Involvement of Dihydroceramide Desaturase in Cell Cycle Progression in Human Neuroblastoma Cells*

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The role of dihydroceramide desaturase as a key enzyme in the de novo pathway of ceramide generation was investigated in human neuroblastoma cells (SMS-KCNR). A novel assay using water-soluble analogs of dihydroceramide, dihydroceramidoids (N-erythro-dhCCPS analogs), was used to measure desaturase activity in situ. Conversion of N-erythro-2-N-[12’-(1’-pyridinium)dodecanoyl]-4,5-dihydrosphingosine bromide (C12-dhCCPS) to its 4,5-desaturated counterpart, N-erythro-2-N-[12’-(1’-pyridinium)dodecanoyl]sphingosine bromide (C12-CCPS), was determined by liquid chromatography/mass spectrometry analysis. The validity of the assay was confirmed using C8-cyclopropenylceramide, a competitive inhibitor of dihydroceramide desaturase. A human homolog (DEGS-1) of the Drosophila melanogaster des-1 gene was recently identified and reported to have desaturase activity. Transfection of SMS-KCNR cells with small interfering RNA to DEGS-1 significantly blocked the conversion of C12-dhCCPS to C12-CCPS. The associated accumulation of endogenous dihydroceramides confirmed DEGS-1 as the main active dihydroceramide desaturase in these cells. The partial loss of DEGS-1 inhibited cell growth, with cell cycle arrest at G0/G1. This was accompanied by a significant decrease in the amount of phosphorylated retinoblastoma protein. Hypophosphorylation was inhibited by tautomycin and not by okadaic acid, suggesting the involvement of protein phosphatase 1. Additionally, we found that treatment of SMS-KCNR cells with fenretinide inhibited desaturase activity in a dose-dependent manner. An increase in dihydroceramides (but not ceramides) paralleled this process as measured by liquid chromatography/mass spectrometry. There were no effects on the mRNA or protein levels of DEGS-1, suggesting that fenretinide acts at the post-translational level as an inhibitor of this enzyme. Tautomycin was also able to block the hypophosphorylation of the retinoblastoma protein observed upon fenretinide treatment. These findings suggest a novel biological function for dihydroceramides.

In addition to their roles as structural components of cell membranes, sphingolipids play important roles as regulators of signal transduction in cell differentiation, proliferation, and apoptosis. One of the most studied sphingolipids is ceramide (Cer) (1–5). Cer is the central building block for sphingolipids. It serves as a precursor for the synthesis of more complex sphingolipids and is generated in cells by multiple pathways. Cer can be produced de novo from serine and palmitoyl-CoA via dihydroceramide (dhCer), followed by its desaturation to Cer by dihydroceramide desaturase. Although many of the enzymes of the de novo pathway and Cer have been extensively investigated, the enzyme dihydroceramide desaturase and its substrate dhCer have not been well characterized.

Dihydroceramide desaturase is responsible for inserting the 4,5-trans-double bond into the sphingoid backbone of dhCer. The enzyme was previously characterized, and an in vitro assay was developed to determine its activity (6–9). A crude enzyme preparation was isolated from rat liver microsomes. In an independent study, a family of sphingolipid Δ4-desaturases (homologs of Drosophila melanogaster des-1 (degenerative spermatocyte gene-1) was identified via a bioinformatics approach (10). These proteins contain three His-containing consensus motifs that are characteristic of a group of membrane fatty acid desaturases. The human homolog of des-1 is now referred to as DEGS-1 (DESI) and was first cloned in 1997 and named membrane lipid desaturase (11). It was identified using a yeast two-hybrid screen. It was reported that overexpression of membrane lipid desaturase inhibits biosynthesis of the epidermal growth factor receptor in Epstein-Barr virus nuclear antigen-expressing 293 human embryonic kidney cells.

Dihydroceramides are believed to be biologically inactive molecules. Treatment of cells with exogenous short chain dhCers has failed to inhibit cell growth and to induce apoptosis (12–14). In this study on dihydroceramide desaturase in SMS-KCNR cells, we used a novel in situ assay to measure the activity of this enzyme using cell-permeable dihydroceramide (dhCCPS analogs) (15). dhCCPS analogs represent

