Ceramide Signaling in Fenretinide-induced Endothelial Cell Apoptosis*

Anat Erdreich-Epstein‡§, Linda B. Tran‡, Nina N. Bowman‡, Hongtao Wang¶, Myles C. Cabot¶, Donald L. Durden‖, Jitka Vlckova‡, C. Patrick Reynolds‡, Monique F. Stins**, Susan Groshen‡‡, and Melissa Millard‡

*From the ‡Division of Hematology-Oncology, Childrens Hospital Los Angeles, Department of Pediatrics and the ‡‡Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California 90027, the ¶John Wayne Cancer Institute at Saint John's Health Center, Santa Monica, California 90404, the ‖Section of Hematology/Oncology, Department of Pediatrics, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Indiana 46202, and the **Division of Infectious Diseases, Department of Pediatrics, Johns Hopkins School of Medicine, Baltimore, Maryland 21205*

Stress stimuli can mediate apoptosis by generation of the lipid second messenger, ceramide. Herein we investigate the molecular mechanism of ceramide signaling in endothelial apoptosis induced by fenretinide (*N*-(4-hydroxyphenyl)retinamide (4-HPR)). 4-HPR, a synthetic derivative of retinoic acid that induces ceramide in tumor cell lines, has been shown to have antiangiogenic effects, but the molecular mechanism of these is largely unknown. We report that 4-HPR was cytotoxic to endothelial cells (50% cytotoxicity at 2.4 μM, 90% at 5.36 μM) and induced a caspase-dependent endothelial apoptosis. 4-HPR (5 μM) increased ceramide levels in endothelial cells 5.3-fold, and the increase in ceramide was required to achieve the apoptotic effect of 4-HPR. The 4-HPR-induced increase in ceramide was suppressed by inhibitors of ceramide synthesis, fumonisin B$_1$, myriocin, and L-cycloserine, and 4-HPR transiently activated serine palmitoyltransferase, demonstrating that 4-HPR induced *de novo* ceramide synthesis. Sphingomyelin levels were not altered by 4-HPR, and desipramine had no effect on ceramide level, suggesting that sphingomyelinase did not contribute to the 4-HPR-induced ceramide increase. Finally, the pancaspase inhibitor, t-butyloxycarbonyl-aspartyl[*O*-methyl]-fluoromethyl ketone, suppressed 4-HPR-mediated apoptosis but not ceramide accumulation, suggesting that ceramide is upstream of caspases. Our results provide the first evidence that increased ceramide biosynthesis is required for 4-HPR-induced endothelial apoptosis and present a molecular mechanism for its antiangiogenic effects.

Stress stimuli such as irradiation, tumor necrosis factor-α, lipopolysaccharide, and some chemotherapy drugs such as doxorubicin mediate apoptosis by generation of the lipid second messenger, ceramide (1–4). We have recently shown that another stress response, endothelial anoikis (apoptosis resulting from the loss of matrix adhesion) is also associated with in-creased ceramide (5). Ceramide can be generated by hydrolysis of membrane sphingomyelin by acid and/or neutral sphingomyelinase and via activation of the *de novo* ceramide synthesis pathway, both of which can promote apoptosis (1, 2, 4, 6–11).

Fenretinide (*N*-(4-hydroxyphenyl)retinamide (4-HPR))[1] is a synthetic derivative of *all-trans*-retinoic acid that induces apoptosis in cancer cell lines and is in clinical trials for adult and pediatric cancers (reviewed in Refs. 11–13). 4-HPR has been shown to possess antiendothelial activity in tissue culture and the chick chorioallantoic membrane model (14, 15). However, whereas Pienta *et al.* (14) demonstrated that 4-HPR was cytotoxic to CPAE bovine artery endothelial cells, Ribatti *et al.* (15) found that it inhibited proliferation but was not cytotoxic to human adrenal gland capillary endothelial cells. These data support an antiangiogenic role for 4-HPR, yet the molecular mechanism of the effects of 4-HPR in endothelial cells remains largely unknown.

To date, the cytotoxic mechanism of 4-HPR has been studied almost exclusively in tumor cell lines, where it appears to function by more than one mechanism (12, 13). In leukemia cells, 4-HPR-mediated apoptosis was associated with decreased levels of *bcl-2* mRNA and was diminished by inhibitors of tyrosine kinases, by inhibitors of RNA and protein synthesis, by activators of protein kinase C, and by antioxidants (16, 17). 4-HPR-mediated apoptosis in leukemia cells was also associated with activation of caspase-3 via a mechanism separate from induction of reactive oxygen species (18) and with increased *de novo* synthesis of ceramide (19). In neuroblastoma cells, 4-HPR induced a mixed caspase-mediated apoptosis and caspase-independent necrosis that was associated with increased reactive oxygen species and increase in intracellular ceramide via *de novo* synthesis (20). Combining 4-HPR with agents that inhibit intracellular ceramide metabolism further increased ceramide levels and was associated with increased cytotoxicity, suggesting that ceramide is a mediator of 4-HPR-induced tumor cell cytotoxicity (21).

In light of the antiendothelial effects of 4-HPR, its potential for antiangiogenic activity (14, 15), and the role of ceramide as an important mediator of endothelial cell apoptosis (5, 22–24), we were interested in the role of ceramide signaling in medi-

[1] The abbreviations used are: 4-HPR, fenretinide; bovine TBMEC, bovine large-T brain microvascular endothelial cells; FBS, fetal bovine serum; human BMEC, human brain microvascular endothelial cells; HPLC, high performance liquid chromatography; HUVEC, human umbilical vein endothelial cells; SPT, serine palmitoyltransferase; ANOVA, analysis of variance; FMK, fluoromethyl ketone; BOC-D-FMK, butyloxycarbonyl-Asp-fluoromethyl ketone.

ating the antiendothelial effect of 4-HPR. In the work reported here we demonstrate that 4-HPR induces a caspase-dependent endothelial apoptosis. We also show that 4-HPR increased endothelial cell ceramide by stimulation of *de novo* synthesis and that *de novo* generated ceramide has a causal role in 4-HPR-mediated apoptosis of human brain microvascular endothelial cells (human BMEC). Last, we establish that ceramide functions upstream of caspases in the ordering of ceramide and caspases in endothelial cells. These results provide the first evidence for endothelial apoptosis by 4-HPR and show that activation of the ceramide pathway is required for this apoptosis, thus presenting a molecular mechanism for the antiangiogenic effects of 4-HPR.

