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H_2O_2 acts on cellular membranes to generate ceramide signaling and initiate apoptosis in tracheobronchial epithelial cells

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SUMMARY

Hydrogen peroxide (H_2O_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_2O_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_2O_2 induced rapid sphingomyelin hydrolysis to ceramide, the elevation of which paralleled the induction of apoptosis. Furthermore, H_2O_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_2O_2-induced apoptosis in tracheobronchial epithelial cells.

Key words: Ceramide, Apoptosis, Oxidant, Bronchial epithelium

INTRODUCTION

H_2O_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

sphingomyelin-specific sphingomyelinases, to generate ceramide. Ceramide then serves as a second messenger in this system, leading to apoptotic DNA degradation.

The sphingomyelin pathway is an ubiquitous, evolutionarily conserved signaling system analogous to the cAMP and phosphoinositide pathways. Sphingomyelin (N-acylsphingosin-1-phosphocholine) is a phospholipid preferentially concentrated in the plasma membrane of mammalian cells (Merrill and Jones, 1990). Sphingomyelin catabolism occurs via the action of sphingomyelin-specific forms of phospholipase C, termed sphingomyelinases, which hydrolyze the phosphodiester bond of sphingomyelin, yielding ceramide and phosphorylcholine. Several forms of sphingomyelinase exist, distinguished by their pH optima (Spence, 1993; Cifone et al., 1994; Schutze et al., 1992; Chatterjee, 1993; Wiegmann et al., 1994; Okazaki et al., 1994). Human and murine acid sphingomyelinase (A-SMase; pH optimum 4.5-5.0) have been cloned and determined to be the products of a conserved gene, while Mg^{2+}-dependent or -independent neutral SMases (pH optimum 7.4) have yet to be molecularly characterized.

Agonists of the ceramide pathway include cytokines such as tumor necrosis factor- (TNFα; Obeid et al., 1993; Jarvis et al., 1994a), interleukin-1 (Andrieu et al., 1995), γ-interferon (Kim et al., 1991); antibodies directed against functional molecules such as Fas/APO-1 (Cifone et al., 1994; Tepper et al., 1995; Martin et al., 1995a) or CD28 (Boucher et al., 1995) proteins; as well as stress-inducing agents such as UV (Verheij et al., 1996) and ionizing radiation (Haimovitz-Friedman et al., 1994; Santana et al., 1996); and antileukemic agents (Strum et al., 1994; Jaffrézou et al., 1996). The observation that cell-permeant synthetic ceramides or natural ceramide (generated by treating cells with bacterial sphingomyelinase) could mimic the biological effects of most ceramide cycle agonists has provided significant weight to the role of ceramide in signal transduction and apoptosis.

There is growing evidence that oxidative stress plays a major role in the control of apoptosis, however, the precise molecular mechanisms of this control is unknown. In this study, we show that micromolar concentrations of H_2O_2 can induce apoptosis in normal tracheobronchial epithelial (TBE) cells. The effect is mediated by the ceramide second messenger which is potently and rapidly generated from the plasma membrane sphingomyelin by H_2O_2 treatment of the TBE cells. These experiments demonstrate unequivocally that apoptotic signaling can be produced via ceramide generation by H_2O_2 interaction with the cell membrane of the lung airway epithelium.

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), Hepes (15 mM, pH 7.2), transferrin (0.1 μM), insulin (10 μM), retinoic acid (0.1μM), hydrocortisone (0.1 μM), and epidermal growth factor (0.01 μM) and plated at a density of 1- to 5×10^3 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 8×10^4 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 deoxynucleotidyl trasferase end-labeling (TUNEL) technique was used for evaluation of airway epithelial cells for DNA fragmentation and the appearance of apoptotic bodies (Gorczyca et al., 1993). Slides were stained with DNA counterstains, bis-benzimide (Hoechst 33258; 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 (Martin 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 4× SSC buffer (Sigma), 15 μg/ml fluorescinated 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 10^4/ml cells 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*-hectanoyl sphingosine) and 1,2-dioctanoyl-sn-glycerol were obtained from Matreya (Pleasant Gap, PA, US), and stock solutions were prepared in dimethyl sulfoxide. C2-dihydroceramide (*N*-acetyl dihydrosphingosine) was obtained from Matreya, and 1,2-dioctanoyl-*sn*-glycero-3-phosphate, which was obtained from Avanti Polar Lipids, were 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 dihydroceramide 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 H_2O_2, 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 N_2 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), 50 mM NaCl, 51 mM imidazole, 1 mM EDTA, 12.5 mM $MgCl_2$, 2 mM dithiothreitol. 0.7% glycerol, 70 μM β-mercaptoethanol, 1 mM ATP, 10 μCi of [γ-^{32}P]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 $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 10 mM Hepes, pH 7.2), and 30 μ1 of 100 mM EDTA. The lower organic phase was dried under N_2. 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 autoradiograptly, and incorporated ^{32}P 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 [^{3}H]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 [^{3}H]choline (1.0 μCi/ml in tissue culture medium) for at least three cell doublings. Incubation with H_2O_2, 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 (50:30:8:3), was identified by iodine vapor staining, and was quantified by liquid scintillation counting. Sphingomyelin mass was verified by lipid phosphorus assay. In brief, sphingomyelin spots were scraped and extracted three times with 500 μl of chloroform:methanol:HCl (200:100:1), and the combined extracts were dried under N_2. Samples were refluxed with 50 μl of 70% perchloric acid for 30 minutes at 180°C. Color reagent (1.0 ml) (0.6 M H_2SO_4, 0.25% ammonium molybdate, 1% ascorbic acid) was added, and samples were incubated at 50°C for 1 hour. A_{700} was read, and phosphorous content was determined by comparison with known quantities at Na_2HPO_4.

