H$_2$O$_2$ acts on cellular membranes to generate ceramide signaling and initiate apoptosis in tracheobronchial epithelial cells

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SUMMARY

Hydrogen peroxide (H$_2$O$_2$) is an inflammatory oxidant which contributes to the pathogenesis of chronic diseases such as lung injury of the respiratory tract, atherosclerosis and cancer. The mechanisms and target sites of this reactive oxidant are mainly unknown. So far there are opposing reports as to whether reactive oxidants inhibit or promote apoptosis. We activated the death pathway in primary tracheobronchial epithelial (TBE) cells with H$_2$O$_2$ (20-200 μM) and observed the morphological changes, DNA laddering patterns, and DNA fragmentation associated with apoptosis. Elevation of ceramide with exogenous ceramide analogs was sufficient for apoptosis induction with the same characteristics and in the same time frame. H$_2$O$_2$ induced rapid sphingomyelin hydrolysis to ceramide, the elevation of which paralleled the induction of apoptosis. Furthermore, H$_2$O$_2$ acted directly on TBE cells membrane preparations devoid of nuclei, stimulating sphingomyelin hydrolysis through a neutral Mg$^{2+}$ dependent sphingomyelinase (SMase). These data suggest that the formation of ceramide from sphingomyelin in the plasma membrane is a key event in H$_2$O$_2$-induced apoptosis in tracheobronchial epithelial cells.

Key words: Ceramide, Apoptosis, Oxidant, Bronchial epithelium

INTRODUCTION

H$_2$O$_2$ and its products, such as hydroxyl radicals and superoxide (Cross et al., 1990; Halliwell and Cross, 1991) are termed ‘oxidative stress’. Collectively, these species possess significant capacity for cellular damage and have been implicated in both the aging process and the pathogenesis of chronic diseases, among them, atherosclerosis, cancer and diseases of the respiratory tract (Halliwell and Gutteridge, 1984). Reactive oxygen intermediates are produced in all mammalian cells, partly as a result of normal cellular metabolism, and partly due to activation of a variety of oxidants-producing enzymes in response to exogenous stimuli. Excessive accumulation of reactive oxidants is toxic (Behl et al., 1994), and the intracellular level of reactive oxidants is therefore tightly regulated by several antioxidants. Although, antioxidant defenses are constitutively expressed in mammalian cells (Halliwell and Cross, 1994), additional responses are mounted when the amount of environmental oxidants exceeds a threshold level, thereby becoming a threat to overall tissue integrity. Apoptosis may be one such cellular adaptive response.

Apoptosis is an essential mechanism for the maintenance of homeostasis in multicellular organisms. This orderly process of programmed cell death selectively eliminates single damaged cells, without perturbing the neighboring tissue. Keeping apoptosis in balance limits the survival of deranged cells thus potentially reducing inflammatory processes. Apoptotic cell death can result either from developmentally controlled activation of endogenous execution programs or from transduction of death signals triggered by a wide variety of exogenous stimuli (Thompson, 1995; Whyte and Evan, 1995). Potential exogenous triggers of apoptosis range from growth factor withdrawal to ligand- or antibody-mediated engagement of specific cell surface receptors capable of transducing lethal signals (Pitti et al., 1996; Wu et al., 1995).

Most of the signaling pathways that trigger apoptosis remain unknown but the morphologic features of apoptosis are typical and well conserved in diverse cell types. Moreover, they are distinct from those occurring during necrosis. This suggests a possible convergence of multiple signaling pathways which ultimately culminate in one common route towards apoptosis. The sphingomyelin/ceramide pathway may constitute that final common step.

Even though most of the studies related to the ceramide signaling pathway were done in the hematopoetic system, particularly in leukemia cell lines, the sphingomyelin pathway is perceived as a ubiquitous signaling system that links specific cell-surface receptors and environmental stresses through to the nucleus (Hannun, 1996). This pathway is initiated by hydrolysis of the phospholipid, sphingomyelin, which is preferentially concentrated in the plasma membrane of mammalian cells. Sphingomyelin hydrolysis occurs within seconds to minutes after stimulation via the action of
**MATERIALS AND METHODS**

**Cell culture**

Airway epithelial cells were grown as described (Robinson and Wu, 1991). Briefly, tracheobronchial tissues from non-human primates were immersed in minimal essential medium (MEM) and treated for 24 hours at 4°C with 0.1% protease. Dissociated cells were recovered by centrifugation, resuspended in growth medium F12 (Gibco) supplemented with penicillin, streptomycin and garamycin (50 mg/ml), Heps (15 mM, pH 7.2), transferrin (0.1 μM), insulin (10 μM), retinoic acid (0.1 μM), hydrocortisone (0.1 μM), and epidermal growth factor (0.01 μM) and plated at a density of 1- to 5×10^5 cells/cm^2. Cells were incubated at 37°C with 5% CO_2 atmosphere. The medium was changed every other day, and a final cell density of 3 to 8×10^5 cells/cm^2 is obtained for primary cultures within 7 to 9 days of incubation. Cells were further passaged once or twice. Subcultures were performed as follows: near-confluent cultures were treated with trypsin (0.05%)-EDTA (1 mM) in phosphate-buffered saline (PBS), pH 7.0. After cells were detached from the plates, an equal volume of trypsin inhibitor solution (1 mg/ml) in F12 medium was added to stop the trypsinization. Cells were recovered by centrifugation and resuspended in culture medium for plating. Cell numbers were determined using a Coulter counter, model ZF (Coulter Electronics) and verified by hemacytometer (Hausser Scientific). Cell viability was assessed by Trypan blue exclusion analysis.