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‡The abbreviations used are: Cer, ceramide; dhCer, dihydroceramide; dhCCPS, dihydroceramidoids(s); siRNA, small interfering RNA; Rb, retinoblastoma protein; 4-HPR, N’(4-hydroxyphenyl)retinamide (fenretinide); C12-CCPS, N-erythro-2-N-[12’-(1’-pyridinium)dodecanoyl]sphingosine bromide (N-erythro-C12-merceramide); C12-dhCCPS, N-erythro-2-N-[12’-(1’-pyridinium)dodecanoyl]4,5-dihydrosphingosine bromide (N-erythro-C12-dihydroceramide); C4, N-erythro-4,5-dimethylthiazol(2-yl)-2,5-diphenyltetrazolium bromide; C8-CPPC, C8-cyclopropenylceramide; LC/MS, liquid chromatography/mass spectrometry; CCPS, ceramidoid(s); RT, reverse transcription; pRb, phosphorylated retinoblastoma protein; PP, protein phosphatase.
a novel class of water-soluble long chain dhCers that can be delivered to cells in culture.

The obtained data demonstrate that DEGS-1 is the main dihydroceramide desaturase active in SMS-KCN KR human neuroblastoma cells and that inhibition of DEGS-1 with small interfering RNA (siRNA) leads to the accumulation of endogenous dhCers with subsequent effects on cell growth, with cell cycle arrest and hypophosphorylation of the retinoblastoma protein (Rb). Activation of ceramide-activated protein phosphatases appears to be involved in this process. Furthermore, we have confirmed that dihydroceramide desaturase is a target for the synthetic retinoid fenretinide (N-(4-hydroxyphenyl)-retinamide (4-HPR)).

**EXPERIMENTAL PROCEDURES**

Reagents—d-erythro-2-N-[12'-(-1”-Pyridinium)dodecanoyl]-sphingosine bromide (d-erythro-C12-,CCPS) and d-erythro-2-N-[12’-(1”-pyridinium)dodecanoyl]-4,5-dihydroceramide bromide (d-erythro-C12-,dhCCPS) were synthesized in the Lipidomics Core Facility of the Medical University of South Carolina (15). d-erythro-2-N-[12’-(-1”-pyridinium)dodecanoyl]-4,5-dihydroceramide bromide (d-erythro-C12-,dhCCPS) were synthesized in the Lipidomics Core Facility of the Medical University of South Carolina (15). 4-HPR, all-trans-retinoic acid, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma. C8-Cyclopropenylceramide (C8-CPPC) was obtained from Matreya, LLC (Pleasant Gap, PA). Okadaic acid and tautomycin were obtained from BIOMOL International, L.P. (Plymouth Meeting, PA).

Cell Lines and Culture Conditions—The SMS-KCN cell line was obtained from Dr. C. Pat Reynolds (Children’s Hospital of Los Angeles) The MCF-7 cell line was obtained from Dr. Chiara Luberto (Medical University of South Carolina). Cells were maintained in growth medium (RPMI 1640) containing 10% fetal calf serum (Invitrogen) at 37 °C in 5% CO2. C8-CPPC was maintained in growth medium (RPMI 1640) containing 10% fetal calf serum (Invitrogen). The MCF-7 cell line was obtained from Dr. Chiara Luberto (Medical University of South Carolina). Anti-DEGS-1 antibody was obtained from Pharmingen. Secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Anti-Rb antibody was obtained from Pharmingen. Secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA).

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**LC/MS Analysis of Endogenous Ceramides and the Cellular Level of Ceramidoid (CCPS) and dhCCPS Analogs**—Advanced analyses were performed in the Lipidomics Core Facility of the Medical University of South Carolina (hcc.musc.edu/research/shared_resources/lipidomics.cfm) on a Thermo Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer operating in a multiple reaction monitoring positive ionization mode as described (15, 16).

**RNA Interference Transfection**—Transient transfections were performed using HiPerFect (Qiagen Inc., Valencia, CA) following the manufacturer’s recommendations. siRNA to human DEGS-1 (GenBank™ accession numbers NM_144780 and NM_003676) and a nonspecific siRNA (Silencer® negative control) were purchased from Ambion, Inc. (Austin, TX). Cells were treated with siRNA at least 24–48 h prior to the addition of dhCCPS analogs. Knockdown of target gene expression was confirmed by reverse transcription (RT)-PCR and Western blotting.