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

H_2O_2 induces apoptosis in tracheobronchial epithelial (TBE) cells

Tracheobronchial epithelial (TBE) cells were treated with 100 μM H_2O_2 and analyzed over time for the presence of ordered DNA fragmentation and apoptotic bodies. Temporal analysis

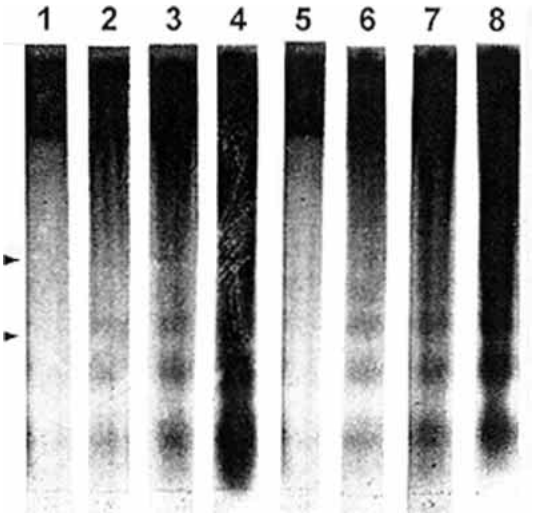


Fig. 1. Agarose gel electrophoresis of DNA ladders induced by H_2O_2 and by a synthetic ceramide analog (C8-ceramide) in tracheobronchial epithelial (TBE) cells. TBE monolayers were treated with 100 μM H_2O_2 or 20 μM C8-ceramide for various time periods. DNA was extracted and subjected to agarose gel electrophoresis and analyzed by Southern blot hybridization with total DNA. Untreated cells (lane 1), and cells treated with 50 μM dihidroceramide (lane 5) were sampled at 24 hours. Cells treated with 100 μM H_2O_2 (lanes 2-4) were sampled at 3, 6, and 24 hours, respectively. Cells treated with 20 μM C8-ceramide (lanes 6-8) were also sampled at 3, 6, and 24 hours, respectively. Arrowheads mark 500- and 1,000-bp locations based on ethidium bromide staining of DNA size markers run on the original gel. This experiment represents one of three similar studies.

of DNA fragmentation by agarose gel electrophoresis indicated that smaller sized fragments (180 bp) increased in abundance up to 24 hours after H_2O_2 treatment (Fig. 1, lanes 1-4). DNA ladders were routinely observed within 12 hours of treatment with H_2O_2 with concentrations as low as 10 μM. Incubation of TBE cells with 100-200 μM H_2O_2 for the indicated time points also resulted in the appearance of typical morphological changes of apoptosis upon staining with the DNA-binding fluorochrome bis-benzimide (Hoechst 33258) (Fig. 2). These changes include nucleoplasmic condensation and chromatin segmentation into apoptotic bodies. A time 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 H_2O_2 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 H_2O_2, and a plateau of 80% apoptotic cells was observed with a dose of 100-200 μM (data not shown). The ED_{50} of H_2O_2 induced apoptosis at 24 hours was 70 μM. Of note is that at higher H_2O_2 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 H_2O_2 treatment. DNA ladders were routinely observed within 12 hours of treatment with H_2O_2 with concentrations as low as 10 μM.

Ceramide mimics H_2O_2 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 H_2O_2 in producing oligonucleosomal DNA fragmentation on agarose gel electrophoresis. Moreover, exposures to 20 μM C8 and C6 ceramide analogs also mimicked H_2O_2 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 H_2O_2. 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 H_2O_2. 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

