Osmotic stress induces both secretion and apoptosis in rat alveolar type II cells

YASMIN S. EDWARDS, LEANNE M. SUTHERLAND, JOHN H. T. POWER, TERENCE E. NICHOLAS, AND ANDREW W. MURRAY

School of Biological Sciences, Faculty of Science and Engineering, and
department of Human Physiology, School of Medicine, Flinders University
of South Australia, Adelaide, South Australia 5001, Australia

Edwards, Yasmin S., Leanne M. Sutherland, John H. T. Power, Terence E. Nicholas, and Andrew W. Murray. Osmotic stress induces both secretion and apoptosis in rat alveolar type II cells. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L670–L678, 1998.—The aim of this study was to analyze the effects of osmotic shock and secretagogues such as ATP and 12-O-tetradecanoylphorbol 13-acetate (TPA) on various intracellular signaling pathways in primary cultures of alveolar type II cells and examine their potential role in regulating events such as secretion and apoptosis in these cells. Sorbitol-induced osmotic stress caused the sustained release of [3H]phosphatidylcholine (3H)PC from primary cultures of rat alveolar type II cells prelabeled with [3H]choline chloride. This release was not dependent on protein kinase C because downregulation of the major protein kinase C isozymes (α, β1, δ, and η) expressed in alveolar type II cells had no effect on 3HPC secretion. Sorbitol, as well as the known secretagogues TPA and ATP, activated extracellular signal-regulated kinase. Although an inhibitor of the extracellular signal-regulated kinase cascade, PD-98059, blocked this activation, it had no effect on the release of 3HPC. Sorbitol and ultraviolet C radiation, but not TPA or ATP, were also found to activate both p38 and stress-activated protein kinase/c-Jun NH2-terminal kinase. Furthermore, both sorbitol and ultraviolet C radiation induced apoptosis in alveolar type II cells as demonstrated by Hoechst 33258 staining of the condensed nuclei, the generation of DNA ladders, and the activation of caspases. The data indicate that multiple signaling pathways are activated by traditional secretagogues such as TPA and ATP and by cellular stresses such as osmotic shock and that these may be involved in regulating secretory and apoptotic events in alveolar type II cells.

sorbitol; 12-O-tetradecanoylphorbol 13-acetate; adenosine 5'-triphosphate; mitogen-activated protein kinase; stress-activated signaling

PULMONARY SURFACTANT is a complex mixture of lipids and proteins that lines the gas-liquid interface of the alveolar compartment where it both stabilizes the alveolus and maintains the fluid balance (23). Surfactant is synthesized in alveolar type II cells and is primarily released via specialized secretory vesicles termed lamellar bodies in response to a variety of secretagogues. These include purinergic (28) and β-adrenergic agonists (6, 24), phorbol esters (15), calcium ionophores (13), fatty acids (1), vasopressin (5), histamine (9), endothelin-1 (31), and lipoproteins (27). Various intracellular signaling pathways are activated by these secretagogues, suggesting that more than one mechanism is involved in controlling the secretory response.

The protein kinase C (PKC) family of intracellular signaling molecules, of which alveolar type II cells have recently been reported to express several subtypes (22), is involved in regulating the release of surfactant. This has been clearly demonstrated in studies using the potent phorbol ester secretagogue 12-O-tetradecanoylphorbol 13-acetate (TPA), which functions as a structural analog of diacylglycerol, the physiological activator of PKC (29). In such experiments, the effect of TPA on secretion is abolished if either PKC is downregulated or PKC inhibitors are present. Similar results are obtained with the purinergic secretagogue ATP, which triggers the biphasic formation of diacylglycerol as a result of receptor-coupled phosphatidylinositol 4,5-bisphosphate hydrolysis and subsequent hydrolysis of phosphatidylcholine (PC) in type II cells (8, 32).

In many cell types, the activation of PKC is accompanied by the activation of the extracellular signal-regulated kinase (ERK) cascade, a member of the mitogen-activated protein kinase (MAPK) family (12). Consequently, it would be predicted that phorbol esters would activate the ERK cascade in alveolar type II cells, although the relationship of this pathway to secretion is unknown. Other members of the MAPK family include the activation of the caspase family of cysteine proteases, condensation of nuclei, and oligosomal fragmentation of DNA.