**RNA Isolation and RT-PCR**—One µg of total RNA (isolated using an RNA isolation kit from Qiagen Inc.) was used in reverse transcription reactions with avian myeloblastosis virus reverse transcriptase and random primers (Promega Corp., Madison, WI) as recommended by the manufacturer. The resulting total cDNA was then used in PCR to measure the mRNA levels of DEGS-1. The mRNA levels of 28 S rRNA were used as internal controls. Linear amplification cycles were determined separately for each gene. The following primers were used for PCR amplification: DEGS-1, 5’-TTCTTTCTGT-AACGCTTTCCAG-3’ (forward) and 5’-TTACTCCAGACC-ATCTCT-3’ (reverse); and rRNA, 5’-TTACAAAGTGGC-CCACTA-3’ (forward) and 5’-GAAGATGGTGACTAT-GCC-3’ (reverse).

The reactions were performed at 95 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min for 30 cycles. The concentrations of RNA primers were used at a 1:6 ratio compared with other primers to achieve linear amplification conditions in the same reactions. The RT-PCR products were quantitated by densitometry using Quantity One one-dimensional analysis software (Bio-Rad). The mRNA levels of DEGS-1 were normalized to the mRNA levels used as internal controls. The amplified fragments were separated on 2% agarose gels and visualized by ethidium bromide staining.

**Immunoblotting**—The protein levels of DEGS-1, phosphorylated Rb (pRb), Rb, and β-actin were determined by Western blot analysis. In brief, total proteins (30 µg/lane) were separated by 10% SDS-PAGE, blotted onto Immobilon™ membranes (Millipore Corp., Billerica, MA), and detected using anti-DEGS-1 (1:2000), anti-pRb (1:1000), anti-Rb (1:1000), or anti-β-actin (1:1000) primary antibody for 1 h, followed by peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:10,000) for 1 h. The proteins were visualized by chemiluminescence using the ECL™ protein detection kit (GE Healthcare) as described by the manufacturer. Anti-DEGS-1 antibody (MLD 3906) was kindly provided by Dr. Gordon N. Gill (University of California, San Diego). Anti-β-actin antibody was purchased from Sigma. Anti-pRb (Ser789) was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Anti-Rb antibody was obtained from Pharmingen. Secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA).
were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Protein levels were quantitated by densitometry using Quantity One one-dimensional analysis software.

Cell Growth Assays—The effects of DEGS-1 siRNA cell growth were detected using a trypan blue exclusion method as described previously (17). SMS-KCNR cells were transfected with 10 nM siRNA to DEGS-1 or nonspecific siRNA. These cells, along with untransfected cells, were replated 24 h after transfection in 6-well plates at a density of 5000 cells/well. At 2, 4, and 6 days after plating, the cells were trypsinized and diluted in phosphate-buffered saline. The floating (dead) cells in the medium and the cells that remained attached to the plates were then counted using a hematocytometer in the presence of trypan blue solution (Sigma) at a 1:1 (v/v) ratio as recommended by the manufacturer. Triplicate wells were used for each treatment.

The effects of increasing concentrations of 4-HPR on cell growth were detected using a trypan blue exclusion method as described previously (17). SMS-KCNR and MCF-7 cells were treated with 0.5 μM C12-dhCCPS for 15 min and 2, 6, and 24 h. Cells were harvested at these time points, and pyridinium-conjugated ceramidoids were measured by LC/MS as described under "Experimental Procedures." The percentages of the total detected pyridinium-conjugated ceramidoids that were converted to C12-dhCCPS are depicted here. The data are representative of the means ± S.D. of at least three independent experiments. The error bars represent S.D., and where not seen, they are smaller than the thickness of the lines on the graphs.

RESULTS

Conversion of 4,5-Dihydroceramides to the Corresponding Ceramides—Positively charged cell-permeable analogs of n-erythro-C12-ceramide (C12-CCPS) and n-erythro-C12-dihydroceramide (C12-dhCCPS) were used as molecular probes to study dihydroceramide desaturase activity by monitoring conversion of C12-dhCCPS to C12-CCPS in intact cells by an LC/MS approach (16). This conversion is depicted in Fig. 1A. SMS-KCNR human neuroblastoma cells and MCF-7 human breast carcinoma cells were incubated with 0.5 μM C12-dhCCPS for 15 min and 2, 6, and 24 h (Fig. 1B). The cells were collected at these time points, and the levels of C12-dhCCPS and C12-CCPS were measured by LC/MS as described under "Experimental Procedures." The percentage of the conversion is shown in Fig. 1B. Desaturation to C12-CCPS was detected as early as 15 min (8 and 11% in SMS-KCNR and MCF-7 cells, respectively), and nearly total conversion was achieved by 24 h, with >80% conversion at 6 h. This assay was also performed with other human cancer cell lines, including lung adenocarcinoma (A549), squamous cell carcinoma (UM-SCC-1), and other neuroblastomas (SK-N-SH, IMR-32, and SK-N-RA), and similar results were obtained (data not shown). These experiments demonstrated that the desaturase is an active enzyme in human neuroblastoma and MCF-7 human breast carcinoma cells.
these cells, proving this approach to be a powerful method to study the activity of this enzyme in intact cells.