**Agarose gel electrophoresis for DNA fragmentation**

Oligonucleosomal fragmentation of genomic DNA was determined as previously described (Wyllie, 1980). Cells (6×10^6 to 12×10^6) were lysed in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K. Digestion was continued for 1-3 hours at 50°C, followed by the addition of RNase A to 0.1 mg/ml and further incubation for 1 hour. Running dye (10 mM EDTA, 0.25% Bromophenol Blue, 50% glycerol) was then added in a 1:6 ratio of dye: sample, and DNA preparations were electrophoresed in 1.5% agarose gels in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 4 V per cm of gel. DNA was visualized by ethidium bromide staining.

**Histochemical detection of nuclear DNA fragmentation and apoptotic bodies**

The terminal deoxynucleotidyltransferase end-labeling (TUNEL) technique was used for evaluation of airway epithelial cells for DNA fragmentation and the appearance of apoptotic bodies (Gorzyczka et al., 1993). Slides were stained with DNA counterstains, bis-benzimide (Hoechst 33342, Sigma), and propidium iodide. The morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome bis-benzimide as previously described (Oberhammer et al., 1992). In brief, 0.5×10^6 to 3.0×10^6 cells were pelleted at 300 g for 10 minutes and washed once with phosphate-buffered saline (PBS). Cells were resuspended in 50 μl of 3% paraformaldehyde in PBS and incubated for 10 minutes at room temperature. The fixative was removed, and cells were washed once in PBS and were resuspended in 15 μl of PBS containing 16 μg/ml bis-benzimide. Following a 15 minute incubation at room temperature, a 10 μl aliquot was placed on a glass slide, and 500 cells per slide were scored for the incidence of apoptotic chromatin changes. The slides were viewed under Nikon SA fluorescence microscope and view-fields were captured by C-Imaging System (Compix, Cranberry Twp., PA). Cells with three or more chromatin fragments were considered apoptotic.

To quantify apoptotic cells TBE cultures were also grown in 24-well tissue culture dishes. Cells were then rinsed twice in PBS (calcium and magnesium free) and incubated in 70% ethanol containing 100 μg/ml Hoechst 33258 (Molecular Probes, Inc) for 30 minutes at RT. This procedure served both to fix the cells remaining in the culture and to stain the DNA. After rinsing twice in PBS, the remaining liquid was aspirated, and the residual fluorescence was quantified in a fluorescent plate reader.

**Annexin V binding to phosphatidylserine (PS) flow cytometry assay**

The presence of apoptotic cells was evaluated by an early change in membrane phospholipid asymmetry associated with cells during the early phases of apoptosis. The loss of cell membrane phospholipid asymmetry is accompanied by the exposure of phosphatidylserine (PS) to the outer membrane as described (March et al., 1995b). Briefly, 10^6 cells were removed from the culture dishes by 2 minutes incubation in 0.05% trypsin. After washes of ice-cold PBS, the cells...
were incubated for 30 minutes at room temperature in the dark in a solution containing 4x SSC buffer (Sigma), 15 μg/ml fluoresceinated avidin, 0.1% Triton X-100 (v/v), and 0.5% nonfat milk. After one additional wash in ice-cold PBS containing 0.1% Triton X-100 (v/v), the cells were suspended at a concentration of 10^6/ml in PBS containing propidium iodide (5 μg/ml) and 0.1% RNase. For FACS analysis, cells were stained by fluorescein isothiocyanate (FITC)-conjugated annexin V and by the fluorescent dye propidium iodide (PI). Cells negative for both PI and annexin V staining are live cells; PI-negative, annexin V-positive staining cells are early apoptotic cells; and PI-positive annexin V-positive staining cells are primarily cells in late stages of apoptosis. A FACScan flow cytometer equipped with a doublet discriminating module (Becton Dickinson & Co.) was used. The data were analyzed using LYSYS II software (Hewlett-Packard Co.). An analysis region was set to exclude cell aggregates, and the green channel was set to score <1% of the signals from untreated control cells. The red (propidium iodide, PI) and green (fluorescein) fluorescence were measured.