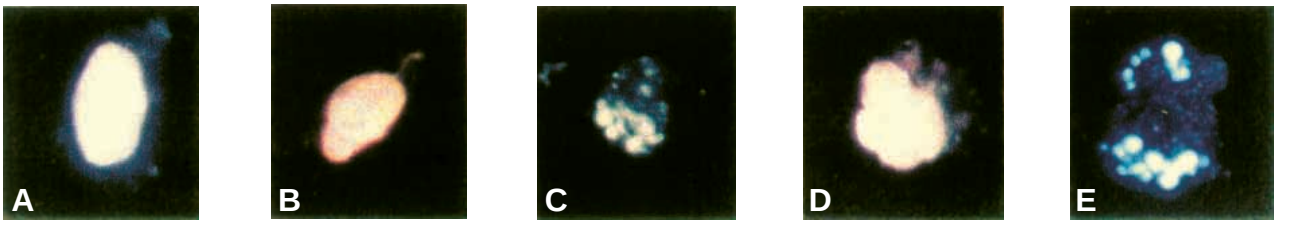
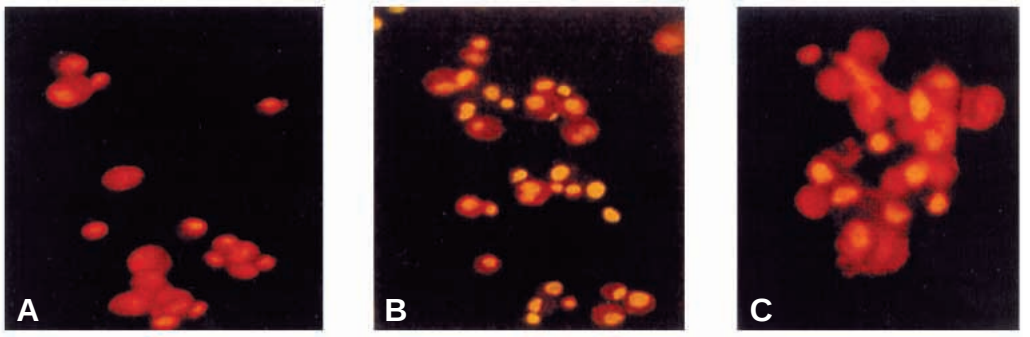


Fig. 2. Progression of nuclear DNA fragmentation in tracheobronchial epithelial (TBE) cells treated with H_2O_2 and with a synthetic ceramide analog (C8-ceramide). (A) TBE monolayers cultured for 24 hours in the absence of treatment; (B) TBEs cultured for 3 hours in the presence of 100 μM H_2O_2; (C) TBEs cultured for 12 hours in the presence of 200 μM H_2O_2; (D) TBEs cultured for 3 hours in the presence of 20 μM synthetic ceramide analog (C8-ceramide); (E) TBEs cultured for 12 hours in the presence of 20 μM C8-ceramide. By using Hoechst 33342 DNA counterstain, total DNA, anti-digoxigenin/fluorescein-labeled and unlabeled DNA was revealed as bright white fluorescence of Hoechst at 346-460 nm. Shown: (A) control cells; (B and D) early stages of apoptosis where nuclear DNA fragments and nucleus budding are revealed as yellow fluorescence; (C and E) late stages of apoptosis ultimately revealing DNA fragments contained within membrane-bounded apoptotic bodies with bright green-blue fluorescence. This experiment represents one of three similar studies.



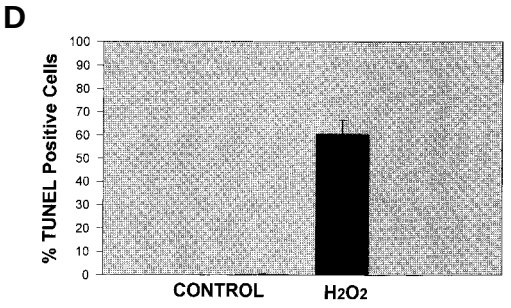


Fig. 3. TUNEL assays of tracheobronchial epithelial (TBE) cells treated with H_2O_2 and with a synthetic ceramide analog (C6-ceramide). (A,B,C) TBE monolayers were untreated (A, control), treated with 100 μM H_2O_2 (B) or with 20 μM synthetic ceramide analogue (C, C6-ceramide). Treatments were terminated 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.

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 streospecific, 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_2O_2 induces increase in intracellular ceramide and decrease in DAG

Ceramide ability to mimic the action of H_2O_2 suggests that it may be a mediator of H_2O_2-induced apoptosis in TBE cells. To investigate this hypothesis, we explored the ability of H_2O_2

