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Cyclic stretch induces both apoptosis and secretion in rat alveolar type II cells

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Abstract We examined the effects of short-term cyclic stretch on both phosphatidylcholine (PC) secretion and apoptosis in primary cultures of rat alveolar type II cells. A 22% cyclic stretch (3 cycles/min) was applied to type II cells cultured on silastic membranes using a Flexercell strain unit. This induced, after a lag period of about 1 h, a small, but significant release of [^{3}H]PC from prelabelled cells. In addition, stretch increased nuclear condensation, the generation of oligosomal DNA fragments and the activation of caspases. Similar responses were triggered by sorbitol-induced osmotic shock, but not by the secretagogue ATP. We conclude that stretch can induce both apoptosis and PC secretion in alveolar type II cells and propose that these diverse responses occur within the lung as a consequence of normal respiratory distortion of the alveolar epithelium.

Key words: Phosphatidylcholine; Secretagogue; Sorbitol; DNA fragmentation; Caspase

1. Introduction

The stability and fluid balance of the lung is largely controlled by a complex mixture of lipids and proteins called pulmonary surfactant, which lines the gas liquid interface of the alveoli. Surfactant is synthesized and stored in epithelial type II cells and is released in response to direct stimulation by various agents including purinergic [1] and β_2-adrenoagonists [2], phorbol esters [3] and calcium [4]. Hyperpnea in animals, induced by altering inspired gas or exercise, also stimulates surfactant release [5], as does a single deep inflation of an isolated perfused lung [6]. Furthermore, the application of a single mechanical stretch to primary cultures of alveolar type II cells stimulates the release of phosphatidylcholine (PC), the major lipid component of surfactant [7]. These latter observations indicate that mechanical distortion of the type II cell, which occurs during respiratory expansion and contraction of the alveolus, is an important secretory stimulus, possibly the predominant physiological trigger for surfactant release.

Mechanical stretch is a form of cellular stress which has been shown to initiate apoptosis in cardiac myocytes [8] and to activate signalling pathways implicated in the regulation of apoptosis in other cell types [9–11]. Apoptosis is a highly orchestrated form of cell death, distinguishable from necrosis by specific structural and biochemical events. Alveolar type II cells reportedly undergo apoptosis as part of normal lung development and maturation [12,13] and as a consequence of acute lung injury [14]. As we have recently shown that type II cells also undergo apoptosis in response to sorbitol-induced osmotic shock and ultraviolet (UV) C radiation [15], we hypothesized that mechanical stretch would initiate both PC secretion and apoptosis in these cells.

We have used a computer-controlled Flexercell strain device to stretch primary cultures of rat alveolar type II cells. We have shown that a short-term cyclic stretch regime induces both PC secretion and events consistent with the onset of apoptosis, including the condensation of nuclei, oligosomal fragmentation of DNA and activation of members of the caspase family of cysteine proteases.

2. Materials and methods

2.1. Cell culture and treatment

Type II cells were isolated from the lungs of adult male Sprague Dawley rats (150–240 g) as previously described [15]. Cells were plated at a density of 0.5×10^6 cells/cm^2 on fibronectin-coated (5 μg/cm^2) 24- or 48-well Falcon multiwell culture plates (Becton and Dickson) or on 25 mm, 6-well Flexcell Flex I/II multiwell culture plates (Flexcell International) in DMEM containing glutamine and supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml of penicillin G, 100 μg/ml of streptomycin sulphate and 10 μg/ml Gentamicin (1×10^6 cells/ml). The cells were incubated for 21 h at 37°C in a humidified atmosphere at 5% CO_2. They were then washed four times with DMEM unsupplemented with serum and treated with 1 mM ATP, 0.4 M sorbitol, or were subjected to cyclic stretch as described below.

2.2. Mechanical stretch

Cells cultured on Flex I Flexcell culture plates, containing flexible elastomer inserts, were subjected to repetitive cycles of stretch and relaxation using a vacuum-operated FX-3000 Flexercell strain unit (Flexcell International). A 22% maximum elongation of the elastomer membrane was maintained for 10 s followed by 10 s of relaxation. This regime was continuously applied to the plated cells for 30 min (3 cycles/min) which were maintained at 37°C in a humidified atmosphere at 5% CO_2. For unstretched control treatments, cells were cultured on Flex II Flexcell culture plates in which the elastomer insert overlaid a rigid polystyrene bottom which prevented transmission of the vacuum force to the cells.

2.3. PC secretion

Alveolar type II cells were cultured in either standard 48-well or Flex I/II culture plates in DMEM containing [^{3}H]choline chloride (1 μCi/ml) for 21 h. After treatment lipids were extracted, radioactivity measured and PC secretion calculated as described previously [15].

