control lungs (A, D) or lungs treated with PTHrP 67–86 antibody (C, F). Arrows mark representative PCNA-positive or BrdU-positive nuclei.]

![Fig. 10. Effects of repeat PTHrP instillation on BrdU incorporation in silica-injured lung. Rats were treated with PTHrP 1–34 or PTHrP 67–86, as described in the legend to Fig. 3 plus an additional intratracheal administration of peptide on day 3 of injury. The animals were given 100 mg/kg BrdU ip 4 h before death on day 4 to label dividing cells. BrdU was revealed in lung sections by immunohistochemistry (brown reaction product in nuclei). PTHrP 1–34 (A) and PTHrP 67–86 (B) significantly reduced BrdU incorporation with this dosing regimen compared with injured animals receiving PBS in place of the experimental treatment (C). The number of BrdU-positive cells per $\times 200$ field was reduced from $13 \pm 1$ in untreated lungs to $3 \pm 2$ and $6 \pm 1$ cells/field in lungs treated with PTHrP 1–34 and PTHrP 67–86, respectively ($P < 0.05$, $n = 3–4$ animals/group).]
We next investigated whether treating lungs with PTHrP peptides at 4 days after injury, the time when levels were reduced, would alter lung phospholipid contents. We found that two of the five peptides, PTHrP 1–34 and PTHrP 67–86, significantly increased BAL total phospholipid and DSPC levels in silica-treated lungs. Supplementary studies demonstrated that both peptides stimulated phosphatidylcholine secretion in cultured type II cells, consistent with previous reports for PTHrP 1–34 (15). Thus, exogenous PTHrP 1–34 and PTHrP 67–86 treatment may alter BAL phospholipid levels in silica injury by direct effects on type II cells (15). It is not clear why these peptides did not augment BAL DSPC in uninjured lung. The effects of the PTHrP peptides may be magnified after silica exposure because the injury results in increased lamellar body size and pneumocyte surfactant phospholipid content (24, 25). The fraction of BAL phospholipid that was DSPC did not increase after treatment with PTHrP 1–34 and PTHrP 67–86, somewhat unexpected if the peptides increased secretion. However, stimulating secretion does not necessarily increase the DSPC fraction. Hyperpnea doubles total BAL phospholipids in rats, for example, without altering the proportion of DSPC (29).

Changes in type II cell proliferation and numbers could also have affected lung phospholipids, but neither PTHrP peptide altered the number of PCNA-positive or alkaline-phosphatase-positive cells in the lung in our first set of experiments. The PCNA and alkaline phosphatase results were somewhat surprising, because we had previously shown that PTHrP 1–34 inhibits type II cell BrdU incorporation after 4 days of lung injury with 85% oxygen (9). Differences in dosing regimens between the two studies might explain the discrepancy in proliferation results. PTHrP 1–34 was administered as a single intratracheal dose after 3.5 days of hyperoxic injury and proliferation was measured ~18 h later. We gave an additional intratracheal dose at the same time point in a second set of silica injury experiments and found that PTHrP 1–34 and PTHrP 67–86 inhibited BrdU incorporation (Fig. 10), as PTHrP 1–34 had in hyperoxic injury. Thus the antiproliferative response to PTHrP can be affected by route of administration and timing. The effects might also depend on the nature of injury, the presence of other growth factors, and the rate of pneumocyte proliferation. PTHrP might exert greater effects when proliferation peaks at 14–28 days after injury compared with 4 days when the rate of type II cell growth is relatively low (28).

We used PTHrP antibodies to decrease lung PTHrP levels, as we have reported previously (16). The PTHrP 1–34 antibody, 1A5, decreased lung PTHrP measured in radioimmunoassays for PTHrP 1–34 and PTHrP 67–86, but the PTHrP 67–86 antibody, 3B8, did not. These results indicate that 1A5 neutralizes a species of PTHrP in silica-injured lung that is larger than PTHrP 1–34. This observation is novel because the known processed forms of PTHrP include PTHrP 1–34, PTHrP 38–94, PTHrP 38–107, and PTHrP 107–139, but no form on the order of PTHrP 1–65 (27). PTHrP can also be secreted as full-length PTHrP 1–141, so PTHrP 1–65 or a related species could be a fragment of full-length PTHrP produced within injured lung. We have no immunoassay for PTHrP 67–86 to confirm the neutralizing activity of 3B8, a low affinity next to 1A5. However, 3B8 had a physiological effect in decreasing phospholipid levels that constitutes indirect evidence of neutralizing PTHrP 67–86.

We expected that the PTHrP antibodies might stimulate type II cell division and that they would either decrease BAL phospholipids through a proliferative effect or augment levels through a proselyctory effect. We observed some of the changes that we had anticipated, but the effects were not consistent. Two lung proliferation markers, PCNA expression and BrdU incorporation, increased after 1A5. This observation indicates that PTHrP 1–34 still had an effect in inhibiting proliferation in the lung at 4 days of silica injury, even though levels were reduced compared with normal lung, and that neutralizing PTHrP 1–34 with 1A5 increased growth. The result is consistent with previous studies in which antibodies to PTHrP 1–34 stimulated type II cell proliferation (16). The lack of an effect on phospholipid levels after 1A5 suggests that increases in type II cell numbers balanced any decrease in phospholipid secretion per cell due to the reduction in PTHrP. The failure of a 3B8 to alter proliferation markers suggests that PTHrP 67–86 levels were not high enough to inhibit growth in the injured lung or that 3B8 did not neutralize enough secreted PTHrP 67–86 to reduce proliferation. A loss of the secretory effect of PTHrP 67–86 with no offsetting increase in type II cell numbers can explain the fall in BAL DSPC levels after 3B8. These effects are admit-