Because there was a >80% conversion of C₁₂-dhCCPS to C₁₂-CCPS at 6 h, this time point was selected to study potential inhibitors and activators of the desaturase enzyme. Prior to testing the action of various compounds on enzyme activity, the cytotoxicity of C₁₂-dhCCPS was examined by MTT assay. There was no significant effect on cell viability of increasing concentrations up to 10 μM a in SMS-KCNR and MCF-7 cells (data not shown). Measurement of endogenous ceramide and dihydroceramide levels was also performed, and no changes in these sphingolipid levels were observed at 6 h (data not shown).

**Effects of the Desaturase Inhibitor C₈-CPPC on Conversion of C₁₂-dhCCPS to C₁₂-CCPS**

At low concentrations, C₈-CPPC inhibits dihydroceramide desaturase, whereas at high concentrations, it also inhibits sphingosine-1-phosphate lyase and serine palmitoyltransferase (21). SMS-KCNR cells were pretreated with increasing concentrations (0, 0.01, 0.1, 0.5, 1, and 2.5 μM) of C₈-CPPC for 30 min, and 0.5 μM C₁₂-dhCCPS was added for 6 h (Fig. 2A). Cells were collected after 6 h, and the conversion to C₁₂-CCPS was measured by LC/MS as described under "Experimental Procedures." The total endogenous levels of Cers and dhCers (B); dihydrophosphoglycerine (dhSph), dihydrophosphoglycerine 1-phosphate (dhSph-1P), sphingosine (Sph), and sphingosine 1-phosphate (Sph-1P) (C); Cer species (D); and dhCer species (E) were measured by LC/MS as described under "Experimental Procedures." The sphingolipid levels were normalized to total lipid phosphate. The data are representative of the means ± S.D. of two independent experiments performed in duplicate. The error bars represent S.D., and where not seen, they are smaller than the thickness of the lines on the graphs.

**FIGURE 2. Effects of C₈-CPPC on desaturase activity.** SMS-KCNR cells were pretreated with increasing concentrations of the dihydroceramide desaturase inhibitor C₈-CPPC for 30 min, and 0.5 μM C₁₂-dhCCPS was then added for 6 h (A). Cells were collected after 6 h, and the conversion to C₁₂-CCPS was measured by LC/MS as described under "Experimental Procedures." The total endogenous levels of Cers and dhCers (B); dihydrophosphoglycerine (dhSph), dihydrophosphoglycerine 1-phosphate (dhSph-1P), sphingosine (Sph), and sphingosine 1-phosphate (Sph-1P) (C); Cer species (D); and dhCer species (E) were measured by LC/MS as described under "Experimental Procedures." The sphingolipid levels were normalized to total lipid phosphate. The data are representative of the means ± S.D. of two independent experiments performed in duplicate. The error bars represent S.D., and where not seen, they are smaller than the thickness of the lines on the graphs.
were elevated; the largest increases occurred for those species that were predominant in this cell line, mainly C16, C24, C24:1, C26, and C26:1 (Fig. 2E). These results verify that C8-CPPC is a potent and total (not showing specificity for dhCer species) inhibitor of dihydroceramide desaturase and that inhibition of this enzyme with C8-CPPC leads to the accumulation of endogenous dhCers.