In the present paper, we examined the effects of osmotic stress (sorbitol) and exposure to UVC radiation in addition to traditional secretagogues (ATP and TPA) on PC secretion, apoptosis, and activation of the MAPK family of signaling molecules in alveolar type II cells. We report that osmotic stress strongly stimulates secretion, induces apoptosis, and activates all members of the MAPK family.

MATERIALS AND METHODS

We obtained [methyl-3H]choline chloride (74.4 Ci/mmol) from Amersham (Sydney, Australia). DMEM was purchased from J RH Biosciences (CommonWealth Serum Laboratories,
Mélanie, Australia) and fetal calf serum was obtained from Trace Biosciences (Sydney, Australia). Elastase and human plasma-derived fibronectin were obtained from Boehringer Mannheim (Mannheim, Germany) and serum-derived rat γ-globulin was purchased from Calbiochem-Novabiochem (Sydney, Australia). TPA was acquired from P-L Biochemicals (Milwaukee, WI), and ATP was purchased from Sigma. PD-globulin was purchased from Calbiochem-Novabiochem Mannheim (Mannheim, Germany) and serum-derived rat plasma-derived fibronectin were obtained from Boehringer Melbourne, Australia) and fetal calf serum was obtained from New England Biolabs. Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD) was purchased from Bachem. Peptide-purified PKCα, βII, δ, and γ antibodies and phospho-specific ERK I/II and phospho-specific SAPK/J NK antibodies were obtained from New England Biolabs, and phospho-specific p38 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep anti-rabbit IgG-horseradish peroxidase conjugate and mouse anti-rabbit IgG-horseradish peroxidase conjugate were purchased from Silexus (Melbourne, Australia). Supported nitrocellulose membrane (0.5 µm) was obtained from Schleicher and Schuell (Dassel, Germany), and enhanced chemiluminescence reagents were obtained from NEN-DuPont.

Isolation and culture of alveolar type II cells. Type II cells were isolated from the lungs of adult male Porton rats (190–260 g) by the method of Dobbs et al. (14). Briefly, the lungs were perfused with buffer A (150 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 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 ± kg) followed by two volumes of buffer B (150 mM NaCl, 5 mM KCl, 2.5 mM phosphate buffer, 2 mM CaCl₂, 1.3 mM MgSO₄, 0.1% glucose, and 10 mM HEPES, pH 7.4; 60 ± kg), each instilled and withdrawn three times. Alveolar type II cells were dissociated from lung tissue by elastase digestion (1,050 U/kg) and were separated from contaminating macrophages by panning on IgG-coated bacteriological plates. We have found that this procedure yields 95% viable alveolar type II cells as determined by trypan blue exclusion (data not shown). Nonadherent type II cells were collected and plated at a density of 0.5 × 10⁶ cells/cm² on fibronectin-coated (4 µg/cm²) 35-mm Corning culture dishes or 24-, 48-, or 96-well Falcon multiwell culture plates in DMEM (1 × 10⁶ cells/ml) containing glutamine and supplemented with 10% (vol/vol) fetal calf serum, 60 µg/ml of penicillin G, 100 µg/ml of streptomycin sulphate, and 10 µg/ml of gentamicin. Plating efficiency was determined by trypan blue exclusion (data not shown). Nonadherent cells were pelleted (300 g for 10 min), lipids were extracted from the medium by the method of Bligh and Dyer (2), with 500 µg/sample of unlabeled PC as the carrier. Lipids were also solvent extracted from the adherent cells after they were combined with the 300-g pellet obtained above. The solvent phases were dried, and radioactivity was measured with liquid scintillation spectrometry. Secretion was calculated as the amount of radioactivity in the medium expressed as a percentage of the total radioactivity measured in the medium and cells combined. We have found that under these conditions >95% of the total radioactivity is associated with PC (data not shown).

Cell viability. Viability of the cells after treatment with the various secretagogues was routinely monitored via the exclusion of either trypan blue or propidium iodide. In selected experiments, the lactate dehydrogenase (LDH) content of the culture medium from treated cells (1 × 10⁶ cells in 24-well plates) was analyzed by measuring the disappearance of NADH at 340 nm in assays carried out by the Department of Medical Biochemistry, Flinders Medical Centre (Adelaide, Australia). The amount of LDH in the medium was calculated as the rate of NADH oxidation using a molar extinction coefficient of 6,200 M/cm. Total cellular LDH was determined by measuring the LDH content of the medium after lysis of the cells with five cycles of freezing and thawing.