EXPERIMENTAL PROCEDURES

Cell Culture—Human brain microvascular endothelial cells (human BMEC (25)) and large-T antigen-transfected bovine microvascular endothelial cells (bovine TBMEC (26)) were a gift from Dr. K. S. Kim, Johns Hopkins School of Medicine (Baltimore, MD). Two different isolates of human BMEC were used. Cells were maintained in RPMI 1640 supplemented with L-glutamine, sodium pyruvate, 10% heat-inactivated fetal bovine serum (FBS), and 10% Nu-Serum™ IV Culture Supplement (Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, MA) as described (5, 25, 26), with the addition of 20 mM HEPES buffer for the human BMEC. Human umbilical vein endothelial cells (HUVEC) (ATCC CRL-1730; passages 16–20) were maintained according to the supplier's recommendations. For experiments, cells were plated and allowed to attach and spread for 4–18 h prior to beginning the experiment. In all cases, floating and attached cells in each sample were both combined for processing at the end of incubation. Both high passage (passage 56) and low passage (passage 16) human BMEC showed characteristic endothelial morphology comparable with primary human BMEC (passage 5). Factor VIII-reactive antigen (Dako, Carpinteria, CA) (25) was expressed at all passages as determined by flow cytometry, although levels decreased in the higher passages. Uptake of fluorescent 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (BTI, Stoughton, MA) remained similar for all passages and further confirmed the endothelial phenotype (25). High and low passage human BMEC were functionally similar, as demonstrated by equivalent expression of intercellular adhesion molecule in response to tumor necrosis factor-α (10 ng/ml) with or without interferon-γ pretreatment and in response to other stimuli (*e.g.* gp120 (0.5 μg/ml), bacterial exposure (*Escherichia coli* E44), or lipopolysaccharide (50 ng/ml)) as determined by enzyme-linked immunosorbent assay (27). In addition, tumor necrosis factor-α- and lipopolysaccharide-induced lactate dehydrogenase release (Sigma kit) were also similar in both the high and low passage human BMEC (data not shown). Experiments were performed using both low passage (passages 12–25) and higher passage (passages 25–55) human BMEC, yielding similar results for cytotoxicity, apoptosis, and ceramide increase.

Reagents—4-HPR was kindly provided by Dr. Sherry Ansher (NCI, National Institutes of Health). Stock solution (10 mM) in ethanol was stored protected from light at −20 °C. BOC-D-FMK were from Enzyme Systems Products, Livermore, CA, and myriocin (ISP-1), and L-cycloserine were from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Benzyloxycarbonyl-VAD-FMK was from BioVision Inc. (Mountain View, CA). [9,10-^{3}H]Palmitic acid (50 Ci/mmol) was from PerkinElmer Life Sciences. L-[^{3}H(G)]Serine (20 Ci/mmol) and [5,6-^{3}H]sphinganine (60 Ci/mmol) were from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Sphinganine (D-*erythro*-dihydrosphingosine) was purchased from Matreya (Pleasant Gap, PA). Lipid standards were from Avanti Polar Lipids, Inc. (Alabaster, AL). Uniplate Silica gel G TLC plates were from Analtech, Inc. (Newark, DE). EN^{3}HANCE® spray was from PerkinElmer Life Sciences. Ecolume scintillation mixture was from ICN Biomedicals, Inc. (Costa Mesa, CA). All other reagents were purchased from Sigma.

Apoptosis Assays—Apoptosis was assessed by staining ethanol-fixed, RNase-treated cells with propidium iodide (50 μg/ml in phosphate-buffered saline containing 5 mM EDTA, 10 min on ice) and identifying cells with a sub-G_0/G_1 DNA content, indicative of apoptosis, using a Coulter Epics ELITE flow cytometer (Coulter, Miami, FL). For morphological assessment of DNA condensation and/or apoptotic bodies, cells were grown on chamber slides and then incubated with the supravital DNA stain Hoechst 33342 (10 μg/ml for 30 min at 37 °C). When using fixed cells (cytospins), the dye used was Hoechst-bisbenzamide 33258 according to the manufacturer's instructions (Sigma). For analysis of apoptosis by

Hoechst staining, ~300–500 cells from a total of 5–10 fields in each sample were counted under UV filter at ×400 magnification.

Endothelial Viability and Cytotoxicity—Cell viability was assessed by uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (thiazolyl blue (MTT)) (5, 28) and confirmed by trypan blue exclusion and a fluorescence-based assay using fluorescein diacetate that selectively accumulates in live cells and is measured by digital imaging microscopy (DIMSCAN) (20, 21).

Radiolabeling and Analysis of Cellular Ceramide, Sphingomyelin, and Glucosylceramide—Ceramide metabolism was studied as described (5, 20) with some modifications. Briefly, endothelial cells (3 × 10^6 cells/10-cm dish) were allowed to attach and spread in medium containing 10% heat-inactivated FBS. Cells were radioactively labeled with [^{3}H]palmitic acid (1 μCi/ml, 10 ml per 10-cm dish), and 4-HPR was added either simultaneously with [^{3}H]palmitic acid or following 6–24 h labeling, as indicated. For experiments to measure sphingomyelin, glucosylceramide, or ceramide in the absence of *de novo* synthesis, cells were prepared by prelabeling with [^{3}H]palmitic acid for 24 h, washing with phosphate-buffered saline, and incubating them for 2 h in fresh growth medium containing 0.1% FBS and lacking isotope. After 2 h, the medium was replaced, and 4-HPR or vehicle control was added for the indicated time.

At the end of incubation, adherent and detached cells were trypsinized, combined, and washed with phosphate-buffered saline (4 °C). Total cellular lipids were extracted using equal volumes of methanol 2% acetic acid (v/v), water, and chloroform. After phase separation by centrifugation, the lower phase was dried under N$_2$ and stored at −20 °C. Lipids were solubilized in chloroform/methanol (2:1, v/v) and analyzed by TLC utilizing commercial lipid standards as markers visualized in iodine vapors, as described (5, 20, 29, 30). The solvent systems used were chloroform/acetic acid (9:1, v/v) for ceramide (5, 20, 29, 30), chloroform/methanol/acetic acid/double-distilled H$_2$O (50:30:7:4, v/v/v/v) for sphingomyelin (29), and chloroform/methanol/ammonium hydroxide (70:20:4, v/v/v) for glucosylceramide (31). Tritium in the TLC-resolved lipid band and total tritium in equal aliquots of the extracted cellular lipids were quantitated by liquid scintillation counting. The amount of ceramide, sphingomyelin, or glucosylceramide was expressed as percentage of cpm in these classes of lipids out of the total lipid tritium in the sample. For radiographs, the TLC plates were sprayed with EN^{3}HANCE® according to the manufacturer's instructions and exposed to film (Hyperfilm; Amersham Biosciences) at −80 °C for 3–7 days.

High Performance Liquid Chromatography (HPLC) for Measurement of Cellular 4-HPR Content—4-HPR content in endothelial cell pellets was determined by HPLC using UV absorbance detection in a modification of the method used by Le Doze *et al.* (32). Weighed pellets of treated cells were homogenized in 1 ml of acetonitrile and centrifuged, and the supernatant was analyzed by HPLC.

Isolation of Microsomal Membranes—Human BMEC cultured in 10-cm dishes were placed on ice, rinsed twice (ice-cold phosphate-buffered saline), and scraped into 0.5 ml of homogenization buffer (20 mM HEPES, pH 7.4, 5 mM dithiothreitol, 5 mM EDTA, 2 μg/ml leupeptin, 20 μg/ml aprotinin). Cell suspensions were sonicated over ice at 20% output, alternating a 15-s sonication with 20-s pause for four cycles, using a Micro Ultrasonic Cell Disrupter from Kontes (Vineland, NJ). Lysates were centrifuged at 10,000 × *g* for 10 min. The postnuclear supernatant was isolated and centrifuged at 100,000 × *g* for 60 min at 4 °C. The microsomal membrane pellet was resuspended in 100 μl of homogenization buffer by sonication for 5 s and was frozen at −80 °C (33).