Role of DEGS-1 in Mediating the Conversion of C12-dhCCPS to C12-CCPS—Although the experiments above revealed the action of a desaturase, it became important to determine whether this was mediated specifically by the primary dihydroceramide desaturase, DEGS-1. Therefore, siRNA against the human desaturase enzyme DEGS-1 was used to attenuate its expression and consequently inhibit its activity. SMS-KCNR cells were transfected with 10 nm DEGS-1 siRNA or nonspecific siRNA (SCR) and collected after 48 h. Control refers to untreated cells. Knockdown of DEGS-1 mRNA levels was confirmed by semiquantitative RT-PCR (left panel). The mRNA levels of 28 S rRNA were used as internal controls. Expression of the DEGS-1 protein was also detected by Western blotting (right panel). Total cell lysates were prepared 48 h after siRNA transfection. Equal amounts of proteins (30 µg) were run on 10% SDS-polyacrylamide gel and blotted onto an Immobilon membrane as described under “Experimental Procedures.” β-Actin was probed to verify equal loading of proteins per lane. The results are representative of at least three independent experiments. β-Actin was probed to verify equal loading of proteins per lane. The results are representative of at least three independent experiments. The error bars represent S.D., and where not seen, they are smaller than the thickness of the lines on the graphs.
Next, the effects of the loss of DEGS-1 on the conversion of dihydroceramide were evaluated (Fig. 3B). SMS-KCNR cells were transfected with siRNA (DEGS-1 or nonspecific) for 48 h and then, along with untransfected (control) cells, incubated with 0.5 μM C12-dhCCPS for 6 h. In the untreated (control) and nonspecific siRNA-transfected cells, 70% of the substrate C12-dhCCPS was converted to C12-CCPS. However, when expression of the enzyme was inhibited with siRNA, the conversion was significantly reduced, with only 4% conversion observed. Similar results were also obtained with MCF-7 cells (data not shown). These results demonstrate that DEGS-1 is the major dihydroceramide desaturase in these cells.

We next examined what effects inhibition of DEGS-1 would exert on endogenous sphingolipids (Fig. 3, C–E). For these experiments, cells were treated with 10 nM DEGS-1 siRNA or nonspecific siRNA for 48 h, and key sphingolipids were quantitated by LC/MS. As expected, there was a 13-fold increase in endogenous dhCers when the enzyme was inhibited, whereas the levels of endogenous Cer were decreased by ~25% and 20% compared with untreated and nonspecific siRNA-transfected cells (Fig. 3, C and D). As seen with C18-CPPC, all dhCer species were elevated, with the largest increases occurring in the ceramide species predominant in these cell lines (C16, C24, C24:1, C26, and C26:1) (Fig. 3E). There were no significant changes seen in endogenous sphingosine, dihydrosphingosine, or sphingosine 1-phosphate levels (data not shown).

A time course study using siRNA against DEGS-1 (Fig. 4A) showed that the DEGS-1 protein was reduced by ~50% by as early as 24 h (data not shown) and persisted even 6 days after siRNA transfection. LC/MS measurement of endogenous Cers and dhCers (Fig. 4, B–D) was performed 2, 4, and 6 days after siRNA transfection and demonstrated the persistent elevation of endogenous dhCers after silencing of the DEGS-1 protein. Endogenous dhCers were elevated by ~18-fold, ~22-fold, and ~8-fold compared those in untreated cells on days 2, 4, and 6 after siRNA transfection, respectively. Taken together, these results demonstrate that DEGS-1 is the major dihydroceramide desaturase active in these cells and that inhibition of this enzyme with siRNA leads to the accumulation of endogenous dhCers.

Effects of Loss of DEGS-1 on Cell Growth—In most cell studies, exogenous dhCers behave as “biological inactive compounds” compared with exogenous ceramides (12–14). To evaluate the biological role of the loss of DEGS-1 (which results in elevation of endogenous dhCers), we examined its effects on cell growth using a trypsin blue exclusion method. SMS-KCNR
cells were transfected with 10 nM DEGS-1 siRNA or non-targeting siRNA (nonspecific). As shown in Fig. 5A, treatment of these cells with DEGS-1 siRNA resulted in a significant inhibition of cell growth (~65%) at days 4 and 6.

We next determined the cell cycle profile of these cells after reduction of DEGS-1 expression at 48 h (Fig. 5B). Loss of DEGS-1 resulted in cell cycle arrest at G0/G1. In DEGS-1 siRNA-treated cells, 72% of the cell population was in G0/G1 phase and 19% in the S phase, whereas in nonspecific siRNA-treated and untreated cells, 34 and 36% of the cell population was in the G1 phase and 44 and 54% in the S phase, respectively.

Because loss of DEGS-1 resulted in G1 arrest, the phosphorylation status of Rb was examined next (Fig. 5C). pRb is critical for cell cycle progression; it regulates the G1/S phase restriction point, thereby controlling entry into the S phase (22). Western blotting was performed, and a >50% decrease in the amount of pRb was seen in cells treated with siRNA to DEGS-1. There was no change in the level of total Rb (Fig. 5C, left panels).
Ceramide has been shown to induce the dephosphorylation of Rb (23–26) and to activate the serine/threonine protein phosphatase protein phosphatase (PP) 1 and PP2A (27, 28). PP1 in particular has been associated with ceramide-mediated Rb dephosphorylation (29). To determine whether a ceramide-activated protein phosphatase is involved in this process, we pretreated cells with either OA (Fig. 5C, center panels), a specific PP2A inhibitor, or tautomycin (right panels), a specific PP1 inhibitor, prior to siRNA transfection. As shown in Fig. 5C, tautomycin was able to inhibit the hypophosphorylation of Rb. Okadaic acid had minimal effects. These data suggest that DEGS-1 activity may be required for normal progression through the cell cycle, and that like ceramide, endogenous dihydroceramides may activate serine/threonine protein phosphatases, specifically PP1.