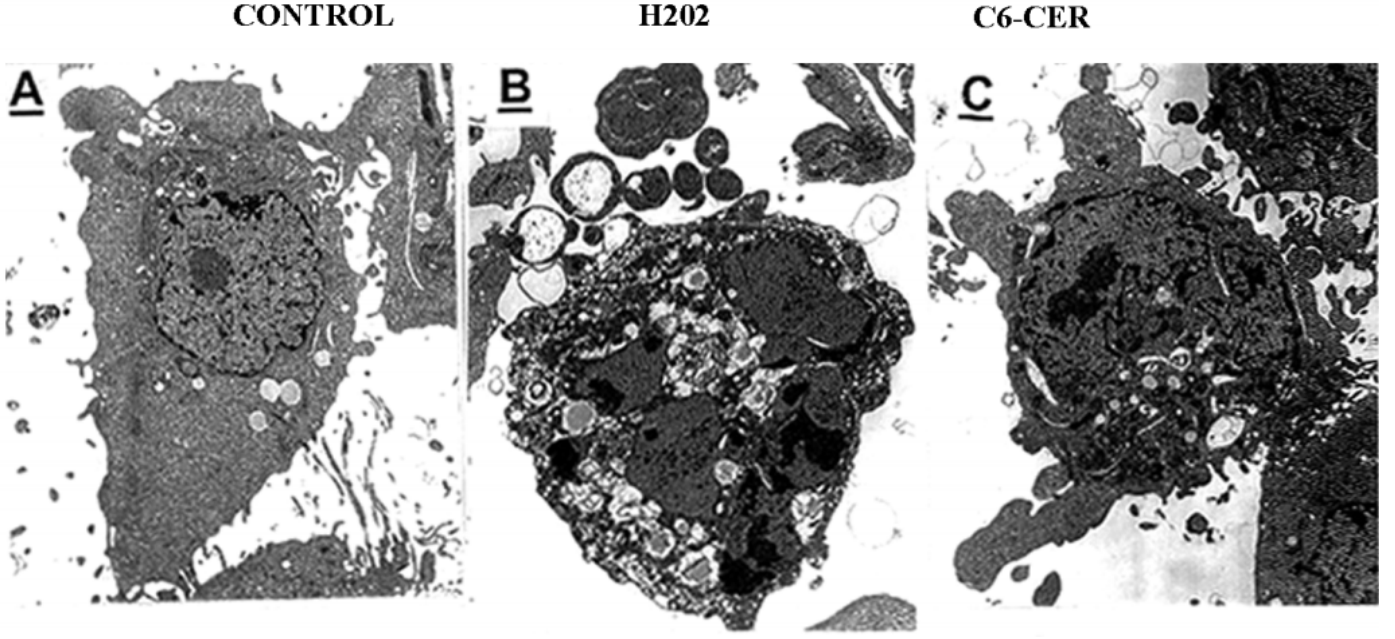


Fig. 4. EM studies of the effects of H_2O_2 and C6-ceramide on cell morphology. TBE monolayers were (A) untreated; (B) treated with 100 μM H_2O_2; and (C) treated with 10 μM synthetic ceramide analog (C6-ceramide). Treatments were terminated after 6 hours by fixing the cells in 2% glutaraldehyde. Shown: (A) control; (B) nucleus heavily fragmented, cytoplasmic vacuoles, membrane blebbing; (C) nucleus fragmented, extensive membrane blebbing. This experiment represents one of three similar studies.

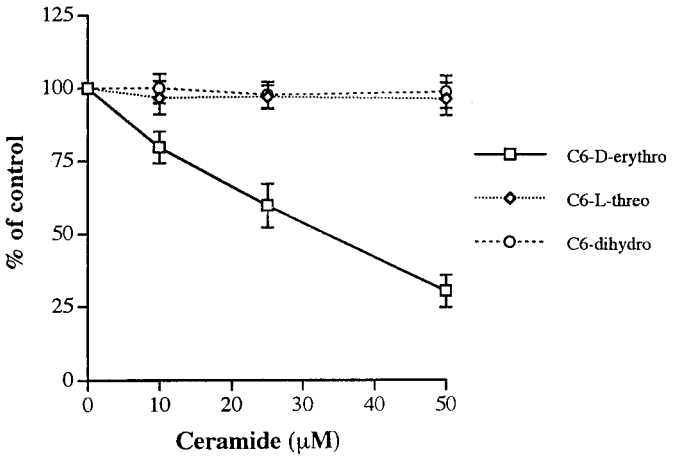


Fig. 5. Ceramide-induced apoptosis in airway epithelial cells is stereospecific. TBE monolayers were treated as indicated with active 20 μM C6-D-erythro-ceramide (C6-D-erythro), 20 μM inactive C6-L-threo-ceramide (C6-L-threo), or 100 μM C6-dihydroceramide (C6-dihydro) for 24 hours. The cells were then 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 ×100. The values represent mean ± s.e.m. of independent triplicate determinations from three separate studies.

to elevate the intracellular levels of ceramide. As shown in Fig. 6 treatment of TBE cells with 100 μM H_2O_2 produced an increase in ceramide levels. Production of ceramide was detectable after 1 minute of H_2O_2 exposure, reached a plateau at 3 minutes, and remained elevated for hours. The rise in ceramide levels was 2 fold: from 127 pmoles per 10^6 control cells to 238 pmoles of H_2O_2 treated cells. Similar quantitative results were obtained in three other studies done with 50 μM H_2O_2. 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 H_2O_2 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 H_2O_2 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 H_2O_2 treatment (Fig. 6). The elevation of ceramide and the decrease of DAG by H_2O_2 were dose-dependent. Statistically significant changes were detected at H_2O_2 concentrations as low 10 μM ($P<0.01$). In parallel with H_2O_2 effects on DAG, we also measured the effects of H_2O_2 on PKC activity (Fig. 7) and found that H_2O_2 exposure of TBE cells inhibited PKC activity, i.e. caused its translocation from the membrane to the cytosol. Similarly, treatments with C6-