Serine Palmitoyltransferase Assay—Enzymatic activity was determined by measuring the incorporation of [^{3}H]serine into 3-ketosphinganine (34, 35). Each tube (0.1-ml final volume) contained 100 μg of microsomal protein in 0.1 M HEPES, pH 8.3, 2.5 mM EDTA, 50 μM pyridoxal phosphate, 5 mM dithiothreitol, and 1.0 mM L-serine. After preincubation at 37 °C for 10 min, the reaction was initiated by the simultaneous addition of palmitoyl-CoA (0.2 mM) and 1.0 μCi of [^{3}H]serine. Control samples contained either boiled microsomes or no protein. After incubation at 37 °C for 7 min, the reaction was terminated by the addition of 0.2 ml of 0.5 N NH$_4$OH. Organic-soluble products were isolated by the addition of 3 ml of chloroform/methanol (2:1), 25 μg of sphingosine carrier, and 2.0 ml of 0.5 N NH$_4$OH. The washed organic phase was isolated, and 1.0 ml was dried under a stream of nitrogen and analyzed by liquid scintillation counting (33).

Ceramide Synthase Assay—Enzymatic activity was determined by measuring the incorporation of [^{3}H]sphinganine into [^{3}H]dihydroceramide (34, 35). Sphinganine in chloroform/methanol (2:1) was dried under nitrogen and dissolved to 10 μM with sonication, in 25 mM HEPES, pH 7.4, 2 mM MgCl$_2$, 0.5 mM dithiothreitol, prior to the addi-
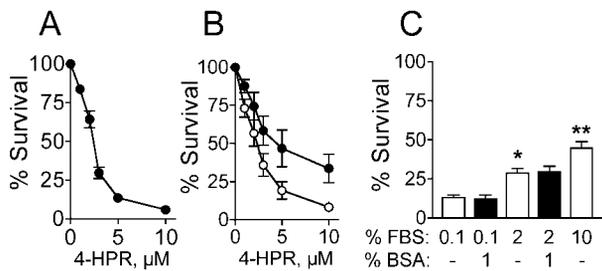
FIG. 1. **Fenretinide was cytotoxic to human BMEC.** Human BMEC (6×10^3 cells/well in a 96-well plate) were incubated with 4-HPR for 24 h. Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *A*, cells were incubated in medium containing 0.1% FBS. Data points are means ± S.E. of 10–21 experiments, performed in 5–10 replicates. $p < 0.001$ by one way ANOVA for 0–10 μM 4-HPR. *B*, cells were incubated in culture medium containing 10% (●) or 0.1% (○) FBS. Data points are means ± S.E. of 3–6 experiments performed in 5–10 replicates. $p < 0.001$ by two-tailed paired *t* test comparing mean survivals in 10% and 0.1% FBS at each 4-HPR concentration in each of the experiments. *C*, cells were incubated with the indicated concentrations of FBS with (■) or without (□) 4-HPR (5 μM) in the presence or absence of 1% heat-inactivated fatty acid-free bovine serum albumin (*BSA*). *Bars* represent means ± S.E. of five replicate samples. *, $p = 0.0023$ for cells in 2% compared with 0.1% FBS; **, $p = 0.013$ for cells in 10% compared with 2% FBS, by unpaired *t* tests. $p =$ not significant in the presence compared with the absence of bovine serum albumin at each of the FBS concentrations, by unpaired *t* tests.

tion of microsomal protein (100 μg) to a final reaction volume of 0.1 ml. Assays were initiated by the simultaneous addition of palmitoyl-CoA (0.1 mM) and 0.5 μCi of [³H]sphinganine followed by incubation at 37 °C for 40 min with gentle shaking. The reaction was terminated by lipid extraction. [³H]dihydroceramide was isolated by TLC and quantitated by liquid scintillation counting.

Statistical Analysis—Statistical analyses were performed using GraphPad Prism version 3.0c for MacIntosh (GraphPad Software, San Diego, CA). Values are given as means ± S.E. When two means were compared, *p* values were based on the *t* test (unpaired or paired, depending on the experimental design). When three or more means were compared, the overall *p* value was based on the F-test from an analysis of variance (ANOVA). If the means were based on doses or times (*i.e.* on a continuum), then the *p* value was based on the test for trend using linear regression. $p < 0.05$ was considered significant.

RESULTS

4-HPR Was Cytotoxic to Endothelial Cells—Since only one of two reports found 4-HPR to be cytotoxic to endothelial cells, we determined the cytotoxicity of 4-HPR in human BMEC (Fig. 1). 4-HPR was cytotoxic to human BMEC in a dose-dependent manner as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with 50% cytotoxicity at 2.4 ± 0.2 μM (S.E.) and 90% cytotoxicity at 5.3 ± 0.5 μM 4-HPR when incubated in medium containing 0.1% FBS (Fig. 1A). Assessment by trypan blue exclusion and DIMSCAN (digital imaging microscopy (20)) revealed similar results.[2] Cytotoxicity was similar in low passage (passages 14–30) and high passage (passages 40–57) human BMEC, with 50% cytotoxicity at a mean 4-HPR of 2.2 ± 0.3 and 2.6 ± 0.2 μM, respectively ($n = 10$ and 11; $p = 0.32$ by unpaired *t* test). With HUVEC, 50 and 90% cytotoxicity was achieved at 3.1 and 9.0 μM 4-HPR, respectively ($n = 10$, $p < 0.001$ by one-way ANOVA), and in bovine TBMEC, 5 μM 4-HPR induced 98% cytotoxicity ($n = 10$, $p < 0.001$ by unpaired *t* test compared with control cells). Thus, 4-HPR was cytotoxic to both human and bovine BMEC as well as in HUVEC.