Lipid analogs
C2-ceramide (N-acetyl sphingosine), C6-ceramide (N-hexanoyl sphingosine), C8-ceramide (N-heptanoyl sphingosine) and 1,2-dioctanoyl-sn-glycerol were obtained from Matreya (Pleasant Gap, PA, US), and stock solutions were prepared in dimethyl sulfoxide. C2-dihydrceramide (N-acetyl dihydrosphingosine) was obtained from Matreya, and 1,2-dioctanoyl-sn-glycerol-3-phosphate, which was obtained from Avanti Polar Lipids, was prepared as stock solutions in 100% ethanol. The final concentrations of dimethylsulfoxide and ethanol in the incubations were 0.2% and 0.1%, respectively, which did not induce apoptosis. All experiments involved both vehicle controls and specificity controls using biologically inactive dihydrceramide analogs or inactive L-threo stereoisomers of the active D-erythro.

Lipid studies
Ceramide was quantified by the diacylglycerol kinase assay as described previously (Goldkorn et al., 1992; Goldkorn, 1996; Dressler et al., 1992; Balaban et al., 1996). In brief, following incubation with H2O2, cells were pelleted by centrifugation (300 g for 10 minutes), washed twice with ice-cold PBS, and extracted with 6 ml of chloroform:methanol:1 N HCl (100:100:1, v/v/v). Lipids in the organic-phase extract were dried under N2 and subjected to mild alkaline hydrolysis (0.1 N methanolic KOH for 1 hour at 37°C) to remove glycerophospholipids. Samples were re-extracted, and the organic phase was dried under N2. Ceramide contained in each sample was resuspended in a 100 μl reaction mixture containing 150 μg of cardiolipin (Avanti Polar Lipids), 280 μM diethylenetriaminepenta-acetic acid (DTPA; Sigma), 51 mM octyl-β-D-glucopyranoside (Calbiochem), 0.7% glycerol, 70 μM β-mercaptoethanol, 1 mM ATP, 10 μCi of [γ-32P]ATP (3,000 Ci/mmol; Dupont New England Nuclear), 35 μg/ml E. coli diacylglycerol kinase (Calbiochem) at pH 6.5. After 30 minutes at room temperature, the reaction was stopped by extraction of lipids with 1 ml of chloroform:methanol:1 N HCl (100:100:1), 170 μl of buffered saline solution (BSS) (135 mM NaCl, 1.5 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, and 10 mM Hepes, pH 7.2), and 30 μl of 100 mM EDTA. The lower organic phase was dried under N2. Ceramide 1-phosphate was resolved by thin-layer chromatography on silica gel 60 plates (Whatman) using a solvent system of chloroform:methanol:acetic acid (65:15:5) and detected by autoradiography, and incorporated 32P was quantified by liquid scintillation counting. The level of ceramide was determined by comparison with a standard curve generated concomitantly of known amounts of ceramide (ceramide type III, Sigma). Diacylglycerol was quantified in a similar manner to ceramide, except the alkaline hydrolysis step was omitted. Changes in sphingomyelin levels were measured by labeling cells to isotopic equilibrium with [3H]choline chloride (79.2 Ci/mmol; Dupont New England Nuclear) as previously described (Goldkorn et al., 1992; Dressler et al., 1992). Cells were incubated with [3H]choline (1.0 μCi/ml in tissue culture medium) for at least three cell doublings. Incubation with H2O2, extraction, and alkaline hydrolysis of dried lipids were identical to those used for ceramide determinations. Sphingomyelin was resolved from residual phosphatidylcholine and lysophosphatidylcholine by thin layer chromatography on silica gel 60 plates using a solvent system of chloroform:methanol:acetic acid:water (100:20:10:10). The combined extracts were dried under N2. Samples were refluxed with 50 μl of 70% perchloric acid for 30 minutes at 180°C. Color reagent (1.0 ml (0.6 M H2SO4, 0.25% ammonium molybdate, 1% ascorbic acid) was added, and samples were incubated at 50°C for 1 hour. An 32P was read, and phosphorous content was determined by comparison with known quantities at Na2HPO4.

Protein kinase C (PKC) activity
Airway epithelial cells were harvested by scraping in cold buffer A (20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 6 mM EDTA, 0.5 mM dithiothreitol (DTT), supplemented with protease inhibitors 0.5 mM PMSF, 50 μg/ml leupeptin, and 20 μg/ml aprotinin). The cells were sonicated for 1 minute in a bath sonicator and centrifuged at 500 g for 5 minutes at 4°C to remove nuclei and whole cells. The cytosolic fraction was separated from the membranes by centrifugation at 100,000 g for 1 hour. Membrane-bound PKC was solubilized by resuspending the pellet in buffer A containing 0.5% Triton X-100 for 20 minutes on ice, and centrifuged for 30 minutes at 100,000 g to remove non-soluble material. Both the cytoplasmic and the solubilized membrane fraction were applied to a 0.2 ml anion exchange chromatography DEAE-cellulose (DE-52) column, washed with buffer B (20 mM Tris-HCl, pH 7.5, 2 mM EDTA and 5 mM EGTA). Bound PKC was eluted batchwise with 500 μl buffer C (buffer B containing 0.15 M NaCl). PKC activity was detected using the PKC enzyme assay kit (Amersham), according to the manufacturer’s instructions, and as previously described (Balaban et al., 1996). 12-O-tetradecanoyl phorbol-13-acetate (TPA) was used as a positive control.