2.4. Determination of apoptosis

Alveolar type II cells were cultured in Flex I/II culture plates. After treatment, the cells were used for both the morphological determination of apoptosis using the nuclear stain Hoechst 33258 (Calbiochem) and the analysis of internucleosomal DNA fragmentation by agarose gel electrophoresis as described previously [15]. The generation of

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DNA fragments was also measured using a cell death detection ELISA kit (Boehringer Mannheim) according to the manufacturer's protocol. The activity of caspase proteases following treatment was determined using the synthetic fluorogenic substrate, *N*-acetyl; λ-Asp-Glu-Val-Asp 7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC; BIOMOL Research Laboratories) as described previously [15].

2.5. Cell viability

Viability of the cells following treatment was routinely monitored via the exclusion of either trypan blue or propidium iodide.

3. Results and discussion

As shown in Table 1, cyclic stretch (3 cycles/min) maintained for 30 min resulted in a small but significant secretion of [^{3}H]PC from alveolar type II cells (panel A) after a lag period of about 1 h (panel B). This was not due to leakage as cells continued to exclude trypan blue for at least 4 h after treatment. The stretch regime used was selected following preliminary experiments which showed that cyclic but not a single stretch stimulus induced detectable PC secretion (data not shown). In these experiments a 22% stretch of the membrane inserts facilitated measurable PC secretion without compromising cell viability or the integrity of the membranes. Larger secretory responses, similar to that reported by Wirtz and Dobbs [7] for type II cells which had been subjected to a membrane stretch of 30%, required degrees of stretch in excess of that which could be reliably applied to membranes using the Flexercell strain unit (data not shown).

The effects of stretch on apoptosis in alveolar type II cells are shown in Figs. 1–3. Short-term cyclic stretch for 30 min resulted in nuclear condensation as determined by staining cells with Hoechst 33258 at 8 h (Fig. 1A). As reported previously [15], treatment with sorbitol (0.4 M) also induced nuclear condensation, while ATP (1 mM) had no effect (Fig. 1A). Increased nuclear condensation in response to sorbitol and stretch was detectable after 4 h (Fig. 1B) and continued for at least 24 h (data not shown). Oligosomal DNA fragmentation consistent with the onset of apoptosis, was evident after 6 h in both stretch- and sorbitol-treated cells, as demonstrated by both agarose gel electrophoresis (Fig. 2A) and ELISA analysis (Fig. 2B). A key biochemical event associated with

Table 1
Secretagogue- and stretch-induced [^{3}H]PC secretion

A.

Treatment	% [^{3}H] secretion
Control	0.78 ± 0.05
ATP	2.50 ± 0.14*
Sorbitol	2.59 ± 0.16*
Stretch	1.03 ± 0.10*
	*$P < 0.001$

B.

Treatment	% [^{3}H] secretion	
	Control	Stretch
1 h	0.53 ± 0.03	0.57 ± 0.04
4 h	0.74 ± 0.02	1.60 ± 0.21*
		*$P < 0.001$

Cells were cultured on Flex I/II plates in the presence of [^{3}H]choline chloride (1 μCi/ml) for 21 h. Cells were washed and incubated for A: 90 min following 30 min of stretch or for 2 h in the presence of either 1 mM ATP or 0.4 M sorbitol; B: 30 min or 3.5 h following 30 min of stretch or for 1 and 4 h with either 1 mM ATP or 0.4 M sorbitol. After treatment total lipids were extracted from both the medium and the cells. Radioactivity was measured and the percent [^{3}H]PC secretion calculated. Data were representatives of five separate experiments, with each point representing mean ± S.E. of three replicate determinations.