Some growth factors can activate survival signaling pathways such as the phosphatidylinositol-3-kinase/Akt cascade to protect them from apoptosis, as shown for insulin- and insulin-like growth factor-1-mediated inhibition of anoikis (36). Therefore, we examined whether FBS could rescue endothelial cells
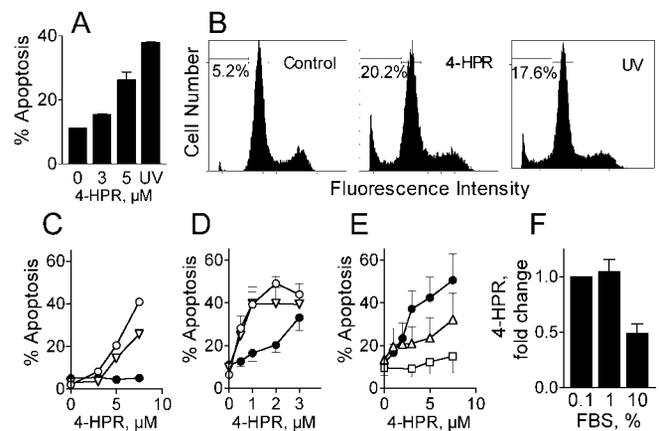
FIG. 2. **Fenretinide-induced apoptosis in human BMEC.** *A–E*, apoptosis (cells with a sub-G_0/G_1 DNA content) was assessed by flow cytometry of permeabilized propidium iodide-stained human BMEC (10^6 cells/10-cm dish) that were incubated with 4-HPR as indicated below. *A*, cells were incubated for 24 h with 4-HPR in culture medium containing 0.1% FBS. UV-irradiated cells harvested 24 h following irradiation (25 mJ; *UV*) were used as positive control. Shown are means ± S.E. of a representative experiment from over 10 performed in triplicates with similar results. $p < 0.001$ by one-way ANOVA for 0–5 μM 4-HPR. *B*, flow cytometry tracings from a representative experiment of cells incubated for 24 h with 4-HPR (5 μM) or vehicle control or following UV irradiation as described for *A*. *C* and *D*, cells were incubated with 4-HPR for 24 h (*C*) or 72 h (*D*) in medium containing 0.1% (○), 1% (▽), or 10% (●) FBS. *C*, representative experiment of three performed under similar conditions; *D*, mean ± S.E. of nine independent experiments. *E*, cells were incubated for 24 (□), 48 (△), or 72 h (●) with 0–7.5 μM 4-HPR in medium containing 10% FBS. Shown are means ± S.E. of three independent experiments. *F*, human BMEC (3×10^6 cells/10-cm plate) were incubated for 6 h with 5 μM 4-HPR in medium containing the indicated concentrations of FBS. Cells were trypsinized and 4-HPR content was determined by HPLC. *Bars*, -fold change of 4-HPR content/g of cells (mean ± S.E.) from three independent experiments. $p = 0.017$ comparing 1 and 10% FBS by unpaired *t* tests.

from 4-HPR-mediated cytotoxicity. Including 1–10% FBS in the medium diminished 4-HPR-induced cytotoxicity in human BMEC, bovine TBMEC, and HUVEC compared with 0.1% FBS when measured at 24 h (Fig. 1, *B* and *C*).[2] The addition of bovine serum albumin to the medium did not suppress the cytotoxic effect of 4-HPR (Fig. 1*C*), indicating that protection by FBS was not due to binding of the drug to the albumin in the FBS.

4-HPR Induced Apoptosis in Endothelial Cells—Protection by serum suggested that 4-HPR could be inducing apoptosis in the endothelial cells. Indeed, human BMEC exposed to 4-HPR demonstrated an increase in the sub-G_0/G_1 DNA content indicative of apoptosis as revealed by flow cytometry (representative experiments shown in Fig. 2, *A* and *B*).
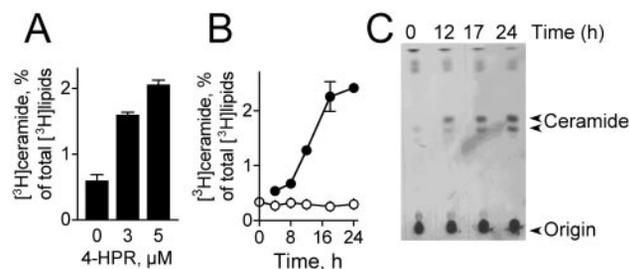
We then examined whether serum protected the cells from 4-HPR-induced apoptosis. At 24 h, 10% FBS completely abrogated the 4-HPR-mediated apoptosis even in the presence of 7.5 μM 4-HPR (Fig. 2*C*). Since in some tumor cell lines induction of apoptosis by 4-HPR requires longer exposures (37, 38), we also examined the apoptotic effect of 4-HPR in the presence of 10% FBS at 48 and 72 h. Indeed, in 1–10% FBS, the apoptotic effect of 4-HPR was restored with longer incubations (Fig. 2, *D* and *E*). To examine the possibility that the delay in apoptosis was due to binding of 4-HPR to the FBS, we determined 4-HPR content in human BMEC in medium containing 0.1–10% FBS. Fig. 2*F* demonstrates that cellular 4-HPR content was similar in cells treated in culture medium with 0.1 and 1% FBS and was decreased in 10% FBS. In order to maintain controlled conditions and minimize overconfluence of cells at the high FBS concentrations during longer

incubations, subsequent experiments were performed in medium containing 0.1–1% FBS.

4-HPR Increased Endogenous Ceramide in Endothelial Cells—Ceramide is thought to mediate endothelial apoptosis by stress stimuli (5, 22–24), and exogenous C_2-ceramide itself can induce endothelial apoptosis (5). Since we observed that 4-HPR-induced apoptosis in endothelial cells, we determined whether this was associated with increased ceramide. 4-HPR effectively increased ceramide levels in human BMEC (Fig. 3) as well as in bovine TBMEC and HUVEC (Table I). In the human BMEC, mean ceramide increase was 5.25-fold ± 0.72 following a 17-h incubation with 5 μM 4-HPR ($n = 12$ experiments in triplicate, $p < 0.001$). The increase in ceramide was observed as early as 4–8 h from the start of exposure to 4-HPR, before cytotoxicity or apoptosis could be detected, and continued to increase up to 24 h (Fig. 3, *B* and *C*). A decrease in several unidentified faster and slower migrating lipid bands was observed in parallel to the increase in [³H]ceramide (Fig. 3*C*). Whereas it is possible that some of these are the lipids that contribute to the increase in ceramide (higher Rf in particular), it is difficult to evaluate the origin due to the large amount of radioactivity.
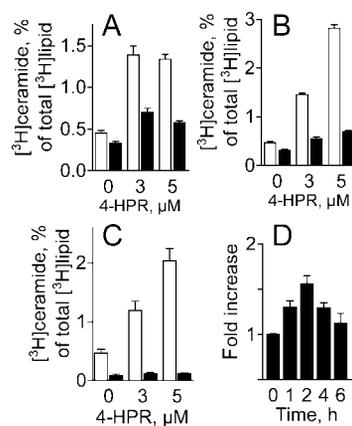
In most experiments, 4-HPR was added to the medium simultaneously with [³H]palmitic acid (Fig. 3). [³H]Palmitic acid rapidly entered both control human BMEC and those incubated with 4-HPR (72 ± 4 and 77 ± 1%, respectively, at 6 h, and 85 ± 2 and 83 ± 5% at 24 h, p = not significant with *versus*

without 4-HPR, by unpaired t tests, $n = 3$). Thus, the increase in ceramide in the presence of 4-HPR was not due to stimulation of uptake of [³H]palmitic acid into the cells. The 4-HPR-induced increase in [³H]ceramide was also observed in human and bovine BMEC and in HUVEC when incubation with [³H]palmitic acid began 17–24 h prior to the addition of 4-HPR and when 10% FBS was included in the medium (Table I). These data indicate that 4-HPR induced generation of ceramide in endothelial cells.