**Effects of Fenretinide on Desaturase Activity**—4-HPR is a synthetic analog of all-trans-retinoic acid. This compound has been reported to increase Cer levels via de novo synthesis within 6 h of treatment (30). We therefore initially aimed at testing whether the inhibition of DEGS-1 by siRNA would block 4-HPR-induced Cer generation and the anti-tumor effects of 4-HPR. First, the levels of endogenous Cer in response to increasing concentrations of 4-HPR (0.5, 1, 2.5, 5, and 10 μM) for 6 h were measured (Fig. 6, A–C). Interestingly, we found that diC12-dhCCPS was added at the same time as 4-HPR or all-trans-retinoic acid. Cells were collected at these time points, and the conversion to C12-dhCCPS was determined by LC/MS. F, the mRNA levels of 28 S rRNA and DEGS-1 in response to 10 μM 4-HPR treatment for 6 and 24 h were measured by semiquantitative RT-PCR. Upper panel, expression of the DEGS-1 protein was detected by Western blotting. Lower panel, total cell lysates were prepared 6 and 24 h after 4-HPR treatment and run on 10% SDS-polyacrylamide gel. Equal amounts of proteins (30 μg) were blotted onto an Immobilon membrane as described under “Experimental Procedures.” β-Actin was probed to verify equal loading of proteins per lane. The results are representative of at least two independent experiments. The error bars represent S.D., and where not seen, they are smaller than the thickness of the lines on the graphs.

**FIGURE 6. Effects of 4-HPR on endogenous sphingolipids and desaturase activity.** A–C, SMS-KCNR cells were treated with increasing concentrations of 4-HPR for 6 h. The total endogenous levels of Cers and dhCers (A), Cer species (B), and dhCer species (C) were measured by LC/MS as described under “Experimental Procedures.” The sphingolipid levels were normalized to total lipid phosphate. The data are representative of the means ± S.D. of three independent experiments. D and E, desaturase activity was measured using our in situ assay. Cells were treated with increasing concentrations of 4-HPR for 2 and 6 h (D) or with 10 μM all-trans-retinoic acid (ATRA) for 6 h (E). C12-dhCCPS was added at the same time as 4-HPR or all-trans-retinoic acid. Cells were collected at these time points, and the conversion to C12-dhCCPS was determined by LC/MS. F, the mRNA levels of 28 S rRNA and DEGS-1 in response to 10 μM 4-HPR treatment for 6 and 24 h were measured by semiquantitative RT-PCR. Upper panel, expression of the DEGS-1 protein was detected by Western blotting. Lower panel, total cell lysates were prepared 6 and 24 h after 4-HPR treatment and run on 10% SDS-polyacrylamide gel. Equal amounts of proteins (30 μg) were blotted onto an Immobilon membrane as described under “Experimental Procedures.” β-Actin was probed to verify equal loading of proteins per lane. The results are representative of at least two independent experiments. The error bars represent S.D., and where not seen, they are smaller than the thickness of the lines on the graphs.
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and 5 μM 4-HPR. An ~15% increase in Cer levels was observed with 0.5 μM 4-HPR. There were no significant changes seen in endogenous sphingosine, dihydrophingosine, or sphingosine 1-phosphate levels (data not shown).

While this study was in progress, Schulz et al. (31) also reported accumulation of dhCers in response to 4-HPR treatments. These findings raised the possibility that 4-HPR results in inhibition of the desaturase. Given these findings, we then tested the ability of 4-HPR to inhibit desaturase activity using the in situ assay for C12-dhCCPS (Fig. 6D). SMS-KCNR cells were treated with increasing concentrations of 4-HPR (0.5, 1, 2.5, 5, and 10 μM) for 2 and 6 h. The substrate C12-dhCCPS (0.5 μM) was added at the same time as 4-HPR, and the conversion to C12-CCPS was measured by LC/MS. 4-HPR inhibited desaturase activity in a dose-dependent manner. Inhibition was observed even at the lowest dose at both 2 and 6 h. In untreated (control) cells, ~36% of the measured pyridinium-conjugated Cers were converted to C12-CCPS at 2 h. The conversion was decreased to ~7, 4, ~2, <1, and <1% in cells treated with 0.5, 1, 2.5, 5, and 10 μM 4-HPR, respectively. At 6 h, there was an ~83% conversion to C12-CCPS in untreated (control) cells. This conversion was decreased to ~42, ~21, ~6, ~2, and ~1% in cells treated with 0.5, 1, 2.5, 5, and 10 μM 4-HPR, respectively.