Preparation of protein extracts and immunoblot analysis. Whole cell protein extracts were prepared from alveolar type II cells as follows. Cells cultured in 35-mm culture dishes for 20 h were washed four times with ice-cold phosphate-buffered saline (PBS) and scraped into 125 µl of sonication buffer (2 mM EDTA, 5 mM EGTA, 0.25 M sucrose, 10 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, 10 mM mercaptoethanol, 0.001% leupeptin, and 20 mM Tris-HCl, pH 7.4) containing 2% Triton X-100. The cells were incubated for 45 min on ice, sonicated (3 × 5 s at 4°C), and centrifuged (100,000 g for 20 min at 4°C). The resulting supernatant was mixed with Laemmli sample buffer (1:2 vol/vol; Ref. 21), heated (5 min at 100°C), and used for immunoblot analysis of total cell proteins.

Soluble protein extracts were prepared from alveolar type II cells as follows. Cells cultured in 35-mm culture dishes for 20 h were washed four times with fresh DMEM and treated with various test substances (see Figs. 1–8). For experiments in which the cells were exposed to UVC radiation, the medium in each well was reduced to 1 ml. After irradiation, the volume of medium in each well was increased to 4 ml, and further incubation of the cells continued for the indicated times (see Figs. 1–8) at 37°C in a humidified atmosphere at 5% CO₂. Incubations were stopped by removing the medium and washing the cells with ice-cold PBS. The cells were scraped into 125 µl of sonication buffer, sonicated (3 × 5 s at 4°C), and centrifuged (100,000 g for 20 min at 4°C). When samples were prepared for immunoblot analysis of phosphorylated ERK, SAPK/J NK, or p38, the following inhibitors were added to the sonication buffer: 10 mM NaF, 1 mM Na₃VO₄, and Sigma 104 phosphatase substrate (1:1,000 vol/vol). The supernatant was mixed with Laemmli sample buffer (1:2 vol/vol), heated (5 min at 100°C), and used for immunoblot analysis of soluble proteins as described below.

Proteins (5–15 µg) determined by the method of Bradford and Beil (4) were separated by gel electrophoresis (21) on SDS-polyacrylamide gels (10% polyacrylamide, 200 V, 0.8 h; Mini-Protein II gel system, Bio-Rad). After electrophoresis, the gels were preequilibrated in transfer buffer (152 mM glycine, 1.3 mM SDS, 20% methanol, and 25 mM Tris; 15 min), after which the proteins were transferred to nitrocellulose membranes (100 V, 1.5 h; Mini Transfer system, Bio-Rad). After transfer, the membranes were incubated with blocking solution (0.1% Tween 20, 5% skimmed-milk powder, and 40 mM Tris-HCl, pH 7.4; 60 min at room temperature) followed by further incubation with renewed blocking solution containing the appropriate primary antibody (45 min at 37°C) (see Figs. 1–8). The membranes were rinsed in washing solution (0.1% Tween 20, 5% skimmed-milk powder, 0.15 mM NaCl, and 20 mM Tris-HCl, pH 7.4; 2 × 5 min and 1 × 15 min) and incubated in blocking solution containing either sheep or mouse anti-rabbit IgG-horseradish peroxidase conjugate.
gate (1:1,000 dilution; 30 min at 37°C). The membranes were again rinsed in washing solution, and immunoreactive bands were detected with enhanced chemiluminescence according to the manufacturer's protocol.

Determination of apoptosis. Morphological determination of apoptosis was analyzed with the nuclear stain Hoechst 33258 combined with the exclusion of propidium iodide. Briefly, cells cultured in 24-well culture plates (1 × 10⁴/well) for 20 h were washed four times in DMEM supplemented with serum and treated with various test substances (see Figs. 1–8). The cells were stained with propidium iodide (10 µg/ml) and Hoechst 33258 (1 mg/ml) before sampling, and apoptotic cells (Hoechst 33258 positive) were distinguished from nonviable cells (propidium iodide positive) and viable cells under phase-contrast or fluorescence microscopy. Apoptosis was expressed as the percentage of the total number of attached cells that showed condensed or fragmented nuclei in four randomly chosen fields of view.

