NO protects alveolar type II cells from stretch-induced apoptosis. A novel role for macrophages in the lung

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Edwards, Yasmin S., Leanne M. Sutherland, and Andrew W. Murray. NO protects alveolar type II cells from stretch-induced apoptosis. A novel role for macrophages in the lung. Am J Physiol Lung Cell Mol Physiol 279: L1236–L1242, 2000.—We have previously shown that mechanical distortion or stretch of alveolar type II (ATII) cells induces both surfactant release and the induction of apoptosis. We hypothesize that nitric oxide (NO) secreted from alveolar macrophages (AMs) prevents cyclic stretch-induced apoptosis. We show that S-nitroso-N-acetyl-D,L-penicillamine (SNAP), a chemical donor of NO, protects cells against nuclear condensation and DNA fragmentation induced by stretch (30% at 60 cycles/min) as well as by sorbitol. SNAP depleted of NO had no protective effect, and the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide blocked the antiapoptotic effect of SNAP. We also show that AMs isolated from rat lung lavage fluid actively synthesize and secrete NO. Using a novel technique in which AMs were cocultured with ATII cells while adhered to floating membrane rafts, we found that NO released from AMs was effective in protecting ATII cells from undergoing apoptosis. We therefore propose that NO secreted by AMs may function as part of a physiological antiapoptotic mechanism that prevents ATII cells from undergoing stretch-induced cell death in the lung.

apoptotic stimuli; antiapoptotic factors; mechanical distortion; S-nitroso-N-acetyl-D,L-penicillamine; sorbitol-induced osmotic shock.

ALVEOLAR TYPE II (ATII) cells synthesize and secrete pulmonary surfactant, a complex mixture of lipids and proteins that maintains fluid balance and stabilizes the alveoli within the lung. Surfactant secretion is triggered by a range of secretagogues, including β-adrenoagonists and ATP, as well as by the mechanical distortion or stretch of ATII cells during breathing (22). Recently, Edwards et al. (6) reported that in addition to phosphatidylcholine (PC) secretion from ATII cells, cyclic stretch (20% at 3 cycles/min) also induced events consistent with the onset of apoptosis, a normal physiological process of cell removal. These events included nuclear condensation and oligosomal fragmentation of DNA (6). Unlike stretch, the secretagogues 12-O-tetradecanoylphorbol 13-acetate and ATP did not induce apoptosis in these cells. The immediate question that arises from this work is whether stretch-induced apoptosis of ATII cells is a physiological phenomenon. Normal respiratory expansion and contraction of the lung are clearly not accompanied by lung damage involving extensive ATII cell apoptosis. This implies that during tidal breathing, the resident ATII cells either are not subjected to proapoptotic stretching forces or do not undergo apoptosis as a result of protective mechanisms that may operate in situ. One such mechanism could include the involvement of antiapoptotic factors present in the alveolar environment of ATII cells.

There are a number of potential antiapoptotic factors that have been examined in different cell types, several of which are reported to be present in the lung. These include insulin growth factor I (9, 28), 5-hydroxyeicosatetraenoic acid (8), keratinocyte growth factor (4), and nitric oxide (NO) (14). Of these, NO has been the most extensively studied, primarily because of its noted vasodilatory properties when inhaled and more recently because of its emerging role as an endogenous regulator of apoptosis in cells and tissues. However, the precise role of NO in the regulation of apoptosis is unclear. Although NO has been reported to exhibit antiapoptotic actions in lung cells (11, 12) and other cell types (15), it has long been attributed with proapoptotic functions that are part of the inflammatory responses triggered in alveolar macrophages (AMs) (10, 21).
In the present paper, we examined the effects of NO on ATII cells induced to undergo apoptosis by cyclic stretch and sorbitol-induced osmotic shock. We show that NO derived from the chemical donor S-nitroso-N-acetyl-d,L-penicillamine (SNAP) attenuates apoptosis in response to both treatments. We also show that AMs obtained from rat lung lavage fluid actively synthesize NO, which is also effective in protecting ATII cells from undergoing apoptosis. This work provides support for the existence of an antiapoptotic mechanism in the lung that involves AM-derived NO and that protects ATII cells from undergoing stretch-induced apoptosis.