We reported previously that treatment of neuroblastoma cells with all-trans-retinoic acid results in generation of long chain ceramides (C24:0 and C24:1) (17). Therefore, the ability of other retinoids such as all-trans-retinoic acid to inhibit desaturase activity was tested with the in situ assay. SMS-KCNR cells were treated with 10 μM all-trans-retinoic acid for 6 h; no inhibition of desaturase activity was detected (Fig. 6E). These results demonstrate the specificity of inhibition of the desaturase by 4-HPR.

To determine the mechanism of action of 4-HPR on desaturase activity, semiquantitative RT-PCR and Western blotting were performed to determine whether the inhibition of the desaturase by 4-HPR was due to decreased transcription or translation of DEGS-1 (Fig. 6F). There were no changes seen in DEGS-1 mRNA (Fig. 6F, upper panel) or protein (lower panel) levels. This suggests that 4-HPR may be a direct and/or post-translational inhibitor of this enzyme.

High concentrations of 4-HPR (>5 μM) have been shown to induce apoptosis and necrosis (30), whereas lower concentrations (3 μM) have been reported to induce G1/S arrest and hypophosphorylation of Rb (32, 33). Because loss of DEGS-1 resulted in cell cycle arrest, we examined what effects lower concentrations of 4-HPR (1 and 2.5 μM) would have on cell growth in an MTT assay (Fig. 7A). SMS-KCNR cells were plated as described under “Experimental Procedures.” Cells were collected on days 2, 4, and 6. As shown in Fig. 7A, low doses of 4-HPR inhibited cell growth in a fashion similar to transfection of cells with DEGS-1 siRNA. There were ~41 and ~47% decreases in growth on days 4 and 8, respectively, with 1 μM 4-HPR. In cells treated with 2.5 μM 4-HPR, there was an ~70% decrease in cell growth on both days. When cells were treated with 5 and 10 μM 4-HPR, marked cell death was observed within 48–72 h (data not shown). We next examined the effects of a low dose of 4-HPR (2.5 μM) on the phosphorylation of Rb (Fig. 7B). The results were similar to those obtained with siRNA-transfected cells (Fig. 5C). After 48 h of 4-HPR treatment, a decrease in the amount of pRb was observed. The hypophosphorylation of Rb was also inhibited by tautomycin. DEGS-1 protein levels were unchanged.

Taken together, these results show that 4-HPR is a potent and rapid inhibitor of dihydroceramide desaturase that induces dihydroceramide (and not ceramide) generation in a dose-dependent manner. It does not have any effects on the mRNA or protein levels of DEGS-1. The hypophosphorylation of Rb involves the activation of PP1.

DISCUSSION

In this study, we used a novel assay to measure dihydroceramide desaturase activity in intact cells employing cationic dhCCPS. This is the first study using siRNA to attenuate dihydroceramide desaturase function. The data presented demonstrate that the human desaturase DEGS-1 is the major desaturase active in SMS-KCNR cells and that inhibition of its function with siRNA, along with the subsequent accumulation of endogenous dhCers, leads to cell cycle arrest with dephosphorylation of Rb. This is the first study to implicate dihydroceramides in serine/threonine protein phosphatase activation.

Because of limitations of the solubility and bioavailability of conventional exogenous dhCers, novel cationic dhCers
(dhCCPS analogs) with high solubility, cell membrane permeability, and cellular uptake were designed and synthesized (15). Measuring the conversion of dhCCPS to the corresponding CCPS counterparts by LC/MS methodology was very accurate and highly reproducible. Because enzyme activity was measured within cells, there was no need for the addition of cofactors.

The in situ assay was able to confirm the utility of the cyclopropene-containing ceramide analog C8-CPPC as an inhibitor of the desaturase in intact cells. Interestingly, there was a decrease in the generation of dhCers with a higher concentration (2.5 μM) of C8-CPPC. This finding may be due to the effect of C8-CPPC on other sphingolipid enzymes (21). Thus, in addition to an siRNA approach, C8-CPPC treatment may be used as a tool to study the function of the enzyme.