Internucleosomal DNA fragmentation in apoptotic cells was analyzed by agarose gel electrophoresis. For gel electrophoresis analysis, cells that were cultured (2 × 10⁶/well) and treated as described above were collected and pelleted by centrifugation (300 g for 10 min). Whole cells were prepared for in-gel digestion, and DNA was separated on a 1.8% agarose gel according to the method of Wolfe et al. (34) together with a 100-bp ladder marker (Pharmacia Biotech, Quarry Bay, Hong Kong). DNA was visualized under UV light after being stained with ethidium bromide (0.5 µg/ml) and photographed.

The activity of caspase proteases was determined by an in vitro assay, modified from that described by Kim et al. (17), with the synthetic fluorogenic substrate N-acetyl-Asp-Glu-Val-Asp 7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC; BIOMOL Research Laboratories). Briefly, alveolar type II cells cultured in 24-well culture plates for 20 h were washed four times in DMEM supplemented with serum and treated with the various test substances (see Figs. 1–8). Cells were scraped into 500 µl of assay buffer (140 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 5 µg/ml of pepstatin, 10 µg/ml of leupeptin, and 100 mM HEPES, pH 7.4) and lysed by five cycles of freezing and thawing. Aliquots of the total cell lysate (50 µg of protein), made up to 990 µl with assay buffer containing 20% glycerol, were transferred to a plastic cuvette. The incubation mixture was maintained at 37°C, and the enzyme reaction was initiated by adding 37 µg of Ac-DEVD-AFC. Caspase activity was calculated from the change in fluorescence measured at 505 nm.

Statistical analysis. Results were analyzed by Student's unpaired t-test.

**RESULTS**

Effect of sorbitol on PC secretion. Sorbitol stimulated the secretion of [³H]PC from alveolar type II cells that had been prelabeled for 20 h with [³H]choline chloride. As shown in Fig. 1, secretion of [³H]PC was concentration dependent (Fig. 1A) and after treatment of the cells with 0.4 M sorbitol was detectable within 30 s, which continued to increase linearly for at least 4 h (Fig. 1B). The cells remained viable after exposure to sorbitol, determined initially by the exclusion of either trypan blue or propidium iodide and supported by the absence of any detectable LDH activity in the medium of sorbitol-treated cells (LDH activity = control value = 0.3 ± 0.17 µmol·min⁻¹·l⁻¹; sorbitol not detected; total cellular LDH = 248 ± 3.5 µmol·min⁻¹·l⁻¹).

Effect of phorbol ester pretreatment on PKC and secretagogue-stimulated PC secretion. Pretreatment of type II cells with 500 nM TPA for 20 h resulted in a marked depletion of immunologically detectable PKC-α, -β₁₁, -β₁, and -γ (Fig. 2A), the major PKC subforms expressed in alveolar type II cells. Both TPA (100 nM) and ATP (1 mM) are moderate secretagogues that produced a reproducible twofold increase in [³H]PC release from type II cells prelabeled with [³H]choline chloride (Fig. 2B). In this same experiment, 0.4 M sorbitol was a potent secretagogue, causing over a threefold increase in [³H]PC release. Phorbol ester pretreatment clearly abolished the stimulatory effects...
of both TPA and ATP on [3H]PC secretion (Fig. 2B). However, as shown in Fig. 2, the secretion of [3H]PC in response to sorbitol was only slightly attenuated after phorbol ester pretreatment.

Effect of secretagogues on ERK, p38, and SAPK/JNK activity. In this series of experiments, the effects of TPA, ATP, and sorbitol on the activity of the MAPK family was examined. As a routine, activation of ERK, p38, and SAPK/JNK was assessed in secretagogue-treated type II cells by Western blot analysis of soluble protein extracts with antibodies that detect the dual-phosphorylated active forms of these kinases. Immunoreactive bands were detected with enhanced chemiluminescence. For [3H]PC secretion, [3H]choline chloride-labeled cells, pretreated with either DMSO or TPA, were washed and then incubated for 2 h in absence (-) or presence (+) of 100 nM TPA, 1 mM ATP, or 0.4 M sorbitol. Incubations were stopped, and total lipids were extracted from medium and cells. Radioactivity was measured, and percent secretion was calculated as described in MATERIALS AND METHODS. Results are expressed as mean ± SE of 3 replicate determinations. *SE values for all treatments except control (−TPA) were rounded off to the nearest 2nd decimal place. Similar results were obtained in 5 separate experiments.