Western blotting of PKC
Protein fractions containing PKC which were eluted from the DE-52 columns (see above) were separated on SDS 10% PAGE, western blotted onto a nitrocellulose membrane, and membranes blocked in 3% BSA in PBS. PKC was detected by incubating the membrane in specific antibodies directed against the various PKC isozymes (1:1,000 in PBS). Bound antibodies were detected using Protein A conjugated horseradish peroxidase. Blots were scanned with an LKB Ultrascan XL densitometer to quantify PKC immunoreactivity, as previously described (Balaban et al., 1996).

Statistical analysis
Statistical analysis was performed by Student’s t-test, and linear regression was performed by the method of least squares.

RESULTS
H2O2 induces apoptosis in tracheobronchial epithelial (TBE) cells
Tracheobronchial epithelial (TBE) cells were treated with 100 μM H2O2 and analyzed over time for the presence of ordered DNA fragmentation and apoptotic bodies. Temporal analysis
course of nuclear fragmentation demonstrated an increase in the number of apoptotic cells, which became apparent 3-12 hour after the addition of 100 μM H2O2 to the culture medium. At 6 hours, nearly 60% of the cells demonstrated apoptotic changes by TUNEL (Fig. 3), and at 24 hours, nearly 80% of the cells demonstrated apoptotic changes. This apoptotic effect was also obtained with as little as 10 μM H2O2, and a plateau of 80% apoptotic cells was observed with a dose of 100-200 μM (data not shown). The ED50 of H2O2 induced apoptosis at 24 hours was 70 μM. Of note is that at higher H2O2 concentrations (such as 300 μM) cells died predominately by necrosis. Temporal analysis of DNA fragmentation by agarose gel electrophoresis (not shown) indicated that smaller sized fragments (180 bp) increased in abundance up to 24 hours after H2O2 treatment. DNA ladders were routinely observed within 12 hours of treatment with H2O2 with concentrations as low as 10 μM.

Ceramide mimics H2O2 in inducing apoptosis in TBE cells

Since previous studies in hematopoetic cells reported that the apoptosis induced by TNFα and Fas is mediated via increase of intracellular ceramide (Obeid et al., 1993; Tepper et al., 1995), we tested whether addition of cell-permeable ceramide analogs can cause apoptosis in TBE cells. Fig. 1 (lanes 6-8) shows that treatment of TBE cells with 20 μM C8-ceramide mimicked H2O2 in producing oligonucleosomal DNA fragmentation on agarose gel electrophoresis. Moreover, exposures to 20 μM C8 and C6 ceramide analogs also mimicked H2O2 in the generation of typical apoptotic changes with bis-benzimide (Fig. 2) and TUNEL (Fig. 3) staining. Similar morphological changes were also observed with the C6-ceramide analogs at a concentration of 10 μM (Fig. 4). Concentrations of synthetic ceramides as low as 5 μM were found to induce apoptosis, and the morphological changes after treatment with ceramide analogs developed more rapidly than those induced by H2O2. At 3 hours of treatment with C6-ceramide, about 60% of the airway epithelial cells were TUNEL-positive, and at 6 hours, nearly 80% of the cells demonstrated apoptotic changes by TUNEL. However, the effects at 6-12 hours were quantitatively comparable for ceramide analogs and for H2O2. In contrast, treatment with 100 μM of the immediate precursor of ceramide, dihydroceramide, which lacks the trans double bond C4-C5 of the sphingoid base
Ceramide mediates \( \text{H}_2\text{O}_2 \)-induced apoptosis

backbone, resulted in no apoptotic response (Fig. 1, lane 5 and Fig. 5). Furthermore, other cell-permeable analogs of lipid second messengers, including 1,2-dioctanoyl-sn-glycerol (the analog of 1,2-diacylglycerol (DAG)), and 1,2-dioctanoyl-sn-glycero-3-phosphate (the analog of phosphatidic acid) did not induce apoptosis (data not shown). Importantly, ceramide-induced cell death is stereospecific, since only the D-erythro isomer, but not the inactive L-threo isomer of C6-ceramide, induced epithelial cell apoptosis in a dose-dependent manner (Fig. 5).

**H\( \text{O}_2 \)** induces increase in intracellular ceramide and decrease in DAG

Ceramide ability to mimic the action of \( \text{H}_2\text{O}_2 \) suggests that it may be a mediator of \( \text{H}_2\text{O}_2 \)-induced apoptosis in TBE cells. To investigate this hypothesis, we explored the ability of \( \text{H}_2\text{O}_2 \), induced epithelial cell apoptosis in a dose-dependent manner (Fig. 5).

