Ceramide signaling in fenretinide-induced endothelial cell apoptosis

Anat Erdreich-Epstein‡,1, 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‡.

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; John Wayne Cancer Institute at Saint John’s Health Center, Santa Monica, California 90404§; Section of Hematology/Oncology, Department of Pediatrics, Herman B Wells Center for Pediatric Research, Indiana School of Medicine, Indianapolis, IN 46202§; Division of Infectious Diseases, Dept Pediatrics, Johns Hopkins School of Medicine, Baltimore, MD 21205**.

To whom correspondence should be addressed:
Anat Erdreich-Epstein, MD, PhD
Childrens Hospital Los Angeles
4650 Sunset Boulevard, Mailstop #57
Los Angeles, California 90027
Telephone: (323) 669-4613; Fax: (323) 664-9455;
E-mail: epstein@usc.edu

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SUMMARY

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 anti-angiogenic 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 B₁, 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 pan-caspase inhibitor, BOC-D-FMK, 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 presents a molecular mechanism for its anti-angiogenic effects.
INTRODUCTION

Stress-stimuli such as irradiation, TNFα, 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 increased 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-HPR²) 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 by (11-13)). 4-HPR has been shown to possess anti-endothelial activity in tissue culture and the chick chorioallantoic membrane model (14,15). However, while Pienta et al demonstrated that 4-HPR was cytotoxic to CPAE bovine artery endothelial cells (14), Ribatti et al found that it inhibited proliferation, but was not cytotoxic to human adrenal gland capillary endothelial cells (15). 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 anti-endothelial 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 mediating the anti-endothelial 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). Lastly, 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 anti-angiogenic 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 addition of 20 mM HEPES buffer for the human BMEC. Human umbilical vein endothelial cells (HUVEC) (ATCC #CRL-1730, passage 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 (#56) and low passage (#16) human BMEC showed characteristic endothelial morphology comparable to 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-Dil-labeled acetylated low density lipoprotein (Dil-ac-LDL; 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 ICAM in response to TNFα (10 ng/ml) with or without IFNγ pretreatment, and in response to other stimuli, e.g. gp120 (0.5 µg/ml), bacterial exposure (E. coli E44), or lipopolysaccharide (50 ng/ml), as determined by ELISA (27). In addition, TNFα- and LPS-induced LDH release (Sigma kit) was also similar in both the high and low passage human BMEC (data not shown). Experiments were performed using both low passage (#12-25) and higher passage (#25-55) human BMEC, yielding similar results for cytotoxicity, apoptosis, and ceramide increase.
**Reagents.** 4-HPR was kindly provided by Dr. Sherry Ansher at the National Cancer Institute. Stock solution (10 mM) in ethanol was stored protected from light at –20°C. BOC-D-FMK, myriocin (ISP-1), and L-cycloserine were from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Z-VAD-FMK was from BioVision Inc. (Mountain View, CA). [9,10-3H-(N)]Palmitic acid (50 Ci/mmol) was from Dupont NEN® Research Products (Boston, MA). L-[3H(G)]Serine (20 Ci/mmol) and [5,6-3H]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). EN3HANCE® spray was from NEN Life Science Products (Boston, MA). Ecolume scintillation cocktail was from ICN Biomedicals, Inc. (Costa Mesa, CA). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

**Apoptosis assays.** Apoptosis was assessed by staining ethanol-fixed, RNAase-treated cells with propidium iodide (50 µg/ml in PBS containing 5 mM EDTA, 10 min on ice) and identifying cells with a sub-G0/G1 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 manufacturer’s instructions (Sigma Chemical Company). For analysis of apoptosis by Hoechst staining approximately 300-500 cells from a total of 5-10 fields in each sample were counted under UV filter at magnification 400 x.

**Endothelial viability and cytotoxicity.** Cell viability was assessed by uptake of MTT (Thiazolyl blue) (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 (3x10^6 cells/10-cm dish) were allowed to attach and spread in medium containing 10% heat inactivated FBS. Cells were radioactively labeled with [³H]palmitic acid (1 µCi/ml, 10 ml per 10-cm dish) and 4-HPR was added either simultaneously with [³H]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 pre-labeling with [³H]palmitic acid for 24 h, washing with PBS, 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 were added for the indicated time.