Fig. 2. Phorbol ester-induced downregulation of protein kinase (PK) C and effect on secretagogue-stimulated [3H]PC secretion. Alveolar type II cells were isolated from a rat lung as described in MATERIALS AND METHODS and cultured in presence of 0.1% DMSO or 500 nM 12-O-tetradecanoylphorbol 13-acetate (TPA) for 20 h in either 35-mm culture dishes for Western blot analysis of PKC (A) or 48-well culture plates in presence of [3H]choline chloride (1 μCi/ml) for [3H]PC secretion (B). For Western blot analysis, whole cell protein extracts were prepared from both DMSO- and TPA-pretreated cells. Extracts were fractionated (24 μg of protein) by gel electrophoresis and analyzed for presence of PKC-α, -βII, -δ, and -η with peptide-purified antibodies. Immunoreactive bands were detected with enhanced chemiluminescence. For [3H]PC secretion, [3H]choline chloride-labeled cells, pretreated with either DMSO or TPA, were washed and then incubated for 2 h in absence (-) or presence (+) of 100 nM TPA, 1 mM ATP, or 0.4 M sorbitol. Incubations were stopped, and total lipids were extracted from medium and cells. Radioactivity was measured, and percent secretion was calculated as described in MATERIALS AND METHODS. Results are expressed as mean ± SE of 3 replicate determinations. *SE values for all treatments except control (−TPA) were rounded off to the nearest 2nd decimal place. Similar results were obtained in 5 separate experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% [3H] PC secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.69 ± 0.001*</td>
</tr>
<tr>
<td>TPA</td>
<td>1.13 ± 0.03</td>
</tr>
<tr>
<td>ATP</td>
<td>1.23 ± 0.03</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>2.43 ± 0.23</td>
</tr>
</tbody>
</table>

Fig. 3. Time course of extracellular signal-regulated kinase (ERK), p38, and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) activity after secretagogue treatment. Alveolar type II cells were isolated from 4 separate rat lung preparations. Each isolate was cultured in six 35-mm dishes for 20 h as described in MATERIALS AND METHODS, washed, and then incubated in absence or presence of 100 nM TPA (A), 1 mM ATP (B), or 0.4 M sorbitol (C) for the indicated times. Soluble protein extracts (10 μg) were fractionated by gel electrophoresis and analyzed for presence of phosphorylated forms of ERK, p38, and SAPK/JNK by immunoblot analysis with peptide-purified, phospho-specific antibodies. Immunoreactive bands were detected with enhanced chemiluminescence. These results are representative of 4 (TPA and ATP) and 2 (sorbitol) separate experiments.
Secretagogues, 0.4 M sorbitol activated all three MAPK subspecies (Fig. 3C). The activation of both ERK and SAPK/JNK was delayed by ∼10 min, whereas activation of p38 was rapid, with a reproducible increase in immunoreactive, phosphorylated p38 observed after 30 s.

The involvement of ERK in sorbitol- as well as in TPA- and ATP-stimulated secretion was examined with PD-98059, an inhibitor of the ERK upstream activating kinase, MAPK/ERK kinase. The effect of PD-98059 on ERK activity is clearly shown in Fig. 4A, in which preincubation of alveolar type II cells for 1 h with PD-98059 completely blocked the accumulation of phosphorylated ERK I/II induced by TPA. However, despite this effect on the ERK pathway, PD-98059 did not prevent [3H]PC release induced by either sorbitol, TPA, or ATP (Fig. 4B).

Induction of apoptosis in alveolar type II cells. The apoptotic responses of alveolar type II cells was initially examined by the uptake of Hoechst 33258 combined with the exclusion of propidium iodide. Results obtained after type II cells treated with sorbitol, the traditional secretagogues TPA and ATP, and UVC radiation, a widely used apoptotic stimulus, were stained as shown in Fig 5. As shown in Fig 5A, there was little detectable nuclear condensation in cells treated for 8 h with either TPA or ATP. However, both sorbitol and, to a lesser degree, UVC radiation caused a distinct increase in the proportion of cells containing condensed nuclei, consistent with the induction of apoptosis in these cells. Increased nuclear condensation in response to sorbitol and UVC radiation was detectable after 4 h of treatment and continued for at least 24 h, although a greater proportion of the total cells at this time had undergone necrosis (Fig. 5B).