MATERIALS AND METHODS

Chemicals and reagents. We obtained [methyl-3H]choline chloride (74.4 Ci/mmol) from Amersham (Sydney, Australia). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from JRH Biosciences (Melbourne, Australia), and fetal calf serum was obtained from Trace Biosciences (Sydney, Australia). Human plasma-derived fibronectin was obtained from Boehringer Mannheim (Mannheim, Germany), elastase purified from porcine pancreas (≥3 U/mg protein) was obtained from Worthington Biochemical (ScirA, Victoria, Australia), and serum-derived rat γ-globulin was purchased from Calbiochem-Novabiochem (Sydney, Australia). ATP, SNAP, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), N-nitro-L-arginine methyl ester, and aminoguanidine were purchased from Sigma.

Culture and treatment of ATII cells. ATII cells were isolated from the lungs of specific pathogen-free adult male Sprague-Dawley rats (150–240 g) as previously described (5). Briefly, the lungs were perfused with buffer A (150 mM NaCl, 5 mM KCl, 2.5 mM NaHPO4-NaH2PO4, 0.2 mM EGTA, 0.1% glucose, and 10 mM HEPES, pH 7.4) for 2 min. The lungs were then lavaged with two volumes of buffer A (60 ml/kg) followed by two volumes of buffer B (150 mM NaCl, 5 mM KCl, 2.5 mM NaHPO4-NaH2PO4, 2 mM CaCl2, 1.3 mM MgSO4, 0.1% glucose, and 10 mM HEPES, pH 7.4; 60 ml/kg), each instilled and withdrawn three times. ATII cells were dissociated from the lung tissue by elastase digestion (1,050 U/kg) and were separated from contaminating macrophages by panning on immunoglobulin G-coated bacteriological plates. We have found that this procedure yields 95% viable ATII cells as determined by trypan blue exclusion (data not shown). For stretch experiments, nonadherent ATII cells were collected and plated at a density of 0.5 × 10^6 cells/cm² on fibronectin-coated (5 μg/cm²) six-well 25-mm Flex I/II or 35-mm BioFlex Flexcell multiwell culture plates (Flexcell International) in HEPES-buffered DMEM that contained glutamine and was supplemented with 10% (vol/vol) fetal calf serum, 60 μg/ml of penicillin G, 100 μg/ml of streptomycin sulfate, and 10 μg/ml of gentamicin (1 × 10⁵ cells/ml; complete DMEM). For selected nonstretch experiments, the cells were plated on 24-well Falcon multiwell culture plates (Becton Dickinson) in sodium bicarbonate-buffered complete DMEM. Plating efficiency was ~70%. The cells were incubated for 21 h at 37°C in a humidified atmosphere with 5% CO₂.

The cell cultures were washed four times with DMEM and treated with 1 mM ATP or 0.4 M sorbitol or subjected to cyclic stretch as described in Mechanical stretch in the absence and presence of SNAP at the concentrations indicated in Figs. 1–3. In selected experiments, the cells were treated in the absence and presence of 0.2 mM PTIO and 0.5 mM dinitrosylated SNAP (dinitro-SNAP), which was prepared by incubating 0.5 mM SNAP in culture medium for 5 days under normal culture conditions to deplete SNAP of NO. Mechanical stretch. ATII cells cultured on Flex I or BioFlex culture plates that contained flexible elastomer inserts were subjected to repetitive cycles of stretch and relaxation with a vacuum-operated FX-3000 Flexercell strain unit (Flexcell International). A 30% maximum elongation of the elastomer membrane was maintained for 0.5 s followed by 0.5 s of relaxation (60 cycles/min). Because the Flexercell apparatus produces a radially nonuniform strain field, the actual stretch force to which the cells are subjected depends on their axial distance from the center of the membrane. To ensure consistency between experiments, only cell cultures demonstrating an even plating distribution across the membrane were used for stretch experiments. This regimen was continuously applied for 2 h to cells that were maintained at 37°C in a humidified atmosphere and was followed by further incubation without stretching for the total treatment times indicated in Figs. 1–4. For unstretched control treatments, the cells were cultured on either BioFlex plates that were not subjected to stretch or Flex I culture plates in which the elastomer insert overlaid a rigid polystyrene bottom that prevented transmission of the vacuum force to the cells.