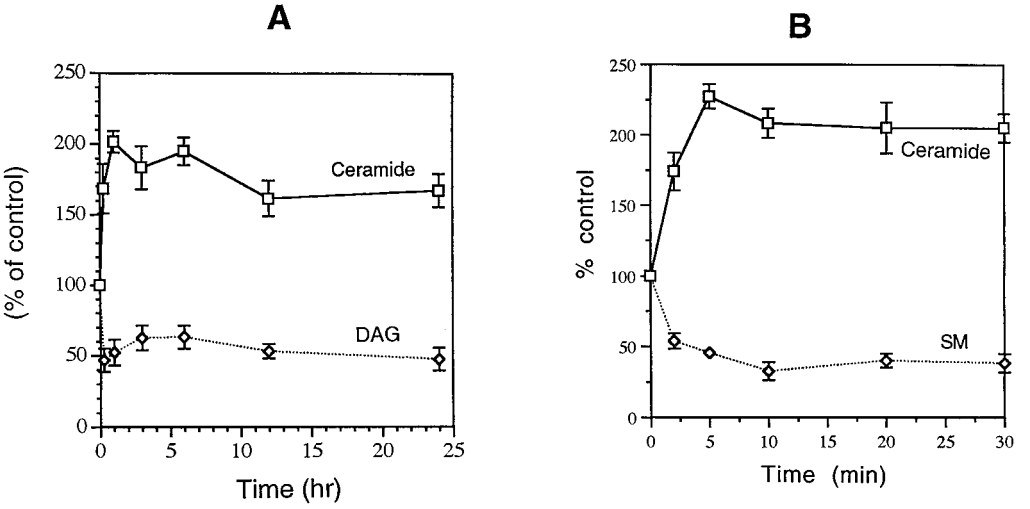


Fig. 6. Changes in ceramide, 1,2-diacylglycerol (DAG), and sphingomyelin levels in response to H_2O_2 exposure of airway epithelial cells. (A) A time course for ceramide and DAG levels: TBE monolayers were treated with 100 μM H_2O_2. At the indicated times, cells were extracted with chloroform:methanol:1 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 H_2O_2 exposure: TBE monolayers were incubated with [^{3}H]cholin (1 μCi/ml) for three cell doubling to label cellular sphingomyelin. Cells were treated with 100 μM H_2O_2. 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.