**Fig. 3.** TUNEL assays of tracheobronchial epithelial (TBE) cells treated with \( \text{H}_2\text{O}_2 \) and with a synthetic ceramide analog (C6-ceramide). (A,B,C) TBE monolayers were untreated (A, control), treated with 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (B) or with 20 \( \mu \text{M} \) synthetic ceramide analogue (C, C6-ceramide). Treatments were terminated after 6 hours by fixing the cells on slides for the TUNEL assay as described in Materials and Methods. (D) To quantify TUNEL positive TBE cells, 500 cells per slide were scored for the incidence of apoptotic chromatin changes. The slides were viewed under Nikon SA fluorescence microscope and view-fields were captured by C-Imaging System (Compix, Cranberry Twp., PA). Cells with three or more chromatin fragments were considered apoptotic. Values reflect the mean ± s.e.m. of quadruplicate determinations.

**Fig. 4.** EM studies of the effects of \( \text{H}_2\text{O}_2 \) and C6-ceramide on cell morphology. TBE monolayers were (A) untreated; (B) treated with 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \); and (C) treated with 10 \( \mu \text{M} \) synthetic ceramide analog (C6-ceramide). Treatments were terminated after 6 hours by fixing the cells in 2% glutaraldehyde. Shown: (A) control; (B) nucleus heavily fragmented, cytoplasmic vacuoles, membrane blebbing; (C) nucleus fragmented, extensive membrane blebbing. This experiment represents one of three similar studies.
to elevate the intracellular levels of ceramide. As shown in Fig. 6 treatment of TBE cells with 100 μM H₂O₂ produced an increase in ceramide levels. Production of ceramide was detectable after 1 minute of H₂O₂ exposure, reached a plateau at 3 minutes, and remained elevated for hours. The rise in ceramide levels was 2 fold: from 127 pmoles per 10⁶ control cells to 238 pmoles of H₂O₂ treated cells. Similar quantitative results were obtained in three other studies done with 50 μM H₂O₂. To assess the maximal possible ceramide response in these epithelial cells, the cells were also treated with an exogenous SMase (Staphylococcus aureus SMase C from Sigma, at 0.5 unit/ml). In order to effectively mimic endogenous intracellular SMase, treatments were also carried out in the presence and in the absence of the bacterial pore-forming protein streptolysin O (40 units/ml). The total observed increase in cellular ceramide reached 250 pmoles and 270 pmoles with treatments of exogenous SMase in the absence and presence of streptolysin O, respectively. Thus, the observed increase in ceramide levels of lung epithelial cells exposed to H₂O₂ was comparable (±10%) to that released by the exogenous SMase treatments. Moreover, treatment of TBE cells with the cell membrane-permeant C6-ceramide or C8-ceramide also resulted in rapid intracellular accumulation of ceramide (not shown), providing evidence that short-chain ceramides do have access to the epithelial cells interior, consistent with results in other cells (Obeid et al., 1993; Tepper et al., 1995; Goldkorn et al., 1992). Both the effects of exogenous SMase and the synthetic ceramide analogs as well as the effect of H₂O₂ on the increase in cellular ceramide levels were specific, in that none of them induced an increase in the level of the lipid second messenger 1,2-diacylglycerol (DAG). In fact, a decrease in endogenous DAG levels was detected with H₂O₂ treatment (Fig. 6). The elevation of ceramide and the decrease of DAG by H₂O₂ were dose-dependent. Statistically significant changes were detected at H₂O₂ concentrations as low 10 μM (P<0.01). In parallel with H₂O₂ effects on DAG, we also measured the effects of H₂O₂ on PKC activity (Fig. 7) and found that H₂O₂ exposure of TBE cells inhibited PKC activity, i.e. caused its translocation from the membrane to the cytosol. Similarly, treatments with C6-

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**Fig. 5.** Ceramide-induced apoptosis in airway epithelial cells is stereospecific. TBE monolayers were treated as indicated with active 20 μM C6-D-erythro-ceramide (C6-D-erythro), 20 μM inactive C6-L-threo-ceramide (C6-L-threo), or 100 μM C6-dihydrococeramide (C6-dihydro) for 24 hours. The cells were then fixed and stained with Hoechst 33258, and residual fluorescence quantified in a fluorescent plate reader. The data are presented as % of control, which represents the residual fluorescence (arbitrary units) in treated wells/residual fluorescence in control wells x100. The values represent mean ± s.e.m. of independent triplicate determinations from three separate studies.