At the end of incubation adherent and detached cells were trypsinized, combined, and washed with PBS (4ºC). 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 N₂ and stored at –20ºC. Lipids were solubilized in chloroform/methanol (2:1, v/v) and analyzed by thin layer chromatography (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/dH₂O (50:30:7:4, v/v) for sphingomyelin (29), and chloroform/methanol/ammonium hydroxide (70:20:4, 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 percent cpm in these classes of lipids out of the total lipid tritium in the sample. For radiographs, the TLC plates were sprayed with EN³HANCE® according to manufacturer’s instructions and exposed to film (Hyperfilm, Amersham Biosciences, Piscataway, NJ) at –80ºC for 3-7 days.

High performance liquid chromatography (HPLC) for measurement of cellular 4-HPR content  4-HPR content in endothelial cell pellets were 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, 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 PBS), and scraped into 0.5 ml homogenization buffer (20 mM HEPES, pH 7.4, 5 mM DTT, 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 4 cycles, using a Micro Ultrasonic Cell Disrupter from Kontes (Vineland, NJ). Lysates were centrifuged at 10,000 x g for 10 min. The postnuclear supernatant was isolated and centrifuged at 100,000 x g for 60 min at 4ºC. The microsomal membrane pellet was resuspended in 100 µl homogenization buffer by sonication for 5 s and was frozen at –80ºC (33).

Serine Palmitoyltransferase Assay. Enzymatic activity was determined by measuring the incorporation of [3H]serine into 3-ketosphinganine (34,35). Each tube (0.1 ml final volume) contained 100 µg microsomal protein in 0.1 M HEPES pH 8.3, 2.5 mM EDTA, 50 µM pyridoxal phosphate, 5 mM DTT, and 1.0 mM L-serine. After preincubation at 37ºC for 10 min, the reaction was initiated by simultaneous addition of palmitoyl CoA (0.2 mM) and 1.0 µCi [3H]serine. Control samples contained either boiled microsomes or no protein. After incubation at 37ºC for 7 min the reaction was terminated by addition of 0.2 ml 0.5 N NH₄OH. Organic-soluble products were isolated by addition of 3 ml chloroform/methanol (2:1), 25 µg sphingosine carrier, and 2.0 ml 0.5 N NH₄OH. 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 [3H]sphinganine into [3H]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 MgCl₂, 0.5 mM DTT, prior to addition of microsomal protein (100 µg), to final reaction volume of 0.1 ml. Assays were initiated by simultaneous addition of palmitoyl CoA
(0.1 mM) and 0.5 μCi [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, California, USA) in consultation with a statistician. Values are given as mean ± standard error of the mean (SEM). 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-value<0.05 was considered significant.
RESULTS

4-HPR was cytotoxic to endothelial cells. As 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 MTT assay, with 50% cytotoxicity at 2.4 ± 0.2 µM (SEM) 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 (unpublished data3). Cytotoxicity was similar in low passage (#14-30) and high passage (#40-57) human BMEC, with 50% cytotoxicity at a mean 4-HPR of 2.2 ± 0.3 µM 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 to 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 IGF-1-mediated inhibition of anoikis (36). Therefore, we examined whether fetal bovine serum (FBS) could rescue endothelial cells 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 to 0.1% FBS when measured at 24 h (Fig. 1B-C and unpublished data3). Addition of BSA to the medium did not suppress the cytotoxic effect of 4-HPR (Fig. 1C) 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-G₀/G₁ DNA content indicative of apoptosis as revealed by flow cytometry (representative experiments shown in Fig. 2A-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. 2C). 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. 2D-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. 2F 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 over-confluence 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 C2-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 1). In the human BMEC mean ceramide increase was 5.25-fold ± 0.72 following 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. 3B-C). A decrease in several unidentified faster- and slower-migrating lipid bands was observed in parallel to the increase in [3H]ceramide (Fig 3C). While 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=ns with vs. 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 addition of 4-HPR, and when 10% FBS was included in the medium (Table 1). 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 B₁ (25 µM), an inhibitor of ceramide synthase, potently inhibited ceramide generation in endothelial cells exposed to 4-HPR (Fig. 4A). 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. 4B-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. 4C). Activity of SPT in absence of 4-HPR was 29.8 ± 0.2 pmol/mg protein/min, and increased by 1.75-fold after 2 h incubation with 10 µM 4-HPR (Fig. 4C), confirming stimulation of de novo ceramide synthesis by 4-HPR. By 6 h of incubation with 4-HPR, SPT activity returned to baseline levels (Fig. 4C). 4-HPR did not induce activation of ceramide synthase, an enzyme downstream of SPT, and at 10 µM even suppressed...
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Effects of 4-HPR on cell viability and apoptosis in human BMEC. To examine the effects of 4-HPR on cell viability and apoptosis, human BMEC were incubated with 4-\text{HPR} (20 