DES family members belong to a desaturase/hydroxylase superfamily that is characterized by three histidine-containing consensus motifs (10). DEGS-1 is the only dihydroceramide desaturase reported to be present in human cells. Its mouse homolog (DES1) was shown to have desaturase activity (34). Human DES2, the human homolog of the mouse Des2 gene, was cloned recently and, like mouse Des2, shown to have dihydroceramide hydroxylase activity (35). Although mouse DES2 has been reported to have both desaturase and hydroxylase activities, no desaturase activity was detected in 293 human embryonic kidney cells overexpressing human DES2 (35). Our data confirm that DEGS-1 is the main dihydroceramide desaturase in human cells because its loss with siRNA blocks its function and increases endogenous dhCers.

The data also show that dihydroceramide desaturase plays an essential role in the regulation of Cer levels and that the function of this enzyme is important in the downstream effects of Cer signaling. Cers have been shown to induce cell cycle arrest and Rb dephosphorylation in multiple cell lines (23–26). Although both serine/threonine protein phosphatases PP1 and PP2A are involved in Rb dephosphorylation, only PP1 has been demonstrated to directly dephosphorylate Rb and to be involved in Cer-mediated Rb dephosphorylation (29, 36). There have been no previous reports of the involvement of dhCers in cell cycle progression. Because the accumulation of dhCers persists even 6 days after siRNA silencing, the cell cycle effects, along with the hypophosphorylation of Rb, are likely due to the endogenous dhCers themselves.

Prior in vitro studies with short chain dhCers failed to activate serine/threonine protein phosphatases (29, 37). This may have been due to their poor solubility and/or short chain length. In this study, tautomycin, a selective PP1 inhibitor, inhibited the hypophosphorylation of Rb associated with dhCer accumulation resulting from siRNA transfection or 4-HPR treatment. Therefore, our results suggest that endogenous dhCers activate PP1.

4-HPR is currently being tested in clinical trials on neuroblastoma, leukemia, lymphoma, and prostate and ovarian cancers. The mechanisms by which 4-HPR mediates anti-proliferative effects are not well understood. It can induce apoptosis independently of retinoid receptor pathways (retinoic acid and retinoid X receptors) (33, 38). Activation of JNK (c-Jun N-terminal kinase) (39) and generation of reactive oxygen species and induction of increased ceramide, gangliosides, and 12-lipoxygenase have all been implicated (30, 40, 41). Our data suggest that the anti-tumor effects of 4-HPR may be related to the accumulation of dhCers and/or more complex dihydrospingolipids. In our experiments, lower concentrations of 4-HPR (<3 μM) decreased cell growth similarly to DEGS-1 siRNA treatment, whereas higher concentrations (5 and 10 μM) at doses associated with reactive oxygen species generation (30) caused cell death.

Our findings revealed marked accumulation of endogenous dhCers via inhibition of dihydroceramide desaturase activity in SMS-KCNR cells treated with 4-HPR. The role and mechanisms of ceramide generation in 4-HPR downstream effects have not been clear, and differences have been reported with different neuroblastoma cell lines and the concentration of 4-HPR used (30, 40, 41). Some of the limitations of these earlier studies were due to the method used for the quantitation of ceramide levels because ceramide was measured by enzymatic or labeling methods with which it was difficult to differentiate Cers from dhCers. Given our experimental data, it is likely that dhCers (and not Cers) were the sphingolipids that were elevated in the previously published studies.

Previous studies on the biological activity of dhCers using their short chain analogs concluded that dhCers are inactive sphingolipids (12–14). The results from this study with dhCCPS analogs suggest a novel biological function for dhCers. Our findings are in agreement with some recent publications showing biological functions of dhCers. Jiang et al. (42) reported marked accumulation of dihydroceramide and dihydrosphingosine upon γ-tocopherol treatment, which preceded apoptosis in LNCaP human prostate cancer cells. Tserng and Griffin (43) also reported C16-dhCer accumulation prior to cell death in HL-60 human leukemia cells.

Further investigation of the profile of complex sphingolipids/glycosphingolipids in response to DEGS-1 siRNA or 4-HPR treatment is warranted. Understanding the regulation of this enzyme has potential for future cancer therapy.

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Note Added in Proof—While these studies were in progress, another paper by Zheng et al. (Biochim. Biophys. Acta (2006) 1758, 1864–1884) has reported that 4-HPR inhibits the desaturase and elevates dihydroceramides.

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