Isolation and culture of AMs. AMs were collected from the lavage fluid obtained during the isolation of ATII cells. Briefly, after the lungs were lavaged, both buffers A and B were combined and centrifuged (300 g for 10 min) to pellet the AMs. The cells were resuspended in complete DMEM and plated at a density of 0.1 × 10⁶ cells/cm² in Falcon culture inserts (0.4-μm pore size). For nonstretch coculture experiments, inserts suitable for use with 24-well multiwell culture plates were used and were cocultured for 21 h with freshly isolated ATII cells. For stretch coculture experiments, the AMs were initially left to adhere to the insert membranes (23.1-mm diameter) for 2 h in complete DMEM. The membranes were then carefully removed from the inserts and floated upside down in the medium of ATII cells cultured on BioFlex plates. Of those cells that adhered to the membrane rafts during the initial 2-h incubation period, ~95% remained attached for the duration of the subsequent coculture phase of the experiment (data not shown). The approximate ratio of AMs to ATII cells during coculture was 0.25:1.

PC secretion. ATII cells were cultured on BioFlex plates in complete DMEM containing [3H]choline chloride (1 μCi/ml). After 21 h, adherent cells were washed four times with HEPES-buffered DMEM and treated with various test substances or subjected to stretch as described in Figs. 1–4. After 2 h, PC secretion was measured as previously described (13).

Measurement of NO. NO release into the culture medium was determined by assaying for nitrite accumulation with a microtiter plate assay based on the Griess reaction (1). Briefly, 50 μl of sample were incubated (10 min at 25°C in the dark) with 1% sulfanilamide in 5% phosphoric acid followed by incubation (10 min at 25°C in the dark) with 0.1% N-1-naphthylethylenediamine dihydrochloride in water. Absorbance was measured at 543 nm, and nitrite concentration was determined with a standard curve of sodium nitrite concentrations ranging from 0 to 100 μM.

Determination of apoptosis. ATII cells cultured and treated as described in Figs. 1–4 were used for the measurement of DNA condensation with the nuclear stain Hoechst 33258 (Calbiochem-Novabiochem) and the analysis of internucleosomal DNA fragmentation with agarose gel electrophoresis as previously described (5). The generation of DNA fragments was also measured with a cell death detection ELISA kit (Boehringer Mannheim) as described in the manufacturer’s protocol.
Table 1. Secretagogue- and cyclic stretch-induced secretion and apoptosis in ATII cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3H]PC Secretion, %</th>
<th>Apoptosis, %</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.8 ± 0.05</td>
<td>5.6 ± 1.6</td>
</tr>
<tr>
<td>ATP</td>
<td>2.4 ± 0.09*</td>
<td>7.9 ± 1.8</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>2.9 ± 0.11*</td>
<td>38.9 ± 2.1</td>
</tr>
<tr>
<td>Stretch</td>
<td>1.8 ± 0.09*</td>
<td>17.7 ± 1.9</td>
</tr>
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Values are means ± SE of triplicate determinations; n = 5 separate experiments. Alveolar type II (ATII) cells were cultured (with and without 1 Ci/ml of [3H]choline chloride) on BioFlex plates for 21 h. Washed, and incubated in the absence and presence of 1 mM ATP or 0.4 M sorbitol or subjected to cyclic stretch for 2 h as described in MATERIALS AND METHODS. For the analysis of [3H]phosphatidylcholine (PC) secretion, total lipids were extracted from both the medium and the cells. Radioactivity was measured, and the percentage of [3H]PC secretion was calculated as the amount of radioactivity in the culture medium as a percentage of total radioactivity measured in the medium and cells combined. For the analysis of apoptosis, cells were stained with Hoechst 33258, and the percentage undergoing apoptosis was determined. *P < 0.001 compared with control.

Statistical analysis. Results were analyzed by one-way ANOVA, Bonferroni test, Dunnett’s test, and Student’s unpaired t-test as indicated in Figs. 1–4.

RESULTS

As shown in Table 1, ATP, sorbitol-induced osmotic shock, and cyclic stretch all induced a significant secretion of [3H]PC from ATII cells prelabeled for 20 h with [3H]choline chloride. Sorbitol (0.4 M) and ATP (1 mM) stimulated a 3.5-fold and a 3-fold release, respectively, after 2 h of treatment, whereas cyclic stretch (30% at 60 cycles/min) induced a more moderate 2-fold increase over this time. Secretion of [3H]PC was unlikely to be due to leakage because the cell membranes appeared to remain intact both during treatment and for at least 4 h after treatment as demonstrated by the sustained exclusion of propidium iodide during the experiments (data not shown). Both sorbitol and cyclic stretch also induced nuclear condensation cells that were measured after 6 h of treatment in ATII by staining the cells with Hoechst 33258 (Table 1). As shown, the percentage of cells exhibiting nuclear condensation increased sixfold after sorbitol treatment and underwent a more moderate threefold increase after cyclic stretch. ATP had no detectable effect on nuclear condensation in ATII cells.