Effect of 4-HPR on sphingomyelin metabolism in human BMEC. To assess whether sphingomyelin hydrolysis contributed to ceramide increase we pre-labeled human BMEC with [\text{3H}]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 to controls following the 24 h incubation with the drug (Fig. 5A). Incubation with desipramine, a nonspecific inhibitor of sphingomyelin hydrolysis, starting 2 h before 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. 5A). Under these conditions there was also no change in levels of cellular glucosylceramide in the presence of 4-HPR (Fig. 5C). Interestingly, despite the removal of the remaining exogenous [\text{3H}]palmitic acid from the medium prior to addition of 4-HPR in these experiments, [\text{3H}]ceramide levels increased in presence of 4-HPR (Fig. 5B). The increase in ceramide was not inhibited by desipramine (Fig. 5B), further supporting 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-induced apoptosis (Fig. 6). Incubation of human BMEC with the ceramide synthase inhibitor, fumonisin B1, at a concentration that effectively suppressed de novo-generated ceramide increase (25 µM; Fig. 4A), completely prevented apoptosis induced by 4-

its activity by up to 25\% (data not shown). However, since SPT is the rate limiting enzyme for de novo ceramide synthesis, and the baseline activity of ceramide synthase (138 \pm 5 \text{ pmol/mg protein/min}) in the human BMEC was more than four times higher than that of SPT (29.8 \pm 0.2 \text{ pmol/mg 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.

\textit{Effect of 4-HPR on sphingomyelin metabolism in human BMEC.} To assess whether sphingomyelin hydrolysis contributed to ceramide increase we pre-labeled human BMEC with [\text{3H}]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 to controls following the 24 h incubation with the drug (Fig. 5A). Incubation with desipramine, a nonspecific inhibitor of sphingomyelin hydrolysis, starting 2 h before 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. 5A). Under these conditions there was also no change in levels of cellular glucosylceramide in the presence of 4-HPR (Fig. 5C). Interestingly, despite the removal of the remaining exogenous [\text{3H}]palmitic acid from the medium prior to addition of 4-HPR in these experiments, [\text{3H}]ceramide levels increased in presence of 4-HPR (Fig. 5B). The increase in ceramide was not inhibited by desipramine (Fig. 5B), further supporting that sphingomyelinase activation did not contribute to the 4-HPR-induced ceramide increase.

\textit{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-induced apoptosis (Fig. 6). Incubation of human BMEC with the ceramide synthase inhibitor, fumonisin B1, at a concentration that effectively suppressed de novo-generated ceramide increase (25 µM; Fig. 4A), completely prevented apoptosis induced by 4-
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HPR (Fig. 6A-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 pan-caspase inhibitor, BOC-D-FMK, and exposed to 4-HPR, apoptosis was inhibited to baseline levels compared to control cells (Fig 7A-C). Z-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. 7A-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. 7D). While BOC-D-FMK effectively inhibited 4-HPR-induced apoptosis (Fig. 7A-C), it had no effect on the 4-HPR-induced ceramide increase in human BMEC (Fig. 7D). 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 TNFα 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, non-sphingomyelinase-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), while 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, that is thought to be biologically different than endothelium 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 pan-caspase 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 [³H]ceramide in human BMEC by inhibitors of de novo ceramide synthesis (Fig. 4A-C). Additionally, 4-HPR activated SPT, the first enzyme in this pathway (Fig. 4D), as was found in neuroblastoma cells (35). In neuroblastoma, a downstream enzyme, ceramide synthase, was also activated by 4-HPR (35), while in the human BMEC it was not. Lack of stimulation of ceramide synthase, or even its mild inhibition, are not expected to prevent an increase in de novo-generated ceramide by 4-HPR, since in the human BMEC ceramide synthase activity was about four times higher than that of SPT at baseline (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 three times higher than SPT at baseline (34). This supports a role for increased SPT activity in 4-HPR-mediated upregulation of ceramide in endothelial cells. In the human BMEC the activation of SPT was early and transient, while 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 pre-labeled with \[^{3}H\]palmitic acid (Fig 5B), conditions where \textit{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 \textit{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 (Fig. 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 \textit{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 \(\mu\)M) were similar or lower than those used in some tumor cell lines (25-100 \(\mu\)M) (39,43). It is possible that other pro-apoptotic 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, that itself may be part of the signaling cascade to ceramide-mediated apoptosis (16,17).