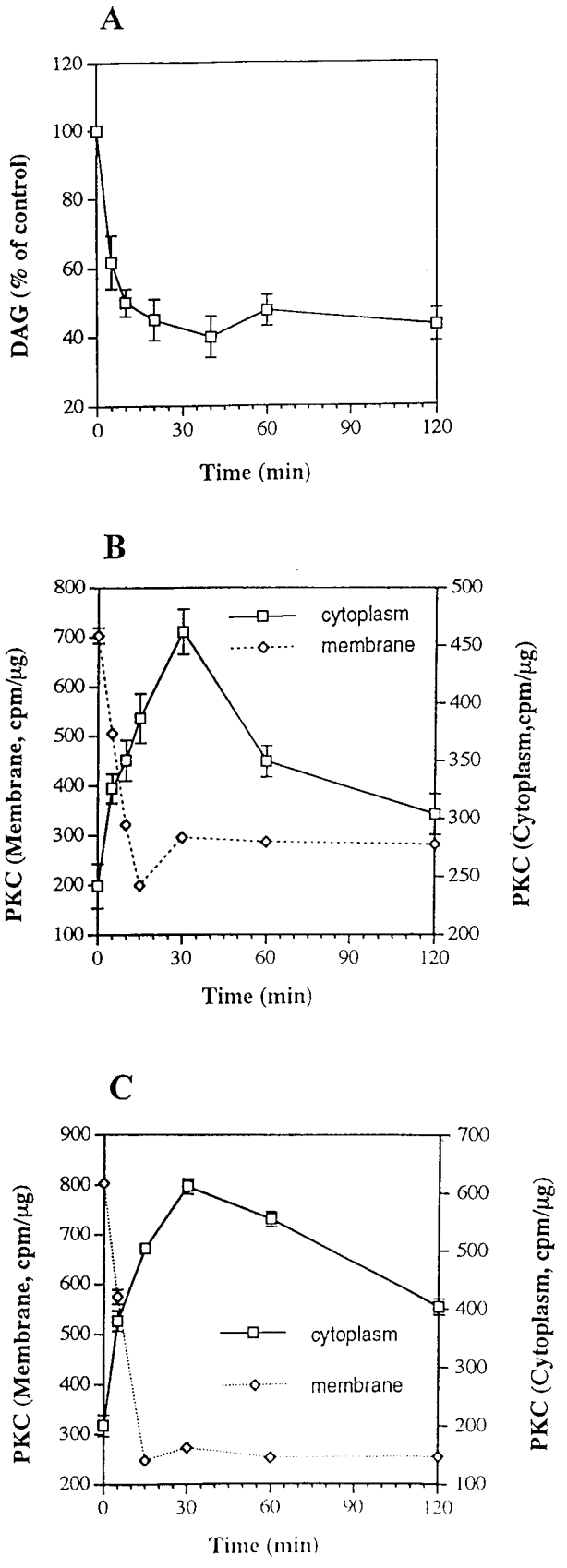


Fig. 7. PKC inhibition by H_2O_2 and C6-ceramide. Airway epithelial cells were treated with 100 μM H_2O_2 (A,B) or with 5 μM C6-ceramide (C). At the indicated times, cells were collected, cytoplasmic and membranal PKC purified, and PKC activity measured (B,C). (A) Alternatively, at the indicated times, cells were extracted with chloroform:methanol: 1 N HCl (100:100:1). Lipid extracts were assayed for DAG levels by the DAG kinase reaction. The values represent mean ± s.e.m. of independent triplicate determinations from three separate studies.

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 H_2O_2 may inhibit PKC by both reducing DAG and increasing ceramide levels in airway epithelial cells.

H_2O_2 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 H_2O_2 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 H_2O_2. We conclude that H_2O_2 induces a complete ceramide cycle in epithelial cells, with peak ceramide accumulation close to that induced by exogenous SMase.

H_2O_2-induced generation of ceramide in cell membrane preparations

To localize the H_2O_2-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 H_2O_2 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 H_2O_2 treatment. The magnitude of this effect was similar to that observed after H_2O_2 exposure of intact TBE cells (Fig. 6). A concomitant reduction in sphingomyelin levels was observed (data not shown). If, however, H_2O_2 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 H_2O_2 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 H_2O_2 on the membrane is sufficient to produce the critical lipid ceramide that transduces the apoptotic signals.

The role of ceramide generation in H_2O_2-induced apoptosis in TBE cells

To evaluate whether there is a cause-and-effect relationship

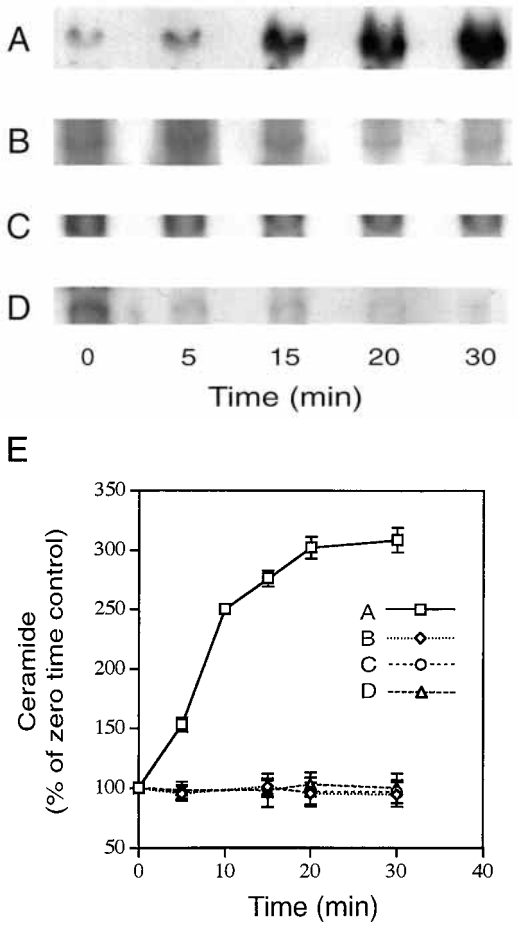


Fig. 8. Effects of H_2O_2 on the ceramide level of nuclei-free membrane fraction. TBE monolayers were detached and resuspended (45×10^6 /ml) in homogenization buffer (50 nM NaF, 5 mM EGTA, and 25 mM Hepes, pH 7.4), disrupted at 4°C with 150 strokes of a tight-fitting Dounce homogenizer and centrifuged for 5 minutes at 500 ***g***. The nuclei-free membrane fraction was either 200 μM H_2O_2 treated at 4°C (A,C,D), or untreated (B). To measure the effects on ceramide levels, 150 μl of membrane samples (1.65 mg/ml) were incubated at 37°C in a reaction mixture containing 30 μl of 3 mM ATP, 30 μl of homogenization buffer, 90 μl deionized water, and 300 μl sphingomyelinase assay buffer (50 mM Hepes, pH 7.4, and 20 mM $MgCl_2$) (A,B), or in a reaction mixture without Mg^{2+} (C). Alternatively membranes were incubated in a complete reaction mixture (+Mg^{2+}), but at 0°C (D). The incubations were terminated and ceramide quantified as described in Fig. 6. The values are derived from triplicate determinations from two experiments. The mean range of ceramide values for control and H_2O_2 treated membrane preparations was 5 and 7%, respectively.