The release of NO into the culture medium from the chemical donor SNAP and its effects on nuclear condensation in ATII cells are shown in Fig. 1. The amount of NO spontaneously liberated from SNAP during the incubation periods was estimated from the amount of nitrite measured in the culture medium with the Griess assay (see MATERIALS AND METHODS). As shown in Fig. 1A, nitrite levels in the medium rose with increasing concentrations of SNAP and continued to accumulate linearly with time up to 24 h (data not shown). In cells treated with sorbitol, the level of nuclear condensation induced after 6 h was decreased when incubated with SNAP (Fig. 1B). This decrease was significant at all of the concentrations of SNAP tested, with a maximal effect obtained at 0.5 mM. A similar trend was observed when stretch was used as the apoptotic stimulus, although a significant decrease in nuclear condensation was only measured in the presence of 0.5 mM SNAP due to replicate variability (Fig. 1C). At 0.5 mM, SNAP continued to protect against both sorbitol- and stretch-induced nuclear condensation for at least 24 h after its addition (nuclear condensation at 24 h: sorbitol alone, 23.7 ± 2.1%;
sorbitol plus SNAP, 64.6 ± 2.2%; stretch alone, 55.1 ± 2.1%; stretch plus SNAP, 72 ± 2%). However, as shown in Fig. 1, B and C, the percentage of cells exhibiting nuclear condensation increased when incubated in the presence of SNAP at concentrations > 0.5 mM, being significantly higher at 5 mM SNAP for both sorbitol-treated (P < 0.05) and stretch-treated (P < 0.01) cells.

As shown in Fig. 2, SNAP also protected ATII cells against DNA fragmentation. This was evident after 6 h in both sorbitol- and stretch-treated cells from the clear decreases in both the levels of DNA laddering detected by agarose gel electrophoresis (Fig. 2A) and the level of nucleosomal enrichment as measured by cell death ELISA (Fig. 2B).

To further examine the role of NO in mediating the protective effect of SNAP, experiments were carried out with dinitro-SNAP, a NO-depleted derivative of SNAP. Dinitro-SNAP, which was generated by prolonged preincubation of SNAP (0.5 mM) in the culture medium (see MATERIALS AND METHODS), had no significant protective effects on either stretch- or sorbitol-induced apoptosis as determined from the percentage of cells exhibiting nuclear condensation (Fig. 3A). In addi-

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tional experiments, PTIO, a stable radical scavenger of NO, was used to deplete the incubation medium of NO. As shown in Fig. 3B, incubating cells with SNAP in the presence of PTIO (5 μM) abrogated the protective effect of SNAP, with PTIO maintaining sorbitol- and stretch-induced nuclear condensation levels close to those found in control cells at 6 h.

AMs isolated from rat lung lavage fluid and cultured on membrane rafts as described in MATERIALS AND METHODS released NO into the culture medium (Fig. 4A). This release, which was measured at 6 h with the Griess assay, continued linearly for up to 48 h (data not shown). As shown in Fig. 4A, NO release was completely blocked by incubation with the combined NO synthase inhibitors Nω-nitro-L-arginine methyl ester (5 μM) and aminoguanidine (5 μM). Coculture experiments were carried out to examine the effects of AM-derived NO on the apoptotic responses of ATII cells. In these experiments, ATII cells were treated with sorbitol or subjected to cyclic stretch in the presence of AMs that were attached to membrane rafts floating in the culture medium. As shown in Fig. 4, B and C, AMs significantly suppressed ATII cell apoptosis in response to both sorbitol (P > 0.001) and stretch (P > 0.01), respectively. This effect was completely blocked when treatments were carried out in the presence of the NO synthase inhibitors that in the case of sorbitol led to a slight although not significant increase in the percentage of ATII cells undergoing apoptosis while in coculture with AMs (Fig. 4B).