**Fig. 6.** Changes in ceramide, 1,2-diacylglycerol (DAG), and sphingomyelin levels in response to H₂O₂ exposure of airway epithelial cells. (A) A time course for ceramide and DAG levels: TBE monolayers were treated with 100 μM H₂O₂. At the indicated times, cells were extracted with chloroform:methanol:1 N HCl (100:100:1). Lipid extracts were assayed for ceramide and DAG levels by the DAG kinase reaction. (B) A time course for changes in Sphingomyelin levels in response to H₂O₂ exposure: TBE monolayers were incubated with [3H]cholin (1 μCi/ml) for three cell doubling to label cellular sphingomyelin. Cells were treated with 100 μM H₂O₂. At the indicated times, cells were extracted with chloroform:methanol:1 N HCl (100:100:1). Lipid extracts were subjected to mild alkaline hydrolysis, and sphingomyelin was resolved by thin-layer chromatography. Baseline sphingomyelin mass was determined by lipid phosphorous measurements. The values represent mean ± s.e.m. of independent triplicate determinations from three separate studies for ceramide and DAG and four experiments for sphingomyelin.
Ceramide mediates H₂O₂-induced apoptosis

Ceramide also triggered PKC translocation from the membrane to the cytosol. On the other hand, exposure to phorbol esters (TPA) or to a synthetic DAG analog caused activation of PKC following its translocation to the membrane. The mechanism of PKC inhibition by ceramide is unknown yet, but H₂O₂ may inhibit PKC by both reducing DAG and increasing ceramide levels in airway epithelial cells.

H₂O₂ induces decrease in intracellular sphingomyelin

Previous studies on the involvement of ceramide in activation of apoptotic pathway by TNFα, Fas, and ionizing radiation reported that intracellular ceramide elevation resulted from hydrolysis of the phospholipid sphingomyelin by a sphingomyelinase (SMase) (Obeid et al., 1993; Kim et al., 1991; Tepper et al., 1995; Haimovitz-Friedman et al., 1994). Activation of SMase and ceramide generation occurred within seconds to minutes after exposure to these agents. Fig. 6 shows that the pattern of ceramide activation after exposure to H₂O₂ did follow the TNFα model, because measurements of ceramide and sphingomyelin levels showed changes in the levels of these lipids within the first 1 minute to 120 minutes after exposure of TBE cells to H₂O₂. We conclude that H₂O₂ induces a complete ceramide cycle in epithelial cells, with peak ceramide accumulation close to that induced by exogenous SMase.

H₂O₂-induced generation of ceramide in cell membrane preparations

To localize the H₂O₂-susceptible SMase, experiments were carried out in a cell free system. Fresh supernatants of nuclei-free membranes were prepared from TBE cells and treated with 200 μM H₂O₂ at 4°C. The membranes were then incubated at 37°C in an assay buffer optimized for neutral sphingomyelinase activity. Under these conditions, the level of ceramide increased within minutes by 2- to 3-fold (Fig. 8). The maximal level of ceramide was achieved 20 minutes after H₂O₂ treatment. The magnitude of this effect was similar to that observed after H₂O₂ exposure of intact TBE cells (Fig. 6). A concomitant reduction in sphingomyelin levels was observed (data not shown). If, however, H₂O₂ treated membranes were incubated at 4°C, or at 37°C in a buffer that did not contain magnesium, which is required for neutral sphingomyelinase activity, the ceramide elevation was not observed at any time up to 30 minutes after treatment. These studies suggest that the effect of H₂O₂ to generate ceramide is mediated via activation of a neutral sphingomyelinase located at the cell membrane. Furthermore, this set of experiments indicates that a direct effect of H₂O₂ on the membrane is sufficient to produce the critical lipid ceramide that transduces the apoptotic signals.

The role of ceramide generation in H₂O₂-induced apoptosis in TBE cells

To evaluate whether there is a cause-and-effect relationship...
obtained with a 15 minute or 1 hour pretreatment with TPA. Fig. 10 demonstrates FACS analyses of changes in the population of apoptotic cells, and shows that TPA also eliminated apoptosis after exposure of cells to 100 μM H₂O₂. While H₂O₂ increased the percentile of apoptotic cells to 90%, preincubation with TPA reduced it to 30%. Hence, activation of PKC by TPA appears to block both the generation of ceramide (Fig. 9) and apoptosis (Fig. 10) induced by H₂O₂ exposure. To strictly prove that ceramide is the critical second messenger in the H₂O₂-induced apoptotic cascade, additional experiments were performed to examine whether selective restoration of ceramide would overcome this inhibition. Therefore, TBE cells were first treated for 30 minutes with 50 ng/ml TPA, then exposed to 100 μM H₂O₂, and subsequently incubated with 20 μM C₂-ceramide. The later step restored the apoptotic response, as demonstrated by the increase in the percentile of apoptotic cells to 60% (Fig. 10). Higher concentrations of C₂-ceramide further restored apoptosis, and 75 μM C₂-ceramide overcame completely the TPA-anti apoptotic effects. This suggests that apoptotic signaling can be produced via ceramide generation by H₂O₂ exposure and that the context of ceramide signaling may determine the ultimate biological response. Therefore, it is concluded that ceramide-mediated apoptosis may be subjected to a transmodulatory control via PKC activation by either DAG or TPA, and that H₂O₂-induced generation of ceramide is a critical and obligatory event in the H₂O₂ induction of the apoptotic cascade in airway epithelial cells.