4-HPR Stimulated de Novo Ceramide Synthesis—Sphingomyelinase activation has been thought to be the main pathway for ceramide generation in apoptosis following stress stimuli (1–4). However, later reports demonstrate that some stimuli can induce apoptosis via *de novo* ceramide synthesis (6, 8, 10). To determine which of these pathways was activated by 4-HPR in human BMEC, we first examined whether 4-HPR-induced ceramide formation could be suppressed by inhibitors of *de novo* ceramide synthesis (Fig. 4). Fumonisin B_1 (25 μM), an inhibitor of ceramide synthase, potently inhibited ceramide generation in endothelial cells exposed to 4-HPR (Fig. 4*A*). Similarly, L-cycloserine (30 μM) and myriocin (0.05 μM), both inhibitors of the rate-limiting enzyme in the *de novo* ceramide synthesis pathway, serine palmitoyltransferase (SPT) (10), potently suppressed the 4-HPR-induced generation of ceramide

FIG. 3. **4-HPR increased endothelial ceramide in a dose- and time-dependent manner.** *A*, 4-HPR and [³H]palmitic acid were added simultaneously to human BMEC (3×10^6 cells/10-cm dish) in medium containing 0.1% FBS. After 16 h, cells were harvested, lipids were extracted, and ceramide was determined by TLC. Results are presented as cpm in [³H]ceramide as a percentage of the total lipid tritium. Shown are means ± S.E. from a representative experiment of multiple repeats performed in triplicates. $p < 0.001$ by one-way ANOVA for 0, 3, and 5 μM 4-HPR. *B*, human BMEC (3×10^6 cells/10-cm dish) were incubated in medium containing [³H]palmitic acid and 0.1% FBS. Vehicle control (○) or 4-HPR (●; 5 μM) were added either at the time of the addition of [³H]palmitic acid (for the 24-h time point) or 6, 12, 16, or 20 h later (to achieve incubations with 4-HPR of 24, 18, 12, 8, and 4 h, respectively). Cells were harvested 24 h after the addition of [³H]palmitic acid. The *x* axis denotes length of incubation with 4-HPR or control. Lipids were processed as described. Shown are means ± S.E. from a representative experiment of three performed in triplicates. p = 0.0060 in cells with 5 μM 4-HPR at 4–24 h, by simple linear regression. *C*, human BMEC were labeled with [³H]palmitic acid and treated with 4-HPR (5 μM) as in *B*. Lipids were extracted, equal amounts (cpm) of total labeled lipids were separated on TLC, and radiographs were developed. The time indicated is length of incubation with 4-HPR.

FIG. 4. **4-HPR stimulated activity of SPT and increased *de novo* synthesis of ceramide in human BMEC.** *A–C*, human BMEC (3×10^6 cells/10-cm dish) were incubated in medium containing 0.1% FBS with inhibitor (fumonisin B_1, 25 μM (A), L-cycloserine, 30 μM (B), and myriocin, 0.05 μM (C); ■) or vehicle control (Me₂SO; □) beginning 2 h before the addition of [³H]palmitic acid and 4-HPR (0, 3, or 5 μM). After overnight incubation with 4-HPR, cells were harvested, lipids were extracted, and ceramide was determined by TLC. Results are presented as cpm in [³H]ceramide, as a percentage of the total lipid tritium. $p < 0.001$ in cells exposed to 4-HPR with inhibitors (■) compared with without them (□), by unpaired t tests for each inhibitor. Shown are means ± S.E. from representative experiments of three, performed in triplicates. *D*, human BMEC were incubated with 4-HPR (10 μM) for the indicated times. Microsomes were isolated, and enzymatic activity of SPT was determined as described under "Experimental Procedures." Results are expressed as -fold change in SPT activity (mean ± S.E.). SPT activity in the absence of 4-HPR was 29.8 pmol/mg of protein/min. Shown is one of two similar experiments, performed in duplicates. p = 0.022 by one-way ANOVA.

TABLE I
Ceramide increase by 4-HPR occurred in three different endothelial preparations and in the presence of 10% FBS

Cells were incubated overnight in the presence of 10% FBS and [³H]palmitic acid. 4-HPR was then added for the time indicated. Lipids were processed, and [³H]ceramide was measured as described under "Experimental Procedures." p values were derived by unpaired t tests, $n = 3$.

| | 6 h | | 24 h | | |
	No 4-HPR	5 μM 4-HPR	No 4-HPR	10 μM 4-HPR	p value
Human BMEC			0.4 ± 0.08^a	0.9 ± 0.06	0.007
Bovine TBMEC	1.0 ± 0.02	2.3 ± 0.04			<0.001
HUV-EC-C	0.89 ± 0.08	1.3 ± 0.09			0.014

[a] [³H]ceramide, percentage of labeled lipids, mean ± S.E.
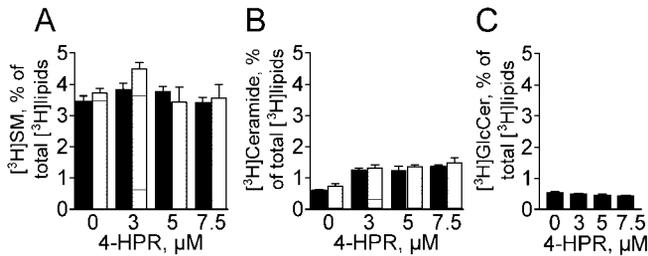
Fig. 5. **Effect of 4-HPR on sphingomyelin levels in endothelial cells.** *A–C*, human BMEC (3×10^6 cells/10-cm dish) were preloaded with [^{3}H]palmitic acid for 24 h, and the isotope was washed out for 2 h. Desipramine (5 μM; *horizontally hatched bars*) or vehicle control (*filled bars*) were added in fresh medium lacking isotope and containing 0.1% FBS, and 4-HPR was added 2 h later for 24 h. Cells were harvested, lipids were extracted, and sphingomyelin (*A; SM*), ceramide (*B*), and glucosylceramide (*C*) were determined by TLC using the solvent systems described under "Experimental Procedures." Results are presented as cpm in either [^{3}H]ceramide, [^{3}H]sphingomyelin, or [^{3}H]glucosylceramide, as a percentage of the total lipid tritium. For sphingomyelin (*A*), there was no effect of 4-HPR or desipramine (*p* = not significant, by two-way ANOVA). However, for ceramide (*B*) there was a strong 4-HPR effect (*p* < 0.001) but no desipramine effect (*p* = 0.13 for the overall effect and *p* = 0.98 for the interaction; all based on two-way ANOVA). *Bars*, means ± S.E. of one of ten experiments with similar results, and comparison with desipramine represents one of two experiments, all performed in triplicate.
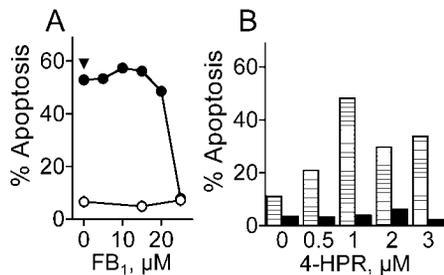
Fig. 6. **Prevention of 4-HPR-induced generation of ceramide suppressed endothelial cell apoptosis.** Fumonisin B$_1$ or vehicle control were added to human BMEC (10^6 cells/10-cm dish) incubated in medium containing 1% FBS, followed 2 h later by 4-HPR or vehicle control. 72 h later, cells were permeabilized, stained with propidium iodide, and analyzed for apoptosis by flow cytometry. *A*, 4-HPR (3 μM) (●), control (○), and UV (25 mJ) (▼), in the presence of 0–25 μM FB$_1$. Data points represent mean percentage of apoptosis of duplicate samples. Error bars (S.E.) are smaller than the *symbols*. *p* < 0.0001 for cells with 4-HPR and 0–25 μM fumonisin B$_1$, by one-way ANOVA. *B*, fumonisin B$_1$ (25 μM) (*filled bars*) and Me$_2$SO (*horizontally hatched bars*) in the presence of 0–3 μM 4-HPR. *p* = 0.016 between cells treated with 4-HPR with fumonisin or without it, by paired *t* test.