Table 3. Effect of PTHrP antibodies on indices of injury and inflammation

<table>
<thead>
<tr>
<th>Injury</th>
<th>Antibody</th>
<th>Lung Wt, g</th>
<th>Protein, μg/ml</th>
<th>Total cells, millions</th>
<th>Macrophages, millions</th>
<th>Neutrophils, millions</th>
<th>Lymphocytes, millions</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Isotype</td>
<td>2.4 ± 0.1</td>
<td>120 ± 6</td>
<td>1.8 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>0.1 ± 0.01</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>None</td>
<td>Anti-PTHrP 1–34</td>
<td>2.5 ± 0.1</td>
<td>110 ± 13</td>
<td>1.4 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>0.1 ± 0.01</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>None</td>
<td>Anti-PTHrP 67–86</td>
<td>2.1 ± 0.2</td>
<td>140 ± 20</td>
<td>3.4 ± 0.7*</td>
<td>2.9 ± 0.6*</td>
<td>0.1 ± 0.01</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>Silica</td>
<td>Isotype</td>
<td>2.4 ± 0.2</td>
<td>540 ± 50</td>
<td>15.9 ± 3.4</td>
<td>14.6 ± 3.2</td>
<td>2.4 ± 1.4</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>Silica</td>
<td>Anti-PTHrP 1–34</td>
<td>2.6 ± 0.1</td>
<td>400 ± 20</td>
<td>14.8 ± 1.8</td>
<td>9.9 ± 1.2</td>
<td>3.8 ± 0.9</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>Silica</td>
<td>Anti-PTHrP 67–86</td>
<td>2.8 ± 0.1</td>
<td>400 ± 60</td>
<td>8.6 ± 1.0*</td>
<td>6.0 ± 0.8*</td>
<td>2.0 ± 0.5</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. corresponding (no injury or silica injury) isotype control group.
tedly complex, and we do not have experimental evidence at this time to construct a complete explanation.

The effects of PTHrP 67–86 on phospholipid levels in silica lung injury are new, but not remarkable. Mid-molecule PTHrP peptides are known to have biological effects in other tissues. For example, PTHrP 67–86 has antimitogenic effects on selected breast carcinoma cell lines (18, 19). We recently demonstrated that PTHrP 1–34 and PTHrP 67–86 both sensitize type II cells to apoptosis after exposure to ultraviolet radiation (13). Both peptides cause a rapid increase in pneumocyte inositol phosphate levels. A receptor for sequences in the region of PTHrP 67–86 has not been identified, but our results suggest that it may be a G protein-coupled receptor, as is the receptor for PTHrP 1–34. PTHrP 67–86 may not be the exact biological ligand of this receptor. We tested PTHrP 67–86 because it is commercially available but have not delineated the sequence requirements for maximal activity on BAL DSPC, DSPC secretion, or type II cell growth.

Our phospholipid measurements are consistent with the findings of other investigators in rat silica lung injury. For example, Kawada and colleagues (17) found that total extracellular phospholipid levels more than double after 4 days of injury in agreement with our results. Dethloff and coworkers (4) found that DSPC represents a similar, but slightly lower percentage of total BAL phospholipid in silica-injured lungs compared with control lungs, 46.8 ± 3.2 vs. 55.4 ± 4.1%, respectively. Finally, several investigators before us have observed an increase in the fractional distribution of BAL phospholipids into phosphatidylinositol in silica injury (1, 3, 17). The reason for this change is unclear but might be a nonspecific manifestation of alveolar injury and inflammation or a change in biosynthetic pathways (1, 17). We found that PTHrP 1–34 and PTHrP 67–86 did not cause significant changes in the distribution of BAL phospholipid species or in the ratio of phosphatidylcholine to sphingomyelin compared with untreated injured lungs, suggesting that the two peptides did not worsen the degree of injury. The inflammation and injury indexes in Table 3 support this statement.

In summary, this study demonstrates that lung PTHrP levels decrease early after silica lung injury. Treating injured lungs with one intratracheal dose and four daily subcutaneous doses of PTHrP 1–34 or PTHrP 67–86 stimulated BAL DSPC levels, possibly through direct effects on secretion by type II cells. Neither peptide affected type II cell proliferation with this dosing regimen, but the addition of an additional intratracheal dose resulted in significant inhibition of growth, consistent with previous effects of PTHrP 1–34 in hyperoxic lung injury. Experiments with neutralizing antibodies resulted in complex effects, either stimulating proliferation or reducing BAL DSPC. These studies establish a regulatory role for PTHrP in DSPC metabolism and type II cell proliferation in silica injury. Biological activity of PTHrP 67–86 in the lung is a new finding. A receptor for this midmolecule PTHrP peptide has not been identified but may be a G protein-coupled receptor.

Kathy Smith and Cheryl Chalberg conducted the immunoassay studies. Devin Duey, Yvette Rascon, and Lacrista Fuqua performed immunohistology and phospholipid assays.

DISCLOSURES

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