As shown in Fig. 6, only sorbitol- and UVC radiation-treated cells generated the oligosomal DNA ladders characteristic of apoptosis after agarose gel electrophoresis. The formation of DNA fragments in response to sorbitol was confirmed in a separate experiment in which a photometric enzyme immunoassay was used to detect the release of oligonucleosomal DNA into the cytoplasmic fraction of cell lysates (Cell Death Detection Kit, Boehringer Mannheim) prepared from type II cells after treatment (data not shown).

Sorbitol caused a marked activation of caspases in alveolar type II cells as measured with a synthetic substrate selective for caspase-3 (Fig. 7). UVC radiation had only a moderate effect on caspase activity in these cells, whereas both ATP and TPA had no detectable effect (Fig. 7). In addition, the activation of caspases in response to sorbitol was completely blocked by Z-VAD, a general inhibitor of caspase activity.

Like sorbitol, UVC radiation stimulated all three MAPK pathways in alveolar type II cells. However, as shown in Fig. 8A, this activation was somewhat more rapid, with immunoreactive bands corresponding to the phosphorylated forms of ERK, p38, and SAPK/JNK appearing after 30 s. Despite this and in contrast to sorbitol, UVC radiation was found to be only a very weak inducer of [3H]PC secretion in alveolar type cells prelabeled with [3H]choline chloride (Fig. 8B).

DISCUSSION

Effect of sorbitol on secretion and PKC activation. The release of pulmonary surfactant from alveolar type II cells is induced by a range of secretagogues that operate through multiple intracellular signaling pathways. The involvement of PKC has been widely examined, and this kinase family is clearly involved in regulating secretion in response to secretagogues such as TPA and ATP. In many cell types, the activation of PKC is accompanied by the activation of MAPK, a kinase family that is variably regulated by a range of stimuli, including cellular stressors such as osmotic shock (3, 19). In the present study, we initially examined the effect of sorbitol-induced osmotic shock on PC secretion and the activation of PKC in primary cultures of alveolar type II cells. In these experiments, sorbitol clearly induced a rapid and sustained secretory response. However, phorbol ester pretreatment, which depleted type II cells of the four PKC isoforms (α, β, 1, 2, and δ) normally expressed and blocked secretion in response to TPA and ATP, had no effect on sorbitol-induced secretion. This suggests that signaling pathways other than PKC are involved in mediating the sorbitol-induced response. In the present study, the secretion of PC measured in response to TPA and ATP...
was routinely approximately twofold higher than that measured under basal conditions. It should be noted that although similar secretory responses have been reported by others (7), there is also another study (32) in which these secretagogues are reported to increase PC secretion by as much as sixfold above basal levels. Such variation between studies is likely to be due to differences in the experimental methodologies used to measure stimulated secretion.

In our experiments, it was important to examine the effects of sorbitol on cell viability because membrane

![Fig. 5. Effect of secretagogues on alveolar type II cell morphology.](image)

**A**
- Control (- DMSO)
- ATP
- TPA
- Sorbitol
- UVC

**B**
- Control (+ DMSO)
- ATP
- TPA
- Sorbitol
- UVC

![Fig. 6. Effect of secretagogues on alveolar type II cell DNA fragmentation.](image)

**M**
- 1600
- 800
- 400

**Fig. 5.** Effect of secretagogues on alveolar type II cell morphology. Alveolar type II cells (4 x 10⁶/well) were isolated from a rat lung and cultured in 6-well culture plates for 20 h as described in MATERIALS AND METHODS. Cells were washed, incubated in either absence or presence of 0.1% DMSO, 100 nM TPA, 1 mM ATP, or 0.4 M sorbitol or exposed to 500 J/m² UV-C radiation for 8 h. Cells were stained with Hoechst 33258 and propidium iodide and photographed under a Nikon inverted fluorescence microscope (magnification x400).