**DISCUSSION**

The role of oxidative stress in apoptosis has generated considerable debate since antioxidants as well as pro-oxidants were shown to inhibit this form of cell death (Clement and Stamnokovic, 1996; Kazzaz et al., 1996; McGowan et al., 1996). On one hand there is growing consensus that reactive oxidants play a key role in the control of apoptosis, although the precise nature of this control is unclear (Bonfoco et al., 1995; Lin et al., 1997; Salgo et al., 1995; Buttke and Sandstrom, 1994). On the other hand there is evidence that oxidative stress, induced by overproduction or decreased elimination of H₂O₂, provides tumor cells with a survival advantage over normal counterparts (Cerutti, 1985). It has also been recently reported that oxidative stress may activate growth-stimulatory responses similar to those induced by hormones and cytokines (Krieger-Brauer and Kather, 1995; Goldkorn et al., 1997, 1998). Indeed, redox control appears to be a broad regulatory system that could allow cells to adapt to a variety of redox-active stimuli, including UV and radiation, and thus may also be involved in signals of hormones and cytokines (Devery et al., 1992; Krieger-Brauer and Kather, 1992; Guy et al., 1993). The lack of evidence for specific receptor type molecules for superoxide or hydrogen peroxide in mammalian cells does not imply their absence, and mechanisms of ‘allosteric’ interaction with possible specific receptors have been suggested (Burdon, 1995). Our studies have recently shown that H₂O₂ affects EGF receptor tyrosine phosphorylation (Goldkorn et al., 1998a), while peroxynitrite (ONO-O⁻) affects EGF receptor dimerization (Van der Vliet et al., 1998). This suggests that reactive oxygen intermediates
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may be involved in cellular signaling pathways via plasma membrane anchored receptors and enzymes.

Signaling pathways involved in apoptosis induction remain largely unknown. The sphingomyelin pathway, initiated by hydrolysis of sphingomyelin in the cell membrane to generate the second messenger ceramide (Goldkorn et al., 1992; Hannun, 1994; Hannun and Obeid, 1995), is thought to mediate apoptosis to TNF$\alpha$ (Obeid et al., 1993; Dbaibo et al.,

Fig. 9. Phorbol esters inhibit H$_2$O$_2$-induced sphingomyelin degradation to ceramide. Cells were cultured as described in Fig. 6, except that TPA (50 ng/ml) or the diluent DMSO was added for 30 minutes before the cells were exposed to 100 $\mu$M H$_2$O$_2$. Sphingomyelin and ceramide levels were quantified as described in Fig. 6. (A) Ceramide in H$_2$O$_2$-treated, and TPA-pretreated cells. (B) Sphingomyelin in H$_2$O$_2$-treated, and TPA-pretreated cells. Values are derived from triplicate determinations from two experiments. The mean range of values for sphingomyelin and ceramide was 2 and 7%, respectively.

Fig. 10. FACS analysis of the restoration of apoptosis by ceramide in airway epithelial cells treated with H$_2$O$_2$ and TPA. TBE cells were cultured as described in Fig. 6, and analyzed by FACS as described in Materials and Methods. The response to fluorescein is plotted on the x-axis and the response to PI is presented on the y-axis. The % stated represents the % apoptosis, which reflects the number of events to the lower right of the vertical axis of the quad-stats plot. Concentrations used for TPA, H$_2$O$_2$ and C2-ceramide were 50 ng/ml, 100 $\mu$M, and 20 $\mu$M, respectively. This experiment represents one of three similar studies.
In conclusion, the present studies directly demonstrate that H$_2$O$_2$ induces apoptotic signaling at the cell membrane of tracheobronchial epithelial (TBE) cells. The immediate events in this pathway involve hydrolysis of sphingomyelin to ceramide by the action of a neutral magnesium-dependent sphingomyelinase. The generation of ceramide was maximal within minutes of cell exposure to H$_2$O$_2$, and was sensitive to physiologic micromolar concentrations of H$_2$O$_2$. The hypothesis that ceramide acts as a second messenger in the pathway of H$_2$O$_2$-induced apoptosis is supported by the fact that the C$_6$- and C$_8$-ceramide analogs were capable of mimicking H$_2$O$_2$ as inducers of the apoptotic response, as has been previously shown in TNFα-induced apoptosis (Obeid et al., 1993; Dbaibo et al., 1993). Additional support for this idea is derived from our studies with phorbol esters. These agents have been shown to block apoptosis induced by TNFα (Obeid et al., 1993; Jarvis et al., 1994), the chemotherapeutic agent ara-C (Grant et al., 1992) and by ionizing radiation (Tomei et al., 1988; McConkey et al., 1989). In TBE cells, phorbol esters similarly blocked H$_2$O$_2$-induced apoptosis and abolished sphingomyelin hydrolysis to ceramide. However, when ceramide increase was reinstanted by addition of exogenous C$_2$-ceramide, the phorbol ester effect to inhibit apoptosis was eliminated, suggesting that ceramide may be an essential factor of the apoptotic cascade when induced by H$_2$O$_2$ in these cells. Furthermore, H$_2$O$_2$-induced hydrolysis of sphingomyelin to ceramide took place in a cell free, devoid of nuclei, extract, and thus seems to be independent of direct H$_2$O$_2$-induced DNA damage. These results provide unequivocal evidence that H$_2$O$_2$ generates apoptotic signaling at the cell membrane. Apoptosis triggered by membrane signals may happen frequently after H$_2$O$_2$ exposure. This mechanism may predominate at the physiologically relevant to low dose range of H$_2$O$_2$, in which unrepaired lethal damage to the DNA may be less common than at the higher doses rarely applicable to physiologic situations.