DISCUSSION

Using a computer-controlled Flexercell strain device to stretch primary cultures of rat ATII cells, we have shown that short-term cyclic stretch, applied as a 30%, 60 cycles/min regimen for 2 h, stimulated both [3H]PC secretion and apoptosis. The secretary response measured after stretch was less than that obtained after treatment with the secretagogue ATP or the proapoptotic stimulus sorbitol and was quantitatively similar to that obtained for the 20%, 3 cycles/min regimen that we have used and that was previously reported by Edwards et al. (6). The induction of nuclear condensation by cyclic stretch and sorbitol was measured after 6 h and is consistent with the earlier work by Edwards et al. in which they showed that cyclic stretch functions as an apoptotic stimulus as well as a trigger for the release of PC from isolated ATII cells. In the current experiments, we elected to use a stretch regimen (30%, 60 cycles/min) that resembles the normal breathing pattern of rats in an endeavor to better model the stretching patterns that ATII cells are likely to encounter in situ. This follows the current trend in which researchers using the Flexercell strain device apply stretch regimens that mimic breathing patterns that are physiologically relevant to the animal origins of the particular cell types on which they are working (25–27, 29). However, it remains difficult to correlate physiological stretch with the degree of stretch applied to ATII cell cultures with the Flexercell apparatus given...
the uncertainty surrounding our current understanding of the types of stretch forces that ATII cells are likely to encounter in vivo.

Clearly, one of the immediate questions raised by our observations is why stretch, responsible for triggering the release of surfactant from ATII cells, also causes these cells to die. Furthermore, what is the physiological relevance of this phenomenon? As an initial step toward addressing these questions, we began to examine the potential role of antiapoptotic factors in preventing apoptosis in stretched ATII cells. Such factors have been identified in other cell systems (3, 7, 13, 18–20, 23, 24) and may be present in the alveolus, originating either from the ATII cells themselves or from neighboring cells such as AMs or alveolar type I (gas-exchange cells). One such factor is NO, which is secreted by AMs and has been reported to have both proapoptotic (10) and antiapoptotic (11, 12) properties in lung cells and in an array of other cell types (2, 16, 17). In the present work, SNAP, a commercially available chemical donor of NO, protected against both sorbitol- and stretch-induced apoptosis in cultured ATII cells. This protection was concentration dependent, and there was evidence that higher concentrations of SNAP (>0.5 mM) led to increased cell death. These results suggest that the reported antiapoptotic (11, 15, 17) and proapoptotic (10, 21) effects of NO may be a concentration-related phenomenon. The protective effect of SNAP is attributable to the NO moiety as shown by the experiments carried out with dinitro-SNAP and PTIO. However, NO is a highly unstable free radical that will rapidly react with oxygen species and transition metals to form derivatives such as nitrate, nitrite, peroxynitrite, and metal-NO adducts (30). Although nitrate and nitrite were not found to protect ATII cells from apoptosis (data not shown), we cannot eliminate other NO derivatives as potential antiapoptotic mediators.

ATII cells are in the close vicinity of AMs in the lung, which are located both in the alveolar compartment and in interstitial regions (14). Because AMs are known to modulate ATII cell responses through secreted regulators such as NO (10), we investigated whether AMs could have an antiapoptotic effect on ATII cells. To do this, AMs were isolated from rat lung lavage fluid and cultured on membrane rafts that were then floated in the medium of ATII cell cultures, enabling us to analyze the effects of diffusible mediators while keeping the two cell populations separate. Using this approach, we showed that AMs suppressed both sorbitol- and stretch-induced apoptosis in ATII cells. Moreover, the ability of AMs to prevent apoptosis was completely inhibited by NO synthase inhibitors that blocked the production of NO. These data therefore show that the antiapoptotic effects of AMs are mediated by NO. However, because AMs secrete a range of molecules, it is not yet known whether secreted cytokines or other diffusible factors are also required for the antiapoptotic effect. However, from our experiments with SNAP and the AM-ATII cell cocultures, the most likely conclusion is that NO plays an important role in modulating stretch-induced cell death. Therefore, in addressing the physiological relevance of stretch-induced ATII apoptosis, we propose that AMs are a potential component of the antiapoptotic mechanism that operates in the lung. Clearly, our in vitro model for examining the effects of stretch on ATII cells eliminates the influence of neuroendocrine factors and complex cell-cell interactions that may modify cell responses in the lung. Such influences and the role that secreted molecules in addition to NO may play in regulating apoptotic responses of ATII cells are also being explored.

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REFERENCES