**Fig. 6.** Effect of secretagogues on alveolar type II cell DNA fragmentation. Alveolar type II cells (2 x 10⁶/well) were isolated from a rat lung and cultured in 24-well culture plates for 20 h as described in MATERIALS AND METHODS. Cells were washed, treated in either absence (lanes 1 and 6) or presence of 100 nM TPA (lane 2), 1 mM ATP (lane 3), 0.4 M sorbitol (lane 4) or exposed to 500 J/m² UV-C radiation (lane 5), and prepared for in-gel digestion and analysis of internucleosomal DNA fragmentation by agarose gel electrophoresis as described in MATERIALS AND METHODS. M, bands corresponding to 1600-, 800-, and 400-bp DNA fragments. DNA was stained with ethidium bromide, and gel was photographed after DNA was visualized under UV light.
rupture, as a consequence of osmotic shock, could potentially account for the observed increase in PC release after sorbitol treatment. There was no detectable release of LDH into the medium of treated cells as well as no observable uptake of either trypan blue or propidium iodide. Because these are all indexes of membrane permeability, these results indicate that the cells remain intact for the duration of the secretion experiments and that the release of radiolabeled PC into the medium is due to activated secretion and not to membrane leakage.

Secretagogue activation of the MAPK pathways. We found that ERK was strongly activated by TPA and ATP as well as by sorbitol in alveolar type II cells. However, this activation appears to be unrelated to PC secretion because PD-98059, which blocked ERK activation, had no effect on the secretion of radiolabeled PC. ERK is known to regulate the downstream activity of various transcription factors (30), and it is possible that secretory signals are linked to the synthesis of surfactant-associated proteins via the ERK pathway. This would support a mechanism in which the processes of surfactant synthesis and secretion are coupled to secretory stimuli via the ERK and PKC pathways, respectively.

Neither TPA nor ATP had any measurable effect on the activity of the SAPK/JNK and p38 pathways. This was in contrast to sorbitol, which strongly activated both SAPK/JNK and p38, suggesting that one or both of these pathways could be involved in regulating sorbitol-induced secretion. In particular, activation of the p38 pathway closely correlated with secretion. Sorbitol induced both p38 activation and secretion within 30 s, whereas activation of SAPK/JNK could not be detected until 10 min. However, it must be noted that the quantitative relationship between activation of the stress pathways and secretion is not strong. This is evident from other studies (10, 16) with UVC radiation.

Fig. 7. Effects of secretagogues on caspase activity. Alveolar type II cells were isolated from a rat lung and cultured in 24-well culture plates (1 × 10⁶ well) for 20 h as described in MATERIALS AND METHODS. Cells were washed and incubated for 6 h in either absence or presence of 0.1% DMSO, 100 nM TPA, 1 mM ATP, or 0.4 M sorbitol or after exposure to 500 J/m² UVC radiation. Medium was removed, cells were scraped into 500 µl of assay buffer, and caspase activity was measured with synthetic fluorogenic substrate Ac-DEVD-AFC as described in MATERIALS AND METHODS. Z-VAD, Z-Val-Ala-DL-Asp-fluoromethylketone. Results are expressed as means ± SE of 3 separate experiments. * For these treatments, results are from 6 separate experiments.

Fig. 8. Effects of UVC radiation of [³H]PC secretion and mitogen-activated protein kinase signaling pathways. A: alveolar type II cells were isolated from a rat lung and cultured in 48-well culture plates in presence of [³H]choline chloride (1 µCi/ml) for 20 h as described in MATERIALS AND METHODS. Cells were washed, either untreated (0 min) or exposed to 500 J/m² UVC radiation as described in MATERIALS AND METHODS, and then incubated for the indicated times. Soluble protein extracts (10 µg) were fractionated by gel electrophoresis and analyzed for presence of phosphorylated forms of ERK, p38, and SAPK/JNK by immunoblot analysis with peptide-purified, phospho-specific antibodies. Immunoreactive bands were detected with enhanced chemiluminescence. Results are representative of 2 separate experiments. B: alveolar type II cells were isolated from a rat lung and cultured in 48-well culture plates for 20 h in presence of [³H]choline chloride as described in MATERIALS AND METHODS. Cells were washed and either untreated (0 min) or exposed to 500 J/m² UVC radiation as described in MATERIALS AND METHODS. Cells were washed and exposed to 500 J/m² UVC radiation as described in MATERIALS AND METHODS. After incubation, total lipids were extracted from medium and cells. Radioactivity was measured, and percent secretion was calculated as described in MATERIALS AND METHODS. Results are expressed as means ± SE of 3 replicate determinations. Similar results were obtained in 2 (A) and 5 (B) separate experiments.
another form of cellular stress that has been shown to activate SAPK/J NK and p38 in other cell systems. Although exposure of type II cells to UVC radiation caused a rapid and sustained activation of all three MAPK pathways, it was found to be only a weak secretory stimulus. However, it is, of course, possible that UVC radiation induces other biological effects that block the secretory response, thereby masking any detectable involvement of SAPK/J NK and p38. It is also possible that other changes associated with cellular stress may be involved in regulating secretion in type II cells. For example, osmotic stress initiates multiple cellular events including the activation of selective ion channels and associated changes in cell volume (26). Similar events may therefore be involved in regulating the secretory responses of alveolar type II cells exposed to sorbitol-induced osmotic shock.