Taken together, our study establishes a causal role for \textit{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.
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FOOTNOTES

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2Abbreviations used: 4-HPR, fenretinide; bovine TBMEC, bovine large-T brain microvascular endothelial cells; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; human BMEC, human brain microvascular endothelial cells; HPLC, high performance liquid chromatography; HUVEC, human umbilical vein endothelial cells; ns, non significant; SM, sphingomyelin; SPT, serine palmitoyltransferase.

3Erdreich-Epstein, A., unpublished data.
FIGURE LEGENDS

Figure 1: Fenretinide was cytotoxic to human BMEC.
Human BMEC (6x10^3 cells per well in 96-well plate) were incubated with 4-HPR for 24 h. Cell viability was assessed using MTT assay.

A. Cells were incubated in medium containing 0.1% FBS. Data points are means ± SEM 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 ± SEM of 3-6 experiments performed in 5-10 replicates. p<0.001 by two-tailed paired t-test comparing mean survivals in 10% and in 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 (5µM) in the presence or absence of 1% heat inactivated fatty-acid-free BSA. Bars represent means ± SEM of 5 replicate samples. *p=0.0023 for cells in 2% compared to 0.1% FBS and **p=0.013 for cells in 10% compared to 2% FBS, by unpaired t-tests. p=ns in presence compared to absence of BSA at each of the FBS concentrations, by unpaired t-tests.

Figure 2: Fenretinide induced apoptosis in human BMEC.

A-E. Apoptosis (cells with a sub-G0/G1 DNA content) was assessed by flow cytometry of permeabilized propidium-iodide stained human BMEC (10^6 cells per 10 cm dish) that were incubated with 4-HPR as indicated below:

A. Cells were incubated 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 mean ± SEM of a representative experiment from over ten 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 in (A).

C-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 ± SEM 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 ± SEM of three independent experiments.

F. Human BMEC (3 x 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 are fold-change of 4-HPR content per gram cells (mean ± SEM) from three independent experiments. \( p=0.017 \) comparing 1% and 10% FBS, by unpaired t-tests.

**Figure 3: 4-HPR increased endothelial ceramide in a dose and time dependent manner.**

**A.** 4-HPR and \(^{[3]}\text{H}\)-palmitic acid were added simultaneously to human BMEC (3x10\(^6\) cells per 10 cm dish) in medium containing 0.1% FBS. After 16 h cells were harvested, lipids were extracted and ceramide was determined by thin layer chromatography (TLC). Results are presented as cpm in \(^{[3]}\text{H}\)ceramide, as a percent of the total lipid tritium. Shown are means ± SEM from a representative experiment of multiple repeats performed in triplicates. \( p<0.001 \) by one-way ANOVA for 0, 3, and 5 \( \mu M \) 4-HPR.

**B.** Human BMEC (3x10\(^6\) cells per 10 cm dish) were incubated in medium containing \(^{[3]}\text{H}\)-palmitic acid and 0.1% FBS. Vehicle control (○) or 4-HPR (●; 5 \( \mu M \)) were added either at the time of addition of \(^{[3]}\text{H}\)-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 addition of \(^{[3]}\text{H}\)-palmitic acid. X-axis denotes length of incubation with 4-HPR or control. Lipids were processed as described. Shown are means ± SEM from a representative experiment of three performed in triplicates. \( p=0.0060 \) in cells with 5 \( \mu M \) 4-HPR at 4-24 h, by simple linear regression.

**C.** Human BMEC were labeled with \(^{[3]}\text{H}\)-palmitic acid and treated with 4-HPR (5 \( \mu M \)) as in (B). Lipids were extracted, equal amounts (cpm) of total labeled lipids were separated on TLC, and radiographs were developed. Time indicated is length of incubation with 4-HPR.