between H_2O_2-induced ceramide generation and the subsequent progression of H_2O_2-induced apoptotic cascade, the effect of 12-O-tetradecanoylphorbol 13-acetate (TPA) on ceramide production and apoptosis was examined. Previous studies have reported that protein kinase C (PKC) activation by phorbol esters abolished programmed cell death in response to various agents that induce apoptosis (Balaban et al., 1996; Jarvis et al., 1994b). Fig. 9 shows that 30 minutes treatment of TBE cells with TPA (50 ng/ml) abolished H_2O_2-induced sphingomyelin hydrolysis to ceramide. Similar results were 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 H_2O_2. While H_2O_2 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 H_2O_2 exposure. To strictly prove that ceramide is the critical second messenger in the H_2O_2-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 H_2O_2, 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 H_2O_2 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 H_2O_2-induced generation of ceramide is a critical and obligatory event in the H_2O_2 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 Stamenkovic, 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 H_2O_2, 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 (Devary 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 H_2O_2 affects EGF receptor tyrosine phosphorylation (Goldkorn et al., 1998a), while peroxynitrite ($ONOO^-$) affects EGF receptor dimerization (Van der Vliet et al., 1998). This suggests that reactive oxygen intermediates

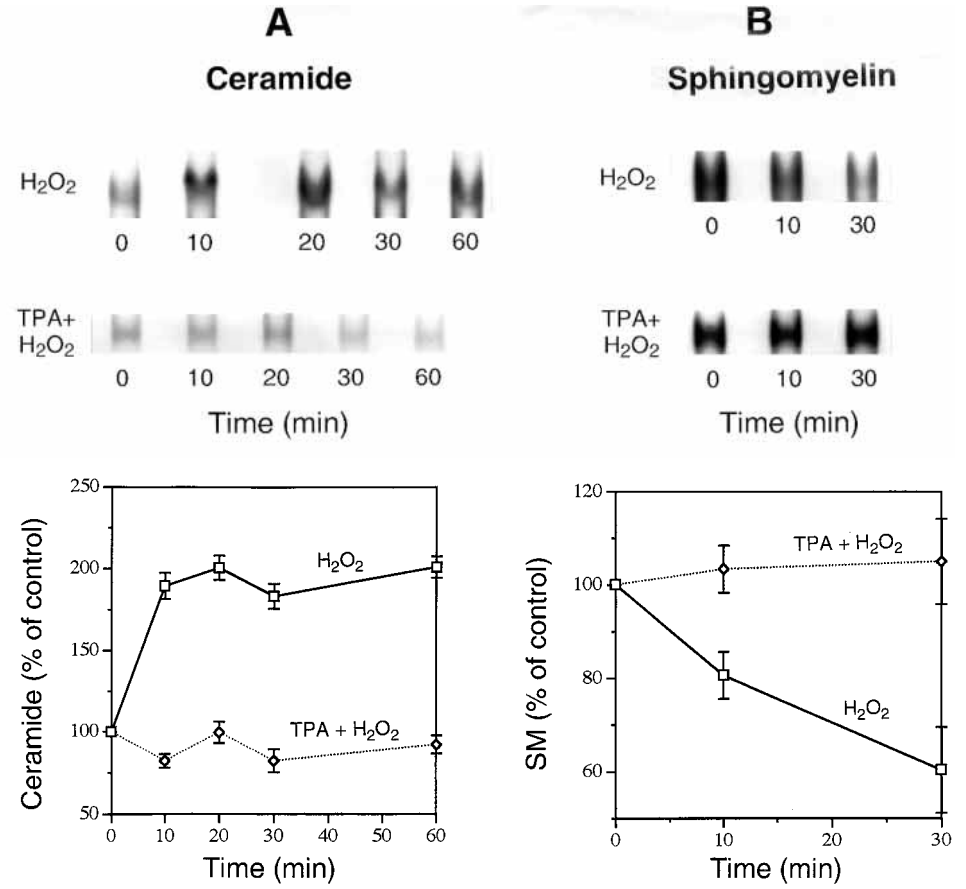


Fig. 9. Phorbol esters inhibit H_2O_2-induced sphingomyelin degradation to ceramide. Cells were cultured as described in Fig. 6, except that TPA (50 ng/ml) or the diluent DMSO was added for 30 minutes before the cells were exposed to 100 μM H_2O_2. Sphingomyelin and ceramide levels were quantified as described in Fig. 6. (A) Ceramide in H_2O_2-treated, and TPA-pretreated cells. (B) Sphingomyelin in H_2O_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.