Sorbitol- and UVC radiation-induced apoptosis of alveolar type II cells. In this study, both sorbitol and UVC radiation were found to induce apoptosis in alveolar type II cells. This is the first time that the stimulation of an apoptotic response has been reported in these cells. Apoptosis is characterized morphologically by nuclear condensation and DNA fragmentation and is routinely visualized in cell systems by staining with Hoechst 33258. We used this stain in combination with propidium iodide, enabling apoptotic cells containing condensed nuclei (Hoechst positive) to be clearly distinguished from nonviable cells also containing condensed nuclei (propidium iodide positive). The apoptosis response induced by sorbitol and UVC radiation was also demonstrated by the generation of internucleosomal DNA fragments that were examined by both agarose gel electrophoresis (Fig. 6) and photometric enzyme immunoassay (data not shown).

One of the characteristic biochemical events associated with apoptosis is the activation of caspases, a family of cysteine proteases, with specificity for aspartate residues in the target proteins (25). Activation of caspases can be determined by following the cleavage of known substrates, by measuring caspase activity with artificial peptide substrates, or by directly monitoring caspase activation by analyzing the cleavage of inactive zymogen precursors with Western blotting. In the present experiments, we analyzed the effects of sorbitol and UVC radiation on the activity of caspases. The substrate used in these experiments has some specificity for caspase-3 but is likely to also react with other members of the caspase family. Clearly, treatment with sorbitol or UVC radiation resulted in a marked increase in caspase activity that was completely blocked by the caspase inhibitor Z-VAD. In a separate experiment, we also determined that Z-VAD blocks both sorbitol- and UVC-induced nuclear condensation (data not shown). Together, these observations establish unequivocally that sorbitol and UVC radiation induce dramatic apoptotic responses in alveolar type II cells.

The activation of the SAPK/J NK and p38 kinase cascades has been linked to the induction of apoptosis in other cell types (16). Consistent with this, we found both sorbitol-induced osmotic shock and UVC radiation to be potent inducers of apoptosis in alveolar type II cells. These results therefore support a role for the SAPK/J NK and p38 pathways in mediating the apoptotic responses of these cells.

The findings presented in this paper extend our current understanding of the signaling pathways that operate in alveolar type II cells after stimulation by secretagogues. Distortion, another form of cellular stress, is widely believed to be the predominant physiological stimulus for surfactant release in the lung (24). Mechanical stretch has also been shown to stimulate the release of PC from primary cultures of alveolar type II cells (33). Furthermore, it has been reported recently that physical stretch can activate stress pathways (18) and induce apoptosis in cultured myocytes (11). In preliminary studies, we have found that the exposure of alveolar type II cells to a single mechanical stretch induces both apoptosis and secretion. We are currently exploring the relationship between these responses and the effect stretch has on PKC and the various MAPK signaling cascades.

This work was supported by a grant from the National Health and Medical Research Council of Australia.

Address for reprint requests: Y. S. Edwards, School of Biological Sciences, Faculty of Science and Engineering, Flinders Univ. of South Australia, GPO Box 2100, Adelaide, South Australia 5001, Australia.

Received 22 December 1997; accepted in final form 9 June 1998.

REFERENCES