(Fig. 4, *B* and *C*). In order to more directly demonstrate the stimulation of *de novo* ceramide synthesis by 4-HPR, we measured activity of SPT in microsomes isolated from endothelial cells that had been exposed to the drug or to vehicle control (Fig. 4*D*). Activity of SPT in the absence of 4-HPR was 29.8 ± 0.2 pmol/mg of protein/min and increased by 1.75-fold after 2 h of incubation with 10 μM 4-HPR (Fig. 4*D*), confirming stimulation of *de novo* ceramide synthesis by 4-HPR. By 6 h of incubation with 4-HPR, SPT activity returned to base-line levels (Fig. 4*D*). 4-HPR did not induce activation of ceramide synthase, an enzyme downstream of SPT, and at 10 μM even suppressed its activity by up to 25% (data not shown). However, since SPT is the rate-limiting enzyme for *de novo* ceramide synthesis and the base-line activity of ceramide synthase (138 ± 5 pmol/mg protein/min) in the human BMEC was more than 4 times higher than that of SPT (29.8 ± 0.2 pmol/mg of protein/min), such change in ceramide synthase was not expected to affect the increase in ceramide induced by 4-HPR. These experiments demonstrate that 4-HPR activated *de novo* ceramide synthesis in endothelial cells.
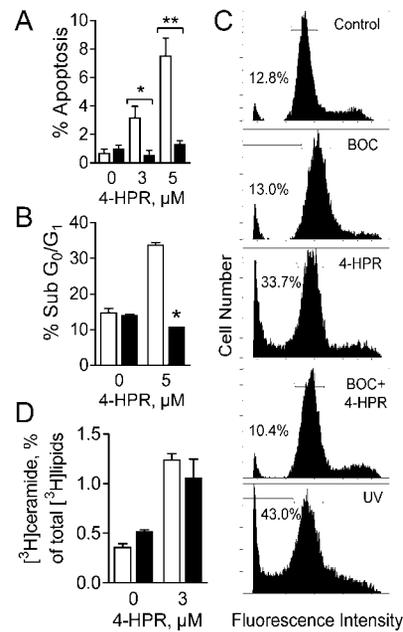
Fig. 7. **4-HPR-induced ceramide generation was independent of caspases in endothelial cells.** Human BMEC (10^5 cells/well in two-well chamber slide (*A*), 10^6 cells/10-cm dish (*B* and *C*), and 3×10^6 cells/10-cm dish (*D*)) were preincubated in medium containing 0.1% FBS with 25 μM BOC-D-FMK (■) or Me$_2$SO (□) for 2 h. 4-HPR or vehicle control were then added for 24 h (*A–C*) or 16 h (*D*). Shown are means ± S.E. of triplicate samples. *A*, cells were fixed, stained with Hoechst-bisbenzamide 33258, and scored for apoptosis (cells with condensed and fragmented nuclei) in a blinded manner. *, *p* = 0.017; **, *p* = 0.001 by unpaired *t* test. *B*, apoptosis was assessed by flow cytometry of propidium iodide-stained fixed cells. Shown are means ± S.E.; *, *p* < 0.001 comparing cells with 5 μM 4-HPR with or without BOC-D-FMK by unpaired *t* test. Shown is one of two experiments with similar results, performed in triplicate. *C*, representative flow cytometry tracings of propidium iodide-stained fixed cells from the experiment shown in *B*. UV-irradiated (25 mJ) human BMEC serve as positive control. *D*, cells were harvested, lipids were extracted, and ceramide was quantitated by TLC. Results are presented as cpm in [^{3}H]ceramide, as a percentage of the total lipid tritium. *p* = 0.42 comparing cells in 3 μM 4-HPR with or without BOC-D-FMK by unpaired *t* test. Shown is one of two experiments with similar results.

Effect of 4-HPR on Sphingomyelin Metabolism in Human BMEC—To assess whether sphingomyelin hydrolysis contributed to ceramide increase, we prelabeled human BMEC with [^{3}H]palmitic acid and measured its decay following incubation with 4-HPR (Fig. 5). Under these conditions, sphingomyelin levels in human BMEC treated with 4-HPR either remained unchanged or increased slightly but did not decrease compared with controls following the 24-h incubation with the drug (Fig. 5*A*). Incubation with desipramine, a nonspecific inhibitor of sphingomyelin hydrolysis, starting 2 h before the addition of 4-HPR, did not alter sphingomyelin levels in the presence of 4-HPR, further suggesting that 4-HPR did not activate sphingomyelinase (Fig. 5*A*). Under these conditions, there was also no change in levels of cellular glucosylceramide in the presence of 4-HPR (Fig. 5*C*). Interestingly, despite the removal of the remaining exogenous [^{3}H]palmitic acid from the medium prior to the addition of 4-HPR in these experiments, [^{3}H]ceramide levels increased in the presence of 4-HPR (Fig. 5*B*). The increase in ceramide was not inhibited by desipramine (Fig. 5*B*), further suggesting that sphingomyelinase activation did not contribute to the 4-HPR-induced ceramide increase.

Inhibition of 4-HPR-induced Ceramide Generation Suppressed Endothelial Apoptosis—To determine whether activation of *de novo* ceramide synthesis has a causal role in 4-HPR-induced endothelial apoptosis, we examined whether suppression of the increase in ceramide could prevent 4-HPR-

Ceramide in Fenretinide-induced Endothelial Apoptosis

induced apoptosis (Fig. 6). Incubation of human BMEC with the ceramide synthase inhibitor, fumonisin B_1, at a concentration that effectively suppressed *de novo*-generated ceramide increase (25 μM; Fig. 4*A*), completely prevented apoptosis induced by 4-HPR (Fig. 6, *A* and *B*). These data provide evidence for a causal role of 4-HPR-induced *de novo*-generated ceramide in endothelial apoptosis.

4-HPR-induced Ceramide Increase Was Independent of Caspase Activation in Endothelial Cell Apoptosis—To determine whether 4-HPR-induced apoptosis in BMEC was mediated by caspases, we examined whether apoptosis could be inhibited by caspase inhibitors. When human BMEC were incubated with the pancaspase inhibitor, BOC-D-FMK, and exposed to 4-HPR, apoptosis was inhibited to base-line levels compared with control cells (Fig. 7, *A–C*). Benzyloxycarbonyl-VAD-FMK, another caspase family inhibitor, also suppressed 4-HPR-induced apoptosis, and similar inhibition by BOC-D-FMK was observed in bovine TBMEC (data not shown). The effect of the caspase inhibitors further confirmed the apoptotic nature of 4-HPR toxicity toward the human BMEC (Fig. 7, *A–C*). In order to determine the ordering of caspase activation and 4-HPR-induced ceramide generation, we incubated human BMEC with BOC-D-FMK in the presence of 4-HPR and analyzed ceramide levels (Fig. 7*D*). Whereas BOC-D-FMK effectively inhibited 4-HPR-induced apoptosis (Fig. 7, *A–C*), it had no effect on the 4-HPR-induced ceramide increase in human BMEC (Fig. 7*D*). Similar results were obtained using bovine TBMEC (data not shown). These data place ceramide upstream of caspases in 4-HPR-induced endothelial apoptosis. Collectively, these data provide evidence for a signaling role for ceramide in 4-HPR-mediated endothelial apoptosis.