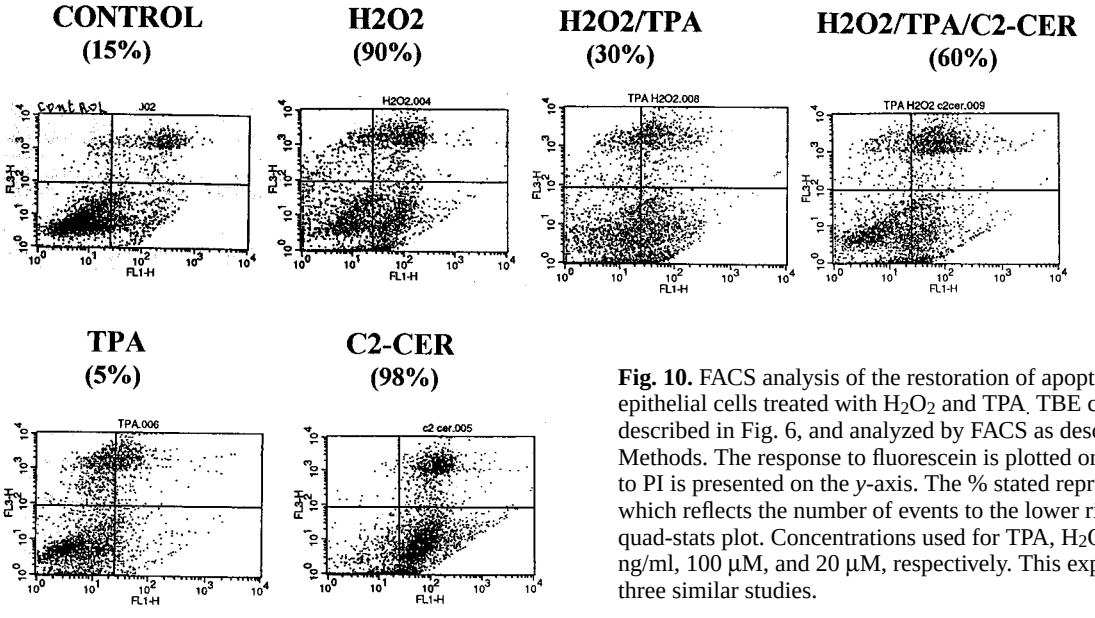


Fig. 10. FACS analysis of the restoration of apoptosis by ceramide in airway epithelial cells treated with H_2O_2 and TPA. TBE cells were cultured as described in Fig. 6, and analyzed by FACS as described in Materials and Methods. The response to fluorescein is plotted on the *x*-axis and the response 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_2O_2 and C2-ceramide were 50 ng/ml, 100 μM, and 20 μM, respectively. This experiment represents one of three similar studies.

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 TNFα (Obeid et al., 1993; Dbaibo et al.,

1993), to Fas ligand (Tepper et al., 1995) and to X-rays (Haimovitz-Friedman et al., 1994). It is not known whether it plays a role in the stimulation of other forms of stress-induced apoptosis. Moreover, most of the studies regarding ceramide signaling and apoptosis were carried out in transformed hematapoetic cells. In this capacity, it has also been shown that non-physiologic, mM concentrations of H_2O_2 raised ceramide levels and induced apoptosis in U937 human monoblastic leukaemic cells (Verheij et al., 1996). Since lung airway epithelial cells are exposed extensively to reactive oxidants, we set up studies aiming to address whether these normal cells are capable of entering apoptosis when exposed to physiologic micromolar concentrations of H_2O_2 and whether the process is mediated by ceramide as a second messenger.

Our present studies show that H_2O_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_2O_2, and was sensitive to physiologic micromolar concentrations of H_2O_2. The hypothesis that ceramide acts as a second messenger in the pathway of H_2O_2-induced apoptosis is supported by the fact that the C6- and C8-ceramide analogs were capable of mimicking H_2O_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_2O_2-induced apoptosis and abolished sphingomyelin hydrolysis to ceramide. However, when ceramide increase was reinstated by addition of exogenous C2-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_2O_2 in these cells. Furthermore, H_2O_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_2O_2-induced DNA damage. These results provide unequivocal evidence that H_2O_2 generates apoptotic signaling at the cell membrane. Apoptosis triggered by membrane signals may happen frequently after H_2O_2 exposure. This mechanism may predominate at the physiologically relevant to low dose range of H_2O_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 C6-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 C2- and C6-ceramide inhibited PKCα activity, while C2 and C6 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 C2-ceramide, blocked PKCα's translocation to the membrane and thus inhibited its activity. Similarly, our present studies in TBE cells demonstrated that H_2O_2 induced ceramide production and inhibition of PKC translocation to the membrane, and that C6-ceramide blocked membrane PKC translocation. Taken together, these observations support a model of a balance between H_2O_2 induction of apoptosis via the sphingomyelin/ceramide pathway and its down-regulation by natural suppresser 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_2O_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_2O_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_2O_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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