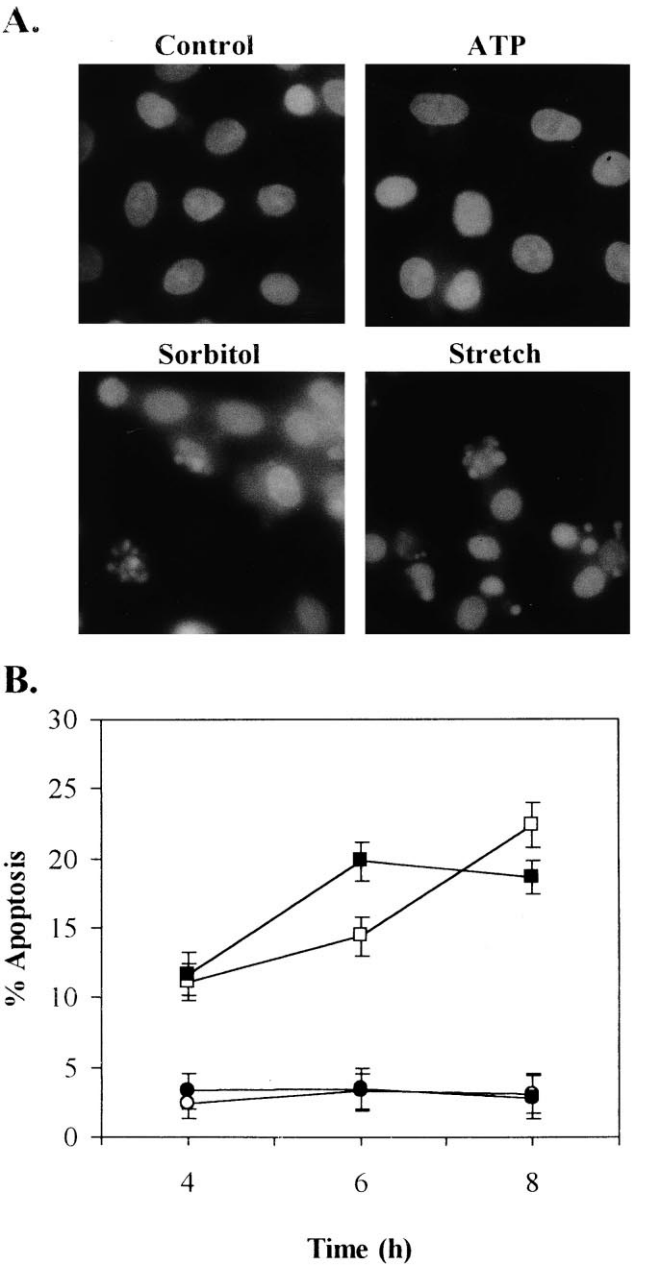
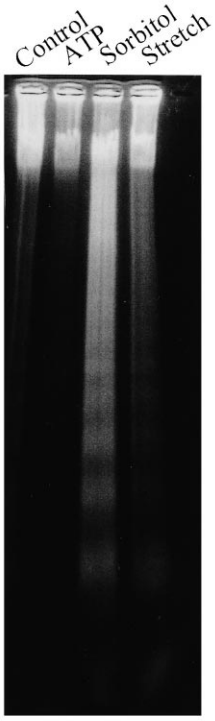


Fig. 1. Effects of secretagogues and stretch on cell morphology. A: Cells cultured on Flex I/II plates were washed and incubated for 7.5 h following 30 min of stretch or for 8 h in the presence of either 1 mM ATP or 0.4 M sorbitol. Cells were stained with Hoechst 33258 and photographed under a Nikon inverted fluorescence microscope (mag. ×400). B: Cells cultured as above were incubated for 3.5, 5.5, or 7.5 h after 30 min of stretch or in the presence of either 1 mM ATP or 0.4 M sorbitol for the indicated times. Cells were stained with Hoechst 33258 and the number of cells undergoing apoptosis scored at the indicated times. Apoptosis is expressed as the percentage of the total number of attached cells that show condensed or fragmented nuclei in four randomly chosen fields of view for untreated cells (○), cells treated with either ATP (●) or sorbitol (□) and cells subjected to stretch (■). At least 800 cells/treatment were counted and results are expressed as means ± S.E. of four replicate experiments.

A.



B.

Treatment	Nucleosome enrichment
ATP	1.30 ± 0.04
Sorbitol	139.20 ± 12.00
Stretch	40.50 ± 0.70

Fig. 2. Effects of secretagogues and stretch on DNA fragmentation. Cells cultured on Flex I/II plates were washed and incubated for 7.5 h following 30 min of stretch or for 8 h in the presence of either 1 mM ATP or 0.4 M sorbitol. A: Cells were prepared for in-gel digestion and analysis of internucleosomal DNA fragmentation by agarose gel electrophoresis. DNA was stained with ethidium bromide, visualized under UV light and the gel photographed. B: Cells were harvested and lysates prepared for analysis of released oligonucleosomal DNA using a cell death ELISA kit. Nucleosomal enrichment factors ($\times 10^{-5}$) were calculated from absorbance readings measured at 405 nm and are expressed for each treatment as mean ± S.E. of three replicate determinations relative to a control.