DISCUSSION

Stress stimuli such as irradiation, lipopolysaccharide, serum starvation, and tumor necrosis factor-α mediate endothelial apoptosis by increasing ceramide (22–24). We have recently shown that endothelial anoikis, the apoptosis resulting from the loss of matrix adhesion, is also associated with increased ceramide (5). Our current results demonstrate that 1) 4-HPR increased endothelial ceramide by *de novo*, nonsphingomyelinase-mediated ceramide synthesis, 2) 4-HPR induced caspase-dependent endothelial apoptosis mediated by ceramide, and 3) ceramide was upstream of caspases in 4-HPR signaling to endothelial apoptosis. These data are the first investigation of the molecular signaling events mediating 4-HPR-induced endothelial cell apoptosis.

To date, induction of apoptosis by 4-HPR has only been described in tumor cell lines (12, 13, 20). Our data now provide evidence for apoptosis by 4-HPR in cultured endothelial cells, achieved at concentrations similar to those that induce apoptosis in tumor cell lines (20, 37–40). Decrease in capillary formation in the chick chorioallantoic membrane by 4-HPR has been demonstrated by two groups, indicating its *in vivo* anti-angiogenic activity (14, 15). However, only one of these groups found 4-HPR (2.5–5 μM, 48 h) to be cytotoxic to endothelial cells (14), whereas the other did not detect cytotoxicity even after 72-h incubation with 10 μM 4-HPR (15). In our experiments, 4-HPR was cytotoxic to the three types of endothelial cell preparations studied in tissue culture. Thus, 4-HPR-mediated endothelial cytotoxicity occurs in four of the five endothelial preparations studied to date, suggesting that its effects may differ depending on the culture conditions and the cells used. Clinical trials demonstrate that 4-HPR does not induce generalized vascular damage in humans (12). This suggests that its anti-endothelial effect may manifest selectively in angiogenic endothelium, which is thought to be biologically different from en-

dothelium in stable vasculature, and thus presents a selective target for antiangiogenic therapies (41).

Ceramide increase in cells undergoing apoptosis has mostly been described to occur independently of caspases (1). However, this pathway may be cell-specific, stimulus-dependent, and affected by culture conditions (1). For some stimuli such as CD95 (Fas/APO-1), ceramide accumulation occurs upstream of effector caspases but is downstream of the initiator caspases (42). Similar to 4-HPR-mediated apoptosis in neuroblastoma and HL-60 cell lines (20, 39), 4-HPR-induced apoptosis in BMEC was dependent on caspase activation. Initial molecular ordering of ceramide in the BMEC utilizing a pancaspase inhibitor placed ceramide upstream of caspase activation, as has been shown for 4-HPR-induced apoptosis in HL-60 cells (39). However, it is still not known whether there is a requirement for the initiator caspases in 4-HPR-mediated ceramide increase in endothelial cells.

Fenretinide caused no detectable sphingomyelin hydrolysis (Fig. 5). Additionally, sphingomyelin and ceramide levels in 4-HPR-treated cells were not altered by desipramine (Fig. 5). Thus, it is unlikely that sphingomyelinase, an important component of ceramide-mediated apoptosis by several other stimuli (9), contributed to 4-HPR-induced generation of ceramide in endothelial cells. Instead, the *de novo* ceramide synthesis pathway was implicated, as was found in neuroblastoma and leukemia cells (19, 21, 35). This was shown by the efficient suppression of 4-HPR-induced increase in [^{3}H]ceramide in human BMEC by inhibitors of *de novo* ceramide synthesis (Fig. 4, *A–C*). Additionally, 4-HPR activated SPT, the first enzyme in this pathway (Fig. 4*D*), as was found in neuroblastoma cells (35). In neuroblastoma, a downstream enzyme, ceramide synthase, was also activated by 4-HPR (35), whereas in the human BMEC it was not. Lack of stimulation of ceramide synthase, or even its mild inhibition, is not expected to prevent an increase in *de novo* generated ceramide by 4-HPR, since in the human BMEC ceramide synthase activity was over 4 times higher than that of SPT at base line (138.0 and 29.8 pmol/mg protein/min, respectively). This is similar to the findings in breast cancer cells, where PSC 833 induced a robust increase in *de novo* ceramide synthesis by stimulation of SPT, the rate-limiting enzyme, without effect on ceramide synthase activity, which was about 3 times higher than SPT at base line (34). This supports a role for increased SPT activity in 4-HPR-mediated up-regulation of ceramide in endothelial cells. In the human BMEC, the activation of SPT was early and transient, whereas ceramide continued to increase for many hours, suggesting that other lipid metabolic pathways may contribute to 4-HPR-induced increase in ceramide. Supporting existence of additional mechanism(s) for 4-HPR-induced ceramide increase, our data show that 4-HPR increased ceramide levels even in BMEC prelabeled with [^{3}H]palmitic acid (Fig. 5*B*), conditions where *de novo* synthesis of [^{3}H]ceramide directly from exogenous [^{3}H]palmitic acid was unlikely. Taken together, these data demonstrate that 4-HPR stimulates *de novo* ceramide synthesis and suggest that other ceramide-generating pathways may also be affected by 4-HPR under the conditions used in these experiments.

Our results further show that fumonisin B_1 efficiently suppressed the 4-HPR-mediated increase in endothelial ceramide, in parallel to prevention of the associated apoptosis (Figs. 4 and 6). This demonstrates a causal role for ceramide in 4-HPR-mediated endothelial apoptosis. This is similar to the mechanism of fenretinide-induced cytotoxicity in leukemia, where inhibitors of *de novo* ceramide synthesis were shown to inhibit apoptosis (39). The concentrations of fumonisin B_1 required to inhibit the 4-HPR-induced increase in ceramide and apoptosis

in the BMEC (25 μM) were similar or lower than those used in some tumor cell lines (25–100 μM) (39, 43). It is possible that other proapoptotic mechanisms not mediated via ceramide are also activated by 4-HPR (12) and contribute to its apoptotic effect in the endothelial cells. One such mechanism is 4-HPR-induced generation of reactive oxygen species described in neuroblastoma and in HL-60 myeloid leukemia, which itself may be part of the signaling cascade to ceramide-mediated apoptosis (16, 17).

Taken together, our study establishes a causal role for *de novo*-generated ceramide in the molecular mechanism of 4-HPR-induced endothelial apoptosis and supports examination of 4-HPR as part of a combined antiangiogenic and anti-tumor approach to cancer therapy.