The specificity of various lipids in inducing apoptosis in lung epithelial cells was determined by treatments with various permeable ceramide synthetic analogs. Isomers, such as dihydro C$_6$-ceramide (which lacks the 4, 5 double bond) did not elicit apoptosis. Moreover, the phospholipid, 1,2-diacylglycerol (DAG) (physiologic activator of protein kinase C (PKC)), did not cause apoptosis as well. When DAG was applied together with ceramide it counteracted ceramide-induced apoptosis, indicating that the context of the ceramide signal determines the ultimate biological response, and that ceramide-mediated apoptosis may be subject to transmodulatory control through DAG/PKC. Therefore, PKC activation may provide an anti-apoptotic mechanism in lung epithelial cells. However, the mechanisms by which PKC activators inhibit ceramide-induced apoptosis are still unknown. It has been shown in other cells (Hannun et al., 1986; Lee et al., 1996; Jones and Murray, 1995), and also in the current studies, in TBE cells, that activation of PKC by DAG or phorbol esters induced its translocation from the cytosol to the membrane (Nishizuka, 1984) and inhibited ceramide-induced apoptosis (Obeid et al., 1993; Jarvis et al., 1994b; Jayadev et al., 1995). On the other hand, it has been reported that ceramide has no effect on PKC activity in vitro (Hannun et al., 1986), but it remained unclear whether ceramide has any effect on PKC in vivo. Indeed, recent studies reported that both C$_2$- and C$_6$-ceramide inhibited PKCα activity, while C$_2$ and C$_6$ dihydro-ceramides did not (Lee et al., 1996). In addition, SMase treatment of mouse epidermal or human skin fibroblast cells, or incubation of these cells with C$_2$-ceramide, blocked PKCα’s translocation to the membrane and thus inhibited its activity. Similarly, our present studies in TBE cells demonstrated that H$_2$O$_2$ induced ceramide production and inhibition of PKC translocation to the membrane, and that C$_6$-ceramide blocked membrane PKC translocation. Taken together, these observations support a model of a balance between H$_2$O$_2$ induction of apoptosis via the sphingomyelin/ceramide pathway and its down-regulation by natural suppressor mechanisms through PKC. According to this hypothesis, spontaneous activation of membrane PKC or its activation by growth factors, may be important in the homeostatic control of redox resistance, while ceramide generation mediates oxidative stress-induced apoptosis.

The mechanism by which H$_2$O$_2$ stimulates sphingomyelin hydrolysis to ceramide is unknown. Moreover, very little is known about the regulation mechanisms of SMases. It has been recently shown by others (Liu and Hannun, 1997) that partially purified magnesium-dependent neutral pH-optimum and membrane-associate sphingomyelinase (N-SMase) is inhibited in vitro by GSH. Since GSH depletion is observed in a variety of cells in the process of cellular apoptosis, it is possible that depletion of GSH may be an important mechanism in activation of N-SMase. Therefore, it is conceivable that H$_2$O$_2$ activates N-SMase by releasing it from GSH inhibition (Goldkorn et al., 1998b), thereby coupling oxidative stress and signaling via products of sphingomyelin hydrolysis to induce apoptosis.

In conclusion, the present studies directly demonstrate that apoptotic signaling can be produced via ceramide generation by H$_2$O$_2$ interaction with the cell membrane of airway epithelial cells.

However, the key events involved in ceramide-triggered apoptosis remain unknown. It has been recently proposed that ceramide is not only a signaling product of oxidative stress, but is also mediating the production of reactive oxidants in the mitochondria (Garcia-Ruiz et al., 1997; Quillet-Mary et al., 1997). These studies pointed to reactive oxygen species generated in the mitochondrial respiratory chain as early major mediators of ceramide-induced apoptosis, suggesting that coupling between oxidative stress and ceramide production is bi-directional: not only oxidants activate ceramide production, but ceramide may also induce generation of reactive oxidants.

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