apoptosis is the activation of caspases, a family of cysteine proteases which participate in a cascade that is triggered in response to apoptotic signals and culminates in the disassembly of the cell [16]. For many stress-related apoptotic stimuli caspase activation is frequently preceded by the release of mitochondrial cytochrome *c* into the cytosol where it binds with Apaf-1 and procaspase-9. This results in the activation of caspase-9 which in turn cleaves procaspase-3 into activate subunits [17–20]. In the present experiments 30 min of cyclic stretch induced a moderate activation of caspases after 6 h (Fig. 3) as measured with a synthetic fluorogenic substrate with some selectivity for caspase-3. Continuous exposure to sorbitol for this period caused a marked activation of caspases, while ATP had no detectable effect. Together these results clearly demonstrate that, as with sorbitol-induced osmotic shock, short-term cyclic stretch induces apoptosis in alveolar type II cells. All of the experiments reported in Figs. 1–3 were carried out in the absence of serum. This was to make the experiments directly compatible with the secretion experiments described in Table 1. Secretion is routinely measured in serum-free medium because of technical difficulties associated with solvent extraction of the lipid in the presence of serum. However in some cells serum starvation is itself an apoptotic stimulus [21]. While this was not the case with alveolar type II cells (see untreated samples in Figs. 1–3), it was possible that the stimulus synergized with serum deprivation to generate apoptosis. However in separate experiments (data not shown) we established that a similar time course and extent of apoptosis was induced by stretch in both the presence and absence of serum. It is worth noting that the presence of serum also had no effect on the degree of surfactant secretion (data not shown).

As a potential physiological phenomenon, stretch-induced apoptosis in alveolar type II cells raises some interesting questions. Firstly, can apoptosis be induced in vivo as a consequence of normal respiratory distortion of the alveoli epithelium. Clearly, our in vitro model for examining the effects of stretch on type II cells is an over-simplification which has eliminated the influence of other cell types. It also does not take into account spatial relationships of type II cells which appear to be preferentially located in corners of the alveoli and hence may be subjected to an exaggerated distortion during breathing. It is therefore difficult to predict and hence mimic, the precise stretch regime which type II cells are subjected to in the lung under physiological conditions. Experiments in which type II cell apoptosis is measured in situ, either in isolated perfused lungs or in whole animals, will help to address the physiological relevance of this response. Another question raised by our observations is, if apoptosis is triggered in type II cells by normal respiratory distortion in vivo, how is this counteracted to prevent massive damage to the lung? It is possible that uncontrolled apoptosis is pre-

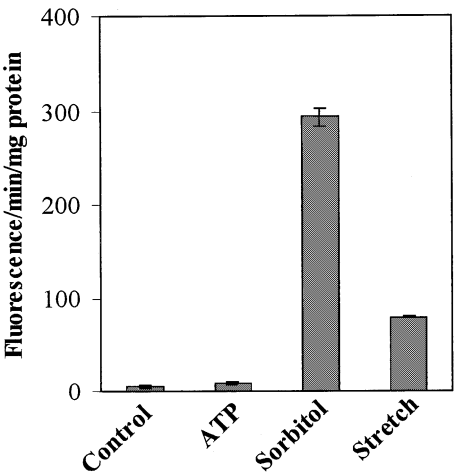


Fig. 3. Effects of secretagogues and stretch on caspase activation. Cells were cultured on Flex I/II plates and incubated for 5.5 h after 30 min of stretch or for 6 h in the presence of either 1 mM ATP or 0.4 M sorbitol. Medium was removed and the cells prepared for the measurement of caspase activity using the substrate Ac-DEVD-AFC. Results are expressed as mean ± S.E. of three separate experiments.

vented by anti-apoptotic factors which are present in the alveolar environment of type II cells, having originated either from the type II cells themselves or from neighboring cells, such as alveolar macrophages or alveolar type I cells. Several possible candidates have been identified in other cell systems, including secreted apoptosis-related proteins [22], IGF-1 [23], arachidonic acid metabolites such as 5-hydroxyeicosatetraenoic acid [24,25] and nitric oxide [26,27]. In preliminary studies we have found that alveolar type II cells secrete an anti-apoptotic factor(s) during prolonged culture which partially protects against both sorbitol- and stretch-induced apoptosis (data not shown). We are currently characterizing this factor(s) and its (their) possible anti-apoptotic role in vivo. It will also be of interest to determine how the same stretch stimulus is able to such diverse events as apoptosis and PC secretion. In other cell types, stretch activates the stress-activated kinases such as c-Jun N-terminal kinase and p38 kinase [9–11]. These pathways have been linked to the induction of apoptosis and we have shown them to be stimulated by osmotic shock in alveolar type II cells [15]. It is not known whether these pathways are also activated in response to short-term cyclic stretch in type II cells or if indeed they play any role in regulating PC secretion. However, in a recent report stretch was shown to activate c-Jun N-terminal kinase in vascular smooth muscle cells through mechanisms involving autocrine ATP stimulation of purinergic receptors [28]. Since ATP is a potent secretagogue, such a mechanism could account for the observed effects of stretch on both apoptosis and secretion in alveolar type II cells. Experiments are currently underway to examine the signalling pathways activated by short-term cyclic stretch and their involvement in regulating both PC secretion and apoptosis in alveolar type II cells.

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