REFERENCES

1. Mathias, S., Pena, L. A., and Kolesnick, R. N. (1998) *Biochem. J.* **335,** 465–480
2. Obeid, L. M., and Hannun, Y. A. (1995) *J. Cell. Biochem.* **58,** 191–198
3. Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. N. (1996) *Nature* **380,** 75–79
4. Hannun, Y. A. (1996) *Science* **274,** 1855–1859
5. Erdreich-Epstein, A., Shimada, H., Groshen, S., Liu, M., Metelitsa, L. S., Kim, K. S., Stins, M. F., Seeger, R. C., and Durden, D. L. (2000) *Cancer Res.* **60,** 712–721
6. Garzotto, M., Haimovitz-Friedman, A., Liao, W. C., White-Jones, M., Huryk, R., Heston, W. D., Cardon-Cardo, C., Kolesnick, R., and Fuks, Z. (1999) *Cancer Res.* **59,** 5194–5201
7. Kroesen, B. J., Pettus, B., Luberto, C., Busman, M., Sietsma, H., de Leij, L., and Hannun, Y. A. (2001) *J. Biol. Chem.* **276,** 13606–13614
8. Perry, D. K., Carton, J., Shah, A. K., Meredith, F., Uhlinger, D. J., and Hannun, Y. A. (2000) *J. Biol. Chem.* **275,** 9078–9084
9. Kolesnick, R. N., Haimovitz-Friedman, A., and Fuks, Z. (1994) *Biochem. Cell Biol.* **72,** 471–474
10. Merrill, A. H. J. (2002) *J. Biol. Chem.* **277,** 25843–25846
11. Kolesnick, R. (2002) *J. Clin. Invest.* **110,** 3–8
12. Reynolds, C. P., and Lemons, R. S. (2001) *Hematol. Oncol. Clin. N. Am.* **15,** 867–910
13. Wu, J. M., DiPietrantonio, A. M., and Hsieh, T. C. (2001) *Apoptosis* **6,** 377–388
14. Pienta, K. J., Nguyen, N. M., and Lehr, J. E. (1993) *Cancer Res.* **53,** 224–226
15. Ribatti, D., Alessandri, G., Baronio, M., Raffaghello, L., Cosimo, E., Marimpietri, D., Montaldo, P. G., De Falco, G., Caruso, A., Vacca, A., and Ponzoni, M. (2001) *Int. J. Cancer* **94,** 314–321
16. Delia, D., Aiello, A., Formelli, F., Fontanella, E., Costa, A., Miyashita, T., Reed, J. C., and Pierotti, M. A. (1995) *Blood* **85,** 359–367
17. Delia, D., Aiello, A., Meroni, L., Nicolini, M., Reed, J. C., and Pierotti, M. A. (1997) *Carcinogenesis* **18,** 943–948
18. DiPietrantonio, A. M., Hsieh, T. C., Juan, G., Traganos, F., Darzynkiewicz, Z., and Wu, J. M. (2000) *Cancer Res.* **60,** 4331–4335
19. O'Donnell, P. H., Guo, W. X., Reynolds, C. P., and Maurer, B. J. (2002) *Leukemia* **16,** 902–910
20. Maurer, B. J., Metelitsa, L. S., Seeger, R. C., Cabot, M. C., and Reynolds, C. P. (1999) *J. Natl. Cancer Inst.* **91,** 1138–1146
21. Maurer, B. J., Melton, L., Billups, C., Cabot, M. C., and Reynolds, C. P. (2000) *J. Natl. Cancer Inst.* **92,** 1897–1909
22. Haimovitz-Friedman, A., Cordon-Cardo, C., Bayoumy, S., Garzotto, M., McLoughlin, M., Gallily, R., Edwards, C. K., III, Schuchman, E. H., Fuks, Z., and Kolesnick, R. (1997) *J. Exp. Med.* **186,** 1831–1841
23. Haimovitz-Friedman, A., Kan, C. C., Ehleiter, D., Persaud, R. S., McLoughlin, M., Fuks, Z., and Kolesnick, R. N. (1994) *J. Exp. Med.* **180,** 525–535
24. Lin, X., Fuks, Z., and Kolesnick, R. (2000) *Crit. Care Med.* **28,** N87–N93
25. Stins, M. F., Gilles, F., and Kim, K. S. (1997) *J. Neuroimmunol.* **76,** 81–90
26. Stins, M. F., Prasadarao, N. V., Zhou, J., Arditi, M., and Kim, K. S. (1997) *In Vitro Cell Dev. Biol.* **33,** 243–247
27. Stins, M. F., Shen, Y., Huang, S. H., Gilles, F., Kalra, V. K., and Kim, K. S. (2001) *J. Neurovirol.* **7,** 125–134
28. Mosmann, T. (1983) *J. Immunol. Methods* **65,** 55–63
29. Lavie, Y., Cao, H., Volner, A., Lucci, A., Han, T. Y., Geffen, V., Giuliano, A. E., and Cabot, M. C. (1997) *J. Biol. Chem.* **272,** 1682–1687
30. Lavie, Y., Cao, H., Bursten, S. L., Giuliano, A. E., and Cabot, M. C. (1996) *J. Biol. Chem.* **271,** 19530–19536
31. Liu, Y. Y., Han, T. Y., Giuliano, A. E., and Cabot, M. C. (1999) *J. Biol. Chem.* **274,** 1140–1146
32. Le Doze, F., Debruyne, D., Albessard, F., Barre, L., and Defer, G. L. (2000) *Drug Metab. Dispos.* **28,** 205–208
33. Holleran, W. M., Williams, M. L., Gao, W. N., and Elias, P. M. (1990) *J. Lipid Res.* **31,** 1655–1661
34. Wang, H., Giuliano, A. E., and Cabot, M. C. (2002) *Mol. Cancer Ther.* **1,** 719–726
35. Wang, H., Maurer, B. J., Reynolds, C. P., and Cabot, M. C. (2001) *Cancer Res.* **61,** 5102–5105
36. Yu, J. T., Foster, R. G., and Dean, D. C. (2001) *Mol. Cell. Biol.* **21,** 3325–3335
37. Puduvalli, V. K., Saito, Y., Xu, R., Kouraklis, G. P., Levin, V. A., and Kyritsis, A. P. (1999) *Clin. Cancer Res.* **5,** 2230–2235
38. Hsieh, T. C., and Wu, J. M. (1997) *Prostate* **33,** 97–104
39. DiPietrantonio, A. M., Hsieh, T. C., Olson, S. C., and Wu, J. M. (1998) *Int. J. Cancer* **78,** 53–61
40. Kalemkerian, G. P., Slusher, R., Ramalingam, S., Gadgeel, S., and Mabry, M. (1995) *J. Natl. Cancer Inst.* **87,** 1674–1680
41. Kerbel, R. S. (2000) *Carcinogenesis* **21,** 505–515
42. Grullich, C., Sullards, M. C., Fuks, Z., Merrill, A. H., Jr., and Kolesnick, R. (2000) *J. Biol. Chem.* **275,** 8650–8656
43. Ciacci-Zanella, J. R., and Jones, C. (1999) *Food Chem. Toxicol.* **37,** 703–712