**Figure 4: 4-HPR stimulated activity of SPT and increased \textit{de novo} synthesis of ceramide in human BMEC.**

**A-C.** Human BMEC (3x10\(^6\) cells per 10 cm dish) were incubated in medium containing 0.1% FBS with inhibitor (A - Fumonisin B\(_1\), 25 \( \mu M \); B - L-cycloserine, 30 \( \mu M \); C – Myriocin, 0.05 \( \mu M \); ■) or vehicle control (DMSO; □) beginning 2 h before addition of \(^{[3]}\text{H}\)palmitic acid and 4-HPR (0, 3 or 5 \( \mu M \)). After overnight incubation with 4-HPR cells were harvested, lipids were extracted, and ceramide was determined by TLC. Results are presented as cpm in \(^{[3]}\text{H}\)ceramide, as a percent of the total lipid tritium. \( p<0.001 \) in cells exposed to 4-HPR with inhibitors (■) compared to without them (□), by unpaired t-tests for each inhibitor. Shown are means ± SEM 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 in Experimental Procedures. Results are expressed as fold change in SPT activity (mean ± SEM). SPT activity in absence of 4-HPR was 29.8 pmol/mg protein/min. Shown is one of two similar experiments, performed in duplicates. p=0.022 by one way ANOVA.

Figure 5: 4-Effect of HPR on sphingomyelin levels in endothelial cells.
A-C. Human BMEC (3x10^6 per 10 cm dish) were preloaded with [3H]palmitic acid for 24 h and the isotope was washed-out for 2 h. Desipramine (5 µM; horizontal-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 extracted, and sphingomyelin (A; SM), ceramide (B) and glucosylceramide (C) determined by TLC using the solvent systems described in Experimental Procedures. Results are presented as cpm in either [3H]ceramide, [3H]sphingomyelin, or [3H]glucosylceramide, as a percent of the total lipid tritium. For sphingomyelin (A) there was no effect of 4-HPR or desipramine (p=ns, 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 are means ± SEM of one of ten experiments with similar results, and comparison with desipramine represents one of two experiments, all performed in triplicate.

Figure 6: Prevention of 4-HPR-induced generation of ceramide suppressed endothelial cell apoptosis.
Fumonisin B₁ or vehicle control were added to human BMEC (10^6 cells per 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, ▼ - UV (25 mj), in presence of 0-25 µM FB₁. Data points represent mean percent apoptosis of duplicate samples. Error bars (SEM) are smaller than the symbols. p<0.0001 for cells with 4-HPR and 0-25 µM fumonisin B₁, by one way ANOVA.
B. Filled bars – fumonisin B₁ (25 µM), horizontal-hatched bars - DMSO, in 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.
Figure 7: 4-HPR-induced ceramide generation was independent of caspases in endothelial cells.

Human BMEC (A: 10^5 cells per well in 2-well chamber slide; B-C: 10^6 cells per 10 cm dish, D: 3x10^6 cells per 10 cm dish) were pre-incubated in medium containing 0.1% FBS with 25 µM BOC-D-FMK (■) or DMSO (□) for 2 h. 4-HPR or vehicle control were then added for 24 h (A-C) or 16 h (D). Shown are means ± SEM 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 ± SEM; *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 extracted, and ceramide quantitated by TLC. Results are presented as cpm in [3H]ceramide, as a percent 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.
## TABLES

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<th>4-HPR:</th>
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<th>24 h</th>
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<td>None</td>
<td>0.4 ± 0.08</td>
<td>0.9 ± 0.06</td>
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<td>5 µM</td>
<td>2.3 ± 0.04</td>
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### Human BMEC
- **bovine TBMEC**: 1.0 ± 0.02
- **HUV-EC-C**: 0.89 ± 0.08

**p**-values were derived by unpaired t-tests, n=3.

### Table 1. Ceramide increase by 4-HPR occurred in three different endothelial preparations and in presence 10% FBS.** Cells were incubated overnight in presence of 10% FBS and [3H]palmitic acid. 4-HPR was then added for the time indicated. Lipids were processed and [3H]ceramide measured as described in Experimental Procedures. p-values were derived by unpaired t-tests, n=3.
FIGURES

FIGURE 1
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FIGURE 2
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**FIGURE 3**
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![Graph A](image)

![Graph B](image)

**FIGURE 4**
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![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)
FIGURE 7
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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

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